ISSUES AND CHALLENGES IN NSCLC IMMUNOTHERAPY

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ISSUES AND CHALLENGES IN NSCLC IMMUNOTHERAPY

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SYSTEMATIC REVIEW
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Efficacy of Immune Checkpoint Inhibitors in Non-small-cell Lung Cancer Patients With Different Metastatic Sites: A Systematic Review and Meta-Analysis

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Background: Organ-specific response patterns reported in previous studies indicate different response toward immune checkpoint inhibitors (ICIs) in non-small-cell lung cancer (NSCLC) patients with different metastatic sites. This study aims to compare the efficacy of ICIs with conventional therapy in NSCLC patients with bone, brain or liver metastases.

Materials and Methods: MEDLINE, Embase, and CENTRAL were searched for studies comparing ICIs with conventional therapy in NSCLC patients with bone, brain or liver metastases. The pooled hazard ratio (HR) of overall survival (OS) and progression-free survival (PFS) among included studies was analyzed using the random effects model.

Results: Eight studies consisting of 988 NSCLC patients were included, 259 with brain metastases and 729 with liver metastases. No available study with bone metastases information was identified. For patients with brain metastases, ICIs significantly improved their OS (HR, 0.57; $P = 0.007$). For patients with liver metastases, both OS (HR, 0.72; $P = 0.006$), and PFS (HR, 0.72; $P = 0.004$) improvements were observed in the ICI treatment arm. Subgroup analysis was conducted based on target of ICIs and treatment regimen. PD-1 inhibitors could benefit patients with liver or brain metastases on OS and PFS (brain metastases: OS, HR, 0.43; $P < 0.001$; liver metastases: PFS, HR, 0.52; $P = 0.003$; OS, HR, 0.66; $P = 0.001$), while PD-L1 inhibitors could not. Patients with brain metastases could only gain OS improvement from ICIs combined with chemotherapy (HR, 0.41; $P = 0.001$), but for patients with liver metastases, the benefit was detected using ICIs single agent (HR, 0.68; $P = 0.012$) or ICIs combined with chemotherapy plus anti-VEGF therapy (HR, 0.52; $P = 0.005$).

Conclusion: ICIs could significantly improve OS in NSCLC patients with brain or liver metastases compared with conventional therapy. Patients with brain metastases could only gain OS benefit from ICIs combined with chemotherapy, while those with liver metastases obtained superior OS from ICIs single agent or ICIs combined with chemotherapy plus anti-VEGF therapy.

Keywords: non-small-cell lung cancer, brain metastasis, liver metastasis, immune checkpoint inhibitor, meta-analysis

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INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality, with 2.1 million cases diagnosed and 1.8 million death every year in the world (1). Non-small-cell lung cancer (NSCLC) accounts for ∼85% of all cases of lung cancer in the United States (2). Emerging therapeutic approaches have improved the prognosis of patients with NSCLC, the most promising among which is immune checkpoint inhibitor (ICI), based on its efficacy on relieving the immune suppression in the tumor microenvironment (TME) (3). Up to date, several ICIs have been approved as the first-line or second-line therapy for the treatment of metastatic NSCLC (4, 5).

Despite the substantial survival improvement of ICIs, identifying the population who can benefit from immunotherapy is still a challenge. Bone, brain, and liver are among the most frequent metastatic sites in NSCLC, with about 34% bone metastases, 39% nervous system metastases and 20% liver metastases reported in a study investigating more than 20,000 cases (6, 7). In addition, population-based studies suggest metastases to bone, brain, and liver conferred poor prognosis (6, 8). Regarding the great therapeutic efficacy of ICIs, whether patients with different metastatic sites can benefit from ICIs uniformly is being intensively investigated. Difference in survival and response according to metastatic sites was observed in multiple retrospective studies (9, 10). A lower organ-specific response rate to nivolumab was observed in liver metastases compared with metastases to lymph nodes (8% vs. 28%) in a retrospective study (9). In a real-world cohort investigating the efficacy of nivolumab in patients with NSCLC, the presence of liver metastases predicted worse overall survival (4.0 vs. 9.0 months, $p < 0.001$), while pulmonary metastasis conferred a better outcome (8.8 vs. 5.6 months, $p = 0.004$) (10). Among different metastatic sites, bone, brain, and liver metastases were generally regarded as independent poor prognostic factors for ICI therapies (11–14). However, these results did not compare the efficacy of ICIs with other conventional treatments. Considering the relatively high cost and potential immune-related adverse effects of ICIs, the therapeutic choice for NSCLC patients with specific metastases is still a problem to be solved. Several phase 3 clinical trials have reported the efficacy of ICIs compared with chemotherapies in subgroups of NSCLC patients with baseline brain or liver metastases (15, 16). Nevertheless, the results were controversial. Early data from the KEYNOTE-189 study suggested patients with baseline brain metastases benefitting from ICI intervention arm while other studies, for example, KEYNOTE-024, reached the opposite conclusion (15, 16).

Therefore, we conducted this meta-analysis to comprehensively investigate whether NSCLC patients with bone, brain or liver metastases could gain more benefits from ICIs compared with conventional treatments.

MATERIALS AND METHODS

Literature Search and Study Selection

The Preferred Reporting Items for Systematic Review and Meta-analyses (PRISMA) statement was used to perform this systematic review and meta-analysis (17). The protocol was registered on the International Prospective Register of Systematic Reviews (PROSPERO) before conducting this study (ID: CRD42020164348). A comprehensive literature search via MEDLINE, Embase and CENTRAL up to May 20, 2020 was performed by two investigators (JRL and KLY) independently. Keywords for the query term included *Lung Neoplasms, NSCLC, Neoplasm Metastasis, checkpoint inhibitor, CTLA-4, PD-1, PD-L1, ipilimumab, atezolizumab, durvalumab, pembrolizumab, nivolumab* (**Supplementary Table 1**). References from published studies were also manually scanned to identify additional relevant trials.

Both inclusion and exclusion criteria were prespecified. The inclusion criteria were listed as follows: (1) patients with histologically or cytologically confirmed NSCLC; (2) studies comparing ICIs (single agent or in combination with chemotherapy or targeted therapy) vs. systematic chemotherapy or targeted therapy or combination of both; (3) available clinical outcomes of patients with baseline bone, brain or liver metastases; (4) any perspective or retrospective studies. The primary outcomes were overall survival (OS) and progression-free survival (PFS). Studies with following characteristics were excluded: (1) duplication of previous studies; (2) publication types such as case report, meta-analysis, and review. For studies with multiple publications, the most recent publication was included. Studies were screened independently by two authors (JRL and KLY). Disagreements were solved by consensus or with a third author (LZ) if necessary.

Data Extraction and Quality Assessment

Data were extracted independently by two authors (JRL and KLY) using a predefined extraction form, including the following information: first author's name, trial name, year of publication, study population, metastatic site, number of patients, intervention, comparison, primary outcomes.

The risk of bias of included studies was independently assessed by two authors (JRL and KLY). Discrepancies were solved by consensus or with a third author (LZ) if necessary. The Cochrane Risk of Bias Tool was used to estimate the quality of randomized controlled trials (RCTs) (18). For retrospective studies or *post-hoc* analysis of subgroups from RCTs, the Newcastle-Ottawa Scale was applied to assess the risk of bias (19). Studies scored ≥ 7 were regarded as being of high quality.

Statistical Analysis

Efficacy of ICIs on outcomes compared to conventional therapy was measured by hazard ratio (HR) with corresponding 95% confidence interval (CI). The random effects model was used to compute the pooled HR of included studies (20). Cochrane Q test and I^2 test were used to evaluate the heterogeneity among included studies, which was considered statistically significant as $P < 0.1$ or $I^2 > 50\%$. Subgroup analyses were conducted based on target of ICIs, and treatment regimen of the intervention group. Sensitivity analysis was performed to assess the bias risk of one single study on the pooled result by a leave-one-out approach. Publication bias was evaluated by Begg's and Egger's test.

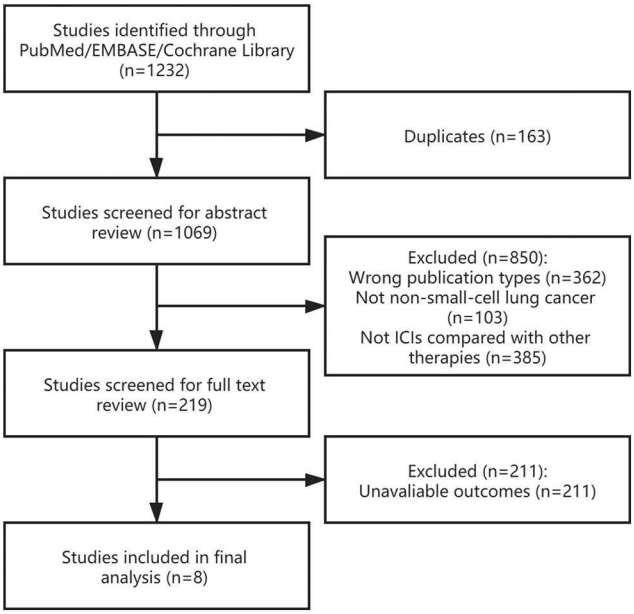

FIGURE 1 | Flowchart diagram of literature search and study selection.

Stata v15.1 (Stata Corporation, College Station, TX, USA) was applied to perform all statistical analyses. *P*-values were two-sided and considered statistically significant if $P < 0.05$ except for the Cochrane Q test.

RESULTS

Eligible Studies and Characteristics

A total of 1,232 studies was initially identified, 163 of which were excluded due to duplications. After screening abstract and full text of references according to the eligible criteria, eight studies were included (15, 21–27). **Figure 1** shows the process of study selection.

The main characteristics of included studies were summarized in **Table 1**. Briefly, 988 cases from eight studies were included, 259 of which with brain metastases, and 729 with liver metastases. No study with available bone metastases information was identified. All the included studies were subgroup analyses of multicenter, randomized, phase 3 trials, published between 2016 and 2019. For metastatic sites, three studies provided OS data of brain metastases (15, 21, 27), while six studies with OS or PFS data of liver metastases (21, 23–27). Two studies included patients who had received 1–2 previous cytotoxic chemotherapy regimens (22, 24), while eligible patients were chemotherapy-naïve in other six studies (15, 21, 23, 25–27). A minimum PD-L1 tumor proportion score of 50% was required in the KEYNOTE-024 study (15), whereas the PD-L1 expression status was not mentioned in other studies. PD-1 inhibitors were applied in three studies (15, 24, 27), while PD-L1 inhibitors were used in 5 studies (21–23, 25, 26). ICI monotherapy were compared with chemotherapy in three studies (15, 22, 24). Four studies applied ICIs combined with chemotherapy vs. chemotherapy alone (21, 25–27), and particularly in one study, ICI was combined with anti-VEGF therapy plus chemotherapy, compared with anti-VEGF therapy plus chemotherapy (23).

The Newcastle-Ottawa Scale was applied to evaluate the risk of bias of included studies. Overall, the methodological quality of all included trials was relatively good (**Table 1**).

Effect of ICIs on Patients With Brain Metastases

A total of three studies with 259 cases was integrated to analyze the effect of ICIs on patients with brain metastases, with OS as the primary outcome. Only KEYNOTE-189 evaluated the efficacy of ICIs on PFS, which was not suitable for data synthesis. The pooled result showed that ICIs were significantly correlated with longer OS than chemotherapy (HR, 0.57; 95%CI, 0.37–0.86; $P = 0.007$) with low statistical heterogeneity (I^2 =34.9%; $P = 0.215$) (**Figure 2**). Subgroup analysis showed that patients with brain metastases could benefit more from PD-1 inhibitors than chemotherapy (HR, 0.43; 95%CI, 0.27–0.69; $P < 0.001$). However, PD-L1 inhibitors did not provide significantly longer OS to this population compared with chemotherapy (HR, 0.74; 95%CI, 0.49–1.13; $P = 0.158$) (**Table 2**). ICI monotherapy did not bring more improvements to patients with brain metastases compared with chemotherapy (HR, 0.71; 95%CI, 0.48–1.04; $P = 0.082$), while ICIs combined with chemotherapy showed a superior OS (HR, 0.41; 95%CI, 0.24–0.67; $P = 0.001$) for this population (**Table 2**).

Effect of ICIs on Patients With Liver Metastases

Five studies provided OS outcome of 590 NSCLC patients with liver metastases, the pooled result demonstrated a superior OS in the intervention arm (HR, 0.72; 95%CI, 0.57–0.91; $P = 0.006$) with relatively low statistical heterogeneity (I^2 =31.7%; $P = 0.210$) (**Figure 3A**). Benefit of OS in the ICI treatment arm compared with control was observed when PD-1 inhibitors were applied (HR, 0.66; 95%CI, 0.51–0.85; $P = 0.001$), but not for PD-L1 inhibitors (HR, 0.80; 95%CI, 0.51–1.26; $P = 0.338$) (**Table 2**). Survival improvements were found to be statistically significant when the intervention arm was ICI single agent (HR, 0.68; 95%CI, 0.50–0.91; $P = 0.012$) or ICI combined with chemotherapy plus anti-VEGF therapy (HR, 0.52; 95%CI, 0.33–0.82; $P = 0.005$), but not for ICIs only combined with chemotherapy (HR, 0.84; 95%CI, 0.63–1.12; $P = 0.324$) (**Table 2**).

Five studies were included for the analysis of PFS of 536 NSCLC patients with liver metastases, indicating patients treated with ICIs have longer PFS than the control group (HR, 0.65; 95%CI, 0.49–0.87; $P = 0.004$) with significant heterogeneity (I^2 =55.7%; $P = 0.06$) (**Figure 3B**). For patients with liver metastases, longer PFS was observed in the ICI arm compared with control, regardless of targets (PD-1: HR, 0.52; 95%CI, 0.34–0.81; $P = 0.003$; PD-L1: HR, 0.69; 95%CI, 0.49–0.97; $P = 0.034$) or the treatment regimen of intervention arm (ICI combined with chemotherapy: HR, 0.73; 95%CI, 0.58–0.92; $P = 0.008$; ICI combined with chemotherapy plus anti-VEGF therapy: HR, 0.41; 95%CI, 0.26–0.62; $P < 0.001$) (**Table 2**).

TABLE 1 | Baseline characteristics of included studies.

Author	Trial name	Year	Study population	No. of baseline liver metastases	No. of baseline brain metastases	Intervention	Comparison	Treatment line	PD-L1 expression	Primary outcomes	Quality
Reck et al. (15)	KEYNOTE-024	2016	Stage IV NSCLC with no sensitizing EGFR mutations or ALK translocations	–	28	Pembrolizumab	Platinum-based chemotherapy	1	>50%	OS	High
Gadgeel et al. (22)	OAK	2019	Squamous or non-squamous NSCLC	–	123	Atezolizumab	docetaxel	≥2	–	OS	High
Jotte et al. (26)	IMpower131	2018	Stage IV squamous NSCLC	139	–	Atezolizumab + carboplatin + nab-paclitaxel	Carboplatin + nab-paclitaxel	≥1 (*)	–	PFS	High
Barlesi et al. (21)	IMpower132	2018	Metastatic non-squamous NSCLC lacking sensitizing EGFR or ALK mutations	73	–	Atezolizumab + carboplatin/cisplatin + + pemetrexed	Carboplatin/cisplatin + + pemetrexed	1	–	OS, PFS	High
Vokes et al. (24)	Checkmate 017 and Checkmate 057	2018	Stage IIIB/IV NSCLC squamous or non-squamous NSCLC	193	–	Nivolumab	Docetaxel	≥2	–	OS	High
West et al. (25)	IMpower130	2019	Stage IV non-squamous NSCLC	100	–	Atezolizumab + carboplatin + nab-paclitaxel	Carboplatin + nab-paclitaxel	≥1 (*)	–	OS, PFS	High
Reck et al. (23)	IMpower150	2019	Stage IV metastatic non-squamous NSCLC	109	–	Atezolizumab + bevacizumab + carboplatin + paclitaxel	Bevacizumab + carboplatin + paclitaxel	≥1 (*)	–	OS, PFS	High
Garassino et al. (27)	KEYNOTE-189	2019	Metastatic non-squamous NSCLC without sensitizing EGFR or ALK mutations	115	108	Pembrolizumab + platinum-based drug + pemetrexed	Placebo + platinum-based drug + pemetrexed	1	–	OS, PFS	High

OS, overall survival; PFS, progression-free survival.

**eligible patients of this study were chemotherapy-naïve. For patients with a sensitizing mutation in the EGFR gene or ALK fusion oncogene, they must have had disease progression or intolerance to treatment with at least one tyrosine inhibitor.*

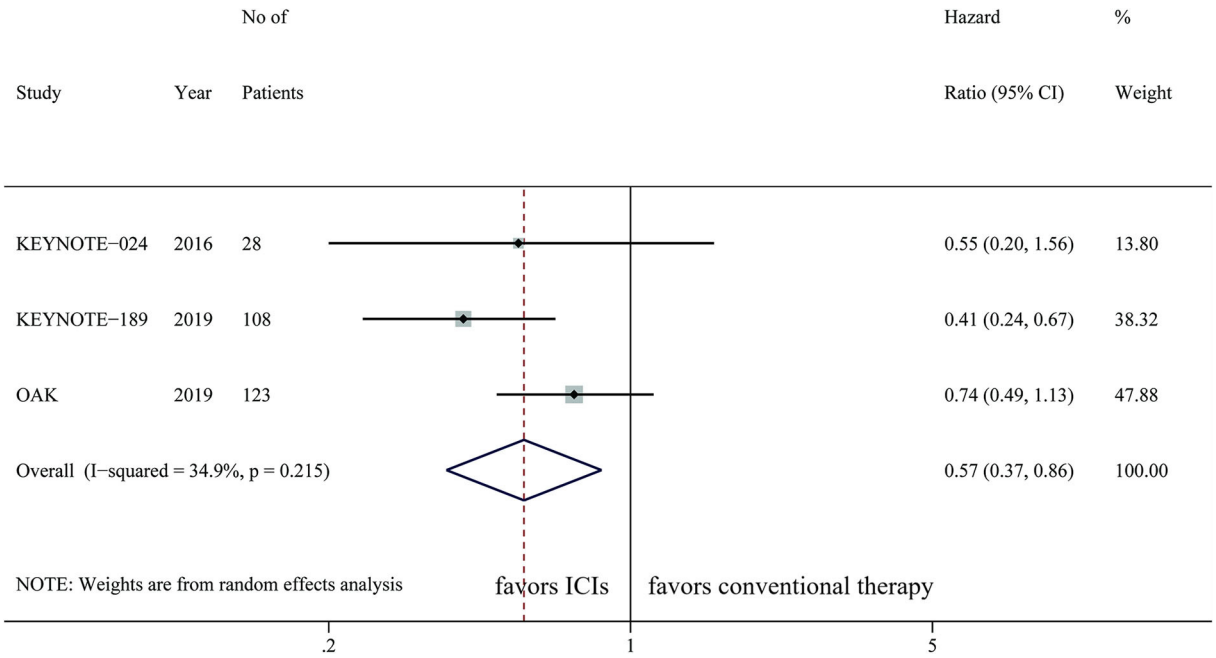

FIGURE 2 | Efficacy of immune checkpoint inhibitors on OS in NSCLC patients with brain metastases.

Sensitivity Analysis and Publication Bias

Sensitivity analysis was conducted using the leave-one-out approach to evaluate the effect of each study on the pooled HR. No single study dominates the final interpretation of the pooled result, indicating a relatively good stability (**Supplementary Figure 1**).

Visual inspection of the Begg funnel plots was symmetry, indicating absence of significant publication bias (**Supplementary Figure 2**). Further tests suggested no statistically significant publication bias was detected in OS for patients with brain metastases (Begg's test, $P = 1$; Egger's test, $P = 0.79$), OS (Begg's test, $P = 0.462$; Egger's test, $P = 0.513$), and PFS (Begg's test, $P = 1$; Egger's test, $P = 0.909$) for patients with liver metastases.

DISCUSSION

One of the major challenges of current cancer immunotherapy is understanding organ-specific tumor immune response (28). The TME differs substantially across various organ sites where the tumor evolves, which in turn influences tumor development and host anti-tumor immune response (29). Previous studies have demonstrated organ-specific response patterns to ICI therapy in metastatic NSCLC, indicating the importance of tumor metastatic sites in guiding immunotherapy strategy (9, 30). However, since many studies have reported the effect of metastatic sites on ICI efficacy, no study has been conducted to comprehensively compare the efficacy of ICIs with conventional systematic therapies in regard of metastatic sites.

This systematic review and meta-analysis aimed to compare the efficacy of ICIs with conventional therapies on NSCLC patients with bone, brain or liver metastases. Our study revealed that NSCLC patients with brain metastases could obtain OS improvements from ICI therapy compared with conventional treatment, and for those with liver metastases, they could benefit from ICIs in terms of both OS and PFS. In this meta-analysis, no eligible studies investigating patients with bone metastases were identified. Although previous studies suggested that bone involvement was independent poor prognostic factor for immunotherapy, the relative benefit of ICIs compared with chemotherapy remains obscure. More randomized controlled trials are required to directly elucidate this issue (10, 14).

Brain metastases are normally considered as a frequent metastatic site of advanced NSCLC with unfavorable prognosis (31). Systematic treatments including targeted treatment and chemotherapy are applied to patients without neurological symptoms, with OS ranging from 5 to 16 months (32). Pivotal clinical trials of ICIs generally excluded patients with symptomatic brain metastases, but those with asymptomatic brain metastases were allowed (33). Several recent studies have demonstrated promising efficacy of ICIs in NSCLC patients with brain metastases. Remarkable disease control rate (DCR) of 39% was observed in a cohort of 409 patients with asymptomatic or controlled brain metastases of non-squamous NSCLC (34). A phase 2 trial reported a brain metastases response of 29.7% in patients treated with pembrolizumab with PD-L1 expression of at least 1% (35). However, these studies were single-arm trials without a control group, making it difficult to decide which treatment is superior. Regarding on this issue, our analysis suggests that patients with asymptomatic brain metastases obtain superior OS under the ICI treatment. Both TME and tumor intrinsic features of brain metastases contribute to this efficacy.

TABLE 2 | Results of subgroup analysis.

Group		No. of studies	Test of association			Test of heterogeneity	
			HR	95% CI	*P*-Value	I^2 (%)	*P*-Value
Brain metastases	**Overall survival**						
	Total	3	0.57	0.37–0.86	0.007	34.9	0.215
	Target of ICIs						
	PD-1	2	0.43	0.27–0.69	<0.001	0	0.616
	PD-L1	1	0.74	0.49–1.13	0.158	–	–
	Treatment regimen						
	ICI monotherapy	2	0.71	0.48–1.04	0.082	0	0.600
	ICI combined with chemotherapy	1	0.41	0.24–0.67	0.001	–	–
Liver metastases	**Overall survival**						
	Total	5	0.72	0.57–0.91	0.006	31.7	0.210
	Target of ICIs						
	PD-1	2	0.66	0.51–0.85	0.001	0	0.742
	PD-L1	3	0.84	0.63–1.12	0.324	26.2	0.258
	Treatment regimen						
	ICI monotherapy	1	0.68	0.50–0.91	0.012	–	–
	ICI combined with chemotherapy	3	0.84	0.63–1.12	0.324	26.2	0.258
	ICI combined with chemotherapy plus anti-VEGF therapy	1	0.52	0.33–0.82	0.005	–	–
	Progression-free survival						
	Total	5	0.65	0.49–0.87	0.004	55.7	0.06
	Target of ICIs						
	PD-1	1	0.52	0.34–0.81	0.003	–	–
	PD-L1	4	0.69	0.49–0.97	0.034	61.1	0.052
	Treatment regimen						
	ICI combined with chemotherapy	4	0.73	0.58–0.92	0.008	15.7	0.313
	ICI combined with chemotherapy plus anti-VEGF therapy	1	0.41	0.26–0.62	<0.001	–	–

HR, hazard ratio; CI, confidence interval; ICI, immune checkpoint inhibitor.

Evidence showed that the integrity of blood-brain barrier (BBB) was compromised in brain metastases, allowing substantial infiltration of immune suppressive cell types, which may also make it possible for antibodies to cross the BBB and functionate (36). Besides, dense infiltration of lymphocytes was observed in specimens of brain metastases, providing the basis for response to ICIs (37). For tumor cell-inherent factors, high mutation load was observed in brain metastases, which is associated with increased frequency of neoantigens and may contribute to improved response to checkpoint inhibition (38). Only three studies with available baseline brain metastases data was included in this analysis. Therefore, large-scale RCTs are further required to reach the conclusion.

Conventional treatment of liver metastases consists of systematic and palliative therapy (39). With the advent of immunotherapy with revolutionary efficacy, however, several studies have demonstrated liver metastases as an independent poor prognostic factor of immunotherapy for NSCLC (11–13). Patients with liver metastases exhibited significantly shorter OS (mOS, 3.12 months) and PFS (mPFS, 1.35 months) compared with those without liver metastases (mOS, 11.37 months; mPFS, 3.75 months) in a retrospective study, with an overall response rate (ORR) of 22.5% (40). One possible explanation is the immunoregulatory hepatic microenvironment. As a major metabolic organ, liver has unique immunoregulatory functions in order to prevent the induction of immunity against innocuous antigens (41). Local hepatic antigen-presenting cells induce T cell tolerance by multiple mechanisms, including clonal elimination, induction of T cell anergy and recruitment of regulatory T cells, and the presence of hepatic sinusoids provides a large immunoregulatory platform for all the interactions (42). This tolerogenic hepatic microenvironment may interfere response of liver metastases toward ICIs. In NSCLC patients with baseline liver metastases treated with PD-1 inhibitor, decreased marginal CD8+ T cells infiltration was observed, in accordance with lower PFS and objective response rates compared with those without liver metastases (13). Despite all the confirmed mechanisms, however, whether patients with liver metastases obtain longer survival from ICI therapies vs. conventional treatments remains controversial. A previous meta-analysis demonstrated superior OS of chemo-immunotherapy in patients with liver involvement, in which three trials regarding liver metastases were included (43). In our analysis consisting of six trials, consistently, superior OS and PFS were observed in the ICI intervention arm, suggesting a preference of ICIs for the therapeutic decision when regarding NSCLC patients with liver metastases.

Subgroup analysis was conducted to identify possible clinical factors influencing the efficacy of ICIs. In terms of ICI target, patients could gain statistically significant OS and PFS benefit from PD-1 inhibitors regardless of metastatic sites, which was

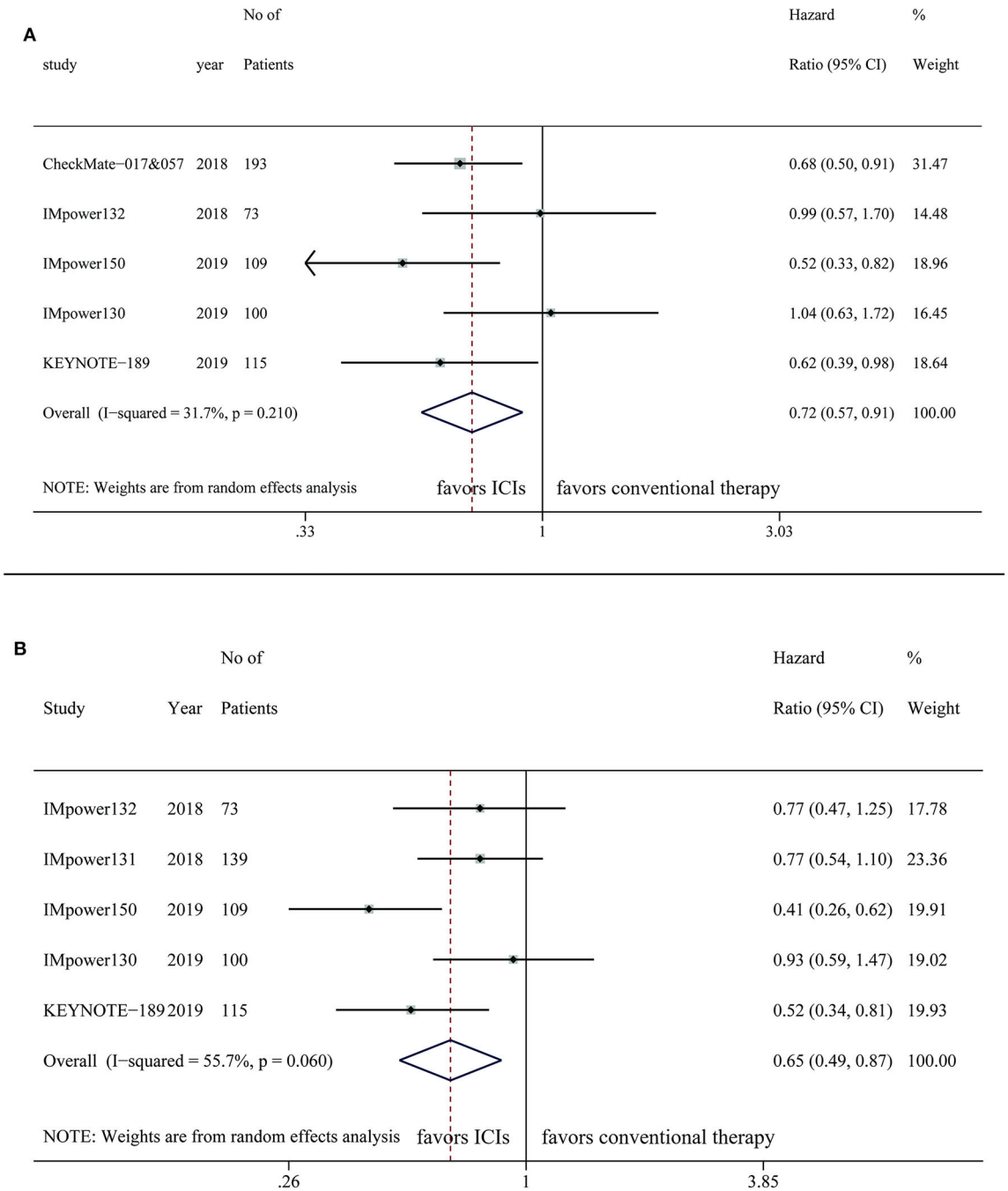

FIGURE 3 | Efficacy of immune checkpoint inhibitors in NSCLC patients with liver metastases on **(A)** OS **(B)** PFS.

not observed in those anti-PD-L1 therapies. At the moment there is no trial directly comparing the efficacy of PD-1 and PD-L1 inhibitors. Two previous large phase 1 studies have suggested PD-1 inhibitor could achieve higher ORR than PD-L1 inhibitor (20%–25% vs. 6%–17%) in patients with advanced solid tumors including NSCLC (44, 45). Furthermore, a recent meta-analysis using paired clinical trials with similar clinical characteristics was conducted to compare the efficacy between PD-1 and PD-L1 inhibitors, suggesting superior OS and PFS benefits of PD-1 inhibitors (46). One possible explanation is that PD-1 inhibitors can block the interactions between PD-1 and PD-L1, as well as PD-L2, which is not viable for PD-L1 inhibitor (47). PD-L2 expression was also identified as a key prognostic factor of ICI treatment in previous studies, and tumors might achieve immune escape through the PD-1/PD-L2 axis under the insufficient blockage of PD-L1 inhibitors (48).

For the choice of single agent or ICI combined with systematic therapy, whether systematic chemotherapy should be combined

with ICI is still under investigation, while results of several studies support this combination. Several clinical trials demonstrated higher ORR in patients treated with combination therapy over ICI single agent (15, 49–51). Besides, a recent meta-analysis showed that chemo-immunotherapy could improve OS and PFS in conditions traditionally thought to be weakly immunogenic (43). As many chemotherapy agents functionalize by damaging DNA structure, they may increase the mutation frequency and neoantigen formation, playing a synergistic role with ICIs and thus increase their efficacy (38). In this analysis, consistently, superior OS was observed ICIs combined with systematic chemotherapy for patients with brain metastases, while the benefit of monotherapy was not statistically significant. This result should be interpreted with caution as only three available studies were included in the analysis. A recent single-arm study has demonstrated clinically meaningful intracranial efficacy of 29.7% in 37 patients treated with pembrolizumab monotherapy (35). We cannot exclude the potential efficacy of ICIs administrated as single agent in patients with brain metastases at present, and the superiority of combination therapy should be validated in larger trials. Currently, several ongoing trials have been investigating the efficacy and safety of ICIs combined with other treatment options in treating patients with brain metastases, such as chemotherapy and radiotherapy (52). We can expect more rigorous evidence for the choice of treatment regimens in the future.

For patients with liver metastases, OS benefit was not observed with ICIs simply combined with chemotherapy, unless the addition of anti-VEGF treatment. Another recent meta-analysis investigating the efficacy of chemotherapy combined with ICIs reached the same conclusion (43). Simple addition of chemotherapy may not act synergistically with ICIs in the context of liver, since cytotoxic chemotherapy also targets proliferating benign cells including immune cells (53). However, the importance of combining anti-VEGF therapy with ICIs should be addressed. VEGF plays an important role in metastatic process to organs with abundant blood supply such as liver. Existing hepatic vessels can be utilized by metastatic cells, and the neovascularization process can be triggered by VEGF, creating the structurally and functionally abnormal tumor vasculature, which in turn facilitates the growth and progression of metastases (54). Bevacizumab-induced tumor vasculature normalization, which promotes T cell infiltration in the TME, may work synergistically with ICI and promotes its antitumor activity (55). Beyond that, in treating NSCLC patients with brain metastases, the application of bevacizumab could also reduce the level of circulating myeloid-derived suppressor cells in peripheral blood, suggesting its potential to induce a more effective anti-tumor microenvironment in metastatic site not just limited to liver (56). Altogether, our study supports ICIs combined with systematic chemotherapy in treating NSCLC patients with brain metastases, and for those with liver metastases, the addition of VEGF blockage to enhance the activity of ICIs is also necessary. It should be noted that based on limited clinical evidence, this suggestion is rather preliminary and exploratory. More prospective large-scale studies are required to further elucidate this problem.

Among other prognostic factors of immunotherapy, PD-L1 expression on tumor or immune cells was the most frequently studied biomarker, and several FDA approvals were linked to a specific PD-L1 threshold (57). This study did not investigate the relationship between PD-L1 expression and efficacy of ICIs in patients with brain or liver metastases, as only the KEYNOTE-024 study mentioned a PD-L1 expression threshold of 50% (15). The predictive value of PD-L1 expression in patients with specific metastases was demonstrated in previous studies (35, 40). In a phase 2 trial evaluating the efficacy of pembrolizumab in treating NSCLC patients with brain metastases, a brain metastasis response of 29.7% was observed in patients with PD-L1 expression of at least 1%, while there was no response in another cohort with PD-L1 expression $<1\%$ or unevaluable (35). However, due to the distinct immune microenvironment of brain metastases, the expression profile of PD-L1 can be pretty heterogenous between primary tumor sites and metastases, demonstrating both temporal and spatial discordance (58, 59). Therefore, although PD-L1 expression may work as a prognostic factor, the response rates of brain metastases can be pretty different from the primary tumor, and while guiding clinical decisions based on PD-L1 expression, biopsy acquisition from metastatic sites should be considered.

Several limitations in this meta-analysis should be acknowledged. First, the number of studies included in this meta-analysis is relatively small. Therefore, the conclusion is preliminary and should be cautiously interpreted, especially for those in subgroup analysis as some subgroups only contain one eligible study. Also, subgroup analysis based on the treatment line was not performed due to insufficient included studies in this meta-analysis. However, we should notice that patients receiving ICIs can be heavily pretreated in real-world clinical practice, and efficacy of immunotherapy is dependent on the line of treatment (10, 60). Second, all the included studies are *post-hoc* exploratory analyses with risk of bias to some extent, as inevitable imbalance of confounding factors presenting between treatment and control arms. Besides, most ongoing and completed clinical trials do not report survival outcomes of patients with specific metastatic sites. Thus, there may be a selection bias to some extent. Up to date, several clinical trials are ongoing investigating ICIs in solid tumor with brain metastases (52). Further investigations are warranted to elucidate organ-specific tumor immune microenvironment, and more randomized trials are required to compare the efficacy of immunotherapy with conventional therapy based on metastatic sites. Precise prognostic biomarkers of organ-specific response should also be identified to guide optimal clinical decisions.

CONCLUSION

In conclusion, current evidence suggests that ICIs can significantly prolong OS in NSCLC patients with brain metastases, and both OS and PFS in those with liver metastases. Although brain and liver metastases are generally regarded as poor prognostic factors for immunotherapy, this study still indicates ICIs are effective therapeutic options for NSCLC patients with these metastatic sites.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

LZ, JL, and KY: conceptualization. JL and KY: data curation and original draft writing. KY: statistical analysis. LZ, JL, KY, ZS, and CB: manuscript review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.01098/full#supplementary-material

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ORIGINAL RESEARCH
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A Prognostic Nomogram Combining Immune-Related Gene Signature and Clinical Factors Predicts Survival in Patients With Lung Adenocarcinoma

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The existence of tumor heterogeneity and complex carcinogenic mechanisms in lung adenocarcinoma (LUAD) make the most commonly used TNM staging system unable to well-interpret the prognosis of patients. Using transcriptome profiling and clinical data from The Cancer Genome Atlas (TCGA) database, we constructed an immune signature based on a multivariate Cox analysis (stepwise model). We estimated the half-maximal inhibitory concentration (IC50) of chemotherapeutic drugs in patients according to the pRRophetic algorithm. Gene-set variation analysis (GSVA) was used to reveal pathway enrichment between groups. Moreover, immune microenvironment landscape was described by single-sample gene-set enrichment analysis (ssGSEA) and CIBERSORT and systematically correlated with genomic of these patients. A prognostic nomogram combining the immune signature and TNM stage to predict the prognosis was developed by multivariate Cox regression. The novel signature with four immune-related genes (MAL, MS4A1, OAS1, and WFDC2) had good robustness, which can accurately distinguish between high- and low-risk patients. Compared with low-risk patients, high-risk patients with a worse prognosis (5-year OS: 46.5 vs. 59.4%, $p = 0.002$) could benefit more from immunotherapy and the application of common chemotherapeutic agents such as cisplatin and paclitaxel (Wilcoxon test, all $p < 0.05$). There were significant differences in tumor immune microenvironment and metabolic pathways between the two groups. Additionally, the constructed nomogram had reliable predictive performance with the C-index of 0.725 (95% CI = 0.668–0.781) in the development set ($n = 500$), 0.793 (95% CI = 0.728–0.858) in the internal validation set ($n = 250$) and 0.679 (95% CI = 0.644–0.714) in the external validation set ($n = 442$). The corresponding calibration curves also showed good consistency. To sum up, we developed an immune-related gene signature and comprehensively evaluated LUAD immune landscape and metabolic pathways. Effective differentiation of high- and low-risk patients and accurate construction of nomogram would be helpful to the development of individualized treatment strategies.

Keywords: lung adenocarcinoma, immune related gene, prognosis, signature, nomogram

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INTRODUCTION

Lung cancer is the most common malignancy, with morbidity and mortality ranking first in the world, according to global data released by the International Center for Cancer Research in 2020 (1). Lung cancer is divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). As the most common subtype of NSCLC (2), lung adenocarcinoma (LUAD) has complex carcinogenic mechanisms and obvious tumor heterogeneity. Due to the continuous improvement in the diagnosis and treatment of LUAD in recent years, especially the rise of immunotherapy, the prognosis of patients has improved significantly. However, the search for new models of diagnosis and treatment to benefit cancer patients has been the focus of oncologists. It is still necessary to further understand the occurrence and progression of LUAD and to identify strong prognostic biomarkers for LUAD.

Immune-related genes have great significance in the immune mechanism and immune function of the body. As we know, cancer is an extremely complex disease involving interactions between tumor and immune system (3). Immunotargeted therapy has played greatly important roles in improving the prognosis of patients with malignant tumors (4, 5). Nevertheless, the treatment can only be applied to some patients, and there are obvious individual differences in the therapeutic effect of this method (6, 7), which further illustrates the existence of tumor heterogeneity and the complexity of carcinogenic mechanisms. The expression of immune-related genes and the density and type of tumor immune infiltrating cells have been widely studied as prognostic biomarkers of lung cancer (8–10). However, the roles of immune-related genes involved in tumor immune microenvironment are still not fully recognized. In this study, a novel immune signature was constructed. We further revealed the differences in the immune microenvironment between high- and low-risk patients and well-predicted the efficacy of chemotherapy and immunotherapy in both groups. In addition, gene-set variation analysis (GSVA) was also used to explore the molecular mechanisms leading to significantly differential prognosis in high- and low-risk patients. Moreover, we developed a nomogram that can accurately predict the prognosis of patients to improve the efficacy of individualized prediction, which may provide a reference for clinicians to formulate more rational treatment strategies.

MATERIALS AND METHODS

Data Source and Preprocessing

The transcriptome profiling data for 535 cases of lung tumor tissue and 59 cases of lung normal tissue were downloaded directly from the Cancer Genome Atlas (TCGA) Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/, updated until March 05, 2020). The same method was also used to extract the corresponding clinical data (including age, sex, T stage, N stage, TNM stage, survival time, and status). Additionally, RNA expression profiles and clinical information of 443 LUAD patients in the GSE68465 dataset (11) were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/).

Acquisition of Immune-Related Genes

The immune-related gene sets (IMMUNE_RESPONSE and IMMUNE_SYSTEM_PROCESS) were extracted from the Molecular Signatures Database (MSigDB) (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp). There were 332 immune-related genes in these two genomes. To increase the available genes, we also downloaded a total of 2,498 immune-related genes in the Gene List from ImmPort (http://www.immport.org/). After deleting duplicate genes, 1,986 genes were finally used for the next analysis. We obtained immune-related genes and their expression profiles in combination with mRNA gene sets extracted from the TCGA database.

Differentially Expressed mRNAs (DEMs) in Lung Normal and Tumor Tissues

DEMs between lung normal and tumor tissues were identified by differential expression analysis using the "limma" package in R (12). $|\log_2$ FC (fold-change)$| > 1$ and $P < 0.05$ were set as the thresholds for screening DEMs. The common DEMs of the two databases (TCGA and GEO) were used for further analysis.

GO and KEGG Enrichment Analyses of the Common DEMs

To explore in depth the possible biological processes (BP), cellular components (CC), molecular functions (MF), and pathways of the common DEMs, we carried out GO and KEGG enrichment analysis utilizing the "clusterProfiler" package in R (13) with a statistical threshold of $p < 0.05$.

Screening of Immune-Related Genes Affecting Prognosis

In order to identify prognosis-related genes, the patients without accurate survival data (e.g., survival time =0 day and unknown) were removed from this study. Finally, 500 patients with detailed survival information were included in the study. Using univariate Cox analysis, we evaluated the association of the common DEMs with OS of LUAD patients. Only these genes with $p < 0.05$ in both two databases (TCGA and GEO) were considered as candidate immune-related genes affecting prognosis.

Construction and Evaluation of Immune-Related Gene Signature

Through a multivariate Cox analysis (stepwise model), we filtered these candidate immune-related genes affecting prognosis. Akaike information criterion (AIC) was used to avoid overfitting. We selected the genes with the highest likelihood ratio and lowest AIC values and estimated the β regression coefficients. Based on

Abbreviations: LUAD, Lung adenocarcinoma; OS, Overall survival; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; CI, Confidence interval; HR, Hazard ratio; ROC, Receiver operating characteristic; AUC, Area under the curve; C-index, Concordance index; CTLA-4, Cytotoxic T-lymphocyte associated 4; PD-1, Programmed cell death 1; PD-L1, Programmed cell death-ligand 1; TMB, Tumor mutational burden; GSVA, Gene-set variation analysis; GSEA, Gene-set enrichment analysis.

the β regression coefficients and expression values of the filtered genes, we calculated the risk score of each sample according to the following formula (14):

$$\text{risk}Score = \sum_{i=1}^{n} Coefi * Expressioni,$$

where Coefi was the β regression coefficients obtained from multivariate Cox analysis and Expressioni was the expression of the immune-related genes in the signature. With the median risk score as the cutoff point, patients were divided into high- and low-risk groups. The Kaplan–Meier method was performed to assess the survival differences between the two groups. To further assess the specificity and sensitivity of the immune-related gene-based signature, the ROC curves were drawn and the corresponding AUC values were also calculated. Additionally, we also used the same method to verify the prognostic performance in the internal and external validation datasets. A specific process for constructing this signature is shown in **Figure 1**.

Evaluation of the Sensitivity of Chemotherapeutic Agents

To predict the half-maximal inhibitory concentration (IC50) of chemotherapy drugs in the high- and low-risk groups of LUAD patients and to infer the sensitivity of the different patients, we used the "pRRophetic" package in R. By constructing the ridge regression model based on Genomics of Drug Sensitivity in Cancer (GDSC) (www.cancerrxgene.org/) cell line expression spectrum and TCGA gene expression profiles, the package could apply pRRophetic algorithm to predict drug IC50 (15).

Prediction of Immunotherapy Efficacy

To explore the relationship between the immune signature and immunotherapeutic efficacy, we adopted two computational methods to infer the immunotherapeutic response of LUAD patients at low and high risk. First, we downloaded the mutation data of LUAD from the TCGA database and calculated the tumor mutational burden (TMB) of each sample. The mutation data was divided into two groups by high- and low-risk samples. Second, an online tool named Tumor Immune Dysfunction and Exclusion (TIDE) (http://tide.dfci.harvard.edu) was applied to

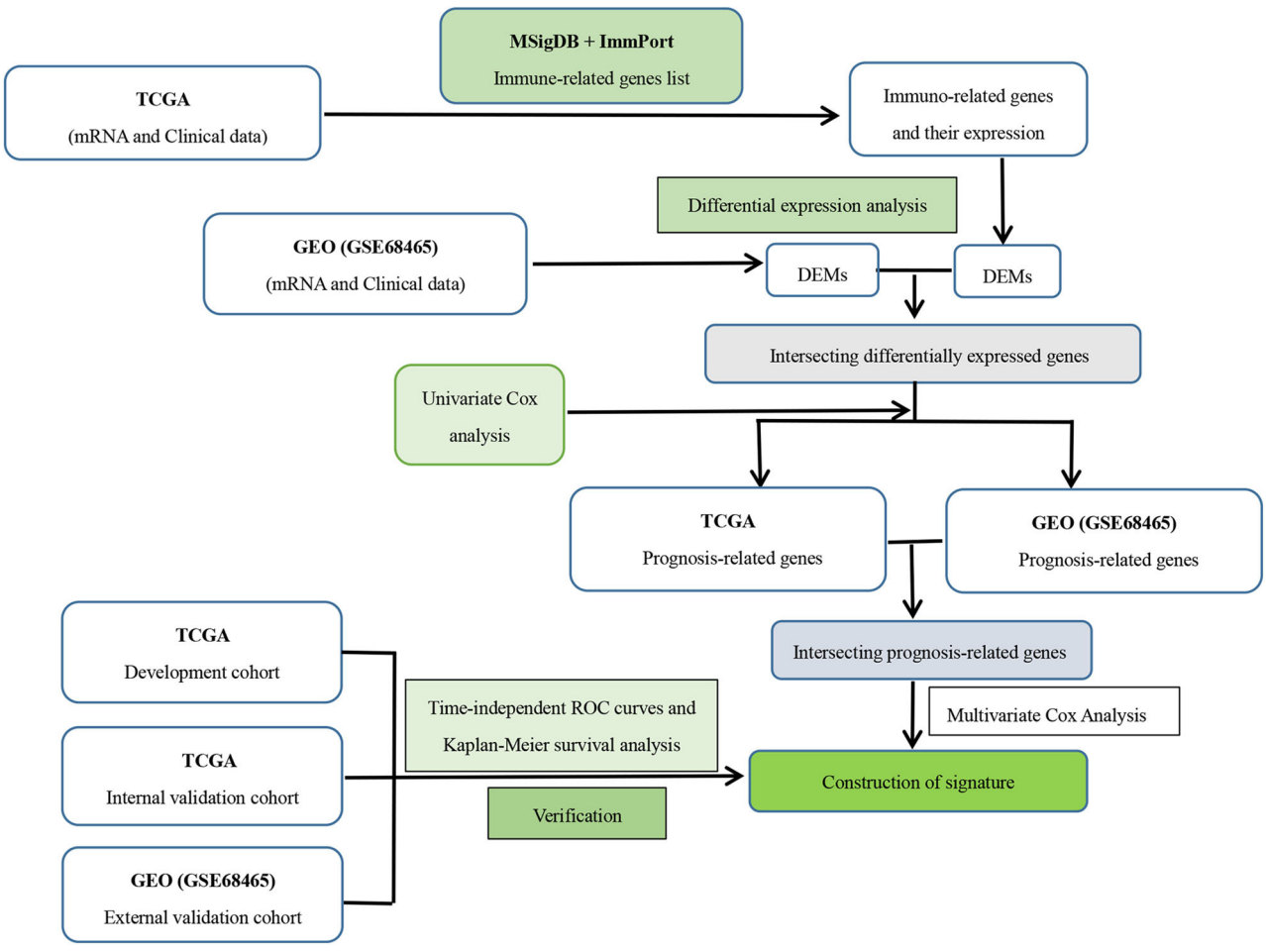

FIGURE 1 | Flowchart on construction and validation of the immune-related gene signature. DEM, differential expressed mRNAs.

infer the anti-PD1 and anti-CTLA4 immunotherapeutic response of each sample based on the transcriptome profiles of the TCGA-LUAD cohort (16).

Exploration of Tumor Immune Landscape

We obtained a set of marker genes related to immune cell types, including different immune cells, immune-related pathways, and functions from Bindea et al. ssGSEA is a feasibility approach, which can apply the characteristics of immune cell population expression to individual cancer samples and can calculate the rank value of each gene according to the expression profile for subsequent statistical analysis (17, 18). In this study, the "GSVA" package in R (19) was utilized with the ssGSEA method. Moreover, the "estimate" package in R was applied to evaluate the immune score, stromal score, and tumor purity of each sample in the high- and low-risk groups.

Moreover, as a wildly proposed computational algorithm, "Cell type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT)" (20) (https://cibersort.stanford.edu) was also used to predict immune-infiltrating cells of each LUAD sample in our study. The proportion of 22 immune-infiltrating cells in each sample can be obtained by inputting the expression data of the samples. Then, the samples with $p < 0.05$ were selected for further analysis. Human leukocyte antigen (HLA) was also applied to validate the differences between the two groups. In addition, Spearman correlation analysis was used to explore the relationship between four immune-related genes and risk score and immune infiltration.

Gene-Set Variation Analysis

Using the "GSEABase" package in R, we applied gene-set variation analysis (GSVA) that was predominantly performed on the 50 hallmark pathways described in the MSigDB, where each pathway-related gene set was trimmed to contain only unique genes to reduce pathway overlap and pathway redundancy and most genomes retained 70% of the genes involved (21). MSigDB is a collection of annotated gene sets for use with GSEA software. The MSigDB gene sets are divided into 8 major collections (H, C1–C7). We downloaded "c5.all.v7.0.symbols" (GO gene sets that contain genes annotated by the same GO term). The C5 collection was divided into three subcollections based on GO ontologies: biological process (BP), cellular component (CC), and molecular function (MF). To reveal pathway enrichment between low- and high-risk patients, we used the "GSVA" package in R (19) to evaluate t score and assign pathway activity conditions. Moreover, "limma" package in R was also applied to display distinctions in pathway activation between low- and high-risk groups.

The Relationship Between Immune-Related Genes and Transcription Factors

We acquired a transcription factor (TF) list from a web application named Cistrome (http://cistrome.org/) and then integrated with the mRNA expression matrix from the TCGA database to derive these TFs' expression level. We examined the correlation between the expression level of the immune-related genes in the signature and each TF using two-sided Pearson correlation coefficients and the *Z*-test. The TFs positively or negatively correlated with the four immune-related genes were considered as immune-related gene-associated TFs (|Pearson correlation coefficients| >0.3 and $P < 0.001$).

Clinical Correlation and Independent Prognostic Analysis

To better understand the impact of the signature and clinical features on patient outcomes, the univariate and multivariate Cox analyses were performed, which may reveal independent prognostic factors in LUAD patients. In addition, the correlation between the immune-related genes in the signature and clinical features was further explored.

Construction and Verification of a Prognostic Nomogram

Based on the multivariate Cox analysis, we developed a nomogram for predicting LUAD prognosis in the TCGA database. This nomogram incorporated two predictors, namely, risk score and TNM stage. To further verify the predictive power of this nomogram, we used the 50% LUAD samples randomly selected from the entire TCGA database as internal validation dataset ($n = 250$) and the GSE68465 dataset from the GEO database as external validation dataset ($n = 442$). The C-index and calibration plots were used to assess the performance of the established nomogram.

Statistical Analysis

All statistical analysis was conducted in R software 3.6.0. All categorical variables were expressed as number (percentage). The Wilcoxon rank-sum test was performed to compare the differences between groups of continuous data. The relationships between immune-related genes and risk score and immune infiltration were determined by the Spearman's correlation analysis. The Kaplan–Meier method was used to survival analysis, where the log-rank test was applied to compare the survival distribution. The Cox proportional hazard model was performed to estimate the β regression coefficient, hazard ratios, *p*-value, and their corresponding 95% confidence interval for each of the selected risk predictors. Based on the multivariate Cox analysis, a nomogram was constructed with the "rms" package in R. The C-index and calibration curve with the bootstrap method were used to evaluate the prediction performance of the nomogram. A $P < 0.05$ was considered statistically significant.

RESULTS

The Common DEMs and Functional Annotation

Of 1,986 immune-related genes obtained from the MSigDB and ImmPort databases, 1,479 genes had corresponding relationships in the TCGA transcriptome. Their expression profiles were used for differential expression analysis (**Figure 2A**). There were 451 differentially expressed genes in lung tumor and normal tissues, of which 237 were upregulated and 214 were downregulated (**Figure 2C**). Also, in the GSE68465 dataset, 2,185

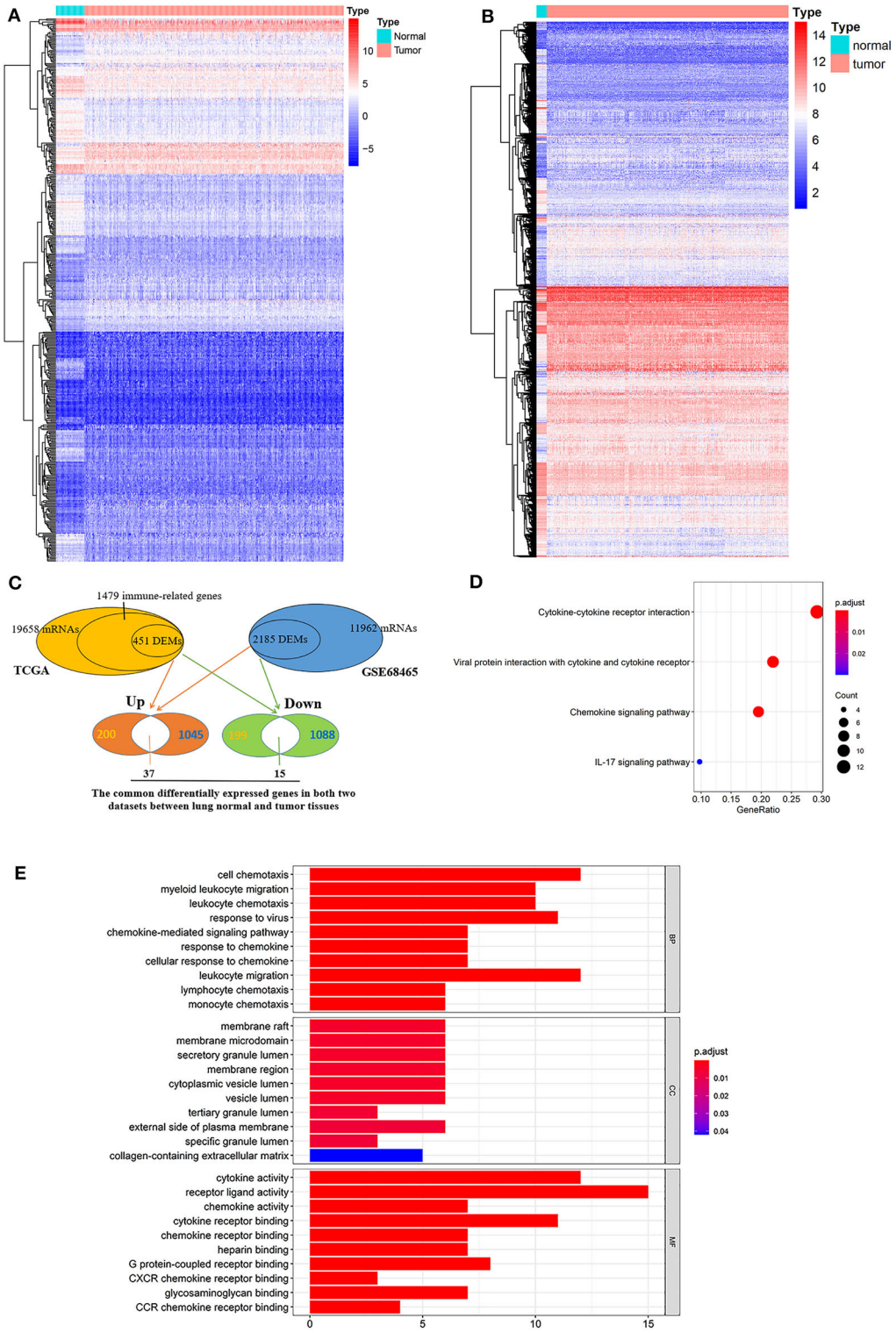

FIGURE 2 | Intersecting genes of differentially expressed genes in two databases and functional enrichment analysis. **(A)** Differential expression analysis of immune-related genes in the TCGA database. **(B)** Differential expression analysis of mRNAs in the GSE68465 dataset. **(C)** Identification of intersection genes for differentially expressed genes in the two datasets. **(D)** KEGG analysis and **(E)** GO enrichment analysis of the common differential expressed genes in two databases.

genes were differentially expressed in lung tumor and normal tissues (1,082 upregulated genes and 1,103 downregulated genes) (**Figures 2B,C**).

GO and KEGG enrichment analysis revealed that there were 4 enriched pathways and 361 GO terms (**Table S1**), of which 4 enrich pathways are shown in **Figure 2D** and the first 30 GO terms are shown in **Figure 2E**. KEGG pathway enrichment analysis pointed out that these genes were involved in cytokine–cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, chemokine signaling pathway, and IL-17 signaling pathway. Moreover, GO enrichment analysis indicated that these genes were enriched in receptor ligand activity, cell chemotaxis, leukocyte migration, cytokine activity, response to virus, etc.

Robustness of the Novel Signature Based on Four Immune-Related Genes

Six immune-related genes (CD79A, MAL, MMP12, MS4A1, OAS1, and WFDC2) were identified to significantly influence patient outcomes (all $p < 0.05$) in both the TCGA (**Figure 3A**) and GSE68465 (**Figure 3B**) datasets and were included in the multivariate Cox analysis (**Table S2**). After the multivariate Cox analysis (stepwise models), there were finally four genes (MAL, MS4A1, OAS1, and WFCD2) included in the signature according to their risk coefficients (**Figure 3C**). Of them, the hazard ratios (HRs) of three genes (MAL, MS4A1, and WFDC2) were <1, indicating that their overexpression was associated with longer OS, while the other gene (OAS1) with HR >1 had the opposite meaning. The expression of these four genes and their relationship to survival are also shown in **Figures 3D,E**. The constructed risk score formula is shown Risk score = $(-0.146 \times \text{ExpressionMAL}) + (-0.227 \times \text{ExpressionMS4A1}) + (0.139 \times \text{ExpressionOAS1}) + (-0.150 \times \text{ExpressionWFDC2})$, through which we estimated the risk score of each patient. Taking the median risk score as the cutoff point, 500 patients were classified into a high-risk group ($n = 250$) and a low-risk group ($n = 250$). The distribution of immune-related genes based on risk score, survival status, and four-gene expression data are shown in **Figures 4A–C** (development set, $n = 500$), **Figures S1A–C** (internal validation set, $n = 250$), and **Figures S2A–C** (external validation set, $n = 442$). The Kaplan–Meier curve analysis in the three datasets obviously demonstrated that patients in the high-risk group had shorter overall survival than those in the low-risk group (log-rank test, all $p < 0.05$; **Figure 4D**, **Figures S1D**, **S2D**). The ROC curves in the development set had a 1-year survival AUC value of 0.718, 3-year survival AUC value of 0.668, and 5-year survival AUC value of 0.652 (**Figure 4E**). The ROC curves in the internal validation set and external validation set also showed the accuracy of the model in predicting 1-, 3-, and 5-year survival (**Figures S1E**, **S2E**). In addition, in three of the four genes (MAL, MS4A1, and WFDC2), their expression value was negatively correlated with the risk score (all cor < -6, all $p < 0.001$), while the other gene (OAS1) was opposite (cor = 0.358, $p < 0.001$). Three datasets also showed the same results (**Figure 4F**, **Figures S1F**, **S2F**).

Response of High- and Low-Risk Patients to Chemotherapy and Immunotherapy

According to the pRRophetic algorithm, we predicted the IC50 of six common chemotherapeutic agents (cisplatin, bleomycin, docetaxel, doxorubicin, gemcitabine, and paclitaxel) in high- and low-risk patients and found that all six drugs had higher IC50 in low-risk patients (Wilcoxon test, all $p < 0.05$; **Figure 5H**). It can be indicated that the high-risk patients were more sensitive to these 6 drugs. In addition, using an online tool TIDE program, TIDE scores were calculated to investigate the effectiveness of immune checkpoint (PD-1 and CTLA-4) inhibitors in immunotherapy in two groups. High-risk patients had markedly lower TIDE score compared with low-risk patients (Wilcoxon test, $p < 0.001$; **Figure 5F**), indicating that high-risk patients may respond better and had better outcome when receiving immune checkpoint (PD-1 and CTLA-4) inhibitors. In addition, the TMB of high- and low-risk patients was investigated in this study. Tumor mutations in both groups are shown in **Figure S3**. The results showed that high-risk patients had higher TMB than low-risk patients (Wilcoxon test, $p < 0.001$; **Figure 5G**).

Differences in Tumor Immune Landscape Between High- and Low-Risk Patients

Comparing the immune infiltration of high- and low-risk groups with two different approaches, we observed that there were significant differences in the components of immune infiltration between the two groups (**Figures 5A,B**). In the high-risk group, the proportions of iDCs, mast cells, type II IFN response, neutrophils, T helper cells, and inflammatory promoting cells were significantly higher than those of the low-risk group (Wilcoxon test, all $p < 0.05$) (**Figure 5A**). Similarly, mast cells, eoshophils, neutrophils, and others had higher infiltrations in high-risk groups (Wilcoxon test, all $p < 0.05$) (**Figure 5B**). Comparing tumor purity and immune score of high- and low-risk patients, we found that LUAD patients in the high-risk group had a lower immune score (Wilcoxon test, $p < 0.001$; **Figure 5E**) and higher tumor purity (Wilcoxon test, $p < 0.001$; **Figure 5D**) than patients in the low-risk group. Human leukocyte antigen (HLA) is a major histocompatibility complex (MHC) in humans, closely related to human immune system function, and it also is an important genetic genome of the human immune system. Thus, we further explored the differences in the expression of HLA-related genes between high- and low-risk patients and found that, in addition to HLA-L and HLA-G, the expression levels of other HLA-related genes were significantly different between high- and low-risk groups, that is, these genes had a higher expression in low-risk patients (Wilcoxon test, all $p < 0.05$; **Figure 5C**). These findings seem to shed light on HLA's important roles in antitumor activity.

We further explored the effects of the four immune-related genes and risk score on the immune infiltration in high- and low-risk patients and found that there was a significant positive correlation between MAL expression level and the infiltration of B cells (cor > 0.4, $p < 0.001$) (**Figure S4A**) and mast cell resting (cor > 0.2, $p < 0.01$) (**Figure S4H**). The MS4A1 expression

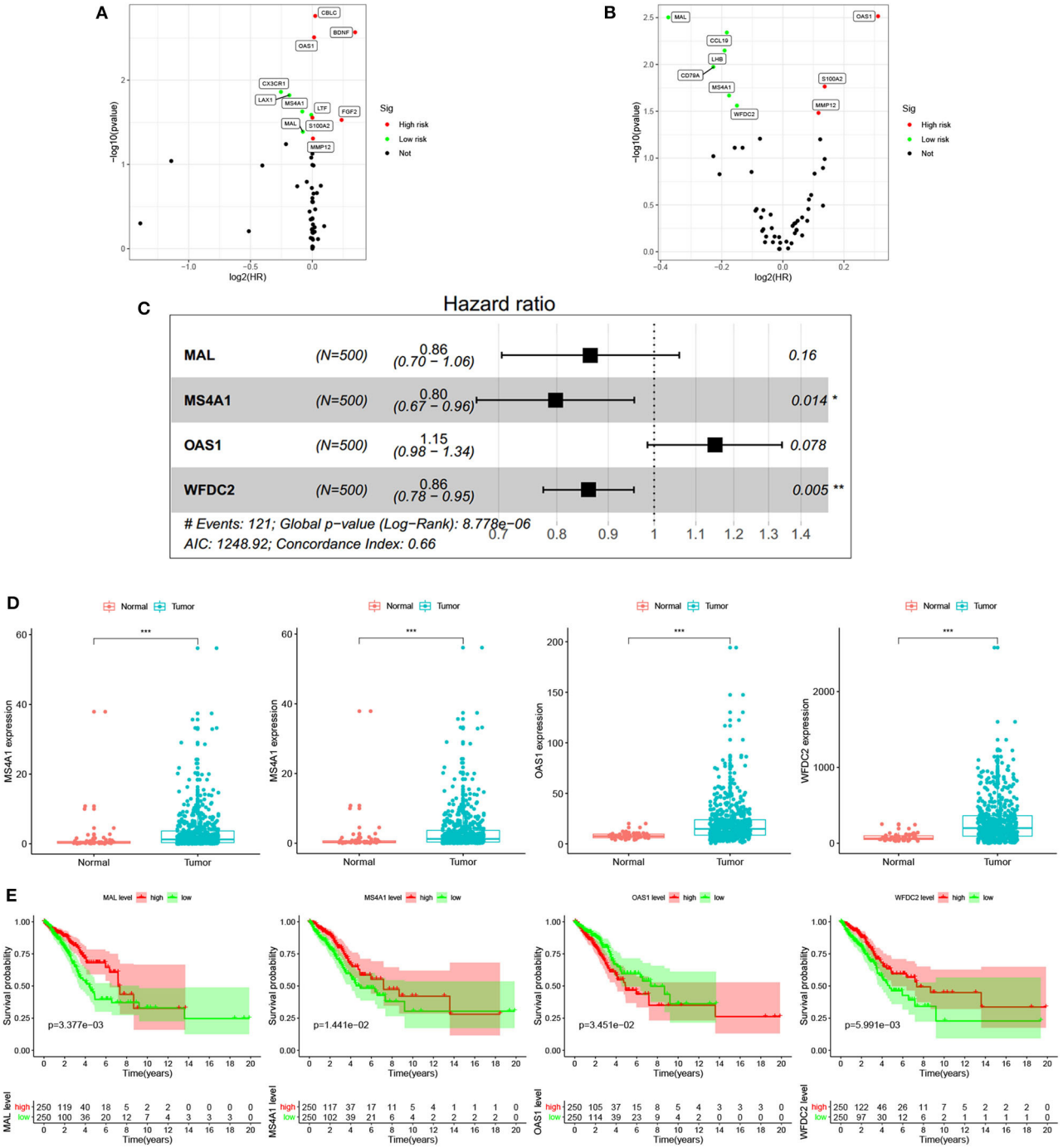

FIGURE 3 | Screening of prognostic immune-related genes and prediction signature construction. Univariate Cox analysis of the common differential expressed genes using the data from the TCGA database **(A)** and using the data from GEO database **(B)**. Each point represents a gene, where the red point represents a high-risk gene whose overexpression is detrimental to patient prognosis, and green dots have the opposite meaning. **(C)** Multivariate Cox analysis of genes with prognostic impact in both datasets using the TCGA database expression matrix and clinical information. **(D)** Differential expression of four immune-related genes (MAL, MS4A1, OAS1, and WFDC2) in the signature between lung normal and tumor tissues. **(E)** Kaplan-Meier survival analysis of four immune-related genes (MAL, MS4A1, OAS1, and WFDC2) in the signature. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

level was significantly positively correlated with B cells (cor > 0.75, $p < 0.001$) (**Figure S4B**) and B cell memory (cor > 0.4, $p < 0.001$) (**Figure S4I**) infiltration. OAS1 expression level and type I IFN response (cor > 0.6, $p < 0.01$) (**Figure S4D**) as well as macrophage M1 (cor > 0.25, $p < 0.01$) infiltration levels (**Figure S4E**) were significantly positively correlated. In addition,

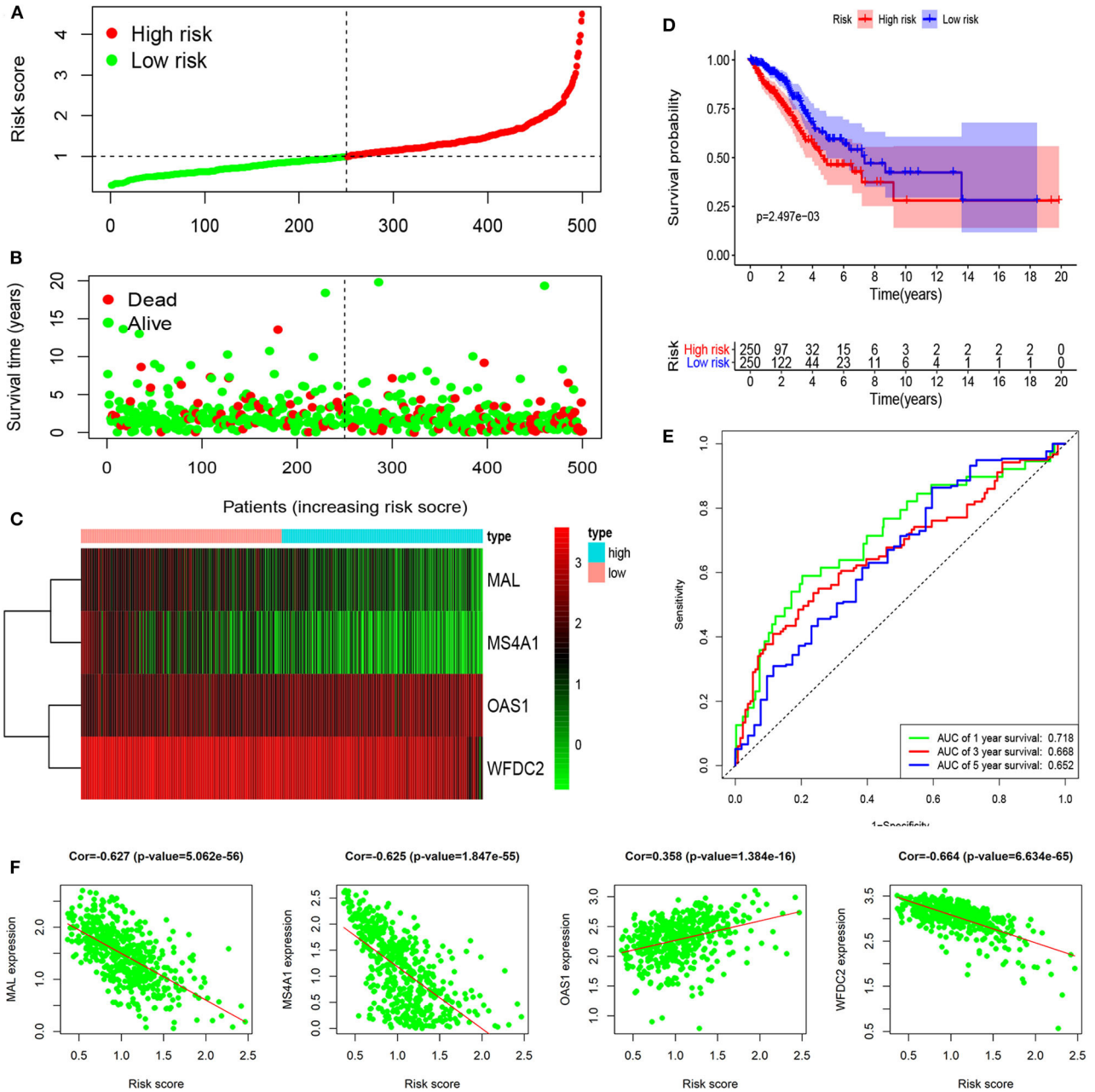

FIGURE 4 | Identification and evaluation of a four-immune-related gene signature to predict OS in the development set. **(A)** The risk score distribution, **(B)** OS status, and **(C)** heat map of the four-immune-related gene signature. **(D)** Kaplan–Meier curves for OS based on the four-immune-related gene signature. The tick marks on the curve represent the censored subjects. The number of patients at risk is listed below the curve. **(E)** The ROC curve analysis of the four-immune-related gene signature for predicting OS. **(F)** Correlation between four immune-related genes and risk score.

WFCD2 expression level was also found to be significantly correlated with iDCs (cor $>$ 0.3, $p < 0.01$) (**Figure S4C**) and T cells CD4 memory activated (cor $<$ -0.2, $p < 0.0025$) (**Figure S4J**) infiltration levels. Moreover, risk score and B cells (cor $<$ -0.5, $p < 0.004$) (**Figure S4F**) as well as NK cells resting (cor $>$ 0.3, $p < 0.005$) (**Figure S4G**) infiltration level showed significantly related.

Differences in Metabolic Pathways Between High- and Low-Risk Patients

Analysis of hallmark pathway gene signatures indicated that signaling pathways converging at various biological processes were obviously different between high- and low-risk patients. Of note, high-risk patients were more relevant in downregulation of Kras signaling, apical surface, bile acid metabolism, and

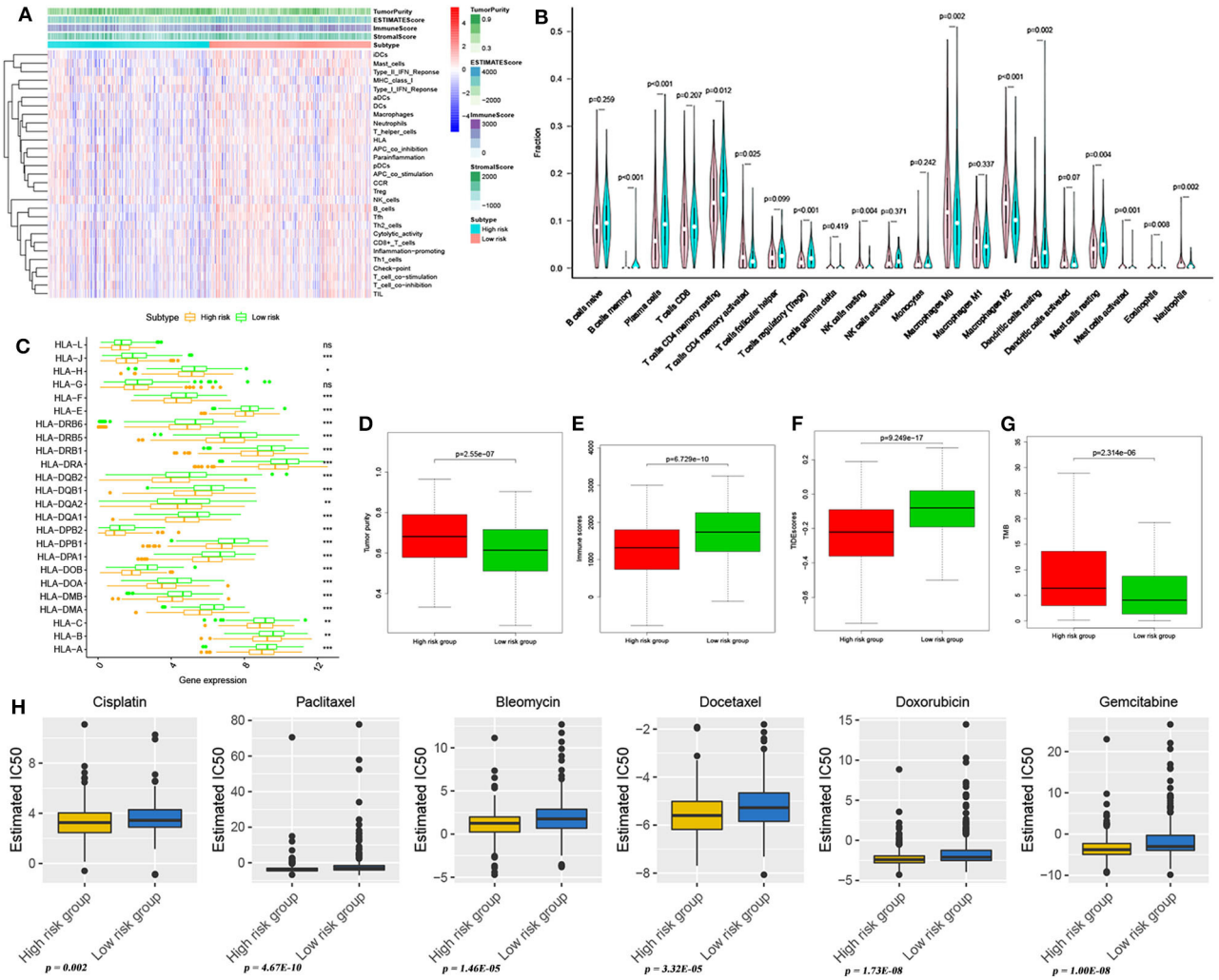

FIGURE 5 | Immune microenvironment landscape and prediction of immunotherapy and chemotherapy effect. **(A)** Immune microenvironment landscape exploration through ssGSEA methods in high- and low-risk patients. **(B)** The comparison of immune infiltration level between high- and low-risk patients, based on CIBERSORT. **(C)** HLA-related gene expression level in high- and low-risk group patients. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. The tumor purity difference **(D)** and immune score difference **(E)** between high- and low-risk patients. Differences in TIDE scores **(F)** and TMB **(G)** between patients in high- and low-risk groups. **(H)** Sensitivity analysis of six common chemotherapeutic drugs in patients at high and low risk.

myogenesis pathways. In comparison, low-risk patients were preferentially related to E2F targets, G2M checkpoint, MYC targets V1, glycolysis, MYC targets V2, unfolded protein response MTORC1 signaling, and PI3K-AKT-MTOR signaling pathways ($|\log_2 FC| > 0.1$, all $p < 0.001$; **Figure 6A**, **Table S3**). In addition, GO gene-set variation analysis revealed that phosphatase activity of inositol triphosphate, inositol polyphosphate 5 phosphatase activity, immunoglobulin complexity, and negative regulation of cell-to-fibroblast growth factor chemotaxis of endogenous lipid antigen MHC IB treatment to present lipid antigen binding and alpha beta T cell receptor complex were enriched in the low-risk patients ($|\log_2 FC| > 0.1$, all $p < 0.001$; **Figure 6B**, **Table S3**).

Transcription Factors Linked to Four Immune-Related Genes

Most transcription factors (TFs) are associated with the cell cycle and play a vital role in the induction of proto-oncogene and tumor suppressor gene. We obtained 318 TFs from the Cistrome program (http://cistrome.org/). By co-expression analysis, we finally identified 45 TFs associated with the four immune-related genes (**Table S4**). Their interrelation is visualized in **Figure 6C**. Of these four genes, the genes co-expressed with the most TFs were MS4A1 ($n = 19$) and WFDC2 ($n = 19$), the least OAS1 ($n = 3$), which could be seen intuitively from the visual network diagram.

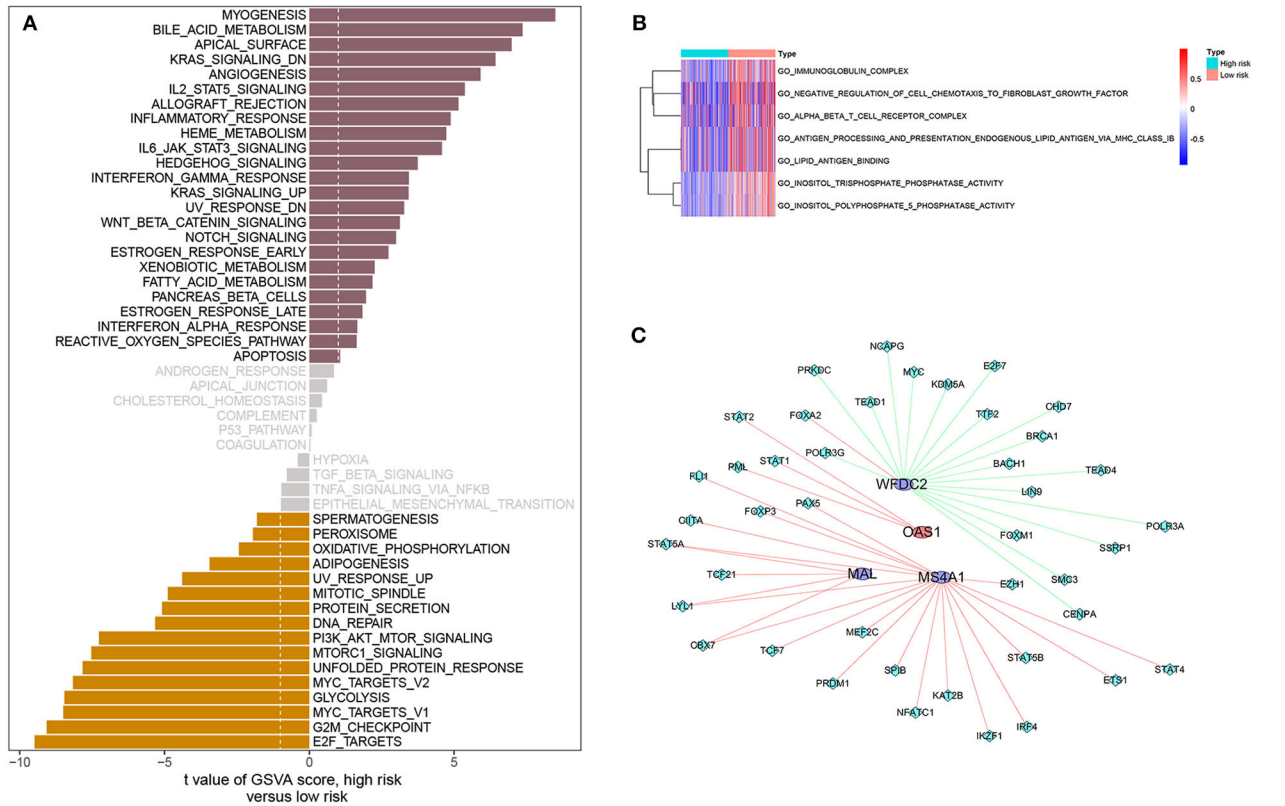

FIGURE 6 | Gene-set variation analysis and correlation between four immune-related genes and TFs. **(A)** Differences in pathway activities scored by GSVA between high- and low-risk patients. T values are shown from a linear model. We set |t| > 1 as a cutoff value. The pink column indicates activated pathways in high-risk patients, and the orange column indicates activated pathways in low-risk patients (DN, down; UV, ultraviolet; v1, version 1; v2, version 2). **(B)** Pathway enrichment analysis based on GO gene sets, including BP, CC, and MF, between high- and low-risk patients. |log2FC| > 0.2 was considered as a cutoff value. Red indicates activated pathways in low-risk patients, and blue indicates activated pathways in high-risk patients. **(C)** Network diagram of four immune-related genes interacting with TFs. The circles represent immune-related genes, where red is for high-risk genes and blue is for low-risk genes. Rhombus represents TFs. Red whip represents positive correlation, and green whip represents negative correlation.

Relationship Between Clinical Factors and Four Immune-Related Genes as Well as Patient Prognosis

On the basis of the obtained sample clinical characteristics (**Table 1**), we performed a univariate as well as a multivariate Cox survival analysis. Risk score was identified to be independent prognostic factors for patients with LUAD in both the TCGA database and GSE68465 dataset (all $p < 0.001$; **Table 2**). Additionally, we also analyzed the correlation between important clinical characteristics and four immune-related genes (**Figure S5**, **Table S5**). There were significant correlations between MAL expression and sex ($p = 0.006$; **Figure S5A**) and T stage ($p = 0.006$; **Figure S5B**). MS4A1 expression was associated with age ($p = 0.024$; **Figure S5C**), sex ($p = 0.001$; **Figure S5D**), lymph-node metastasis ($p = 0.008$; **Figure S5E**), T stage ($p < 0.001$; **Figure S5F**), and TNM stage ($p < 0.001$; **Figure S5G**). In addition, significant correlations were observed between OAS1 expression and lymph-node metastasis ($p = 0.001$; **Figure S5H**) and TNM stage ($p = 0.048$; **Figure S5I**). WFDC2 expression was associated with TNM stage ($p = 0.040$; **Figure S5J**). Specific correlations between the four genes and clinical factors are shown in **Table S5**. Overall, MAL was expressed higher in women and stage T1&T2 patients. MS4A1 was expressed higher in women and the older (>65 years), stage N0, stage T1&T2, and stage I&II patients, while in the patients with lymph-node metastasis and advanced TNM stage, OAS1 had higher expression. Additionally, WFDC2 higher expression was associated with earlier TNM stages.

Predictive Performance of the Established Nomogram

Based on the four-immune-related gene signature (risk score) and clinical factor (TNM stage), we constructed a nomogram to predict patients' prognosis in the TCGA database (**Figure 7A**). According to the multivariate Cox analysis, each factor (in the nomogram) was assigned a score, then the total nomogram score was obtained from the sum of individual scores of all predictors. In association with the total score, 3- and 5-year survival of patients can be estimated by projecting the total points downward. In the present study, we used a bootstrap method

to verify the developed nomogram with the C-index of 0.725 (95% CI = 0.668–0.781) in the development set ($n = 500$), 0.793 (95% CI = 0.728–0.858) in the internal validation set ($n = 250$), and 0.679 (95% CI = 0.644–0.714) in the external validation set ($n = 442$), which suggested that the predictive model had good predictive performance. Furthermore, the calibration curves in three datasets also showed good consistency compared with the ideal model, further indicating that the nomogram was stable in predicting the prognosis of LUAD patients (**Figures 7B–D**).

TABLE 1 | Basic clinicopathologic features.

Characteristics	Subsets	TCGA development set ($n = 500$) (*N*, %)	TCGA internal validation set ($n = 250$) (*N*, %)	GEO external validation set ($n = 442$) (*N*, %)
Age (years)	<65	219 (43.8)	115 (46.0)	214 (48.3)
	≥65	271 (54.2)	129 (51.6)	229 (51.7)
	Unknown	10 (2.0)	6 (0.24)	0 (0.00)
Sex	Female	270 (54.0)	122 (48.8)	220 (49.6)
	Male	230 (46.0)	128 (51.2)	223 (50.4)
T stage	T1&T2	434 (86.8)	218 (87.2)	401 (90.6)
	T3&T4	63 (12.6)	31 (12.4)	40 (9.0)
	Tx	3 (0.6)	1 (0.4)	2 (0.4)
N stage	N0	324 (64.8)	157 (62.8)	229 (67.5)
	N1&N2&N3	165 (33.0)	87 (34.8)	141 (31.9)
	Nx	11 (2.2)	6 (2.4)	3 (0.6)
TNM stage	I&II	387 (77.4)	190 (76.0)	371 (83.8)
	III&IV	105 (21.0)	54 (21.6)	69 (15.6)
	Unknown	8 (1.6)	6 (2.4)	3 (0.6)
Risk score	High risk	250 (50.0)	124 (49.6)	221 (50.0)
	Low risk	250 (50.0)	126 (50.4)	221 (50.0)

DISCUSSION

As one of the malignancies with high morbidity and mortality, lung cancer is a public health concern (22, 23). Due to tumor heterogeneity and complex oncogenic mechanisms in LUAD, it is extremely challenging to develop individualized treatment strategies and accurately predict patient prognosis (24, 25). Increasing researches (26–29) have proved that the prognosis of cancer patients was closely related to tumor microenvironment. Immune responses in tumor microenvironments are also considered important determinants of tumor invasiveness and progression. This study constructed a novel immune signature with good robustness, which could accurately distinguish high- and low-risk patients. On this basis, this study explored the tumor immune microenvironment in high- and low-risk patients and revealed that the high-risk patients had higher tumor purity and lower immune score. Tumor purity and immune score were considered to be important factors affecting the prognosis of cancer patients (30–32). Tumor purity refers to the percentage of tumor cells in the tumor immune microenvironment. Some studies have reported that poor prognosis was closely related to low tumor purity in glioma (30) and colorectal cancer (31). Contrary to the poorer prognosis of low tumor purity in glioma and colorectal cancer, Wang et al. (32) observed that patients with low LUAD purity tended to have a better prognosis. This finding was in line with that of our study. Low tumor purity was associated with different outcomes in different cancer patients, which seemed to indicate that the patterns of occurrence and progression of different tumors were also quite different. In our study, high-risk patients with high tumor purity had poor prognosis. We believe that the survival difference between high- and low-risk patients might be due to higher frequency mutations in key pathways and changes in the tumor microenvironment associated with tumor purity.

TABLE 2 | Univariate and multivariate Cox analysis of the four-immune-related gene signature and clinical risk factors.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
TCGA				
Age (years)	1.012 (0.993–1.032)	0.213	1.020 (1.001–1.040)	0.044
Sex (male vs. female)	0.916 (0.636–1.321)	0.639	0.766 (0.525–1.118)	0.167
T stage (T3&T4 vs. T1&T2)	2.119 (1.315–3.414)	0.002	1.151 (0.669–1.981)	0.612
N stage (N1&N2&N3 vs. N0)	3.161 (2.186–4.569)	<0.001	2.096 (1.354–3.266)	0.001
TNM stage (III&IV vs. I&II)	3.054 (2.099–4.445)	<0.001	1.747 (1.073–2.846)	0.025
Risk score	2.029 (1.654–2.489)	<0.001	1.921 (1.526–2.418)	<0.001
GSE68465				
Age (years)	1.017 (1.004–1.031)	0.013	1.018 (1.004–1.032)	0.010
Sex (male vs. female)	1.224 (0.946–1.584)	0.124	1.051 (0.808–1.368)	0.712
T stage (T3&T4 vs. T1&T2)	1.521 (1.036–2.231)	0.032	1.371 (0.860–2.186)	0.185
N stage (N1&N2&N3 vs. N0)	1.441 (1.107–1.866)	0.007	1.445 (1.057–1.977)	0.021
TNM stage (III&IV vs. I&II)	1.362 (0.983–1.885)	0.063	0.926 (0.588–1.460)	0.742
Risk score	208.6 (21.04–2068.4)	<0.001	192.3 (18.00–2053.7)	<0.001

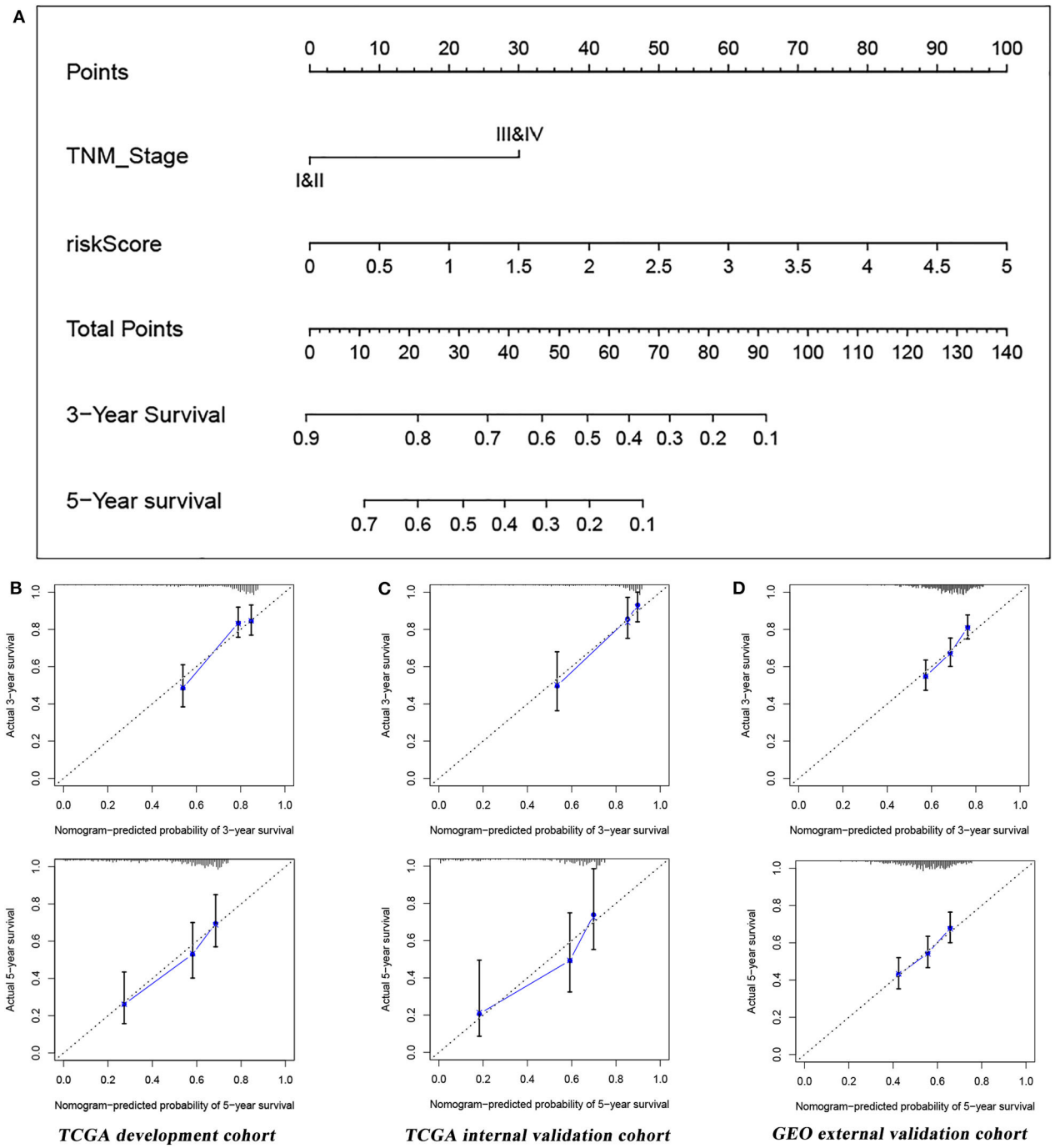

FIGURE 7 | (A) A nomogram combining TNM stage and the four-immune-related gene signature for predicting LUAD prognosis. **(B)** Calibration curves of the nomogram for the probability of OS at 3 and 5 years in the TCGA development set. **(C)** Calibration curves of the nomogram for the probability of OS at 3 and 5 years in the TCGA internal validation set. **(D)** Calibration curves of the nomogram for the probability of OS at 3 and 5 years in the GEO external validation set.

Additionally, all kinds of cells, cytokines, and chemokines that interact with tumor cells in the tumor microenvironment, especially immune cells, are increasingly recognized as important roles in the body against tumors. Immune score, which can promote the quantification of immune components (such as immune cells) in tumors, can significantly affect the prognosis of patients. In several studies (32–35), it has been confirmed that high immune scores were associated with better prognosis. Similarly, high-risk patients with worse prognosis had lower immune scores in our study, which further suggested the validity

and accuracy of the signature constructed in this study in identifying high-risk patients. Besides, high-risk patients were significantly different from low-risk patients in terms of immune infiltrating cell types, for example iDCs, mast cells, type II IFN response, neutrophils, T helper cells, macrophages M1, and inflammatory promoting cells. Four immune-related genes (MAL, MS4A1, OAS1, and WFDC2) in the signature were also associated with the immune infiltration. As explained by Aran et al. (36), we also thought that the inflammatory response caused by immune cells might promote the mutation of tumor cells, which in turn affected the prognosis of patients. Therefore, it is still an important part of the future research on LUAD to explore the specific mechanism of tumor immune microenvironment on prognosis.

Since the patients with refractory malignant tumors, including lung cancer (4, 5), benefit significantly from immune checkpoint inhibitors, immunotherapy is becoming a new therapeutic option for cancer patients. TMB and immune checkpoint levels (e.g., PD-1, PD-L1, CTLA4) are considered biomarkers for predicting the efficacy of immunotherapy. As a marker for evaluating the effectiveness of immunotherapy (37), the effect of TMB has been confirmed in the treatment of malignant tumors with mismatch repair defects by the PD-1/PD-L1 antibody (38, 39). TIDE is a completely new computational framework designed by Jiang et al. (16), which can integrate the two immune escape mechanisms of tumor (immune dysfunction and rejection) and is believed to be a substitute for a single biomarker to effectively predict the efficacy of immune checkpoint inhibitors. In view of this, this study assessed the effect of immunotherapy of high- and low-risk patients from two aspects (TMB and TIDE scores) and speculated that high-risk patients may benefit more from immunotherapy. It is worth noting that although immunotherapy can bring good benefits to some lung cancer patients, some patients still do not show the desired results after using immune checkpoint inhibitors (6, 7). The current bottleneck in the treatment of lung cancer also makes such patients have to return to traditional chemotherapy to improve the prognosis. Thus, the mRNA expression data from the TCGA database was used to explore the sensitivity of patients at high and low risk to traditional chemotherapeutic agents (cisplatin, bleomycin, docetaxel, doxorubicin, gemcitabine, and paclitaxel). This study indicated that using the same drugs, the high-risk patients may perform better than the low-risk patients. Currently, platinum-based chemotherapy is the standard regimen for the treatment of advanced LUAD. However, the mechanism of drug resistance and the existence of heterogeneity also make the effect of drug therapy unsatisfactory. This study revealed the sensitivity of high- and low-risk patients to six chemotherapeutic drugs including cisplatin, which would provide a visual field for researchers to develop drugs with high therapeutic index or high efficacy.

In addition, this study found that there were significant differences in metabolic pathways between high- and low-risk patients, such as immunoglobulin complexity and negative regulation of cell-to-fibroblast growth factor chemotaxis of endogenous lipid antigen MHC IB treatment to present lipid antigen binding and alpha beta T cell receptor complex. This indicated that the pathogenesis of LUAD was a complex biological process driven by specific gene and epigenetic changes. Moreover, abnormal regulation of multiple genes can promote the occurrence and development of LUAD through different mechanisms. Differences in metabolic pathways between high- and low-risk patients based on the established signature have not been previously reported in LUAD, indicating that the four immune-related genes in the signature and varied gene sets in GSVA between high- and low-risk patients have the potential to be further investigated for deeper analysis. In general, these findings may provide a new perspective for researchers and clinicians in finding breakthroughs in further molecular mechanism studies.

For clinical application, good biomarkers should be those that can accurately predict prognosis for patients, distinguish patients with different risks, and thus assist clinicians to make the most reasonable treatment plan in time. Nomogram may be a good choice for this purpose. Nomogram, a visual statistical tool, was wildly used in prognostic assessment of cancer patients (40, 41). In this study, combining the immune signature and TNM stage, a prognosis nomogram with excellent performance was constructed. This nomogram incorporated two important predictors (risk score and TNM stage). The TNM stage of patients may be easy to obtain, while the acquisition of another predictor (risk score) required knowledge of the expression of four immune-related genes (MAL, MS4A1, OAS1, and WFDC2) in tumor tissues, which undoubtedly increased the burden of nomogram application. This appears to be a common problem for most molecular diagnostic or prognostic models. How to simplify the clinical application of predictive models is a question for researchers and clinicians to consider. We believe that the development of molecular detection technology in the future is bound to improve this dilemma. The nomogram may be used routinely in the future.

The four immune-related genes in the signature have previously been shown to be potential biomarkers. Relevant researches have reported that deletion of MAL gene expression was associated with the development and progression of many malignancies in humans, such as cervical cancer, ovarian cancer, oral cancer, laryngeal cancer, breast cancer, esophageal cancer, gastric cancer, bowel cancer, and renal cancer (42–49). MS4A1, also known as CD20, is a member of the MS4A gene family. MS4A1 (CD20) is an important marker of B cell differentiation and an important target for immunotherapy in lymphoma (50). Anti-CD20 monoclonal antibody (rituximab) became the first monoclonal antibody approved for cancer treatment in 1997, and it could kill tumor cells by complement-dependent and antibody-dependent cytotoxicity. Along with the development of antibody humanization and Fc segment modification, the therapeutic spectrum is not only limited to lymphoma but also includes chronic lymphoblastic leukemia, acute lymphoblastic leukemia, solid tumor, and immune-related diseases (51–54). OAS1 is an important component of the immune system and has significant antiviral effects. It is worth noting that the relationship between these three genes and LUAD is hardly reported in the literature; however, this study found that all three genes were associated with prognosis in patients with LUAD, and there were significant

differences in gene expression in patients with different clinical features. In combination with the relationship between these three genes and other tumors and the findings of this study, we believe that these three immune-related genes could affect the immune microenvironment of LUAD and might be involved in the occurrence and progression of LUAD. Additionally, along with further exploration, researchers found that WFDC2 presented a high expression state in lung cancer (55–58) and recognized that WFDC2 as a serum tumor marker had important clinical application in the early diagnosis of lung cancer and the monitoring of a curative effect (59). Nevertheless, the expression of WFDC2 in LUAD and its relationship with patients prognosis were rarely reported. This study found that WFDC2 was significantly associated with TNM stage and prognosis in LUAD patients. In general, these four immune-related genes may play key roles in the development of LUAD. This study provided preliminary evidence that these genes were closely related to the clinical features and prognosis of LUAD, which would provide new research directions and ideas for finding new gene therapy targets and developing new antitumor drugs.

There are some limitations in this study. First, although the signature and nomogram constructed in this study using massive data from TCGA and GEO databases have reliable robustness, the nature of retrospective analysis still exists. Second, we explored the immune microenvironment landscape and molecular mechanisms in patients at different risks and predicted their effects of immunotherapy and chemotherapy, but the study still lacked experimental verification.

To make a long story short, this study identified and validated a novel immune-related gene signature comprising four genes (MAL, MS4A1, OAS1, and WFDC2) in LUAD patients, which can serve as a prognostic predictor of LUAD patients. Additionally, the signature can indicate the sensitivity of LUAD patients to chemotherapeutic agents (cisplatin, bleomycin, docetaxel, doxorubicin, gemcitabine, and paclitaxel) as well as immune checkpoint inhibitors and provide new clinical applications for LUAD patients. Furthermore, the established nomogram with good robustness can accurately predict the prognosis of LUAD patients, which may help doctors make more rational treatment decisions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The study was approved by the Institutional Research Committee of Zhongnan Hospital of Wuhan University. Data of our present study was downloaded from the open databases TCGA and GEO, so there was no informed consent from participants. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CS, WH, and ZD designed the study. CS analyzed the data and prepared the manuscript. All authors were substantially involved in the research, acquisition of data, analysis, manuscript preparation, and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.01300/full#supplementary-material

Figure S1 | Identification and evaluation of a four-immune-related-gene signature to predict OS in the TCGA internal validation set. **(A)** The risk score distribution, **(B)** OS status and **(C)** heatmap of the four-immune-related-gene signature. **(D)** Kaplan-Meier curves for OS based on the four-immune-related-gen signature. The tick-marks on the curve represent the censored subjects. The number of patients at risk is listed below the curve. **(E)** The ROC curve analysis of the four-immune-related-gene signature for predicting OS. **(F)** Correlation between four immune-related genes and risk scores.

Figure S2 | Identification and evaluation of a four-immune-related-gene signature to predict OS in the GEO external validation set. **(A)** The risk score distribution, **(B)** OS status and **(C)** heatmap of the four-immune-related-gene signature. **(D)** Kaplan-Meier curves for OS based on the four-immune-related-gen signature. The tick-marks on the curve represent the censored subjects. The number of patients at risk is listed below the curve. **(E)** The ROC curve analysis of the four-immune-related-gene signature for predicting OS. **(F)** Correlation between four immune-related genes and risk scores.

Figure S3 | Tumor mutations in LUAD patients in TCGA database.

Figure S4 | Correlation of four immune-related genes and risk scores with immune-infiltrating cells. Correlation between MAL **(A)**, MS4A1 **(B)**, WFDC2 **(C)**, OAS1 **(D)**, risk score **(F)** and immune infiltration, based on the ssGSEA approach. Correlation between OAS1 **(E)**, risk score **(G)**, MAL **(H)**, MS4A1 **(I)**, WFDC2 **(J)** and immune-infiltrating cells, based on CIBERSORT.

Figure S5 | Correlation analysis between four immune-related genes and clinical features. There were significant correlations between MAL expression and sex **(A)** and T stage **(B)**. MS4A1 expression was associated with age **(C)**, sex **(D)**, lymph-node metastasis **(E)**, T stage **(F)**, and TNM stage **(G)**. In addition, significant correlations were observed between OAS1 expression and lymph-node metastasis **(H)** and TNM stage **(I)**. WFDC2 expression was associated with TNM stage **(J)**.

Table S1 | GO and KEGG enrichment analysis on the common differentially expressed gene.

Table S2 | Univariate Cox analysis of intersection differentially expressed gene in the TCGA and GEO databases.

Table S3 | Gene set variation analysis in the high- and low-risk groups.

Table S4 | 45 transcription factors associated with the four immune-related genes.

Table S5 | The relationship between four immune-related genes and clinical features.

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Immune-Related Adverse Events and Corticosteroid Use for Cancer-Related Symptoms Are Associated With Efficacy in Patients With Non-Small Cell Lung Cancer Receiving Anti-PD-(L)1 Blockade Agents

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Background: Immune-related adverse events (irAEs) have been associated with improved efficacy in advanced non-small cell lung cancer (NSCLC) patients receiving anti-PD-(L)1 blockade agents, while the concurrent use of corticosteroids seems to worsen it. We evaluated outcomes in advanced NSCLC patients treated with anti-PD-(L)1 blockade agents in relation to the presence of irAEs and the reasons for using corticosteroids: whether for palliative cancer-related reasons or for the management of irAEs.

Methods: Clinical outcomes in advanced NSCLC patients treated with anti-PD-(L)1 blockade agents were calculated with regard to the presence of irAEs and the use of corticosteroids. A landmark analysis was performed to avoid immortal time bias due to the time-dependent nature of irAEs.

Results: Out of a total of 267 patients, the 56.9% of patients who experienced irAEs had significantly improved outcomes. In the landmark analysis, median progression-free survival (PFS) was 12.4 months for patients with irAEs vs. 4.1 months for patients without irAEs ($p < 0.001$), while median overall survival (OS) was 28.2 vs. 12.5 months, respectively ($p < 0.001$). Likewise, objective response and disease control rates were significantly higher in patients experiencing irAEs: 48.6 vs. 22.8% and 77.1 vs. 39.6% ($p < 0.001$), respectively. Median OS was significantly shorter for patients receiving ≥10 mg of prednisone equivalent daily for cancer-related symptoms than for the rest of patients (<10 mg prednisone equivalent daily or for management of irAEs): 6 vs. 15.9 months ($p < 0.001$).

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Conclusions: IrAEs were associated with improved efficacy in advanced NSCLC patients when a landmark analysis was applied. Patients receiving corticosteroids had significantly poorer outcomes when they were used for cancer-related symptoms.

Keywords: immune-related adverse events, immunotherapy, advanced NSCLC, corticosteroids, efficacy

INTRODUCTION

Immunotherapy has become established as a new standard-of-care for multiple solid malignancies, including non-small cell lung cancer (NSCLC). Among other strategies, immune-checkpoint blockade agents targeting the inhibitory pathways of the immune cascade have resulted in an increase in the response against tumor cells (1, 2).

Examples of these agents are nivolumab and pembrolizumab, monoclonal antibodies against the programmed cell death-1 receptor (PD-1) and atezolizumab, durvalumab, and avelumab, against its ligand PD-L1. Most of these agents have been approved in different settings for the treatment of patients with NSCLC (3–8), which has led the scientific community to advance by exploring combinations of anti-PD-(L)1 blockade agents with chemotherapy and/or other immune-checkpoint blockade to achieve better results (9–11).

Due to their mechanism of action, anti-PD-(L)1 blockade agents can induce inflammatory side effects known as immune-related adverse events (irAEs), which are not triggered by conventional cytotoxic anticancer agents. The most commonly reported irAEs are those affecting the skin, the gastrointestinal tract and the thyroid gland, though any organ or system may be involved, including the lung, liver, and the hypophysis (12) IrAEs are generally mild, but ∼10% of cases are severe and may require immunosuppressors and/or treatment discontinuation (13, 14).

However, immunotherapy raises several questions that remain unknown. First, a positive correlation between the presence of irAEs and the efficacy of immunotherapy has been postulated, suggesting that a proper management of such events might be required to maximize the therapeutic effect of these drugs and to avoid treatment interruption (15–23). Second, the activity of anti-PD-(L)1 blockade agents in patients receiving corticosteroids or antibiotics during immunotherapy is controversial, and their use and safety in certain groups, such as patients with brain metastasis, needs to be defined (24–26).

We performed a retrospective study to investigate irAEs profiles and their association with clinical activity in patients with advanced NSCLC treated with anti-PD-(L)1 blockade agents using landmark and multivariable analyses. Additionally, we evaluated the efficacy of immunotherapy with regard to the use of corticosteroids and antibiotics.

MATERIALS AND METHODS

The medical records of all patients with advanced NSCLC treated with anti-PD-(L)1 blockade agents at two tertiary institutions in Spain between March 2013 and August 2018 were reviewed. All patients starting an anti-PD-(L)1 blockade agent alone or in combination with chemotherapy or an anti-CTLA-4 (cytotoxic T-lymphocyte antigen 4) in any treatment line were included.

The end of follow-up was December 31, 2018. The study was approved by the local institutional review board.

Patients were evaluated for objective response rate (ORR), disease control rate (DCR), duration of response (DoR), progression-free survival (PFS), and overall survival (OS). Tumor responses were assessed as per clinical practice using the Response Evaluation Criteria for Solid Tumors (RECIST) version 1.1 every 8–12 weeks (27).

IrAEs were defined as adverse events with a potential immunologic basis that required close monitoring and/or potential intervention with immunosuppressives or hormone replacement. Patient symptoms and physical exploration and laboratory data were assessed at every cycle. Thyroid function was evaluated at baseline and every 6 weeks thereafter. irAEs severity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0, as part of routine clinical practice (28).

Patient data were obtained from a unified database in which clinical and pathological characteristics and toxicity were accurately recorded.

ORR data included patients with partial or complete response, and DCR data included partial response, complete response, and stable disease. PFS and OS were measured as the time from the start of anti-PD-(L)1 blockade agent to documented disease progression or death owing to any cause (PFS) or to death (OS). Patients with no events were censored on the date of the last follow-up. Those patients who were not evaluable for response were not included in the ORR assessment but were included in the PFS and OS evaluations.

Corticosteroid usage within 1 month before the initiation or during anti-PD-(L)1 therapy, and the administration of antibiotics from 3 months before the start of anti-PD-(L)1 therapy to 3 months after finishing were also recorded. The reason for corticosteroid use was specified, distinguishing between management of irAEs or the palliative treatment of cancer-related symptoms. Types of corticosteroid and the prednisone equivalent daily dose, as well as type of antibiotics, were also collected. Transient corticosteroids given along with a chemotherapy combination were not registered.

To analyze efficacy according to the dose of corticosteroids patient were divided into two groups: those receiving prednisone equivalent $\geq$10 mg daily and those receiving prednisone equivalent $\leq$10 mg daily (including patients that did not receive corticosteroids).

Statistical Analysis

Survival curves were estimated using the Kaplan-Meier method and compared with the log-rank test. Taking into account the immortal time bias due to the time-dependent nature of irAEs, we performed tests at 2.4-months for PFS and 5.9-months for OS landmark analyses including only patients manifesting disease control or those who were alive at these time points. Consequently, 92 patients were excluded from the PFS landmark analysis ($n = 175$) and 100 patients were excluded from the OS analysis ($n = 167$). Landmark times were pre-defined before the start of data analysis and were determined from the median PFS and OS data of patients with no irAEs (29, 30). In addition, 6 and 12-month landmark analyses were also performed as complementary evaluations.

Odds ratios were used for ORR and DCR. Univariable and multivariable Cox proportional hazard regression models were adopted to determine hazard ratios (HR).

Two multivariable analyses were performed. First, to determine the influence of clinical characteristics (such as age, smoking status, Eastern Cooperative Oncology Group [ECOG] performance status [PS], brain, and liver metastases, presence of irAEs, toxicity grade, use of prednisone equivalent ≥10 mg daily, use of antibiotics and treatment line), and second, to assess the influence of different types of irAEs on OS.

To describe our population, numbers and percentages were used for qualitative variables, while medians and interquartile ranges (IQR) were calculated for ordinal and quantitative variables with an asymmetric distribution.

All *p*-values were based on a two-sided hypothesis, and those <0.05 were considered statistically significant. Statistical analyses were carried out using IBM SPSS Statistics for Windows, version 25.0 (IBM SPSS Inc., Chicago, IL, USA).

RESULTS

Patient Characteristics

We included 267 consecutive patients with advanced NSCLC treated with anti-PD-(L)1 blockade agents. Baseline characteristics of patients are described in **Table 1**. The median age was 66.1 years (range 26.7–85.2, IQR 14.3), 69.7% were male and 30.3% female. The majority of patients were current or former smokers (74.2%) and baseline ECOG PS was 0–1 in 85% of patients. Non-squamous was the most common histology (67.8%). Brain metastases were present in 15.7% of patients and liver metastases in 15.4%. PD-L1 expression analysis in tumor

TABLE 1 | Patients characteristics and comparison and the presence of irAEs.

Category	Total $n = 267$ (%)	irAEs $n = 152$ (%)	No irAEs $n = 115$ (%)	*P*-value
Gender				
Male	186 (69.7)	108 (71.1)	78 (67.8)	0.593
Female	81 (30.3)	44 (28.9)	37 (32.2)	
Age	66.1 years	66.4 years	65.8 years	0.362
median (range)	(26.7–85.2, IQR 14.3)	(26.7–85.2, IQR 15.3)	(38.8–81.0, IQR 13.7)	
Smoking status				
Non- or light smoker	26 (9.7)	13 (8.6)	13 (11.3)	0.533
Current or former smoker	241 (90.3)	139 (91.4)	102 (88.7)	
ECOG PS				
0–1	227 (85)	136 (89.5)	91 (79.1)	0.024
2	40 (15)	16 (10.5)	24 (20.9)	
Histological subtype				
Squamous	86 (32.2)	49 (32.2)	37 (32.2)	1.000
Non-squamous	181(67.8)	103 (67.8)	78 (67.8)	
Treatment line				
1st line	81 (30.3)	60 (39.5)	21 (18.3)	<0.001
≥2nd line	186 (69.7)	92 (60.5)	94 (81.7)	
Immune-checkpoint blockade schedule				
Monotheraphy	209 (78.3)	112 (73.7)	97 (84.4)	0.082
Combination with chemotherapy	33 (12.3)	24 (15.8)	9 (7.8)	
Combination with anti-CTLA-4	25 (9.4)	16 (10.5)	9 (7.8)	
Treatment duration	2.75 m	4.8 m	1.8 m	<0.001
median (range)	(0.03–56.4, IQR 6.2)	(0.03–56.4, IQR 9.8)	(0.03–46.2, IQR 2.7)	
Prednisone equivalent ≥10 mg/day use				
No	133 (49.8)	66 (43.4)	67 (58.3)	0.019
Yes	134 (50.2)	86 (56.6)	48 (41.7)	
Antibiotics use				
No	126 (47.2)	63 (41.4)	63 (54.8)	0.036
Yes	141 (52.8)	89 (58.6)	52 (45.2)	

irAEs, immune-related adverse events; IQR, interquartile range; ECOG PS, Eastern Cooperative Oncology Group performance status; CTLA-4, cytotoxic T-lymphocyte antigen 4.

samples was available from 135 patients (50.6%), and expression was low (PD-L1 expression 1–49%) in 52 (38.5%), high (PD-L1 $\geq$ 50%) in 41 (30.4%), and negative (PD-L1 < 1%) in 42 (31.1%).

Anti-PD-(L)1 Blockade Treatment and irAEs Characteristics

Eighty-one patients (30.3%) received anti-PD-(L)1 blockade agents as first line treatment, 131 (49.1%) as second line treatment and 55 (20.6%) as third line treatment or beyond. Anti-PD-(L)1 blockade agents were given alone (78.3%) or in combination with chemotherapy (12.3%) or with an anti-CTLA-4 agent (9.4%). Nivolumab (44.2%) and pembrolizumab (25.6%) were the most commonly used types of anti-PD-(L)1 blockade agents, followed by atezolizumab (17.2%).

One hundred and fifty-two patients (56.9%) experienced a total of 255 irAEs. The median number of irAEs per patient was one (range 0–5, IQR 1), and 64 patients (24%) experienced two or more irAEs. The most common irAEs was skin toxicity (35.6%), followed by diarrhea (16.5%) and hypothyroidism (10.2%). According to the CTCAE terminology, 149 irAEs (58.4%) were grade 1, 65 (25.5%) were grade 2, 33 (12.9%) were grade 3 and three (1.2%) were grade 4. There were five treatment-related deaths (2%): four due to pneumonitis and one due to hepatitis. IrAEs were more frequent in patients receiving immunotherapy in first line treatment (74.1%) than in second line treatment or beyond (49.5%) ($p < 0.001$). No differences were observed in the presence of grade $\geq$3 irAEs according to the treatment line ($p = 0.342$). A trend to a higher rate of grade $\geq$3 irAEs was also seen in patients receiving immune blockade combination regimens (50%) in contrast to single-agent anti-PD-1 and anti-PD-L1 (21.6 and 20%, respectively) ($p = 0.058$).

Endocrine toxicity was significantly higher with a combination of immune blockade agents (36%) than with single-agent anti-PD-1 (8.6%) or anti-PD-L1 (8.9%) ($p = 0.034$). A trend to a higher rate of pneumonitis was seen with combinations of immune blockade agents (20% vs. 6.5 and 10.7%) ($p = 0.101$), and a greater number of cases of arthritis was observed with anti-PD-1 blockade agents (11.3%) than with anti-PD-L1 (3.6%) or than with a combination of immune blockade agents (4%) ($p = 0.099$). Global median time to irAEs onset was 7.6 weeks (0.1–123.4, IQR 13.3). A description of irAEs and median onset time are detailed in **Table 2**.

The median duration of treatment with anti-PD-(L)1 blockade agents was significantly longer in patients who experienced irAEs than in those who did not: 4.8 months (range 0.03–56.4, IQR 9.8) vs. 1.8 months (range 0.03–46.2, IQR 2.7) ($p < 0.001$). Comparisons between patients regarding the presence of irAEs can be found in **Table 1**.

Two hundred and eighteen patients (82%) discontinued treatment with anti-PD-(L)1 blockade agents. The most common reason given was progressive disease in 145 patients (66.5%), followed by the presence of irAEs in 44 patients (20.2%). Twenty-nine patients (13.3%) stopped treatment due to other causes, such as deterioration in their general condition or complications unrelated to disease progression. Pneumonitis (34.1%), endocrine dysfunction (29.5%), and diarrhea (22.7%) were the irAEs most frequently associated with treatment discontinuation. Thirty of the 44 patients who stopped anti-PD-(L)1 therapy due to toxicity presented irAEs grade $\geq$3 (68.2%). By the time of data analysis, 83.9% of irAEs had been resolved.

Association Between irAEs and Treatment Outcomes

At the time of data analysis, the median follow-up time was 8.5 months (range 0.3–56.4, IQR 10.6) and the median duration of treatment with anti-PD-(L)1 blockade agents was 2.8 months (range 0.1–56.4, IQR 6.2). The median OS and PFS of the study population were 12.4 months (95% confidence interval [CI], 10.1–14.7) and 4.2 months (95% CI, 3.1–5.3), respectively. In first line setting, the median OS was 19.4 months (95% CI, 11.9–27.0) and the median PFS was 9.8 months (95% CI, 5.4–14.2). As expected, patients receiving anti-PD-(L)1 therapy as second line treatment or beyond had significantly poorer outcomes, with a median OS of 9.0 months (95% CI, 7.0–11.1) (HR 1.89; 95% CI, 1.29–2.79; $p = 0.001$) and a median PFS of 3.3 months (95% CI, 2.6–4.1) (HR 1.78; 95% CI, 1.27–2.51; $p = 0.001$). No differences were found between patients receiving anti-PD-(L)1 blockade agents in monotherapy or combination with anti-CTLA-4 or chemotherapy in first line setting, neither in terms of OS ($p = 0.177$) nor in PFS ($p = 0.343$).

The landmark analysis showed that PFS was significantly longer in patients experiencing irAEs than in those without irAEs: 12.4 months (95% CI, 1.9–22.9) vs. 4.1 months (95% CI, 2.6–5.6), (HR 0.43; 95% CI, 0.28–0.64; $p < 0.001$). Similarly, OS among patients with irAEs was significantly higher: 28.2 months (95% CI, not achieved) vs. 12.5 months (95% CI, 10.8–14.2) (HR 0.38; 95% CI, 0.24–0.59; $p < 0.001$) (**Figures 1A,B**).

Six and 12 months landmark analyses were also performed to provide complementary information. The median OS at both time-points also favored patients with irAEs: 12.9 months (95% CI, 11.3–14.5) vs. 28.2 months (95% CI not calculated) (HR 0.39; 95% CI, 0.25–0.61; $p < 0.001$), and 19.6 months (95% CI, 15.2–23.9) vs. not reached (HR 0.33; 95% CI, 0.17–0.64; $p = 0.001$), respectively. Landmark analyses for PFS at 6 and 12 months could not be calculated since no event happened after these time-points in the no-irAEs group.

Of note, the ORR was significantly higher in patients who experienced irAEs than in those without irAEs: 48.6 vs. 22.8% (odds ratio [OR] 0.31; 95% CI, 0.18–0.55; $p < 0.001$). DCR was also significantly better when irAEs were present: 77.1 vs. 39.6% (OR 0.20; 95% CI, 0.11–0.34; $p < 0.001$).

The landmark analysis was also applied when comparing ORR regarding the development of irAEs. Landmark analysis at 8 and 10 weeks showed that ORR was significantly higher in the irAEs group of patients. At 8 weeks, 54.8 vs. 28% (OR 0.32; 95% CI, 0.15–0.68; $p = 0.004$) and at 10 weeks, 62.3 vs. 36.7% (OR 0.35; 95% CI, 0.14–0.85; $p = 0.028$). However, no differences were detected when greater time-point were used, probably because the progressive decline in number of patients who show first response later in time (**Table S1**).

Though median time to response was slightly shorter in the no-irAEs group [8 weeks (1.3–122.6, IQR 5.3) vs. 9.8 weeks

TABLE 2 | Description of immune-related adverse events.

Types of irAEs	All patients, $n = 267$ (%)			Median onset time (range), weeks
	All grades $n = 255$[a] (95.5)	Grade 3–5 $n = 41$ (15.3)	irAEs requiring prednisone equivalent ≥10 mg/d[d] $n = 63$ (23.6)	
Cutaneous				
Rash	45 (17)	3 (1.1)	5 (2)	10.8 (0.3–145)
Pruritus	46 (17.2)	0	3 (1.1)	
Diarrhea	42 (15.7)	6 (2.2)	8 (3)	8.9 (0.1–89.7)
Endocrine dysfunction				
Hypothyroidism	26 (9.7)[e]	1 (0.4)	1 (0.4)	16 (1.7–106)
Hyperthyroidism	6 (2.2)	0	2 (0.7)	
Adrenal insufficiency	4 (1.5)	2 (0.7)	3 (1.1)	
Pneumonitis	23 (8.6)	12[b] (4.5)	19 (7.1)	16.7 (0.9–189.4)
Hepatitis	14 (5.3)	4[c] (1.5)	7 (2.6)	5.7 (0.4–33)
Mucositis	2 (0.7)	1 (0.4)	0	2.7 (1.6–3.9)
Arthritis	24 (9)	0	4 (1.5)	11 (0.3–123.4)
Others	23 (8.6)	12 (4.5)	11 (4.1)	Not calculated
Hemolytic anemia	1	1	1	
Thrombocytopenia	2	2	1	
Flu-like	4	0	0	
Nephritis	4	1	2	
Vitiligo	1	0	0	
Pancreatitis	1	1	1	
Myopericarditis	2	2	1	
Myositis	3	1	1	
Vasculitis	1	0	1	
Aseptic meningitis	1	1	1	
Encephalitis	2	2	2	
Miasteniforme syndrome	1	1	0	

irAEs, immune-related adverse events.

[a] *Total number of irAEs.*

[b,c] *Four cases of pneumonitis and one case of hepatitis were grade 5.*

[d] *High-dose steroid pulse therapy (metilprednisolone at 1g/day) for 3 days followed by metilprednisolone (1 to 2 mg/kg) treatment for several weeks was administered in one case of grade 3 colitis. No patient received other types of immunosupressors.*

[e] *Eleven patients required thyroid hormone replacement therapy.*

(1.9–117.4, IQR 11.3) ($p = 0.004$)], DoR was significantly longer in patients with irAEs: 6.1 months (range 0.5–50, IQR 10.6) vs. 2.6 months (range 0.2–51.9, IQR 3.8) ($p < 0.001$). As mentioned previously, 44 patients (22.2%) discontinued treatment due to irAEs. Within this group, 29 patients (65.9%) did not progress after stopping immunotherapy, in contrast to the 28.7% (64/223) of patients in the group of patients who did not discontinue treatment due to toxicity ($p < 0.001$).

Association Between the Use of Corticosteroids and Efficacy

The most commonly used types of corticosteroids were prednisone (39.7%) and dexamethasone (34.9%). The median dose of prednisone equivalent was 50 mg daily (range 5–1,250 mg, IQR 53.4). The median duration of corticosteroid treatment was 59 days (range 0.5–83.0, IQR 159). No differences in corticosteroid usage were observed in patients receiving first line therapy (53.1%) vs. second line or beyond (55.4%) ($p = 0.790$).

One hundred and forty-six patients (54.7%) received corticosteroids during therapy with anti-PD-(L)1 blockade agents, of whom 134 patients (91.8%) required ≥10 mg of prednisone equivalent per day: 59 patients (44%) for the treatment of irAEs, and 75 patients (56%) for the management of cancer-related symptoms, including asthenia (8.2%) and anorexia (6.3%), symptomatic bone metastases (13.4%), symptomatic brain metastases (36.3%), dyspnea (14.8%), and chronic obstructive pulmonary disease (COPD) management (21%). No other chronic illness required steroid therapy in our study population. Only seven patients started corticosteroids within the 30 days before immunotherapy initiation, and all patients continued corticosteroids therapy during anti-PD-(L)1 therapy (**Figure S1**). Patients receiving corticosteroids for cancer-related symptoms presented significant differences compared to the rest of the population: there was a higher proportion of patients with ECOG PS 2 (24.1 vs. 10.9%; $p = 0.009$) receiving second line therapy or beyond (83.1 vs. 63.6%; $p = 0.001$) or as a single-agent instead of a combination regimen (90.4 vs. 72.8%; p 0.002). No differences were observed regarding

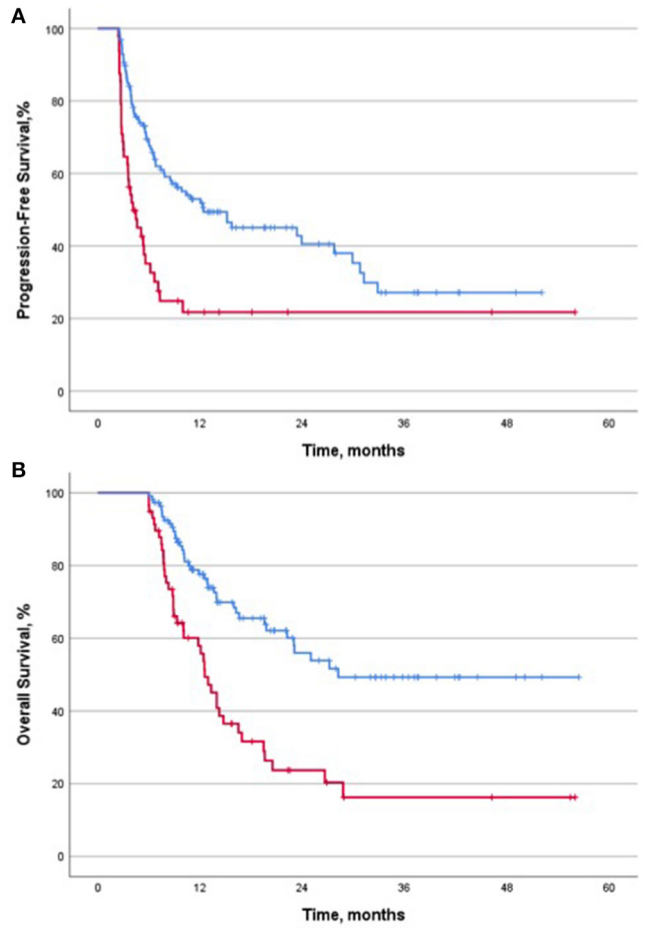

FIGURE 1 | Landmark analysis according to the presence of irAEs. **(A)** Progression-free survival and the presence of irAEs ($n = 175$). **(B)** Overall survival and the presence of irAEs ($n = 167$). Kaplan-Meier curves with 2.4 months landmark analysis for progression-free survival **(A)** and 5.9 months for overall survival **(B)** in patients with or without irAEs. Abbreviations: PFS, progression-free survival; OS, overall survival; irAEs, immune-related adverse events; CI, confidence interval; HR, hazard ratio; m, months.

the presence of liver or brain metastases. Interestingly, patients receiving corticosteroids for cancer-related symptoms presented a lower incidence of irAEs (38.6 vs. 65.2%; $p < 0.001$) (**Table 3**).

Median OS was significantly longer in the group of patients that received <10 mg prednisone equivalent daily or no corticosteroids ($n = 133$) than in the group of patients that received ≥10 mg prednisone equivalent daily ($n = 134$): 14.7 months (95% CI, 11.1–18.3) vs. 8.3 months (95% CI, 6.9–9.8) (HR 0.66; 95% CI, 0.48–0.90; $p = 0.010$) (**Figure 2A**). No differences in PFS were observed. Median OS was significantly shorter in patients receiving ≥10 mg prednisone equivalent daily for cancer-related symptoms ($n = 75$) than in the rest of the study population (patients who did not receive corticosteroids or <10 mg prednisone equivalent daily and those who received them for the management of irAEs, $n = 192$): 6 months (95% CI, 4.4–7.5) vs. 15.9 months (95% CI, 11.2–20.7) (HR 2.28; 95% CI, 1.63–3.20; $p < 0.001$). No differences in terms of PFS were observed.

No differences in OS were found between patients who started corticosteroids for cancer-related symptoms in the 30 days before starting immunotherapy or after starting anti-PD-(L)1 blockade: 5.2 months (95% CI, 0.3–4.6) vs. 6.4 months (95% CI, 1.1–4.3) ($p = 0.898$).

It is important to highlight that no significant differences were observed in OS between patients who received no corticosteroids or <10 mg prednisone equivalent daily and those who received ≥10 mg for the management of irAEs ($p = 0.314$) (**Figures 2B,C**). However, when analyzing the outcomes of patients with irAEs, median OS was greater in patients receiving <10 mg of prednisone equivalent daily than those receiving ≥10 mg for toxicity management: not reached vs. 19.5 months (95% CI, 10.7–28.4) (HR 2.05; 95% CI, 1.13–3.73; $p = 0.016$).

In our study, a duration of corticosteroids ≥30 days correlated with a better outcome in terms of OS: 12.1 months (95% CI, 8.3–15.8) vs. 4.6 months (95% CI, 2.4–6.7) ($p = 0.001$). The same happened when the cut-off point was changed to 15 days: 9.3 months (95% CI, 5.3–13.2) vs. 5.1 (95% CI, 2.4–7.9) ($p = 0.007$). No differences in terms of PFS were detected ($p = 0.746$ and $p = 0.726$ for a cut-off of 30 and 15 days, respectively). However, when analyzing results in terms of corticosteroid use, both OS and PFS were higher in patients receiving ≥10 mg prednisone equivalent for irAEs management. In terms of OS, patients treated with ≥10 mg of prednisone equivalent for irAEs management during ≥30 days presented the highest survival rates: 23 months (95% CI, 11.5–34.6) (p 0.001). Notably, no differences were detected when steroid therapy was given for cancer-related symptoms.

Association Between the Use of Antibiotics and Efficacy

One hundred and forty-one patients (52.8%) received antibiotics. Quinolone (37.6%) and penicillin (33.3%) were the most commonly used groups of antibiotics. Of note, the group of patients experiencing irAEs received significantly more antibiotics (58.6 vs. 45.2%, $p = 0.036$).

However, no relation was found between the use of antibiotics and efficacy of anti-PD-(L)1 blockade agents, with a median OS of 10.2 months (95% CI, 6.4–13.9) in patients receiving antibiotics vs. 12.5 months (95% CI, 9.9–15.0) in patients not receiving antibiotics ($p = 0.924$), and a median PFS of 3.8 months (95% CI, 0.9–1.9) vs. 4.4 months (95% CI, 0.7–2.9), respectively ($p = 0.454$).

Multivariable Analysis

Multivariable analyses revealed that the presence of irAEs was the variable most strongly associated with a better response rate and OS (**Table 4A** and **Table S2A**), with an OR and HR of 0.36 and 0.32, respectively. In addition, in the multivariable analysis of types of irAEs, cutaneous, endocrinological, and rheumatological irAEs were found to be significantly associated with increased ORR and OS (**Table 4B** and **Table S2B**). Pruritus and arthritis were the irAEs subtypes with the lowest HR.

TABLE 3 | Comparison of patient characteristics between patients receiving corticosteroids for cancer-related symptoms and patients not receiving corticosteroids or received corticosteroids for management of irAEs*.

Category	Total $n = 267$ (%)	Corticosteroids for cancer-related symptoms $n = 83$ (%)	Corticosteroids for irAEs or prednisone equivalent <10 mg/d * $n = 184$(%)	*P*-value
Gender				
Male	186 (69.7)	60 (72.3)	126 (68.5)	0.568
Female	81 (30.3)	23 (27.7)	58 (31.5)	
Age	66.1 years	63.6 years	66.7 years	0.742
median (range)	(26.7–85.2, IQR 14.3)	(38.8–85.2, IQR 13.3)	(26.7–83.7, IQR 14.2)	
Smoking status				
Non- or light smoker	26 (9.7)	9 (10.8)	17 (9.2)	0.662
Current or former smoker	241 (90.3)	74 (89.2)	167 (90.8)	
ECOG PS				
0–1	227 (85)	63 (75.9)	164 (89.1)	0.009
2	40 (15)	20 (24.1)	20 (10.9)	
Histological subtype				
Squamous	86 (32.2)	25 (30.1)	61 (33.2)	0.673
Non-squamous	181(67.8)	58 (69.9)	123 (66.8)	
Treatment line			67 (36.4)	
1st line	81 (30.3)	14 (16.9)	117 (63.6)	0.001
≥2nd line	186 (69.7)	69 (83.1)		
Immune-checkpoint blockade schedule				
Monotherapy	209 (78.3)	75 (90.4)	134 (72.8)	0.002
Combination with chemotherapy	33 (12.3)	3 (3.6)	30 (16.3)	
Combination with anti-CTLA-4	25 (9.4)	5 (6)	20 (10.9)	
Presence of irAEs				
No	115 (43.1)	51 (61.4)	64 (34.8)	<0.001
Yes	152 (56.9)	32 (38.6)	120 (65.2)	
Presence of brain metastases				
No	225 (84.3)	65 (78.3)	160 (87)	0.101
Yes	42 (15.7)	18 (21.7)	24 (13)	
Presence of liver metastases				
No	226 (84.6)	67 (80.7)	159 (86.4)	0.272
Yes	41 (15.4)	16 (19.3)	25 (13.6)	
Prednisone equivalent ≥10 mg/day use				
No	133 (49.8)	8 (9.6)	125 (67.9)	<0.001
Yes	134 (50.2)	75 (90.4)	59 (32.1)	
Antibotic use				
No	126 (47.2)	32 (38.6)	94 (51.1)	0.064
Yes	141 (52.8)	51 (61.4)	90 (48.9)	

irAEs, immune-related adverse events; IQR, interquartile range; ECOG PS, Eastern Cooperative Oncology Group performance status; CTLA-4, cytotoxic T-lymphocyte antigen 4.
Includes patients that did not received corticosteroids.

Variables such as ECOG PS ≥ 2, the presence of liver metastasis, the use of corticosteroids, and receiving anti-PD-(L)1 therapy as a second line treatment or beyond were related to poorer outcomes, specially regarding to OS (**Table 2A**). The HR for OS regarding corticosteroid use for cancer-related symptoms was >2 (HR = 2.40), though it gave a result of 1.81 when corticosteroids were used for irAEs management. Notably, no association was found between OS or ORR and the presence of brain metastasis. No differences were found regarding the grade of irAEs (grade 1–2 vs. grade 3–4 irAEs, excluding grade 0), nor in terms of OS ($p = 0.198$) nor in regard to ORR ($p = 0.349$).

DISCUSSION

As far as we are aware, this is one of the largest studies to assess the association between the presence of irAEs and the efficacy of anti-PD-(L)1 blockade agents in advanced NSCLC patients. Statistically significant differences were observed in terms of OS, PFS, ORR and DoR between patients experiencing irAEs and those who did not. Of note, a landmark analysis was performed to minimize the immortal time bias potentially associated with time-dependent factors such as the development of irAEs.

In our study the incidence of irAEs was 56.9%, which is higher than previously reported (13–23). This could be explained

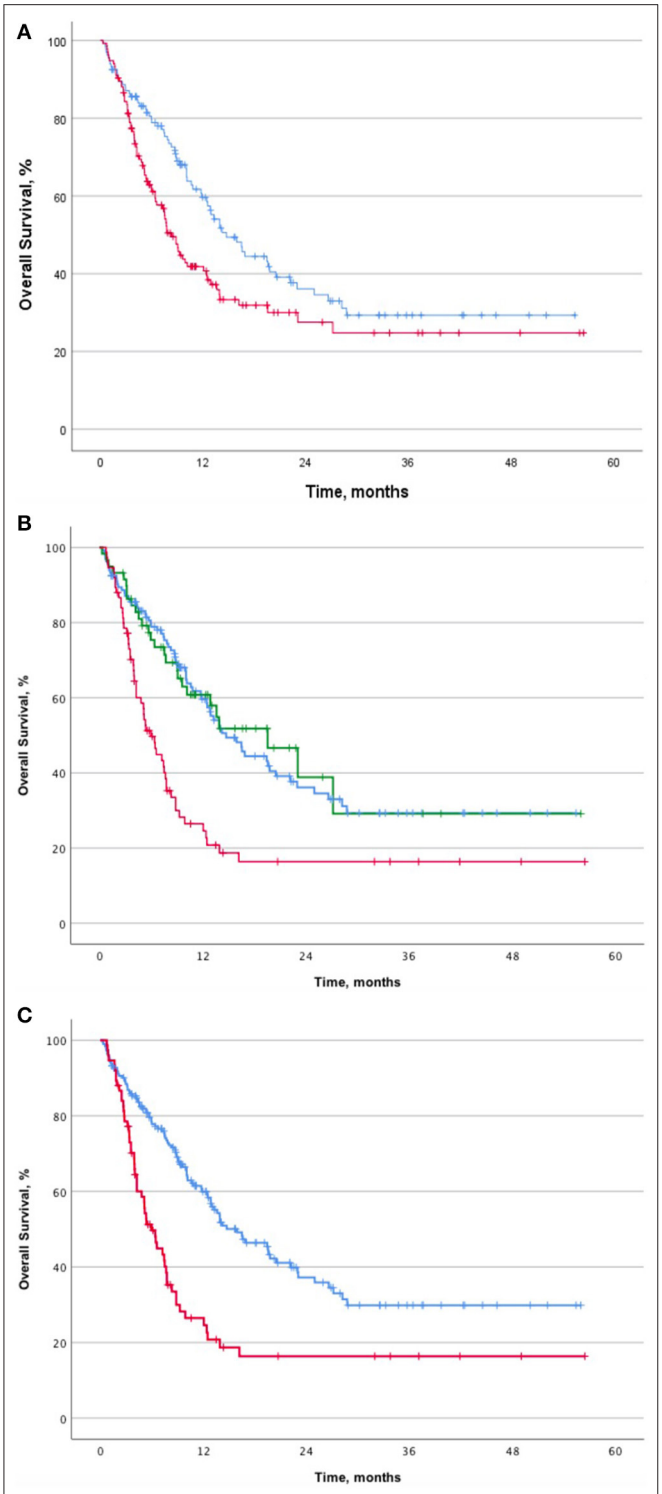

FIGURE 2 | Survival analysis with regard to corticosteroid use (≥10 mg of prednisone equivalent daily). **(A)** Overall survival and the use of ≥10 mg of prednisone equivalent daily ($n = 267$). **(B)** Overall survival and the reason for corticosteroid use ($n = 267$). **(C)** Overall survival and corticosteroid use ($n = 267$). Abbreviations: PFS, progression-free survival; OS, overall survival; irAEs, immune-related adverse events; PDNe, prednisone equivalent; CI, confidence interval; HR, hazard ratio; m, months. Kaplan-Meier curves of patients

(Continued)

FIGURE 2 | treated with anti-PD-(L)1 blockade agents on the basis of reported corticosteroid usage (≥10 mg of prednisone equivalent) in terms of OS **(A)**. OS comparison in patients who did not received any corticosteroids, those who received them for irAEs treatment or cancer-related symptoms management **(B)**. OS comparison according to the use of corticosteroids: for management of cancer-related symptoms and the rest of the population **(C)**. [a]Use of ≥10 mg of prednisone equivalent daily for irAEs management. [b]Use of ≥10 mg of prednisone equivalent daily for cancer-related symptoms.

TABLE 4 | Multivariable analysis of overall response rate according to clinical features (A) and type of irAEs (B).

Variable	Odds ratio	95% CI	*P*-value
(A). Overall response rate and clinical features ($n = 267$)			
Presence of irAEs			
No	0.36	0.20–0.65	0.001
Yes			
Treatment line			
1st line	2.41	1.36–4.28	0.003
≥2nd line			
(B). Overall response rate and type of irAEs ($n = 267$)			
Rash			
No	0.34	0.17–0.69	0.002
Yes			
Endocrine dysfunction			
No	0.38	0.18–0.81	0.012
Yes			
Arthritis			
No	0.28	0.11–0.72	0.008
Yes			

CI, confidence interval; irAEs, immune-related adverse events.

mainly because this is a real-world data study and, in addition, 30.3% of patients received anti-PD-(L)1 blockade agents in a first line setting. Patients receiving anti-PD-(L)1 blockade agents as a second line therapy or beyond experienced significantly fewer irAEs than those treated in the first line. These findings are in line with those observed in KEYNOTE-010 and KEYNOTE-024 trials showing a higher rate of toxicity in treatment naïve patients who are probably less immunosuppressed than pre-treated patients (5, 8). A significantly higher rate of endocrinological toxicity was observed with the combination of immune blockade agents. This was also reported by a group of experts in endocrinopathies. In their review, hypophysitis was more common with anti-CTLA-4 agents, whereas thyroid dysfunction was more frequent with anti-PD-1 agents. The combination of these agents appeared to increase the risk of immune-related endocrinopathies, which may be related to a more frequent association between CTLA-4 polymorphisms and autoimmune endocrinopathies in comparison with PD-1 polymorphisms (31).

The landmark analysis showed longer PFS and OS, and more importantly, a greater ORR, in patients experiencing irAEs, which corroborates the influence exerted by the development of immune-mediated toxicity on immunotherapy efficacy. The longer duration of treatment in patients experiencing irAEs might be explained by a lower percentage of treatment

discontinuation in this group (75 vs. 91%, $p = 0.001$). This raises the question of whether the survival advantage attributed to the presence of irAEs is a reflection of the increased toxicity associated with a longer duration of treatment or a direct result of the irAEs themselves. Recently, both prospective and retrospective data have suggested that this relation is independent of guarantee-time bias, mainly because the majority of patients developed irAEs within the first 2–8 weeks after treatment initiation, supporting the predictive value of irAEs over treatment duration (22, 23).

The presence of irAEs itself was the strongest variable associated with better outcomes in a multivariable analysis, both according to ORR (OR 0.36; 95% CI, 0.20–0.65; $p = 0.001$) and OS (HR 0.32; 95% CI, 0.22–0.46; $p < 0.001$). Additionally, endocrine dysfunction, rash/pruritus, and arthritis were significantly associated with increased ORR and OS. Pruritus and arthritis presented the most favorable HR. Consistent with our findings, two studies have also suggested that thyroiditis and skin toxicity are related to longer OS (32, 33). Of note, no association was found between OS or ORR and the severity of irAEs.

Corticosteroids are the mainstay in the management of irAEs, though they are also a common symptomatic treatment in advanced NSCLC patients. Prednisone equivalent $\geq$10 mg daily for the symptomatic treatment of cancer-related symptoms at the time of initiation or during anti-PD-(L)1 blockade treatment was associated with significantly poorer outcomes than for patients who did not receive corticosteroids or those who received them to manage irAEs. These results are in line with those reported recently in a study carried out on 640 patients with advanced NSCLC receiving single-agent anti-PD-(L)1 blockade treatment (25). In that publication, a multivariable analysis including smoking status, ECOG, and history of brain metastases showed that baseline corticosteroid use was significantly associated with decreased ORR, PFS, and OS. Our multivariable analysis assessed similar prognostic factors, but also included the treatment line as a variable that could influence outcomes. Corticosteroid use for palliation of cancer-related symptoms and anti-PD-(L)1 therapy in the second line or beyond were strong, independent variables associated with a poorer outcome. Patients receiving corticosteroids for cancer-related symptoms showed a higher percentage of patients with an ECOG PS of 2 and treatment in the second line or beyond with single-agent. On the whole, these findings suggest that baseline corticosteroid use may simply identify a group of patients with a higher volume or more aggressive disease or with basal illnesses that worsen cancer prognosis. The same inference has been made in a recently published study in which the authors concluded that the worst outcome associated with $\geq$10 mg of prednisone equivalent daily at the time of immunotherapy seemed to be driven by a poor-prognosis subgroup of patients who received corticosteroids as a palliative treatment (26). Moreover, previous data suggest that the positive correlation between the presence of irAEs and efficacy of anti-PD-(L)1 blockade agents in advanced NSCLC is not hampered by the use of corticosteroids for the treatment of irAEs, concluding that its use in this context should not be restricted for fear of loss of any outcome advantage. In contrast, our study showed a greater median OS in patients receiving $<$10 mg of prednisone equivalent than those receiving $\geq$10 mg for toxicity management. The median duration of $\geq$10 mg prednisone equivalent had a significant positive impact on the efficacy of anti-PD-(L)1 blockade agents when given for irAEs management, but this finding might be explained by irAEs itself playing a role as a confounding factor.

Regarding the implications of the use of antibiotics, no relation was found between the use of antibiotics (3 months before, during or 3 months after the end of anti-PD-(L)1 therapy) and immunotherapy efficacy. These results differ from those observed in a cohort of 249 advanced NSCLC, renal cell and urothelial carcinoma patients, in which 28% of patients received antibiotics within 2 months before or 1 month after the initiation of anti-PD-(L)1 blockade agents, for whom both PFS and OS were significantly shorter (34). However, those results were based on 69 out of 249 patients, so although informative, they are insufficient to draw any firm conclusion. In addition, the different time period over which the use of antibiotics was analyzed makes it difficult to compare results. The prospective analysis of the effect of antibiotics on the efficacy of anti-PD-(L)1 therapy might help to understand the relation between them and the period of time when the use of antibiotics might be discouraged.

Anti-PD-(L)1 therapy interruption due to irAEs is an issue that concerns us all. In our study, the 65.9% of patients who discontinued treatment due to irAEs and did not progress contrasts with the 28.7% of patients who did not interrupt immunotherapy due to toxicity and did not progress. These results are in line with a *post-hoc* analysis of the Checkmate-067 trial in patients with advanced melanoma, in which both PFS and OS were similar after 4 years regardless of discontinuation of treatment due to irAEs (35). Taken together, these data suggest that treatment interruption due to irAEs does not seem to compromise the efficacy of anti-PD-(L)1 blockade agents.

This study has several limitations that could be addressed in future research. First, its retrospective design and the need for a longer follow-up period to fully assess long-term outcomes. Second, the heterogeneity of treatment strategies included in this study, which may influence the efficacy of immunotherapy and the frequency of irAEs. Third, the low frequency of some irAEs subtypes may limit the evaluation of their relationship with efficacy. A prospective study including a larger cohort of patients with the same treatment strategy would help to overcome those limitations and adequately assess the real impact of corticosteroids use by accounting for potential confounding factors.

In conclusion, our results indicate that the presence of irAEs is associated with anti-PD-(L)1 blockade agents efficacy in patients with advanced NSCLC. This is one of the most extensive studies to date to reveal an association between the presence of irAEs and the efficacy of immunotherapy in advanced NSCLC when landmark and multivariable analyses are applied. Corticosteroid use of $\geq$10 mg of prednisone equivalent daily was associated with significantly poorer outcomes when given for patients' cancer-related symptoms. No significant differences were observed in terms of efficacy

between patients that did not receive corticosteroids or who received <10 mg prednisone equivalent daily and those who received ≥ 10 mg for the management of irAEs. No relation was found between antibiotics and outcomes of anti-PD-(L)1 blockade agents.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital de la Santa Creu i Sant Pau. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MR and MM contributed to the conception and design of the study, data acquisition, statistical analysis, interpretation of the data, and writing of the manuscript. JM and RG-C contributed to the acquisition and interpretation of the data and revision of the manuscript. IG and LC contributed to the statistical analysis and interpretation of the data. JS, GA, PG, IS, ABarb, and ABarn contributed to the acquisition of the data. All authors reviewed and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.01677/full#supplementary-material

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Cost-Effectiveness Analysis of Nivolumab Plus Ipilimumab vs. Chemotherapy as First-Line Therapy in Advanced Non-Small Cell Lung Cancer

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Background: The CheckMate 227 trial has indicated that nivolumab plus ipilimumab compared with chemotherapy significantly increases long-term survival in the first-line setting of advanced non-small-cell lung cancer (NSCLC).

Methods: A Markov model was built to estimate the cost and effectiveness of nivolumab plus ipilimumab vs. chemotherapy as the first-line therapy in patients with advanced NSCLC based on outcomes data from the CheckMate 227 trial. We calculated the cost and health outcomes at a willingness-to-pay (WTP) threshold of $150,000 per quality adjusted life year (QALY) in populations with different programmed death ligand 1 (PD-L1) expression levels (≥50, ≥1, and <1%) or a high tumor mutational burden (TMB) (≥10 mutations per megabase). Sensitivity analysis were used to test the model stability.

Results: The outcomes showed that the incremental costs and QALYs by using nivolumab plus ipilimumab were $124180.76 and 1.16, $70951.42 and 0.53, $144093.63 and 0.83 for the advanced NSCLC patients with a PD-L1 expression ≥50%, ≥1%, and <1%, which led to an incremental cost-effective ratio (ICER) of $107403.72, $133732.20, and $172589.15 per QALY, respectively. For patients with a high TMB, nivolumab plus ipilimumab contributed an extra 2.04 QALYs at a cost of $69182.50 per QALY.

Conclusion: Nivolumab plus ipilimumab as first-line therapy makes a better cost-effective strategy than chemotherapy in advanced NSCLC patients with PD-L1 expression levels ≥50% and ≥1% or a high TMB, at a willingness-to-pay threshold of $150,000 per QALY, but not in the patients with a PD-L1 expression <1%.

Keywords: cost-effectiveness, nivolumab, ipilimumab, chemotherapy, non-small cell lung cancer

INTRODUCTION

Around the globe, lung cancer is the leading cause of cancer incidence and mortality, with 2.1 million new lung cancer cases and 1.8 million deaths worldwide (1–4). Up to 61% of patients with NSCLC had advanced disease at the time of diagnosis, with a 5-years survival rate of 18% (5, 6). Platinum-based chemotherapy doublet or pembrolizumab monotherapy for patients with a high level of tumor PD-L1 expression (≥1%) were the standard first-line therapy for advanced NSCLC without treatable driver mutations (7–10).

Nivolumab, the fully human immunoglobulin G4 monoclonal antibody inhibitor of programmed death-1 (PD-1), and ipilimumab, a fully human immunoglobulin G1 monoclonal antibody that targets the cytotoxic T-cell lymphocyte antigen-4 (CTLA-4) checkpoint receptor, are immune checkpoint inhibitors with distinct but complementary mechanisms of action. In preclinical and clinical settings, the combination of nivolumab plus ipilimumab has presented enhanced activity over nivolumab monotherapy's, which has been approved for the treatment of metastatic melanoma and renal-cell carcinoma (11–16). In the pivotal phase three trial CheckMate 227, the first-line therapy using nivolumab plus ipilimumab brought about a longer duration of overall survival (OS) than that of patients with advanced NSCLC using chemotherapy, regardless of PD-L1 expression levels (17, 18). Nivolumab plus ipilimumab was subsequently approved as the first-line treatment for patients with metastatic NSCLC, PD-L1 ≥ 1%, without EGFR or ALK genomic tumor aberrations by the United States (US) Food and Drug Administration (FDA) in May, 2020.

To our knowledge, it is still unclear whether the use of first-line nivolumab and ipilimumab would be cost-effective for advanced NSCLC with different PD-L1 expression levels. This study aims to evaluate the cost-effectiveness of nivolumab plus ipilimumab vs. chemotherapy in previous untreated advanced NSCLC patients without driver alterations that can be targeted. The cost-effectiveness analyses were conducted, respectively, in three populations with different PD-L1 expression levels (≥50, ≥1, and <1%) or patients with a high tumor mutational burden (TMB) (≥10 mutations per megabase), using the most recently reported data from CheckMate 227 (17–19).

MATERIALS AND METHODS

Model Structure

A Markov model was constructed on the basis of outcomes data from the CheckMate 227 trial to evaluate the costs and effectiveness of using nivolumab plus ipilimumab vs. chemotherapy as first-line therapy for advanced NSCLC from the US payer's perspective. The Markov model cycle length was 6-weeks and the time horizon were 20-years. We adopted a 3% discount rate per year for both costs and outcomes (20). The total costs, life years (LYs), quality adjusted life years (QALYs), and incremental cost-effective ratios (ICERs) were calculated in each treatment strategy. The Markov model was constructed via TreeAge Pro 2018 (TreeAge Software Inc., Williamstown, MA).

The model structure included three states to represent the progression of advanced NSCLC: progression-free survival (PFS), progressive disease (PD), and death (**Supplementary Figure 1**). Patients were treated with nivolumab plus ipilimumab or chemotherapy in the PFS state until progression. All patients could continue subsequent treatment until death if any disease progression or unacceptable toxic effects occurred. Grades 3 or 4 adverse events (AEs) with a ≥1% frequency reported in CheckMate 227 trial were included.

Model Survival and Progression Risk Estimates

The estimates of OS for the nivolumab plus ipilimumab group and for the chemotherapy group were based on the OS curves from CheckMate 227 trial. The GetData Graph Digitizer (version 2.25; http://www.getdata-graph-digitizer.com/index.php) was applied to extracting the data points from the OS Kaplan-Meier curves reported in the CheckMate 227 trial, and these data points were then used to fit parametric survival models. The Weibull survival curves matched the number of patients in three states including PFS, PD and death overtime, as the Weibull distribution was flexible and widely used in cancer survival analysis according to Akaike information criterion. Then, we estimated the shape parameter (γ) and the scale parameter (λ) from this fit, and applied Kaplan-Meier curves by using R software package (http://www.r-project.org) and the method of Hoyle et al. (21). With the mean OS time denoted as S(t), the cause-specific mortality M at cycle t can be computed:

$$\mathrm{M} = \frac{\mathrm{S}(t) - S(t\ +\ 1)}{S(t)}, \tag{1}$$

while $\mathrm{S}(t) = \exp(-\lambda t^{\gamma})$ $(\lambda > 0; \gamma > 0)$.

Finally, OS rates in each cycle were:

1-exp (Scale*(_stage) ∧Shape-Scale*(_stage+1) ∧Shape)

The progression risks for nivolumab plus ipilimumab group and chemotherapy group were estimated by the same approach. We used this measure to evaluate the OS rate and PFS rate for two groups, that is, patients with three PD-L1 expression levels (≥50, ≥1, and <1%) and those with a high TMB (≥10 mutations per megabase).

Utility Estimates

Utility was adopted to measure patient's preference for living at a particular health state that is often referred to as QALYs (0 stood for death and 1 for perfect heath), which reflected the impacts of the disease-related health states. We used utilities of 0.784 and 0.693 for the patients with nivolumab plus ipilimumab and chemotherapy as the first-line therapy, respectively, based on the patient-reported outcomes results from CheckMate 227 trial (19). The previously published utility of 0.473 for the NSCLC patients receiving subsequent treatment was used (22).

Cost Inputs

This study only takes into account direct medical costs, included drug, radiographic examination, administration and AEs costs. The patients in nivolumab plus ipilimumab group were treated

TABLE 1 | Model parameters: baseline values, ranges, and distributions for sensitivity analysis in patients with different PD-L1 expression (≥50, ≥1, and <1%).

Variable	Baseline value	Range		Reference	Distribution
		Minimum	Maximum		
Weibull survival model in nivolumab plus ipilimumab group with PD-L1 ≥ 50%					
PFS	Shape = 0.53605, Scale = 0.27369	-	-	(17)	-
OS	Shape = 0.658678, Scale = 0.112585	-	-	(17)	-
Weibull survival model in chemotherapy group with PD-L1 ≥ 50%					
PFS	Shape = 1.10045, Scale = 0.17421	-	-	(17)	-
OS	Shape = 0.868245, Scale = 0.093252	-	-	(17)	-
Weibull survival model in nivolumab plus ipilimumab group with PD-L1 ≥ 1%					
PFS	Shape = 0.51890, Scale = 0.36477	-	-	(17)	-
OS	Shape = 0.79585, Scale = 0.09415	-	-	(17)	-
Weibull survival model in chemotherapy group with PD-L1 ≥ 1%					
PFS	Shape = 0.97117, Scale = 0.21656	-	-	(17)	-
OS	Shape = 0.97371, Scale = 0.07182	-	-	(17)	-
Weibull survival model in nivolumab plus ipilimumab group with PD-L1 < 1%					
PFS	Shape = 0.66247, Scale = 0.29319	-	-	(17)	-
OS	Shape = 0.764844, Scale = 0.098260	-	-	(17)	-
Weibull survival model in chemotherapy group with PD-L1 < 1%					
PFS	Shape = 1.19322, Scale=0.16572	-	-	(17)	-
OS	Shape = 1.023457, Scale = 0.085114	-	-	(17)	-
Proportion of tumor histologic type in chemotherapy PD-L1 ≥ 50% and ≥1%					
Non-squamous	70.8	-	-	(17)	-
Squamous	29.2	-	-	(17)	-
PD-L1 < 1%					
Non-squamous	75.3	-	-	(17)	-
Squamous	24.7	-	-	(17)	-
Proportion of treatment discontinuation PD-L1 ≥ 50% and ≥1%					
Nivolumab plus ipilimumab	0.422	-	-	(17)	-
Chemotherapy	0.625	-	-	(17)	-
PD-L1 < 1%					
Nivolumab plus ipilimumab	0.519	-	-	(17)	-
Chemotherapy	0.570	-	-	(17)	-
Risk for main adverse events in nivolumab plus ipilimumab group with PD-L1 ≥ 50% and ≥1%					
Risk of rash	0.023	0.0184	0.0276	(17)	
Risk of diarrhea	0.015	0.012	0.018	(17)	
Risk of fatigue	0.02	0.016	0.024	(17)	
Risk of decreased appetite	0.01	0.008	0.012	(17)	
Risk of anemia	0.013	0.0104	0.0156	(17)	
Risk for main adverse events in chemotherapy group with PD-L1 ≥ 50% and ≥1%					
Risk of anemia	0.106	0.0848	0.1272	(17)	Beta
Risk of neutropenia	0.07	0.056	0.084	(17)	Beta
Risk of neutrophil count decreased	0.085	0.068	0.102	(17)	Beta
Risk of nausea	0.018	0.0144	0.0216	(17)	Beta
Risk of fatigue	0.01	0.008	0.012	(17)	Beta
Risk of decreased appetite	0.01	0.008	0.012	(17)	Beta
Risk of vomiting	0.026	0.0208	0.0312	(17)	Beta
Risk for main adverse events in nivolumab plus ipilimumab group with PD-L1 < 1%					
Risk of diarrhea	0.022	0.0176	0.0264	(17)	Beta
Risk of fatigue	0.011	0.0088	0.0132	(17)	Beta
Risk of anemia	0.137	0.1096	0.1644	(17)	Beta
Risk for main adverse events in chemotherapy group with PD-L1 < 1%					
Risk of anemia	0.137	0.1096	0.1644	(17)	Beta
Risk of neutropenia	0.115	0.092	0.138	(17)	Beta
Risk of nausea	0.027	0.0216	0.0324	(17)	Beta

(Continued)

TABLE 1 | Continued

Variable	Baseline value	Range		Reference	Distribution
		Minimum	Maximum		
Risk of fatigue	0.022	0.0176	0.0264	(17)	Beta
Risk of decreased appetite	0.016	0.0128	0.0192	(17)	Beta
Risk of vomiting	0.016	0.0128	0.0192	(17)	Beta
Risk of diarrhea	0.011	0.0088	0.0132	(17)	Beta
Nivolumab plus ipilimumab group subsequent therapy proportion in PD-L1 ≥ 50% and ≥1% population					
Radiotherapy	0.174	-	-	(17)	-
Chemotherapy	0.316	-	-	(17)	-
Post-study nivolumab	0.04	-	-	(17)	-
Targeted therapy	0.053	-	-	(17)	-
Chemotherapy group subsequent therapy proportion in PD-L1 ≥ 50% and ≥1% population	0.244	-	-	(17)	-
Radiotherapy					
Chemotherapy	0.275	-	-	(17)	-
Post-study nivolumab	0.325	-	-	(17)	-
Pembrolizumab	0.081	-	-	(17)	-
Targeted therapy	0.043	-	-	(17)	-
Nivolumab plus ipilimumab group subsequent therapy proportion in PD-L1 < 1% population	0.182	-	-	(17)	-
Radiotherapy					
Chemotherapy	0.422	-	-	(17)	-
Targeted therapy	0.064	-	-	(17)	-
Chemotherapy group subsequent therapy proportion in PD-L1 < 1% population	0.167	-	-	(17)	-
Radiotherapy					
Chemotherapy	0.344	-	-	(17)	-
Post-study nivolumab	0.301	-	-	(17)	-
Targeted therapy	0.091	-	-	(17)	-
Utility	0.784	0.74	0.828	(19)	Beta
Utility PFS in nivolumab plus ipilimumab					
Utility PFS in chemotherapy	0.693	0.642	0.743	(19)	Beta
Utility progressive disease	0.473	0.166	0.568	(22)	Beta
Patients' weight, kg	70	-	-	(23)	Beta
Body surface area, m^2	1.84	-	-	(23)	Beta
Drug cost, $/per cycle					
Nivolumab	17517.15	14013.72	21020.58	(17, 24)	Gamma
Ipilimumab	10718.96	8575.17	12862.75	(17, 24)	Gamma
Pemetrexed	12782.85	10226.28	15339.42	(17, 24)	Gamma
Gemcitabine	92	73.6	110.4	(17, 24)	Gamma
Carboplatin	56.55	45.24	67.86	(17, 24)	Gamma
Cisplatin	51.78	41.42	62.14	(17, 24)	Gamma
Pembrolizumab	19755.6	15804.48?	23706.72?	(17, 24)	Gamma
Post-study nivolumab	17517.15	14013.72	21020.58	(17, 24)	Gamma
Radiotherapy	15899.24	12719.39	19079.09	(25)	Gamma
Targeted therapy	12615.68	10092.54	15138.82	(17, 26)	Gamma
Subsequent chemotherapy	238.74	190.99	286.49	(17, 24)	Gamma
Expenditures on main adverse events, $	7969.56	6375.65	9536.47	(27)	Gamma
Anemia					
Neutropenia	32,995	24,746	41,244	(28)	Gamma
Neutrophil count decreased	32,995	24,746	41,244	(28)	Gamma
Fatigue	0	-	-	(29)	Gamma
Rash	13,376	10700.8	16051.2	(30)	Gamma
Diarrhea	10,301	8240.8	12361.2	(30)	Gamma
Decreased appetite	9,711	7768.8	11653.2	(30)	Gamma
Vomiting	10,301	8240.8	12361.2	(30)	Gamma
Nausea	10,301	8240.8	12361.2	(30)	Gamma
Administration $/per cycle	139.61	111.69	167.53	(31)	Gamma
CT $/per cycle	231	208	254	(32)	Gamma
Laboratory $/per cycle	315	252	378	(33)	Gamma
Discount rate	0.03	-	-	(20)	-

CT, compute tomography; NSCLC, non-small-cell lung cancer; OS, overall survival; PD-L1, programmed death ligand 1; PFS, progression-free survival.

TABLE 2 | Baseline results in nivolumab plus ipilimumab and chemotherapy groups in PD-L1 expression ≥50, ≥1, and <1% populations.

Strategies and scenarios	Total cost, $	LYs	QALYs	ICER per LY [a]	ICER per QALY [b]
PD-L1 ≥ 50%					
Nivolumab plus ipilimumab	390218.01	4.69	2.81	67925.33	107403.72
Chemotherapy	266037.25	2.87	1.66	-	-
PD-L1 ≥ 1%					
Nivolumab plus ipilimumab	318368.06	3.43	2.13	100527.95	133732.20
Chemotherapy	247416.64	2.73	1.60	-	-
PD-L1 < 1%					
Nivolumab plus ipilimumab	314172.48	3.63	2.20	105334.45	172589.15
Chemotherapy	170078.85	2.26	1.36	-	-

a, Compared to chemotherapy ($/LY); b, Compared to chemotherapy ($/QALY).
ICER, incremental cost-effectiveness ratio; LY, life-year; PD-L1, programmed death ligand 1; QALY, quality-adjusted life-year.

with nivolumab (3 mg per kilogram of body weight every 2 weeks) plus ipilimumab (1 mg per kilogram every 6 weeks). The chemotherapy group were treated with platinum-doublet chemotherapy every 3 weeks for up to four cycles{non-squamous NSCLC were treated with pemetrexed (500 mg/m^2 of body surface area) plus cisplatin (75 mg/m^2) or carboplatin (area under the concentration-time curve [AUC], 5 or 6), and for squamous NSCLC, with gemcitabine (1,000 or 1,250 mg/m^2) plus cisplatin (75 mg/m^2) or gemcitabine (1,000 mg/m^2) plus carboplatin (AUC, 5)}(17, 18). After four cycles of platinum-doublet chemotherapy, patients with non-squamous NSCLC were received pemetrexed as maintenance therapy (500 mg/m² every 3 weeks) (**Table 1** and **Supplementary Table 3**).

We used a standard AUC, 6 mg/mL/min, and assumed male sex, 65 years old, body weight, 70-kg, height, 178-cm, body surface area, 1.84 m^2, and serum creatinine, 1 (23). The price was derived from the Centers for Medicare & Medicaid Services and published articles, and the details were demonstrated in **Table 1** and **Supplementary Table 3** (24–26, 31–33). The costs of radiographic examination covered computed tomography (CT) (every 6 weeks after treatment and every 9 weeks after progression) (32). Grade 3 or higher AEs with a frequency of >1% were included. The costs related to AEs were calculated by multiplying the incidence of serious AEs by the costs of managing serious AEs per event. AEs costs were derived from previously published studies (27–30). All information regarding the drugs dose, costs were listed in **Table 1** and **Supplementary Table 3**.

Sensitivity Analysis

One-way sensitivity analyses were performed to test the corresponding ICERs by varying each input parameter within a plausible range, as shown in **Table 1**. One thousand Monte Carlo simulations were performed to conduct the probabilistic sensitivity analysis by inputting values drawn from their statistical distributions. The probabilistic sensitivity analysis were conducted to estimate the probability of nivolumab plus ipilimumab being cost-effective compared with platinum-doublet chemotherapy in three populations with PD-L1 expression ≥50, ≥1, and <1% and in patients with high TMB at a willing-to-pay (WTP) threshold of $150,000 (31).

RESULTS

Base Case Results

In the first-line setting of advanced NSCLC patients without driver alterations that can be targeted, nivolumab plus ipilimumab dwarfed chemotherapy with an additional 1.83 LYs, 0.71 Lys, and 1.37 LYs in the PD-L1 expression ≥50, ≥1, and <1% populations, respectively. When compared to chemotherapy, the mean incremental costs and QALYs of nivolumab plus ipilimumab were $124180.76 and 1.16, $70951.42 and 0.53, $144093.63 and 0.83 for the patients with a PD-L1 expression ≥50, ≥1, and <1%, respectively. Resulting an ICERs of $107403.72 per QALY in PD-L1 ≥50% population, $133732.20 in PD-L1 ≥1% population and $172589.15 in PD-L1 <1% population (**Table 2**). For patients with a high TMB, the use of nivolumab plus ipilimumab cost an additional $141255.72, provided an additional 2.04 QALYs and an ICER of $69182.50 per QALY compared with chemotherapy (**Supplementary Table 4**).

Sensitivity Analysis

The results of univariable sensitivity analysis in populations with three PD-L1 expression levels were showed in **Figure 1**. The cost of nivolumab plus ipilimumab and the utility of PD possessed the greatest influences on ICERs, which were similar in three populations with PD-L1 expression ≥50, ≥1, and <1%. Nivolumab plus ipilimumab as first-line therapy in advanced NSCLC can be cost-effective compared with chemotherapy if the cost of nivolumab was cut by 21% or the cost of ipilimumab down by 24% in patients with a PD-L1 expression <1% at a WTP threshold of $150,000 per QALY.

Probabilistic sensitivity analysis suggested that, compared with chemotherapy, nivolumab plus ipilimumab yield 65.3%, 55.2%, and 43.1% probability of cost-effectiveness at a WTP threshold of $150,000 per QALY for patients with a PD-L1 ≥50%, ≥1% and <1% respectively (**Figure 2** and **Supplementary Figure 2**). There was an 81.1% chance that nivolumab plus ipilimumab was cost-effective for patients with a high TMB (**Figure 3** and **Supplementary Figure 3**).

As the results of the subgroup analyses demonstrated, the ICER of nivolumab plus ipilimumab could be most cost-effective for patients with male, squamous histologic type, bone or central

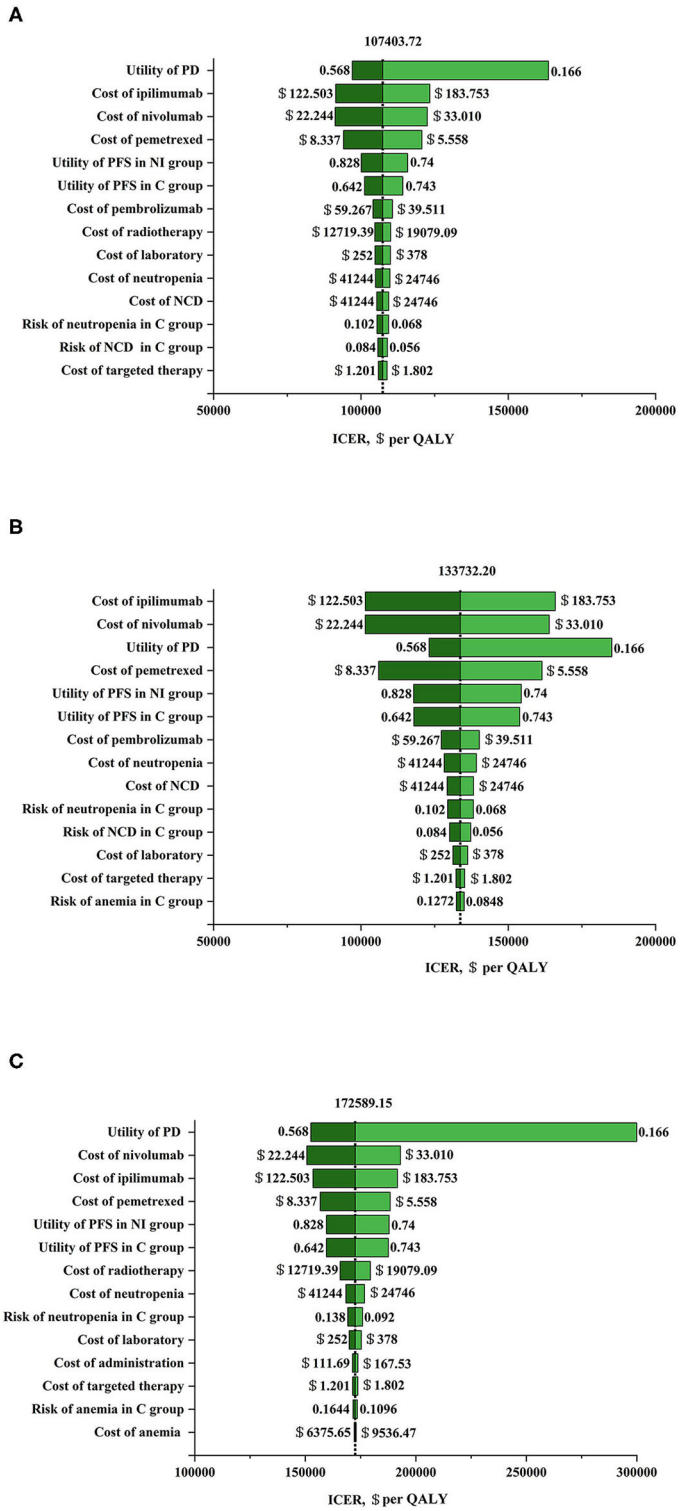

FIGURE 1 | Tornado diagram for one-way sensitivity analysis. **(A)** Nivolumab plus ipilimumab vs. chemotherapy in programmed death ligand 1 (PD-L1) ≥ 50% population. The parameters tested in this one-way sensitivity analysis were displayed in the right of the figure. The vertical dotted line represents incremental cost-effective ratio (ICER) $107403.72/ quality adjusted life year (QALY) (the results of baseline analysis). **(B)** Nivolumab plus ipilimumab vs. chemotherapy in PD-L1 ≥ 1% population. The parameters tested in this one-way sensitivity analysis were displayed in the right of the figure. The vertical dotted line represents ICER $133732.20/QALY (the results of baseline analysis). **(C)** Nivolumab plus ipilimumab vs. chemotherapy in PD-L1 < 1% population. The parameters tested in this one-way sensitivity analysis were displayed in the right of the figure. The vertical dotted line represents ICER $172589.15/QALY (the results of baseline analysis). C group, chemotherapy group; NCD, neutrophil count decreased; NI group, nivolumab plus ipilimumab group; PD, progressive disease; PFS, progression-free survival.

nervous system metastases, regardless of PD-L1 expression levels (**Supplementary Tables 1**, **2**).

DISCUSSION

The phase 3 study CheckMate 227 was the first trial that showed positive results in dual checkpoint inhibition (anti-CTLA-4 and PD-1) in the field of lung cancer. Previous studies suggested that combination immunotherapy with nivolumab plus ipilimumab could be considered a cost-effect choice in intermediate- and poor-risk patients with metastatic renal cell carcinoma in the US (31, 34). However, it is unclear whether treatment with nivolumab plus ipilimumab as first-line therapy for patients with advanced NSCLC is cost-effective.

The current study is the first cost-effectiveness analysis of nivolumab plus ipilimumab vs. chemotherapy in previously untreated advanced NSCLC patients with different PD-L1 expressions (≥50, ≥1, and <1%) and a high TMB. Case-based results indicated that the ICERs of nivolumab plus ipilimumab vs. chemotherapy were $107403.72, $133732.20, and $172589.15 per additional QALY in PD-L1 ≥50, ≥1, and <1% populations, respectively. The one-way sensitivity analyses revealed that the cost of nivolumab plus ipilimumab and the utility value of PD were the greatest influence factors in all PD-L1 populations. The probabilistic sensitivity analyses depicted a high likelihood that nivolumab plus ipilimumab would be considered a cost-effective choice at a WTP threshold of $150,000 per QALY in the PD-L1 ≥50 and 1% populations, whereas it is not cost-effective in the PD-L1 < 1% populations. Further analysis indicated that the nivolumab plus ipilimumab strategy would be cost-effective at a WTP threshold of $150,000 per QALY for PD-L1 < 1% populations by reducing the cost of nivolumab or ipilimumab. In addition, irrespective of tumor PD-L1 expression levels, the ICER of nivolumab plus ipilimumab vs. chemotherapy was $69182.50 for high TMB populations. On the other hand, the results of subgroup analysis exhibited that the ICER of nivolumab plus ipilimumab could be improved by selecting patients in accordance with clinical and pathological parameters (**Supplementary Tables 1**, **2**).

In history, PD-L1 expression has been regarded as a major biomarker of response to PD-1 and PD-L1 inhibitors in light of mechanism of action. The outcomings of a *post hoc* analysis from CheckMate 026, nonetheless, implied that the application of TMB as a predictive biomarker instead of or in addition to PD-L1 expression may be conducive to selecting patients with advanced NSCLC who embrace great possibility of reaping benefits of immunotherapy (35). TMB is an emerging biomarker of immunotherapy outcomes for lung cancer (35–39). The results of CheckMate 568 showed the TMB of more than 10 mutations per megabase could be used as an effective cutoff value for selecting the most likely responding patients (40). It was observed in clinical experience that tumor PD-L1 expression and TMB had no significant correlation between the two biomarkers. Similarly, the analysis results obtained by Hellmann MD et al. attested that first-line treatment with nivolumab plus ipilimumab provided clinical benefits for patients with NSCLC and a high

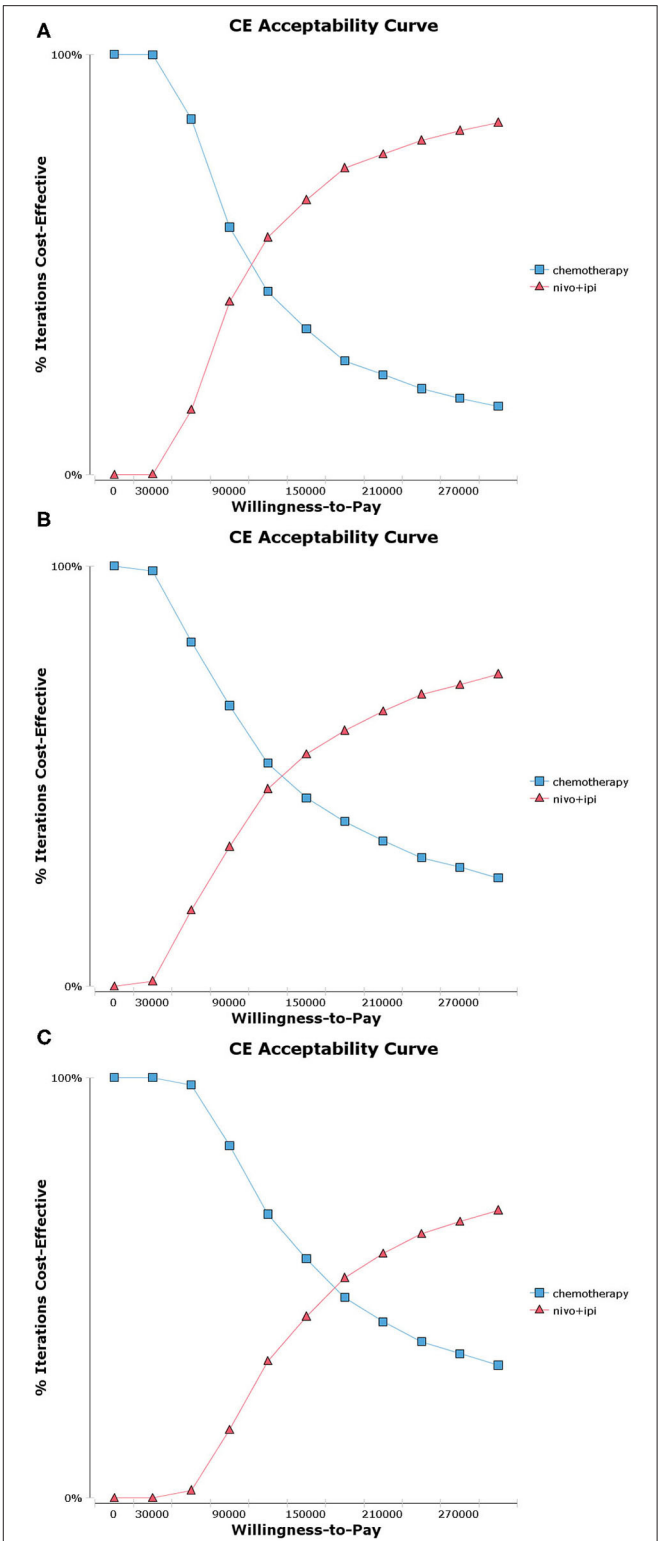

FIGURE 2 | Acceptability curves for the choice of nivolumab plus ipilimumab and chemotherapy treatment strategies at different willingness-to-pay (WTP) thresholds in patients with advanced NSCLC. **(A)** Nivolumab plus ipilimumab vs. chemotherapy in programmed death ligand 1 (PD-L1) ≥ 50% population. **(B)** Nivolumab plus ipilimumab vs. chemotherapy in PD-L1 ≥ 1% population. **(C)** Nivolumab plus ipilimumab vs. chemotherapy in PD-L1 < 1% population.

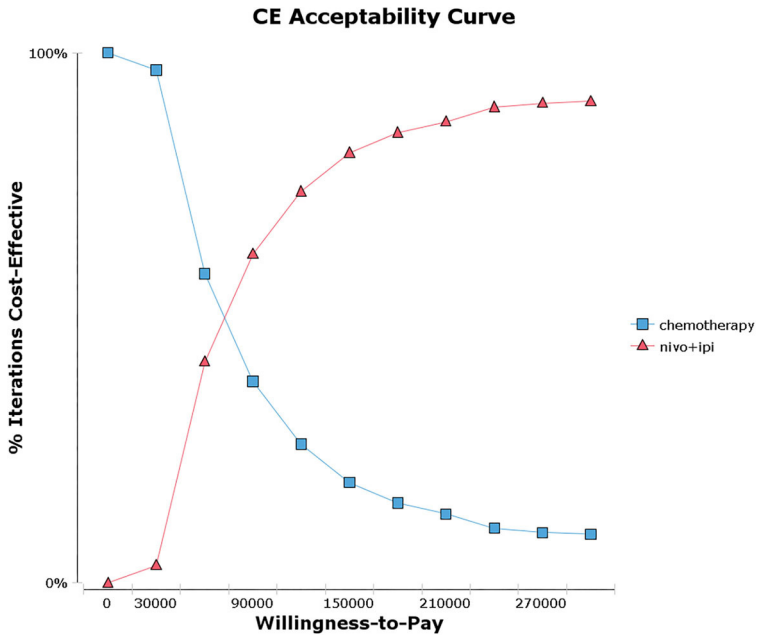

FIGURE 3 | Acceptability curves for the choice of nivolumab plus ipilimumab and chemotherapy treatment strategies at different willingness-to-pay (WTP) thresholds in patients with advanced NSCLC and high tumor mutational burden (TMB).

TMB (≥10 mutations per megabase), regardless of their tumor PD-L1 expression levels (18). We confirmed this in current analysis, as the ICER was $69182.50 in advanced NSCLC patients with a high TMB, irrespective of their tumor PD-L1 expression levels, which was lower than the values of ICER in three PD-L1 expression populations (≥50, ≥1, and <1%). Moreover, the results of probabilistic sensitivity analyses revealed an 81.1% chance of nivolumab plus ipilimumab vs. chemotherapy being cost-effective in patients with a high TMB, which is higher than those of the three PD-L1 expression populations (≥50, ≥1, and <1%). Despite that nivolumab plus ipilimumab provided the greatest absolute survival for patients with a high TMB in CheckMate 227, yet the clinical benefits of nivolumab plus ipilimumab and those of chemotherapy were similar in patients regardless of their TMB. The unexpected impacts of TMB on the overall survival of patients receiving chemotherapy may be the cause of these results (41–45). Thus, the benefits of nivolumab plus ipilimumab may be overestimated or underestimated in our analysis. Therefore, it is necessary to understand the functions of TMB as a biomarker before including it into clinical practice.

Pembrolizumab monotherapy, pembrolizumab plus chemotherapy and atezolizumab combined with bevacizumab and chemotherapy are the first-line immunotherapy in advanced NSCLC. The following are some related cost-effectiveness analyses based on cases in the US. Atezolizumab combined with bevacizumab and chemotherapy was not a cost-effective choice for patients with advanced NSCLC (46). In contrast, pembrolizumab plus chemotherapy was estimated to be cost-effective in advanced NSCLC from the US payers' view (47, 48). Pembrolizumab monotherapy for patients with PD-L1 ≥ 1% is the only chemotherapy-spared therapy approved by US FDA for the first-line treatment of advanced NSCLC patients based on the trial of KEYNOTE-042 (10). The cost-effectiveness analysis of KEYNOTE-042 displayed that ICERs of $136228.82, $160625.98, and $179530.17 per QALY for advanced NSCLC patients with PD-L1 ≥50, ≥20, and ≥1%, respectively, and that pembrolizumab monotherapy was cost-effective in patients with PD-L1 ≥ 50% but not in the ≥20 and 1% populations at a WTP threshold of $150,000 per QALY in the US (49). In our analysis, it estimated that compared with chemotherapy, nivolumab plus ipilimumab was cost-effective in advanced NSCLC patients with PD-L1 ≥ 50% and PD-L1 ≥ 1% but not in the PD-L1 < 1% populations at a WTP threshold of $150,000 per QALY. It means that the indication of nivolumab plus ipilimumab has expanded compared with that of PD-1 inhibitor monotherapy alone. Moreover, treatment with nivolumab plus ipilimumab is a cost-effective choice for patients with PD-L1 ≥ 1% who desire chemotherapy-free treatment. In addition, regardless of the PD-L1 expression levels, nivolumab plus ipilimumab was cost-effective in patients with a high TMB. However, due to the absence of head-to-head trials of pembrolizumab vs. nivolumab plus ipilimumab, caution should be in place before drawing any conclusion of which treatment would be more cost-effective.

Our model estimated the cost and effect over the entire runtime of the model, and then obtained results. However, we noted that the survival curves of the nivolumab plus ipilimumab group and the chemotherapy group started to cross ∼6 months after treatment, which indicated that the efficacy of the chemotherapy group was better than that of the nivolumab plus ipilimumab group within the first 6 months. It

was consistent with the survival data provided by KEYNOTE-042 (10, 50). This may indicate that patients receiving nivolumab plus ipilimumab treatment, if not gaining benefits from combination immunotherapy, would progress rapidly or die within 6 months of treatment. These statistics suggest that in unsegmented populations, these patients receiving immunotherapy are likely to risk rapid progress or death. This may lead our model to overestimate the benefits of treatment with nivolumab plus ipilimumab in this treatment period.

We also discovered that the mortality risk in patients with PD-L1 expression of 1–49% had no statistical significance (hazard ratio 0.94, 95% CI 0.75–1.18) when compared to that in the PD-L1 $\geq$ 50% (hazard ratio 0.70, 95% CI 0.55–0.90) and $\geq$1% (hazard ratio 0.79, 95% CI 0.65–0.96) populations. And further analysis has found that the populations with PD-L1 $\geq$ 1% did not exclude the PD-L1 $\geq$ 50% populations, and that the majority of benefits in the former were manifested in the latter. Thus, it may overestimate the cost-effectiveness of benefits of nivolumab plus ipilimumab in patients with PD-L1 expression of 1–49%. Careful deliberation is called for should these patients be to use combination immunotherapy. Furthermore, patients with PD-L1 expression of 1–49% were not estimated in our analysis, because the trial of CheckMate 227 did not report enough survival data on these patients.

Like any other models, there are some limitations in our analysis. First, the survival data shows a dramatic tail on the OS curve in CheckMate 227, and the benefits in the dual checkpoint inhibition group will become more significant when compared to those of the chemotherapy with a long-term follow-up. It may underestimate the benefits of combined immunotherapy because our study is based on data of a 29.3-months follow-up. Moreover, our model adds too much weight to PFS and PD. However, most cost-effectiveness analyses of immune checkpoint inhibitors were based on Markov model (31, 34, 46, 51). Thus, the results of the present study should be interpreted with discretion, especially those of the PD-L1 $<$ 1% populations. Second, immunotherapy-related AEs are rare, and the cost of treatment in such cases is rather high. Therefore, more cases of immunotherapy-related AEs would be conducive to more accurate evaluation of AE cost for patients using nivolumab plus ipilimumab. Besides, the benefits of nivolumab plus ipilimumab would be overestimated in this model. Third, considering the model hypothesis, the exploratory nature of the subgroup analyses and the small sample subgroup size, the results of the subgroup analyses in the current study should be analyzed with caution. Fourth, standard data was commonly used in our model to estimate drug dose, and it should be adjusted according to the patients' physical conditions which may generate bias.

CONCLUSION

When compared to chemotherapy, nivolumab plus ipilimumab as first-line treatment is cost-effective in advanced NSCLC patients with PD-L1 $\geq$ 50%, PD-L1 $\geq$ 1% or a high TMB but is not cost-effective in PD-L1 $<$ 1% population in view of US payers.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The current research was underlain by model techniques and literature reviews; no written consent was required as per the ethics committee of the Xiangya Hospital of Central South University (Changsha, People's Republic of China).

AUTHOR CONTRIBUTIONS

HH, LS, and JH conceived and designed the experiments. HH, LS, LY, and DD performed the experiments. HH, LS, LY, DD, JH, YS, and ML analyzed the data. SZ, JH, and DC contributed reagents, materials, and analysis tools. HH, LS, and JH wrote the manuscript. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Supplementary Figure 1 | Markov states. To illustrate the disease development process of advanced non-small cell lung cancer (NSCLC).

Supplementary Figure 2 | The results of Monte Carlo probabilistic sensitivity analysis. **(A)** Nivolumab plus ipilimumab vs. chemotherapy in programmed death ligand 1 (PD-L1) $\geq$ 50% population. **(B)** Nivolumab plus ipilimumab vs. chemotherapy in PD-L1 $\geq$ 1% population. **(C)** Nivolumab plus ipilimumab vs. chemotherapy in PD-L1 $<$ 1% population.

Supplementary Figure 3 | The results of Monte Carlo probabilistic sensitivity analysis of nivolumab plus ipilimumab vs. chemotherapy in patients with high tumor mutational burden (TMB).

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REVIEW
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Adverse Effects of Anti-PD-1/PD-L1 Therapy in Non-small Cell Lung Cancer

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Currently, immunotherapy has shown great efficacy in clinical trials, and monoclonal antibodies directed against immune checkpoint PD-1/PD-L1 have shown encouraging results in first-line or second-line treatment of non-small cell lung cancer patients. Meanwhile, anti-PD-1/PD-L1 immune checkpoint drugs combined with other treatments, such as chemotherapy, targeted therapy as well as anti-CTLA-4 checkpoint therapy, are considered an attractive treatment with higher efficacy. However, toxicity associated with PD-1/PD-L1 blockade is worth attention. Understanding the adverse effects caused by anti-PD-1/PD-L1 immunosuppressive agents is vital to guide the clinical rational use of drug. In this review, we summarized the adverse effects that occurred during the clinical use of anti-PD-1/PD-L1 inhibitors in the treatment of non-small cell lung cancer and discussed how to effectively manage and respond to these adverse reactions.

Keywords: adverse effects, PD-1/PD-L1, immunotherapy, immune checkpoint inhibitor, non-small cell lung cancer

INTRODUCTION

Currently, cancer is still a key threat to human health (1). Among them, lung cancer is the leading cause of cancer-related deaths worldwide, and about 80% of lung cancer is non-small cell lung cancer (NSCLC), with poor prognosis (2, 3). Encouragingly, the blockade of immune checkpoints against PD-1/PD-L1 has dramatically changed the treatment prospects for patients with NSCLC (4–6). The traditional treatments of cancer are mainly target at the cancer cells themselves, while the main goal of tumor immunotherapy is to enhance or restore the monitoring and killing effect of the body's immune system on tumors (7–9). There are many immune checkpoint molecules in the body, which are involved in maintaining the body's immune balance and its own immune tolerance (10). Among them, PD-1 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) are classic co-inhibitory molecules that suppress the immune response (11–13). Tumor cells overexpress the immunosuppressive surface ligand PD-L1, which interacts with T cell molecules, leading to T cell failure (14, 15). Knowledge based on the immune escape mechanism of cancer cells has led to the development of immunological checkpoint inhibitors (16, 17).

In recent years, immune checkpoint inhibitors (ICIs) have been widely used in tumor immunotherapy (18, 19). ICIs based on the PD-1/PD-L1 axis have been proved to exhibit promising therapeutic effects in a variety of advanced cancers (20–22). For example, the anti-PD-1

ICIs nivolumab and pembrolizumab have shown exciting results in the treatment of metastatic melanoma and NSCLC (23, 24). Moreover, anti-PD-L1 antibody durvalumab, atezolizumab as well as avelumab have also shown anti-tumor activity in a number of tumor types. However, it is worth noting that as the immune system is reactivated, the body's immune tolerance imbalance occurs (10). Immunotherapy leads to the emergence of novel toxic features, known as immune-related adverse events (irAEs), by reactivating the immune system (14). Although severe irAEs are rare, they can be life-threatening without intervention and proper management (25, 26). In addition, it has also been reported that the combined use of PD-1/PD-L1 ICIs with chemotherapeutics or other targeted therapies leads to the emergence of new toxic reactions (14). Therefore, raising our awareness of these adverse events (AEs) is critical to optimize the clinical efficacy and safety of these new immunotherapeutic.

In this review, we summarized the adverse reactions of the five FDA-approved targeted PD-1/PD-L1 immune checkpoint drugs currently used in the clinic when used alone or in combination with other treatments in NSCLC patients. We aim to raise awareness of the clinical manifestations, diagnosis, and management of these toxic reactions through our summary.

MECHANISM OVERVIEW OF PD-1/PD-L1 BLOCKADE

PD-1, also known as CD279, is a type I transmembrane protein of the immunoglobulin superfamily (27). As a transmembrane protein, PD-1 inducibly expressed on the surface of activated T cells, B cells, NKT cells and antigen presenting cells (APC) (15, 28). PD-1 interacts with two major ligands, PD-L1 and PD-L2, resulting in disruption of intracellular signaling and down-regulation of effector T cell function (18, 29). The binding affinity of PD-1 and PD-L1 is three times than of PD-1 and PD-L2 (30). Studies showed that PD-1 is expressed in multiple type of cells, including T cells, B cells, dendritic cells, monocytes as well as tumor-infiltrating lymphocytes (TILs), while PD-L1 is expressed in cancer cells and APC (31, 32). PD-L1 expression is mainly affected by Toll-like receptors (TLRs) (33, 34). TLR-mediated PD-L1 regulation is dependent on activation of MEK/ERK kinase, which enhances PD-L1 messenger RNA (mRNA) transcription by nuclear factor kappa B (35). PD-L1 interacts with PD-1 expressed on T cells, leading to the negative regulation of effector T cell activation, thereby causing cancer cells to secrete the proinflammatory cytokines, such as TNF-α, IL-2, and IFN-γ, and become more aggressive (30). IFN-γ receptors 1 and 2 are also involved in the regulation of PD-L1 expression, primarily through JAK/STAT-mediated IRF-1 activation (35). In addition, other immunosuppressive cells in the tumor microenvironment (TME), such as regulatory T cells, tumor-associated macrophages and myeloid-derived suppressor cells, also express PD-1 to maintain a highly immunosuppressive microenvironment (**Figure 1**) (36, 37).

ADVERSE EFFECTS BASED ON PD-1/PD-L1 BLOCKADE FOR NSCLC THERAPY

To date, several anti-PD-1/PD-L1 immune checkpoint agents have been approved for the treatment of NSCLC, including two anti-PD-1 drugs pembrolizumab and nivolumab, as well as three anti-PD-L1 drugs atezolizumab, durvalumab and avelumab (38, 39). Blocking of PD-1/PD-L1 immune checkpoint leads to the development of new toxicities by reactivation of the immune system, also known as irAEs (26). These irAEs may affect multiple organ systems and tissues, with clinical manifestations of autoimmune-like/inflammatory side effect that may cause damage to the skin, lungs, gastrointestinal tract, liver, endocrine glands, and skeletal muscle (12). In addition, the most common treatment-related adverse events (TRAEs) include fatigue, fever/chillness and infusion reactions (9). Furthermore, rare and serious TRAEs have been reported, including immune-related encephalitis (40), myasthenia gravis (41), acute renal failure/interstitial nephritis (42), and myocarditis (43). Here, we list the TRAEs caused by PD-1 and PD-L1 inhibitors in the treatment of NSCLC in **Tables 1**, **2**, respectively, both monotherapy and combination therapy are included.

COMPARISON OF THE TOXICITY SPECTRUM BETWEEN PD-1 AND PD-L1 INHIBITORS IN THE TREATMENT OF NSCLC

At present, although various PD-1 and PD-L1 ICIs have shown activity in NSCLC, it is meaningful to analyze and compare the differences in their toxicity profiles (69). According to the results of a systematic meta-analysis by Pillai et al., there was no significant difference in the overall incidence of AEs between the PD-1 treatment group ($n = 3284$) and the PD-L1 treatment group ($n = 2460$) (69–71). However, any grade of irAEs in the PD-1 treatment group was slightly higher than the PD-L1 treatment group (16 vs. 11%; $p = 0.07$) (69). The most common AE of PD-1 and PD-L1 inhibitors is fatigue (19 vs. 21%, $p = 0.4$), while the most common irAE is hypothyroidism (6.7 vs. 4.2%; $p = 0.07$) (69). It was worth noting that in patients receiving PD-1 inhibitors, the incidence of pneumonitis was significantly higher than in the PD-L1 agents treatment group (4 vs. 2%; $P = 0.01$) (69, 70). Therefore, clinicians should be more alert to lung inflammation in NSCLC patients receiving PD-1 blockade therapy (69).

At present, there is no systematic study to analyze the differences in the toxic and side effects of PD-1/PD-L1 inhibitors alone or in combination with other therapies for NSCLC. However, the current clinical trial data seems to indicate that the overall incidence of AEs of PD-1/PD-L1 inhibitor monotherapy is lower than that of combination therapy. For example, any grade of TRAEs that occurred with pembrolizumab monotherapy was 70.9% (24), while pembrolizumab combined with chemotherapy showed a higher incidence of TRAEs (98.2%) (50). Several other

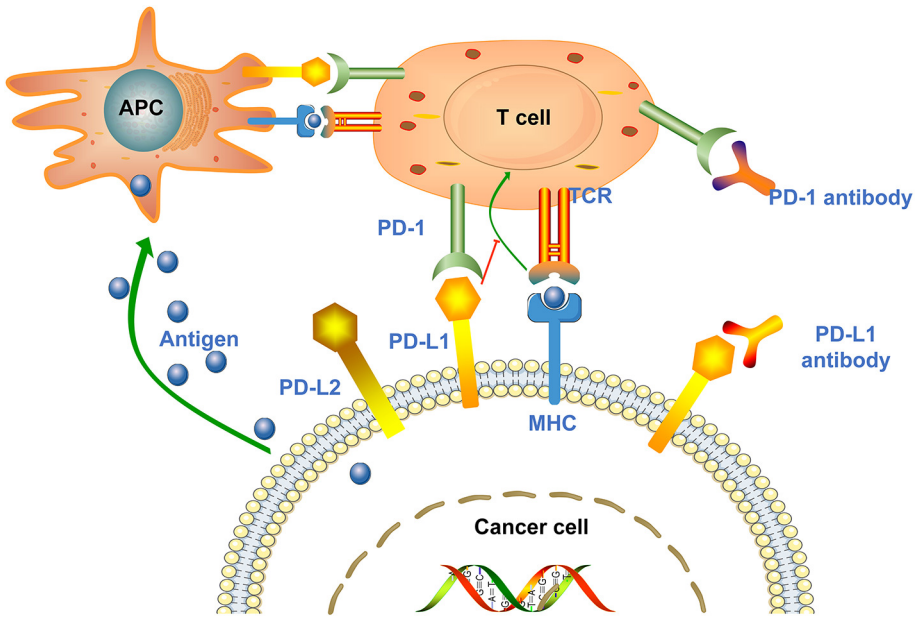

FIGURE 1 | Mechanisms of cancer cell mediated immune escape. Antigen presenting cells (APCs) absorb antigens released by cancer cells and present them to T cells to promote T cells activation and high expression of PD-1. Upon T cell activation, the PD-1 receptor binds to PD-L1/PD-L2 expressed on the surface of cancer cells and suppresses the immune response. In addition, tumor cells can also present antigens directly to activated T cells in the context of MHC. Anti-PD-1/PD-L1 antibodies can block the above process and enhance the body's immune response.

clinical trials of PD-1/PD-L1 inhibitors that have been approved for the treatment of NSCLC also showed the same trend (54, 56).

MANAGEMENT OF ORGAN-SPECIFIC TOXICITIES CAUSED BY ANTI-PD-1/PD-L1 TREATMENT

Skin-Related Adverse Events

Rash and pruritus are the most common skin irAEs that occur in NSCLC patients receiving anti-PD-1/PD-L1 immune checkpoint treatment (12). Skin-related irAEs usually occur after the second cycle of the patient's clinical course (72, 73). Other dermatological lesions include vitiligo, skin capillary hyperplasia (CCEP), lichenoid and bullous pemphigoid (74). Despite frequent reports of immune-related skin AEs, the incidence of skin AEs of grade III or higher is low, and life-threatening AEs are only occasionally reported, but still deserve attention (74). For PD-1/PD-L1 monotherapy, the incidence of treatment-related skin AEs of any grade is ∼7–31%, and the incidence of grade III or higher AEs is lower. Existing clinical trial data showed that the incidence of skin-related AEs of anti-PD-1 monotherapy was slightly higher than that of anti-PD-L1 monotherapy (11–31 vs. 7–19%) (24, 53, 67, 75). In addition, the emergence of skin toxicity caused by pembrolizumab seems to be more frequent than other anti-PD-1/PD-L1 agents (24, 67, 75). However, there was no significant difference in the incidence of skin-related AEs between anti-PD-1/PD-L1 monotherapy and combination therapy (24, 47). Recent studies have shown that patients with complete/partial remission have a higher incidence of skin adverse reactions than patients with stable/progressive disease, suggesting that skin AEs may be a positive prognostic factor for patients, but more prospective studies are still needed to further verify this kind of association (76, 77). However, a basic skin examination is necessary for patients using ICIs, especially those with previous inflammatory skin diseases. Standard dermatological assessments include skin biopsies, kidney and liver function tests, serum tryptase as well as immunoglobulin E levels (74).

For mild (grade I–II) maculopapular patients, it may be managed successfully with moderate potency topical steroids to affected areas and/or oral prednisone 0.5–1 mg/kg/day (78). For grade III–IV maculopapular, immunotherapy may be temporarily held and patients should be treated with high potency topical steroids to affected areas and oral prednisone 0.5–1 mg/kg/day (increase dose up to 2 mg/kg/day if no improvement) (79). In addition, topical emollients, oral antihistamines and lidocaine patches are effective for pruritus. For patients with severe pruritus, the GABA agonists (gabapentin, pregabalin) are useful, and aprepitant or omalizumab can be used in refractory cases (79).

Respiratory System Related Adverse Events

Anti-PD-1/PD-L1 immunotherapy also frequently occurs respiratory system-related AEs, especially for patients with lung cancer, the incidence of such AEs seems to be higher (69). Among them, immune-related pneumonia is the most common. Pneumonia is defined as focal or diffuse inflammation of the lung parenchyma, including pulmonary sarcoidosis and organizing

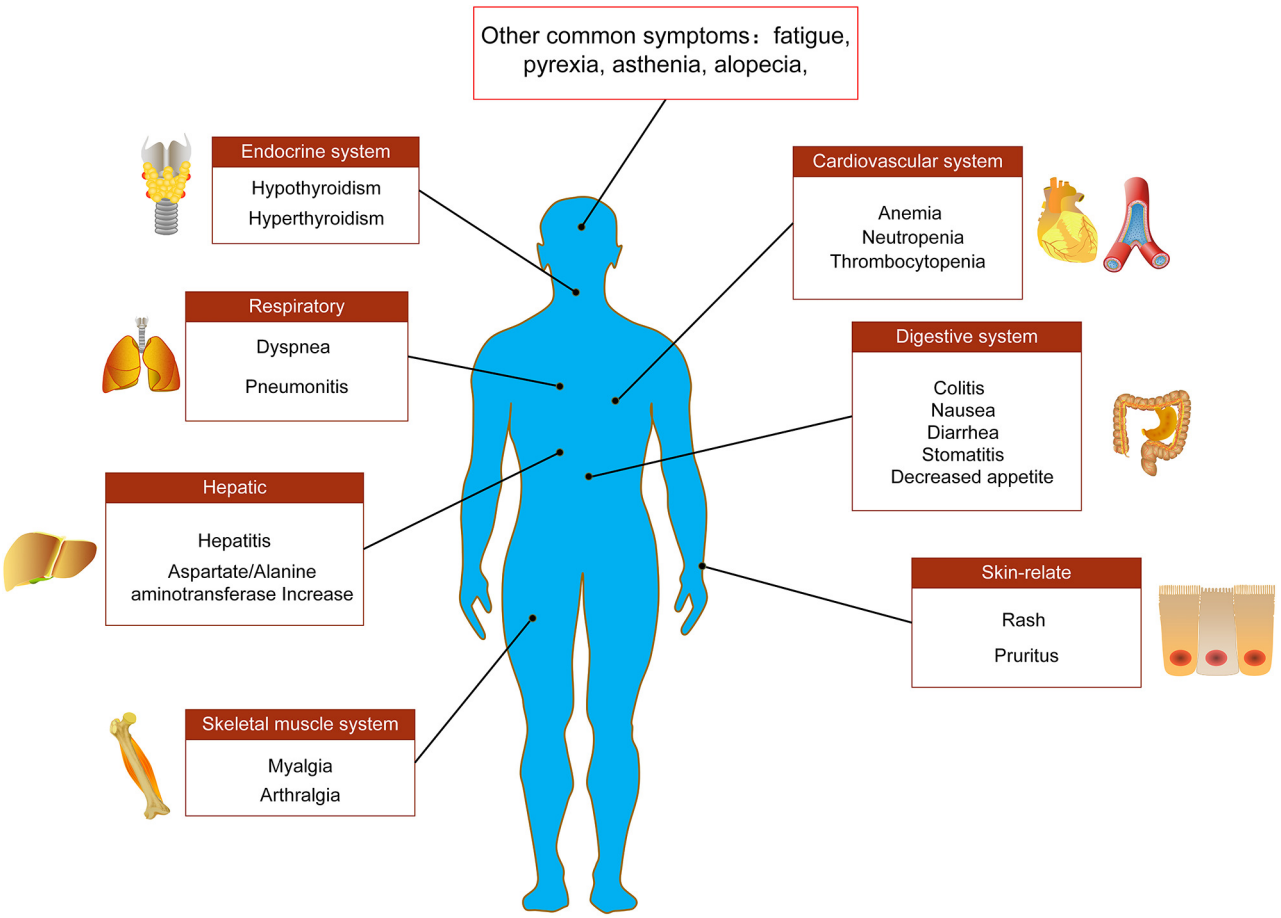

FIGURE 2 | Main adverse events of PD-1/PD-L1 immunotherapy. Adverse events associated with PD-1/PD-L1 immune checkpoint inhibitors in the treatment of NSCLC involve multiple tissues and organs, including endocrine system, respiratory system, digestive system, cardiovascular system, skeletal muscle system, liver, and skin-related adverse reactions.

inflammatory pneumonitis (80). Once pneumonia occurs, it may endanger the life of the patient, so active interventions should be taken (12, 80). The incidence of pneumonia is generally 7.4–24.3 months after the start of treatment. The clinical symptoms are mainly dry cough, dyspnea, fever, and chest pain (12, 81). It is worth noting that the combination of ICIs and other drugs at risk of pneumonia will increase the incidence of pneumonia (82). Chen et al. (83) reported an unpredictable but relatively severe radiation recall pneumonitis (RRP), which was induced by anti-PD-1 inhibitor camrelizumab 2 years after radiotherapy. This indicated that previous radiotherapy combined with subsequent anti-PD-1 immunotherapy may result in overlapping damage to lung (83). Moreover, patients with other underlying lung diseases, such as COPD and pulmonary fibrosis, should be more alert to the occurrence of pneumonia (84, 85).

Chest CT is a key method for diagnosing pneumonia. The imaging features are ground-glass lesions and/or disseminated nodular infiltrates (12, 86). According to the management of the latest NCCN guidelines, any level of immune-related pneumonia should hold immunotherapy, and patients with severe pneumonia should permanently discontinue immunotherapy. Patients with mild (grade I) pneumonia need to re-evaluate arterial oxygen saturation (both resting and active) and repeat chest CT in 4 weeks or as clinically indicated for worsening symptoms (78, 87). For grade II or higher pneumonia should first rule out bacterial infections, such as nasal swab for potential viral pathogens, sputum culture, blood culture, and urine antigen test to detect pneumococcus and legionella (87). Additionally, bronchoscopy and bronchoalveolar lavage are necessary. If the infection cannot be completely ruled out, empiric antibiotics can be used. Management is guided by clinical symptoms, such that grade II pneumonia patients can be taken orally or intravenously prednisone/methylprednisolone 1–2 mg/kg/day (86, 87). Severe cases require hospitalization and intravenous methylprednisolone 1–2 mg/kg/day. Other forms of immunosuppression may be considered, such as infliximab, mycophenolate mofetil or intravenous immunoglobulin, if corticosteroids remain ineffective after 48 h of treatment (86, 87).

TABLE 1 | Adverse effects base on anti-PD-1 antibodies.

Agent	Phase	Clinical Trials.gov	No. of patients	Therapy schedule	TRAEs (Any grade)	Treatment-related serious AEs (grade 3–5)	References
Pembrolizumab	I	NCT01295827	495	2 or 10 mg/kg, Q3W or 10 mg/kg, Q2W	Total: 70.9%, $n = 351$ Fatigue (19%, $n = 96$) Pruritus (11%, $n = 53$) Decreased appetite (11%, $n = 52$) Rash (10%, $n = 48$) Arthralgia (9%, $n = 45$) Diarrhea (8%, $n = 40$) Nausea (8%, $n = 37$) Hypothyroidism (7%, $n = 34$)	Total: 9.5%, $n = 47$ Decreased appetite (1%, $n = 5$) Asthenia (1%, $n = 5$) Dyspnea (4%, $n = 19$) Pneumonitis (2%, $n = 9$)	(24)
			101	2 or 10 mg/kg, Q3W or Q2W	Total: 85%, $n = 86$ Fatigue (28%, $n = 28$) Pruritus (15%, $n = 15$) Hypothyroidism (14%, $n = 14$) Rash (14%, $n = 14$) Arthralgia (12%, $n = 12$) Nausea (12%, $n = 12$) Dyspnea (9%, $n = 9$) Diarrhea (8%, $n = 8$)	Total: 12%, $n = 12$ Hypertension (1%, $n = 1$) Colitis (1%, $n = 1$) Dehydration (1%, n=1) Dyspnea (1%, $n = 1$) Pneumonitis (1%, $n = 1$)	(44)
Pembrolizumab	III	NCT02220894	636	200 mg, Q3W	Total: 63%, $n = 399$ Hypothyroidism (11%, $n = 69$) Fatigue (8%, $n = 50$) Pruritus (7%, $n = 46$) Rash (7%, n=46) Alanine aminotransferase increased (7%, $n = 45$) Pneumonitis (7%, $n = 43$) Decreased appetite (6%, $n = 40$) Hyperthyroidism (6%, $n = 37$)	Total: 18%, $n = 113$ Pneumonitis (3%, $n = 20$) Alanine aminotransferase increased (1%, $n = 9$) Hypothyroidism (<1%, $n = 1$) Fatigue (<1%, $n = 3$)	(45)
Pembrolizumab	II/III	NCT01905657	691	2 or 10 mg/kg, Q3W	Total: 64%, $n = 441$ Fatigue (28%, $n = 95$) Decreased appetite (24%, $n = 79$) Nausea (20%, $n = 68$) Rash (22%%, $n = 73$) Diarrhea (13%, $n = 46$) Asthenia (12%, $n = 39$) Stomatitis (6%, $n = 20$) Anemia (7%, $n = 24$)	Total: 14%, $n = 98$ Fatigue (3%, $n = 10$) Decreased appetite (<2%, $n = 4$) Nausea (<2%, $n = 3$) Diarrhea (1%, $n = 2$)	(46)
Pembrolizumab	III	NCT02142738	154	200 mg, Q3W	Total: 73%, $n = 113$ Diarrhea (14%, $n = 22$) Pyrexia (10%, n=16) Fatigue (10%, $n = 16$) Nausea (10%, $n = 15$) Decreased appetite (9%, $n = 14$) Anemia (5%, $n = 8$) Constipation (4%, $n = 6$) Vomiting (3%, $n = 4$)	Total: 27%, $n = 41$ Diarrhea (4%, $n = 6$) Anemia (2%, $n = 3$) Fatigue (1%, $n = 2$)	(47)
Pembrolizumab + pemetrexed + carboplatin	II	NCT02039674	59	Pembrolizumab 200 mg, Q3W plus chemotherapy	Total: 93%, $n = 55$ Fatigue (61%, $n = 36$) Nausea (56%, $n = 33$) Anemia (20%, $n = 12$) Vomiting (25%, $n = 15$) Rash (25%, $n = 15$) Decreased appetite (19%, $n = 11$) Diarrhea (20%, $n = 12$) Increased aspartate (17%, $n = 10$)	Total: 39%, $n = 23$ Fatigue (3%, $n = 2$) Acute kidney injury (3%, $n = 1$) Anemia (12%, $n = 7$) Neutropenia (3%, $n = 2$) Decreased neutrophil count (3%, $n = 2$)	(48)
Pembrolizumab + pemetrexed + platinum-based drug	III	NCT02578680	405	Pembrolizumab 200 mg, Q3W plus chemotherapy	Total: 99%, $n = 404$ Nausea (56%, $n = 225$) Fatigue (46%, $n = 187$) Anemia (41%, $n = 165$) Constipation (35%, $n = 141$)	Total: 67%, $n = 272$ Anemia (16%, $n = 66$) Neutropenia (15.8%, $n = 64$) Thrombocytopenia (8%, $n = 32$) Asthenia (6%, $n = 25$)	(49)

(Continued)

TABLE 1 | Continued

Agent	Phase	Clinical Trials.gov	No. of patients	Therapy schedule	TRAEs (Any grade)	Treatment-related serious AEs (grade 3–5)	References
					Diarrhea (31%, $n = 125$) Decreased appetite (28%, $n = 114$) Neutropenia (27%, $n = 110$) Vomiting (24%, $n = 98$)	Fatigue (6%, $n = 23$) Diarrhea (5%, $n = 21$) Nausea (4%, $n = 14$)	
Pembrolizumab + carboplatin + paclitaxel or nab-paclitaxel	III	NCT02775435	278	Pembrolizumab 200 mg, Q3W plus chemotherapy	Total: 98%, $n = 273$ Anemia (53%, $n = 148$) Alopecia (46%, $n = 128$) Neutropenia (38%, $n = 105$) Nausea (36%, $n = 99$) Thrombocytopenia (31%, $n = 85$) Diarrhea (30%, $n = 83$) Decreased appetite (25%, $n = 68$) Constipation (23%, $n = 64$)	Total: 70%, $n = 194$ Anemia (16%, $n = 43$) Neutropenia (23%, n=63) Thrombocytopenia (7%, $n = 19$) Diarrhea (4%, $n = 11$) Decreased appetite (2%, $n = 6$)	(50)
Nivolumab	III	NCT01642004	131	3 mg/kg, Q2W	Total: 58%, $n = 76$ Fatigue (16%, $n = 21$) Decreased appetite (11%, $n = 14$) Asthenia (10%, $n = 13$) Nausea (9%, $n = 12$) Diarrhea (8%, $n = 10$) Arthralgia (5%, $n = 7$) Pneumonitis (5%, $n = 6$) Pyrexia (5%, $n = 6$)	Total: 7%, $n = 9$ Fatigue (1%, $n = 1$) Decreased appetite (1%, $n = 1$) Leukopenia (1%, $n = 1$)	(51)
Nivolumab	III	NCT01673867	287	3 mg/kg, Q2W	Total: 69%, $n = 199$ Fatigue (16%, $n = 46$) Nausea (12%, $n = 34$) Decreased appetite (10%, $n = 30$) Asthenia (10%, $n = 29$) Diarrhea (8%, $n = 22$) Peripheral edema (3%, $n = 8$) Myalgia (2%, $n = 7$) Anemia (2%, $n = 6$)	Total: 10%, $n = 30$ Fatigue (1%, $n = 3$) Nausea (1%, $n = 2$) Asthenia (<1%, $n = 1$) Diarrhea (<1%, $n = 2$)	(52)
Nivolumab	II	NCT01721759	117	3 mg/kg, Q2W	Total: 74%, $n = 87$ Fatigue (33%, $n = 38$) Asthenia (12%, $n = 14$) Nausea (15%, $n = 18$) Diarrhea (10%, $n = 12$) Decreased appetite (19%, $n = 22$) Rash (11%, $n = 13$) Anemia (6%, $n = 7$) Pneumonitis (5%, $n = 6$)	Total: 17%, $n = 20$ Fatigue (4%, $n = 5$) Diarrhea (3%, $n = 3$) Rash (1%, $n = 1$) Pneumonitis (1%, $n = 1$) Anemia (1%, $n = 1$)	(53)
Nivolumab	I	NCT01454102	52	3 mg/kg, Q2W	Total: 71%, $n = 37$ Fatigue (29%, $n = 15$) Rash (19%, $n = 10$) Nausea (14%, $n = 7$) Diarrhea (12%, $n = 6$) Pruritus (12%, $n = 6$) Arthralgia (6%, $n = 3$) Constipation (6%, $n = 3$) Hypothyroidism (6%, $n = 3$)	Total: 19%, $n = 10$ Rash (4%, $n = 2$) Diarrhea (2%, $n = 1$) Pneumonitis (2%, $n = 1$)	(54)
Nivolumab + Ipilimumab	I	NCT01454102	38	Nivolumab 3 mg/kg, Q2W + ipilimumab 1 mg/kg, Q12W	Total: 82%, $n = 31$ Pruritus (24%, $n = 9$) Diarrhea (21%, $n = 8$) Nausea (16%, $n = 6$) Fatigue (16%, $n = 6$) Increased amylase (16%, $n = 6$) Maculopapular rash (13%, $n = 5$) Pyrexia (13%, $n = 5$) Rash (16%, $n = 6$)	Total: 37%, $n = 14$ Increased lipase (8%, $n = 3$) Pneumonitis (5%, $n = 2$) Diarrhea (3%, $n = 1$) Fatigue (3%, $n = 1$) Rash (3%, $n = 1$)	(55)

(Continued)

TABLE 1 | Continued

Agent	Phase	Clinical Trials.gov	No. of patients	Therapy schedule	TRAEs (Any grade)	Treatment-related serious AEs (grade 3–5)	References
			39	Nivolumab 3 mg/kg Q2W + ipilimumab 1 mg/kg Q6W	Total: 72%, $n = 28$ Pruritus (13%, $n = 5$) Diarrhea (21%, $n = 8$) Nausea (16%, $n = 6$) Fatigue (23%, $n = 9$) Maculopapular rash (10%, $n = 4$) Pyrexia (5%, $n = 2$) Rash (10%, $n = 4$) Decreased appetite (13%, $n = 5$)	Total: 33%, $n = 13$ Adrenal insufficiency (5%, $n = 2$) Colitis (5%, $n = 2$) Nausea (3%, $n = 1$) Fatigue (3%, $n = 1$) Maculopapular rash (3%, $n = 1$)	(55)
Nivolumab +cisplatin + gemcitabine or paclitaxel	I	NCT01454102	56	5 or 10 mg/kg, Q3W + chemotherapy	Total: 95%, $n = 53$ Fatigue (71%, $n = 40$) Nausea (46%, $n = 26$) Decreased appetite (36%, $n = 20$) Alopecia (30%, $n = 17$) Anemia (27%, $n = 15$) Rash (27%, $n = 15$) Diarrhea (21%, $n = 12$)	Total: 45%, $n = 25$ Pneumonitis (7%, $n = 4$) Fatigue (5%, $n = 3$) Acute renal failure (5%, $n = 3$) Anemia (4%, $n = 2$) Neutropenia (4%, $n = 2$)	(56)
Nivolumab + ALT-803	Ib	NCT02523469	21	Nivolumab 3 mg / kg, Q2W + ALT-803 6,10,15 or 20μg/ kg, Q1W	Injection-site reaction (90%, $n = 19$) Flu-like symptoms (71%, $n = 15$) Fever (67%, $n = 10$) Chills (29%, $n = 6$) Nausea (38%, $n = 8$) Pain (33%, $n = 7$) Dizziness (24%, $n = 5$)	Fatigue (10%, $n = 2$) Lymphocytopenia (10%, $n = 2$) Fever (5%, $n = 1$) Anemia (5%, $n = 1$) Abdominal pain (5%, $n = 1$)	(57)

Digestive System Related Adverse Events

Colitis and diarrhea are the most common gastrointestinal toxicity during the treatment of anti-PD-1/PD-L1 immunotherapy (24). Other gastrointestinal adverse reactions include decreased appetite, nausea, vomiting, constipation (24). Colitis clinically involves clinical or imaging evidence of abdominal pain symptoms and colon inflammation, while diarrhea refers to an increase in stool frequency (72). In immune checkpoint blocking therapy, the incidence of gastrointestinal AEs with anti-CTLA-4 treatment is higher than that with anti-PD-1/PD-L1 therapy (72). Moreover, anti-PD-1/PD-L1 agents combined with chemotherapy drugs will increase the incidence of gastrointestinal AEs (any grade) (23, 56). In general, the incidence of grade III–IV colitis/diarrhea is about 5% and life-threatening cases are rarely reported (12). In clinical management of immune-related colitis and diarrhea AEs, stool evaluation should be performed to rule out any possible bacterial, viral pathogen, and parasitic infections (88). For mild diarrhea or colitis, it is useful to oral loperamide or diphenoxylate/atropine for 2–3 days and hydration (78, 88). Moderate or severe colitis/diarrhea should hold immunotherapy. Patients with grade 3 may consider re-use of anti-PD-1/PD-L1 therapy after toxicity has been relieved, but patients with grade IV should permanently discontinue immunotherapy (78). Patients with grade IV may be successfully managed by using prednisone/methylprednisolone (1–2 mg/kg/day). If the symptoms do not improve, consider adding infliximab or vedolizumab within 2 weeks. Severe cases should be hospitalized to provide supportive treatment (78).

Hepatic Toxicities

Among NSCLC patients receiving anti-PD-1/PD-L1 immunotherapy, the incidence of immune-related hepatitis is approximately 5%, while the incidence of severe hepatitis (grade III-IV) is <2% (89). The median time to onset is usually 6–14 weeks from the first taking of anti-PD-1/PD-L1 drugs, but may occur within a few months after starting treatment or even stopping treatment (89). Any asymptomatic elevations in alanine aminotransferase (ALT) or aspartate aminotransferase (AST) enzymes levels should consider immune-related hepatitis (78, 90). Some patients occasionally observe elevated levels of bilirubin, usually without obvious symptoms. In addition, liver biopsy is the gold standard for diagnosing and evaluating the degree of autoimmune hepatitis and liver injury (90). The clinical symptoms of immune-mediated hepatitis include hepatomegaly, portal and periportal inflammation, lymphadenomegaly, and infiltrating eosinophils, lymphocytes as well as plasma cells (90). Before treatment of immune-related hepatitis, viral etiology (hepatitis A, hepatitis B or C, and emergency hepatitis E virus), disease-related liver dysfunction, and other drug-induced transaminase elevations should be excluded. Ultrasound or magnetic resonance cholangiopancreatography can be considered to rule out liver metastases or gallstones of cancer (78). For mild to moderate hepatitis (grade I–II), immunotherapy can be continued or suspended according to the patient's condition, and liver function tests (LFTs) are closely monitored. Patients with grade III–IV hepatitis should permanently discontinue immunotherapy and use prednisone/methylprednisolone 1–2 mg/kg/day. If the steroid

TABLE 2 | Adverse effects base on anti-PD-L1 antibodies.

Agent	Phase	Clinical Trials.gov	No. of patients receiving anti-PD-L1 agent	Therapy schedule	TRAEs (Any grade)	Treatment-related serious AEs (grade 3–5)	References
Atezolizumab	II	NCT02031458	659	1,200 mg, Q3W	Total: 65%, $n = 429$ Fatigue (19%, $n = 122$) Diarrhea (11%, $n = 71$) Nausea (11%, $n = 73$) Pruritus (10%, $n = 65$) Pyrexia (8%, $n = 54$) Decreased appetite (8%, $n = 53$) Asthenia (8%, $n = 50$) Rash (8%, $n = 50$)	Total: 12%, $n = 82$ Fatigue (1%, $n = 7$) Nausea (1%, $n = 4$) Asthenia (1%, $n = 3$) Rash (1%, $n = 9$)	(58)
Atezolizumab	I	NCT01375842	89	1-20 mg/kg or 1,200 mg, Q3W	Total: 76%, $n = 68$ Fatigue (20%, $n = 18$) Nausea (16%, $n = 14$) Decreased appetite (14%, $n = 12$) Asthenia (10%, $n = 9$) Rash (9%, $n = 8$) Dyspnea (8%, $n = 7$) Diarrhea (8%, $n = 7$) Headache (7%, $n = 6$)	Total: 11%, $n = 10$ Fatigue (2%, $n = 2$) Dyspnea (2%, $n = 2$) Nausea (1%, $n = 1$) Vomiting (1%, $n = 1$)	(59)
Atezolizumab	III	NCT02008227	609	1,200 mg, Q3W	Total: 94%, $n = 573$ Fatigue (27%, $n = 163$) Decreased appetite (24%, $n = 143$) Cough (23%, $n = 141$) Nausea (18%, $n = 108$) Diarrhea (15%, $n = 94$) Asthenia (19%, $n = 116$) Dyspnea (19%, $n = 118$) Anemia (12%, $n = 70$)	Total: 37%, $n = 227$ Fatigue (3%, $n = 17$) Dyspnea (3%, $n = 15$) Anemia (2%, $n = 14$) Asthenia (1%, $n = 8$) Back pain (1%, $n = 7$)	(60)
Atezolizumab	II	NCT01846416	137	1,200 mg, Q3W	Total: 70%, $n = 96$ Fatigue (27%, $n = 37$) Decreased appetite (15%, $n = 21$) Nausea (15%, $n = 20$) Diarrhea (10%, $n = 13$) Pyrexia (8%, $n = 11$) Pruritus (7%, $n = 10$) Arthralgia (7%, $n = 9$) Rash (7%, $n = 9$)	Not mentioned	(61)
Atezolizumab + carboplatin + paclitaxel or pemetrexed or nab-paclitaxel	I	NCT01633970	76	Atezolizumab 15 mg/kg, Q3W + chemotherapy	Not mentioned	Total: 72%, $n = 55$ Neutropenia (38%, $n = 29$) Anemia (21%, $n = 16$) Fatigue (11%, $n = 8$) Thrombocytopenia (8%, $n = 6$) Febrile neutropenia (7%, $n = 5$) Neutrophil count decreased (7%, $n = 5$) Platelet count decreased (5%, $n = 4$) Dehydration (5%, $n = 4$)	(62)

(Continued)

TABLE 2 | Continued

Agent	Phase	Clinical Trials.gov	No. of patients receiving anti-PD-L1 agent	Therapy schedule	TRAEs (Any grade)	Treatment-related serious AEs (grade 3–5)	References
Atezolizumab + bevacizumab + carboplatin +paclitaxel	III	NCT02366143	393	Atezolizumab 1,200 mg, Q3W + chemotherapy	Total: 94.4%, *n* = 371 Alopecia (47%, *n* = 183) Peripheral neuropathy (36%, *n* = 141) Nausea (30%, *n* = 119) Fatigue (22%, *n* = 88) Decreased appetite (20%, *n* = 77) Anemia (18%, *n* = 70) Diarrhea (18%, *n* = 70) Constipation (17%, *n* = 65)	Total: 59%, *n* = 230 Neutropenia (14%, *n* = 54) Decreased neutrophil count (9%, *n* = 34) Febrile neutropenia (9%, *n* = 36) Hypertension (6%, *n* = 25) Anemia (6%, *n* = 24) Decreased platelet count (5%, *n* = 20)	(63)
Durvalumab	II	NCT02087423	444	10 mg/kg, Q2W	Total: 58%, *n* = 256 Fatigue (11%, *n* = 50) Hypothyroidism (8%, *n* = 36) Asthenia (7%, *n* = 31) Nausea (6%, *n* = 28) Pruritus (6%, *n* = 28) Diarrhea (6%, *n* = 27) Vomiting (3%, *n* = 14) Anemia (2%, *n* = 9)	Total: 9%, *n* = 40 Fatigue (<1%, *n* = 2) Vomiting (<1%, *n* = 2) Pneumonitis (1%, *n* = 4) Gamma-glutamyltransferase increased (1%, *n* = 4)	(64)
Durvalumab	III	NCT02125461	475	10 mg/kg, Q2W	Total: 67.8%, *n* = 322 Fatigue (13%, *n* = 62) Hypothyroidism (11%, *n* = 65) Diarrhea (10%, *n* = 46) Pneumonitis (9%, *n* = 43) Rash (8%, *n* = 37) Pruritus (7%, *n* = 33) Hyperthyroidism (6%, *n* = 30) Asthenia (6%, *n* = 28)	Total: 12%, *n* = 56 Pneumonitis (1%, *n* = 6) Asthenia (<1%, *n* = 3) Dyspnea (<1%, *n* = 3)	(65)
Durvalumab +Tremelimumab	I	NCT02000947	102	Durvalumab 10-20 mg/kg, Q4W + Tremelimumab 1-3 mg/kg, Q12W	Total: 80%, *n* = 82 Diarrhea (32%, *n* = 33) Colitis (12%, *n* = 12) Pruritus (21%, *n* = 21) Rash (15%, *n* = 15) Hypothyroidism (10%, *n* = 10) Pneumonitis (5%, *n* = 5) Rash maculopapular (4%, *n* = 4)	Total: 42%, *n* = 43 Diarrhea (11%, *n* = 11) Colitis (9%, *n* = 9) Pneumonitis (4%, *n* = 4) Enteritis (1%, *n* = 1) Hypothyroidism (1%, *n* = 1)	(66)
Avelumab	I	NCT01772004	184	10 mg/kg, Q2W	Total: 77%, *n* = 142 Fatigue (25%, *n* = 46) Infusion-related reaction (19%, *n* = 34) Nausea (13%, *n* = 23) Decreased appetite (7%, *n* = 13) Diarrhea (7%, *n* = 13) Chills (7%, *n* = 12) Hypothyroidism (6%, *n* = 11)	Total: 13%, *n* = 23 Elevated lipase (2%, *n* = 3) Infusion-related reaction (1%, *n* = 2) Dyspnea (1%, *n* = 2) Elevated amylase (1%, *n* = 1) Autoimmune neutropenia (1%, *n* = 1)	(67)

(Continued)

TABLE 2 | Continued

Agent	Phase	Clinical Trials.gov	No. of patients receiving anti-PD-L1 agent	Therapy schedule	TRAEs (Any grade)	Treatment-related serious AEs (grade 3–5)	References
Avelumab	III	NCT02395172	393	10 mg/kg, Q2W	Total: 64%, $n = 251$ Infusion-related reaction (15%, $n = 59$) Decreased appetite (9%, $n = 34$) Fatigue (7%, $n = 29$) Asthenia (7%, $n = 29$) Diarrhea (6%, $n = n = 24$) Nausea (5%, $n = 20$) Myalgia (2%, $n = n = 6$) Mucosal inflammation (1%, $n = 2$)	Total: 12%, $n = 47$ Infusion-related reaction (1%, $n = 5$) Lipase increased (1%, $n = 3$) Asthenia (<1%, $n = 1$) Fatigue (<1%, $n = 1$)	(68)

treatment does not improve after 3 days, consider adding an additional immunosuppressant mycophenolates, but should not use infliximab as its potential hepatotoxicity (78).

Endocrine System Related Adverse Events

The endocrine system contains many important organs of the human body, such as hypothalamus, pituitary, thyroid, adrenal glands, and pancreas. The endocrine toxicity caused by PD-1/PD-L1 ICIs may affect any axis (12). Hypophysitis, thyroiditis, hypothyroidism, hyperthyroidism, and adrenal insufficiency are common immune-related endocrine diseases (44). Among patients with NSCLC, hypothyroidism is the most common endocrine toxicity, with an incidence of 5–15% (44). Since the clinical symptoms of immune endocrine disease are non-specific, such as fatigue, headache, and nausea. Cancer patients are often accompanied by such symptoms. Therefore, the diagnosis of immune-mediated endocrine toxicity is clinically challenging (12). Clinically, endocrine diseases such as central hypothyroidism and pituitary inflammation are diagnosed by evaluating biochemical indicators such as morning cortisol, ACTH (adreno-cortico-tropic-hormone), FSH (follicle-stimulating hormone), LH (luteinizing hormone), TSH (thyroid stimulating hormone), free T4, and DHEA-S (91). For patients with hypothyroidism, the thyroid hormone replacement therapy may be useful, and closely monitor the level of TSH is necessary (every 4–6 weeks) (78). If TSH > 10, levothyroxine should be used to make TSH reach the reference range or age-appropriate range. Patients with hyperthyroidism can be treated with standard antithyroid drugs. In addition, pituitary inflammation with obvious symptoms can be considered with prednisone/methylprednisolone 1–2 mg/kg/day for treatment (78). Primary adrenal insufficiency occurs less frequently in irAEs related to PD-1/PD-L1 blockade therapy, but in rare cases an adrenal crisis may occur (91). It should hold the immunotherapy and perform intravenous corticosteroid as well as supplement aggressive fluid and electrolyte when such AEs occur (91). Most endocrine-related toxicity is effective through hormone replacement therapy, without holding PD-1/PD-L1 immune checkpoint treatment.

Skeletal Muscle System Related Adverse Events

Some tumor patients receiving anti-immunity checkpoint treatment will also have skeletal muscle system-related AEs, but musculoskeletal symptoms are also present in the tumor patients themselves, therefore more attention should be paid to distinguishing (81). Overall, the majority of immune-related muscle AEs in patients with NSCLC are mild (grade I-II). The diagnosis of inflammatory arthritis is mainly by evaluating the degree of joint involvement, X-ray and joint ultrasound (78). Moreover, it is necessary to check the creation kinase/aldolase and troponin levels. NSCLC patients have the most reported immune-related muscle adverse reaction is myalgia (43). Patients with mild pain can continue immunotherapy and continuously monitor serial aldolase/creatine kinase levels, but moderate or severe pain should hold immunotherapy, using prednisone 1–2 mg/kg/day for treatment and considering muscle biopsy (72).

MANAGEMENT OF OTHER COMMON ADVERSE EVENTS

Fatigue

Fatigue widely occurs in patients with NSCLC who are treated with PD-1/PD-L1 immune checkpoint blockade (12). Overall, for NSCLC patients receiving anti-PD-1/PD-L1 monotherapy or combination therapy, ∼6–71% of patients reported treatment-related fatigue (any grade), but the incidence of grade III/IV is low (<5%) (24, 45, 56). Compared with anti-PD-1/PD-L1 monotherapy, PD-1/PD-L1 ICIs combined with other therapies (chemotherapy, targeted therapy, anti-CTLA-4 therapy) significantly increased the incidence of fatigue side effects (6–33 vs. 13–71%) (47, 54, 75). However, it is worth noting that fatigue symptoms are sometimes caused by immune-related endocrine toxicity. For example, early symptoms of hypothyroidism can also cause fatigue (81). Therefore, the treatment of fatigue should consultation based on abnormalities, and the use of low-dose steroids is allowed (78). In addition, moderate physical activity and psychosocial intervention can also help relieve fatigue symptoms (72). For severe fatigue, consideration should be given to whether tumor disease progression or other medical diseases occur (78).

Pyrexia/Chills and Infusion Reactions

Anti-PD-1/PD-L1 immune checkpoint therapy may cause cytokine release and non-specific over-activation of the immune system, which may lead to symptoms of pyrexia, chill and infusion reactions in patients (81). Approximately 5–18% of patients with NSCLC develop immune-related pyrexia during treatment. It can be managed by using antipyretics, such as acetaminophen or non-steroidal anti-inflammatory drugs (78). For grade I–II infusion reactions, it can resume infusion or reduce the infusion rate after the symptoms disappear, and consider premedication with acetaminophen, famotidine, and diphenhydramine with future infusions. For grade III infusion reactions, the immunotherapy should be permanently discontinued, and intravenous antihistamine or corticosteroid drugs are required (74, 78).

MANAGEMENT OF RARE BUT SERIOUS ADVERSE EVENTS

Immune-Related Encephalitis

Immune-related encephalitis is a rare and poorly understood irAE, with an incidence of <1% in cancer patients undergoing immune checkpoint blockade therapy, but it may be fatal (92). Therefore, it is necessary to increase its awareness for effective management. A multicenter cohort retrospectively analyzed the clinical, biological, and radiological characteristics of nine immune-related encephalitis in NSCLC patients undergoing anti-PD-1/PD-L1 treatment (40). The most common clinical symptoms of these patients include fever, confusion, and cerebellar ataxia (40). In addition, it was found that the levels of white blood cell increased, without any bacterial and viral infection. One patient's brain MRI examination showed that the limbic system is involved, which is fatal (40). The most important management of immune-related encephalitis is early treatment with corticosteroids (prednisone 1–2 mg/kg/day). Severe cases should permanently discontinue immunotherapy (78).

Myasthenia Gravis

The immune-related myasthenia gravis is also a rare but serious neurotoxicity caused by anti-PD-1/PD-L1 treatment (43, 91). The average onset time of the patient's symptoms appeared within 6 weeks of starting treatment (range 2–12 weeks) (93). Treatment-related reports of myasthenia gravis in NSCLC patients receiving PD-1 monoclonal antibodies seem to be more common than those receiving PD-L1 agents (41, 94, 95). A 63-year-old female patient with stage IV NSCLC adenocarcinoma, who failed conventional chemotherapy (disease progression) and subsequently used pembrolizumab, was diagnosed with myasthenia gravis after two cycles of treatment (41). The clinical symptoms are bilateral eyelid drooping, extraocular muscle paralysis, shortness of breath, and fatigue (41). Moreover, two patients with NSCLC who received nivolumab reported myasthenia gravis, and the onset time was within 2–3 cycles after the start of treatment (94, 95). Moderate and severe autoimmune myasthenia gravis should permanently discontinued immunotherapy, as well as oral pyridostigmine 30 mg TID and gradually increase to maximum of 120 mg four times a day as tolerated and based on symptoms (93). In addition, considering low-dose oral prednisone 20 mg daily and gradually increase the dose (not more than 100 mg/day) if necessary. Severe cases should use methylprednisolone 1–2 mg/kg/day and consider adding rituximab (375 mg/m^2 weekly for 4 treatments or 500 mg/m^2 every 2 weeks for 2 doses) if refractory to plasmapheresis or intravenous immunoglobulin (IVIG) (93).

Acute Renal Failure/Interstitial Nephritis

The main manifestation of kidney injury is elevated serum creatinine levels, and patients usually develop acute renal failure and interstitial nephritis (96). According to reports, the possible mechanism of kidney damage induced by ICIs is that drugs or drug metabolites activate circulating T cells, which binding to carrier proteins and form drug-carrier immune complexes to obtain immunogenicity (97). When these immune complexes are presented as a local antigen to the kidney, they trigger a hypersensitivity reaction through the release of cytokines, leading to the occurrence of kidney damage (97). In NSCLC patients, a phase I study (NCT01454102) of nivolumab combined with platinum-based dual chemotherapy reported 3 cases of grade 3 acute renal failure. In addition, Koda et al. (42) reported a 67-year-old stage IV acute tubulointerstitial nephritis caused by nivolumab monotherapy in patients with NSCLC. For the management of acute renal failure/interstitial nephritis, creatinine, and urine protein levels should be closely monitored (once every 3–7 days), and prednisone 0.5–1 mg/kg/day may be useful (42). Patients with severe kidney injury should permanently discontinue immunotherapy and use prednisone/methylprednisolone 1–2 mg/kg/day. Conduct renal biopsy and nephrology consultation if necessary. Moreover, add one of the following drugs, azathioprine, cyclophosphamide, cyclosporine, infliximab, and mycophenolate, if the symptoms

still not improve after treated with steroids for more than 1 week (42).

Myocarditis

Immune-mediated cardiotoxicity, myocarditis, is a rare but serious side effect in NSCLC patients receiving anti-PD-1/PD-L1 immune checkpoint treatment, which needs to be recognized as soon as possible for better management (98–100). A case report showed that a 75-year-old NSCLC patient suffered a drug-induced AE of myocarditis during the ninth cycle of nivolumab treatment, and its clinical symptoms were dyspnea and acute chest pain (98). After treatment with ACE-inhibitors, β-blockers and diuretics as well as prednisolone (1 mg/kg/day), the cardiac function of patient was significantly improved (98). Similarly, Gibson et al. (101) reported that a 68-year-old female NSCLC patient receiving nivolumab developed autoimmune myocarditis. The patient's electrocardiogram showed sustained ventricular tachycardia and ectopic ventricular beats (101). In addition to the use of corticosteroids for the treatment of myocarditis, other immunosuppressive agents such as anti-thymocyte globulin, infliximab and mycophenolate can also be added if necessary (**Figure 2**).

PREVENT OR REDUCE THE FREQUENCY OF ADVERSE EVENTS

Potential Predictive Biomarkers Related to Adverse Effects

The effective management strategy for irAEs is early detection and early intervention. Therefore, it is crucial to find biomarkers that can predict the occurrence of AEs during immunotherapy (102). Recently, a study performed by Kurimoto and his colleague found that serum thyroglobulin, thyroid autoantibodies and early changes in the levels of certain cytokines (increased levels of IL-1β, IL-2, and GM-CSF and decreased levels of IL-8, G-CSF, MCP-1) may indicate the development of autoimmune thyroiditis AEs (103). Similarly, thyroid peroxidase (TPO) and thyroglobulin antibody levels are associated with hypothyroidism in NSCLC patients receiving nivolumab treatment (104). Oyanagi et al. (105) reported that the increase in serum protein RANTES is a potential predictive biomarkers of the onset of irAEs in NSCLC patients who treated with nivolumab. In addition, the increase levels of serum C-reactive protein (CRP) are associated with a higher incidence of irAEs, but not with the severity of irAEs and the affected organ (106). For rare but severe immune-mediated myocarditis, several potential predictive biomarkers have also been found, such as serial troponin, miR-30c (107, 108).

Baseline Examination Before Immunotherapy Initiation

By comparing the changes of certain biochemical indicators and imaging features of tissues and organs before and after immunotherapy, it can help clinicians to quickly judge any irAEs that may occur (109). Routine baseline assessments include physical examination (height, weight, heart rate, blood pressure, and other general symptoms), imaging examination (chest CT, brain MRI) as well as laboratory tests (blood routine, blood biochemistry, blood glucose, total bilirubin, TSH, free T4, LH, FSH, testosterone, cortisol, ACTH, infectious disease screening, etc.) (109). In addition, carefully ask patient and family the history of autoimmune disease, infectious disease and organ specific diseases are necessary. Clinicians also need to inform patients of potential side effects of immune checkpoint blockade therapy, whether during or after treatment (73). Patients should also promptly feedback any new symptoms of discomfort.

PERSONALIZED MANAGEMENT

Tumor patients of different races, genders, and ages experience different irAEs profiles and severity, therefore precise care according to the patient's personal situation is conducive to reduce the incidence of AEs (110). Elderly people with lung cancer usually have comorbidities and polypharmacy, therefore adequate clinical monitoring is required (110). However, Hakozaki et al. (111) showed that polypharmacy was not associated with irAEs but was associated with higher rate of unexpected hospitalizations during anti-PD-1/PD-L1 treatment in early NSCLC patients (aged $\geq$ 65 years) in Japanese. Studies have also shown that immune-related fatigue is more common in elderly patients with lung cancer (aged $\geq$75 years) (49.1 vs. 40.2%), but no other differences in irAEs are observed, and it is not recommended to adjust the dosage of elderly patients (109, 110). Given the small number of elderly patients involved in most immune checkpoint blockade studies, the toxicity data for this group is limited and further studies are needed (112). PD-1/PD-L1 blockade may aggravate or reactivate certain existing viral infectious diseases, therefore patients with a history of chronic viral infections (such as HBV, HCV or HIV) should be excluded from clinical trials (109). Due to the ability of IgG to cross the placental barrier, ICI is not recommended for pregnant and lactating women unless the clinical benefit of the patient outweighs the potential risk (109). Most initial clinical trials of PD-1/PD-L1 blocking therapy are conducted in Caucasians or mix races (113). In recent years, more and more clinical trials of anti-PD-1/PD-L1 agents have been conducted in Asian populations (113). The analysis results of Yang et al. (113) showed that in cancer patients with PD-1/PD-L1 blockade therapy, the AEs of any grade with different prevalences between Asian populations and Western/international populations included fatigue, diarrhea, nausea, rash, vomiting, and hypothyroidism. Overall, we still need to develop more sophisticated medical tools in the future to achieve the best management strategy for irAEs in cancer patients.

CONCLUSION

The therapy based on PD-1/PD-L1 immune checkpoint blockade show a better tolerated than traditional standard chemotherapy in NSCLC patients, but the AEs of these drugs are different from traditional cytotoxic therapy. Therefore, it is necessary to increase

awareness of these treatment-related toxic reactions for better management. These adverse reactions involved different tissues and organs in the human body, causing toxic reactions ranging from mild fatigue to severe, life-threatening liver and lung toxicity (115, 116). Compared with traditional chemotherapy, AEs caused by anti-PD-1/PD-L1 treatment were usually of low grade, with relatively good patient tolerance and fewer deaths. However, due to the rapid onset of AEs, so timely medical care was crucial, especially for the elderly patients, these toxic reactions should be more carefully monitored to prevent possible complications.

In conclusion, our review summarizes common and rare adverse reactions based on anti-PD-1/PD-L1 therapy in the treatment of NSCLC. Overall, adverse reactions caused by anti-PD-1/PD-L1 immunotherapy were usually low-grade and most patients were better tolerated. However, there were still some serious and even life-threatening adverse events related to treatment. Therefore, healthcare workers should be alert to the occurrence of such AEs to better monitor and manage these adverse reactions.

AUTHOR CONTRIBUTIONS

CS, HW, and YL wrote the first draft of the manuscript. QG, LZ, JL, and WZ organized the structure of the manuscript. JZ, XZ, and YY contributed conception of the work. All authors have read and agreed to the published version of the manuscript.

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Immune-Stromal Score Signature: Novel Prognostic Tool of the Tumor Microenvironment in Lung Adenocarcinoma

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Background: Immune and stromal cells in the tumor microenvironment (TME) significantly contribute to the prognosis of lung adenocarcinoma; however, the TME-related immune prognostic signature is unknown. The aim of this study was to develop a novel immune prognostic model of the TME in lung adenocarcinoma.

Methods: First, the immune and stromal scores among lung adenocarcinoma patients were determined using the ESTIMATE algorithm in accordance with The Cancer Genome Atlas (TCGA) database. Differentially expressed immune-related genes (IRGs) between high and low immune/stromal score groups were analyzed, and a univariate Cox regression analysis was performed to identify IRGs significantly correlated with overall survival (OS) among patients with lung adenocarcinoma. Furthermore, a least absolute shrinkage and selection operator (LASSO) regression analysis was performed to generate TME-related immune prognostic signatures. Gene set enrichment analysis was performed to analyze the mechanisms underlying these immune prognostic signatures. Finally, the functions of hub IRGs were further analyzed to delineate the potential prognostic mechanisms in comprehensive TCGA datasets.

Results: In total, 702 intersecting differentially expressed IRGs (589 upregulated and 113 downregulated) were screened. Univariate Cox regression analysis revealed that 58 significant differentially expressed IRGs were correlated with patient prognosis in the training cohort, of which three IRGs (*CLEC17A*, *INHA*, and *XIRP1*) were identified through LASSO regression analysis. A robust prognostic model was generated on the basis of this three-IRG signature. Furthermore, functional enrichment analysis of the high-risk-score group was performed primarily on the basis of metabolic pathways, whereas analysis of the low-risk-score group was performed primarily on the basis of immunoregulation and immune cell activation. Finally, hub IRGs *CLEC17A*, *INHA*, and *XIRP1* were considered novel prognostic biomarkers for lung adenocarcinoma. These hub genes had different mutation frequencies and forms in lung adenocarcinoma and participated in different signaling pathways. More importantly, these hub genes were significantly correlated with the infiltration of CD4+ T cells, CD8+ T cells, macrophages, B cells, and neutrophils.

Conclusions: The robust novel TME-related immune prognostic signature effectively predicted the prognosis of patients with lung adenocarcinoma. Further studies are required to further elucidate the regulatory mechanisms of these hub IRGs in the TME and to develop new treatment strategies.

Keywords: tumor microenvironment, lung adenocarcinoma, prognostic model, TCGA database, ESTIMATE algorithm

INTRODUCTION

Lung cancer is still the leading disease worldwide in terms of the threat to human life and health (1, 2), and lung adenocarcinoma is the most common pathological subtype. Studies in the past decade have reported that tyrosine kinase inhibitors (TKIs) targeting epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and ROS proto-oncogene 1 (ROS1) are potential therapeutic targets for lung adenocarcinoma, upon genotyping (3–5). Molecular-targeted therapy based on these sensitive targets has considerably enhanced overall survival (OS) among patients with lung adenocarcinoma; however, this therapy is not suitable for all patients with lung adenocarcinoma. Furthermore, drug resistance is common among patients receiving molecular-targeted therapy, and their prognosis is poor (6, 7). Nonetheless, numerous studies have led to the advancement of immunotherapy for several cancers, including lung adenocarcinoma. Immunotherapy is different from targeted therapy; it has more durable clinical benefits. Furthermore, some antibodies used for immunotherapy have been successfully approved as first- and second-line treatments for advanced lung adenocarcinoma (8). In particular, the immune system reportedly plays an important role in the pathogenesis and prognosis of lung adenocarcinoma (9). Therefore, it is essential to understand the immune prognostic signature of lung adenocarcinoma.

Previous studies have investigated the prognostic role of immune-related genes (IRGs) in lung adenocarcinoma from the ImmPort database (10, 11); however, this database contains published data on IRGs, thus potentially not accounting for all IRGs. Moreover, these studies have reported no correlation between prognostic factors and OS among certain subgroups of patients with lung adenocarcinoma, indicating that this association is largely unknown. One of the important reasons may be the complex prognostic behavior of tumors; furthermore, when considering the characteristics of IRGs directly associated with tumors, it is also important to focus on the tumor microenvironment (TME) (12). The TME is closely associated with tumorigenesis and patient prognosis (13, 14). Moreover, accumulating evidence indicates that tumor-infiltrating immune cells and stromal cells (15, 16), as the primary nontumor components of the TME, play a significant role in lung cancer prognosis. These findings highlight the importance of understanding the association between the TME and lung adenocarcinoma prognosis. The development of a prognostic model of IRGs based on the TME might provide novel insights into the generation of a more accurate prognostic system.

Accurate management and appropriate personalized therapies for lung adenocarcinoma are required in accordance with prognostic stratification. Moreover, an enhanced understanding of IRGs involved in the TME would help elucidate their regulatory mechanisms in the TME and develop new treatment strategies. With advancements in machine learning, the ESTIMATE algorithm has been used to investigate IRGs in the TME, based on the immune and stromal scores of the TME, and to generate a TME-related immune prognostic model (17). Moreover, this algorithm can effectively predict the prognosis of patients with various cancers. Accordingly, in this study, we determined the immune and stromal scores of tumors using the ESTIMATE algorithm and developed a novel TME-related immune prognostic model of lung adenocarcinoma.

Abbreviations: IRGs, immune-related genes; TME, tumor microenvironment; TCGA, The Cancer Genome Atlas data; DAVID, Database for Annotation, Visualization and Integrated Discovery.

MATERIALS AND METHODS

Acquisition of TCGA Data

Normalization of RNA sequence data, in terms of level 3 fragments per kilobase of exon per million fragments mapped (FPKM) reads, was performed for 594 samples obtained from The Cancer Genome Atlas (TCGA) database, including 535 adenocarcinoma and 59 normal lung samples, before December 15, 2019. Thereafter, the Ensemble IDs were converted to gene symbols in accordance with human gene annotations. Furthermore, clinical data of lung adenocarcinoma patients were obtained and merged into a single matrix for subsequent analysis. Patients with an incomplete follow-up duration or recorded date of death of any cause were excluded. Finally, 494 lung adenocarcinoma patients with expression profiles and clinical data were included.

Immune Score and Stromal Score in the TME

Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data (ESTIMATE) is a tool for predicting and estimating infiltrating immune and stromal cells in tumor tissues based on gene expression profiles. Herein, the ESTIMATE algorithm was used to analyze the characteristics of specific gene expression in immune and stromal cells for each tumor sample to predict their immune and stromal scores. Thereafter, the immune and stromal scores were analyzed using the estimate package in R software.

Screening of Differentially Expressed IRGs

Based on the median immune and stromal scores, patients with lung adenocarcinoma were divided into two groups: high and low immune/stromal score groups. Significant differences in OS between the high and low immune/stromal score groups were analyzed. On the basis of the significant differences in patient prognosis, differentially expressed IRGs between the two groups were assessed using the Limma package in R software. Finally, intersecting differentially expressed IRGs in both groups were considered for further analysis. A log(fold change) of >2 and an adjusted *p*-value of <0.05 were considered cutoffs. Heat maps and Venn diagrams were generated using R.

GO and KEGG Pathway Enrichment Analyses of Differentially Expressed IRGs

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed to understand gene functional annotation and functional enrichment, respectively. Common differentially expressed IRGs of GO and KEGG annotation were performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID), an online database (https://david.ncifcrf.gov/), to predict functional domains and their biological implications. Fisher's exact test was performed to analyze pathways, diseases, and functions. A *p*-value of <0.05 indicated the significance of GO terms and KEGG pathway enrichment in genes herein. Furthermore, the top 10 GO terms and KEGG pathway enrichment results were mapped using Hmisc and ggplot2 in R software.

Construction of Prognostic Model for Lung Adenocarcinoma in the Training Set

A total of 494 patients with lung adenocarcinoma in the TCGA dataset were randomly divided into a training set and testing set at a ratio of 7:3 (training cohort, 346 patients; testing cohort, 148 patients).

First, univariate Cox proportional hazard regression analysis was performed to screen out prognostic IRGs in the training cohort (iteration = 1,000), with $p < 0.05$ indicating statistical significance. Second, the key IRGs were further selected from among significant prognostic IRGs on univariate analysis, through least absolute shrinkage and selection operator (LASSO) regression, a powerful tool for developing refined prognostic models, fitting generalized linear models, selecting variables, and regularizing complexity, using R software. Key IRGs were subjected to multivariate Cox regression analysis. Finally, the risk score formula was developed in accordance with the key IRGs identified through LASSO analysis.

Evaluation of the Prognostic Model in the Training Set

After the expression value of each specific gene was included, the risk score formula for each patient was weighted by its estimated regression coefficient on LASSO regression analysis. On the basis of the best separation of risk score, patients were divided into high-risk-score and low-risk-score groups. Survival differences between the two groups were assessed by Kaplan–Meier survival curves using log-rank tests. ROC curves were used to assess the accuracy of model prediction. Furthermore, LASSO regression analysis was performed to examine the role of the risk score in predicting clinical outcomes. Furthermore, the association between risk score and clinical stage was analyzed.

To further determine whether the independent prognostic model could be used as an independent prognostic factor, univariate and multivariate Cox regression analyses were performed to analyze the predictive value of age, sex, stage, TNM stage, and predictive model. In the univariate analysis, the correlation between some independent variables and dependent variables was considered, and some correlations might be masked by the influence of confounding factors. To avoid omitting important predictors of prognosis, the threshold in univariate analysis was relaxed to $p < 0.1$, while the *p*-value in multivariate analysis was still 0.05.

GSEA Analysis of Differences in Pathway Enrichment in the Training Set

To investigate the differences in the putative mechanism between the high-risk-score and low-risk-score groups, gene set enrichment analysis (GSEA) was performed to comprehensively analyze the differences in function enrichment. GSEA is a computational method of determining whether an a priori defined set of genes is significantly different between two biological states. The number of permutations was set to 1,000, and the permutation type was set to phenotype. In this study, all genes in the training set were sequenced according to the degree of differential expression in the high-risk-score group and the low-risk-score group. GSEA was used to comprehensively analyze differences in gene pathway enrichment between the two groups.

Validation of the Prognostic Model in the Testing Cohort

The prognostic model was further validated for the testing cohort ($n = 148$).

Similarly, the risk score of each patient was weighted on the basis of the risk score. Thereafter, based on the best separation of the risk score, patients in the testing cohort were divided into high-risk-score and low-risk-score groups. A Kaplan–Meier survival curve and ROC curve analysis were performed in the testing however.

Functional Analysis of IRG Signatures in the Model

To further analyze the mutation characteristics and the putative functional mechanisms of these hub IRGs in lung adenocarcinoma, gene expression profiles of patients with lung adenocarcinoma were imported from the following datasets: TCGA database (Broad, Cell 2012), Lung Adenocarcinoma (MSKCC, Science 2015), Lung Adenocarcinoma (TCGA, Firehose Legacy), Lung Adenocarcinoma (TSP, Nature 2008), and Non-Small-Cell Cancer (MSKCC, Cancer Discov 2017). A

TABLE 1 | Baseline characteristics of patients with lung adenocarcinoma.

	Training set		Testing set		*p*-value
	High score (*N* = 194)	Low score (*N* = 152)	High score (*N* = 91)	Low score (*N* = 57)	
Age (years)					
≥60	132	116	68	42	0.131
<60	62	36	23	15	0.767
Gender					
Male	92	67	55	14	0.002
Female	102	85	36	42	0.213
AJCC stage					
Stage I	95	99	35	34	0.802
Stage II	47	29	28	13	0.488
Stage III	41	17	18	4	0.312
Stage IV	9	4	9	4	1.000
NA	2	3	1	2	0.850
T stage					
T1	53	70	22	22	0.429
T2	108	68	52	28	0.577
T3	22	9	11	3	0.593
T4	9	5	5	0	0.257
TX	2	0	1	0	NA
N stage					
N0	114	115	50	39	0.305
N1–3	74	34	40	16	0.701
NX	6	3	1	2	0.523
M stage					
M0	133	92	60	40	0.880
M1	8	4	9	4	1.000
MX	51	54	23	13	0.112
NA	2	2	0	0	NA
Survival Time (days)	653.45 ± 36.96	840.44 ± 82.79	714.41 ± 95.64	953.81 ± 118.03	NA
Survival status					
Alive	113	124	51	44	0.323
Dead	81	28	40	13	0.873

combined study of five datasets including 1,825 patients were included in this study.

GSCALite is an online cancer genomic analysis tool that integrates cancer genomics data for 33 cancer types from the TCGA and normal tissue data from GTEx (http://bioinfo.life.hust.edu.cn/web/GSCALite/), enabling gene set pathway analysis in data analysis. In this study, GSCALite was used to analyze the pathway of hub genes.

The TIMER database is a comprehensive tool for analyzing the immune cell infiltrates in tumors. The abundances of six immune infiltrates (B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells) were estimated using the TIMER algorithm. In this study, the TIMER database was further applied to analyze the correlation between hub genes and immune cells. The correlation of hub gene expression with immune infiltration level was visualized in lung adenocarcinoma using the Gene module. The scatterplots were generated and displayed after hub genes and cancer type were submitted successfully, showing the purity-corrected partial Spearman's correlation and statistical significance.

Statistical Analysis

All statistical analyses were conducted with the R language (version 3.6.1). All statistical tests were bilateral, and $p < 0.05$ was statistically significant.

RESULTS

Study Design and Workflow Overview

A total of 594 samples were obtained, including 535 adenocarcinoma and 59 normal lung samples. Data for a total of 494 lung adenocarcinoma patients with clinical information were retrieved (**Table 1**). The workflow for constructing and

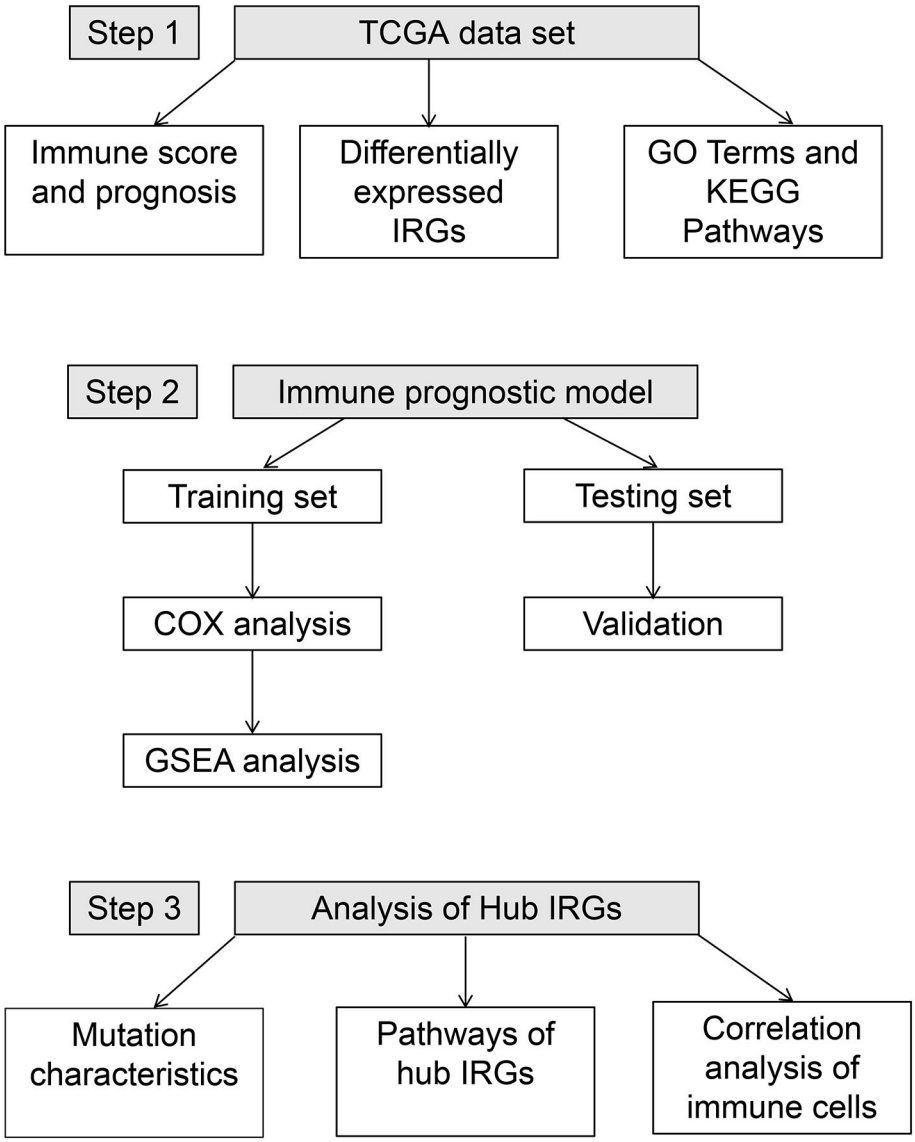

FIGURE 1 | Workflow of the construction and verification of the immune-stromal score prognostic signature.

verifying the immune-related prognostic model is shown in **Figure 1**.

Immune Score and Stromal Score in the TME

The immune and stromal scores were analyzed using the ESTIMATE algorithm. The immune and stromal scores of lung adenocarcinoma are shown in **Supplementary Table 1**. On the basis of the median value of immune and stromal scores, patients with lung adenocarcinoma were divided into two groups: the high immune/stromal score group and the low score group. These results show that the high immune score group had better OS than the low immune score group for lung adenocarcinoma. In terms of stromal score, although patients with high stromal score had better prognosis than those with low stromal score, the difference was not statistically significant (**Figures 2A–C**).

Screening of Differentially Expressed IRGs

According to the criteria of a log(fold change) of >2 and an adjusted p-value of <0.05, our results showed that a total of 3,034 genes with significant differentially expressed IRGs were screened, including 2,521 upregulated IRGs and 513 downregulated IRGs. Among them, 1,394 differentially expressed IRGs (1,092 upregulated IRGs and 302 downregulated IRGs) were included in the immune score group (**Supplementary Table 2**), and 1,640 differentially expressed IRGs (1,429 upregulated IRGs and 211 downregulated IRGs) were included in the stromal score group (**Supplementary Table 3**). Heat maps and

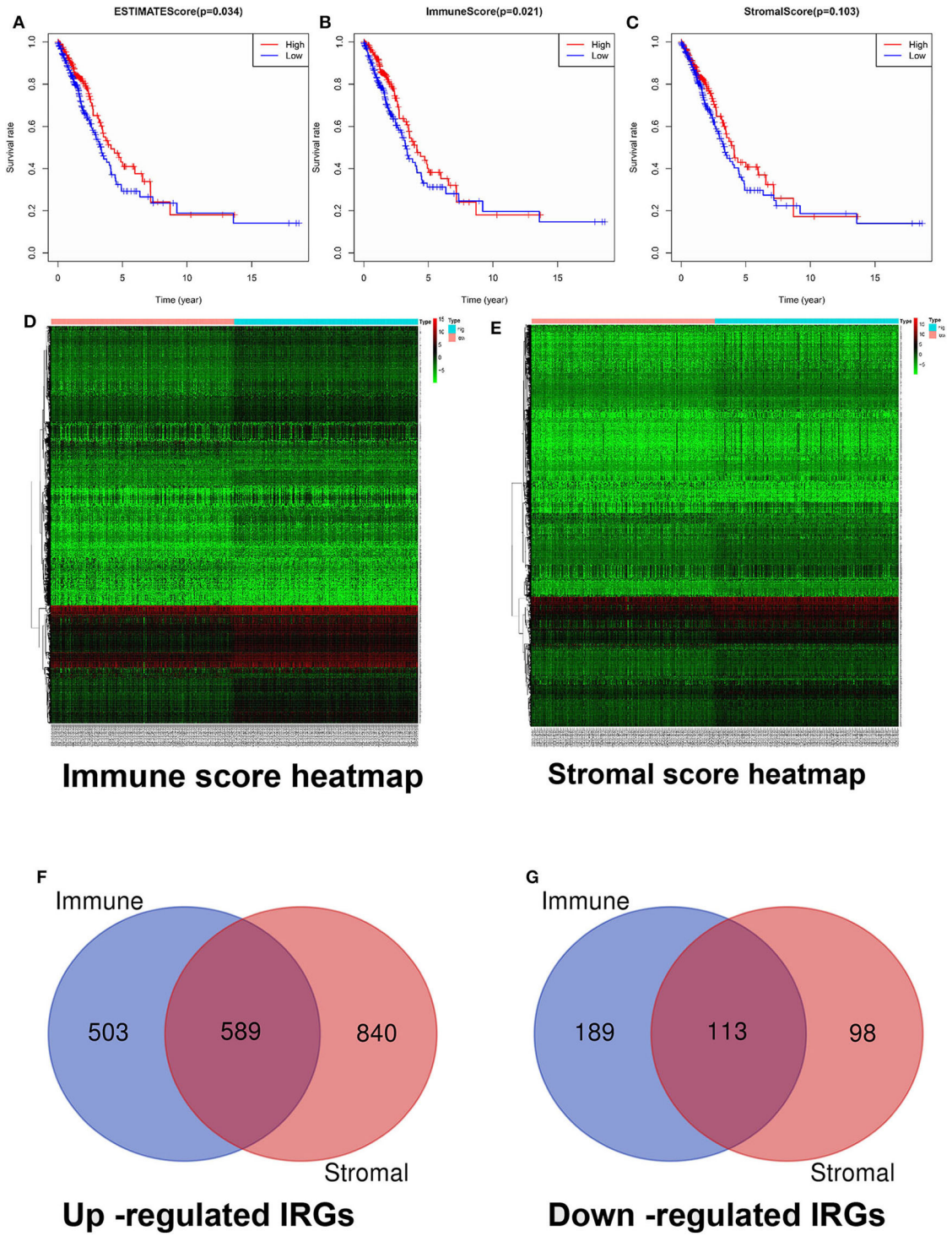

FIGURE 2 | Screening and identification of differentially expressed IRGs. **(A)** Relationship between comprehensive immune score and OS. **(B)** Relationship between immune score and OS. **(C)** Relationship between stromal score and OS. **(D)** Heatmap of immune score groups. **(E)** Heatmap of stromal score groups. **(F)** The Venn diagram of the intersection of up-regulated IRGs between the immune and stromal score groups. **(G)** The Venn diagram of the intersection of down-regulated IRGs between the immune and stromal score groups. OS, overall survival; IRGs, immune-related genes.

Venn diagrams are displayed in **Figures 2D–G**. In total, 702 intersecting differentially expressed IRGs (589 upregulated and 113 downregulated) in both groups are indicated in **Figures 2F,G** (**Supplementary Table 4**).

GO Terms and KEGG Pathway Enrichment Analysis of Differentially Expressed IRGs

GO terms are divided into three parts: biological processes, cellular components, and molecular functions. GO analysis

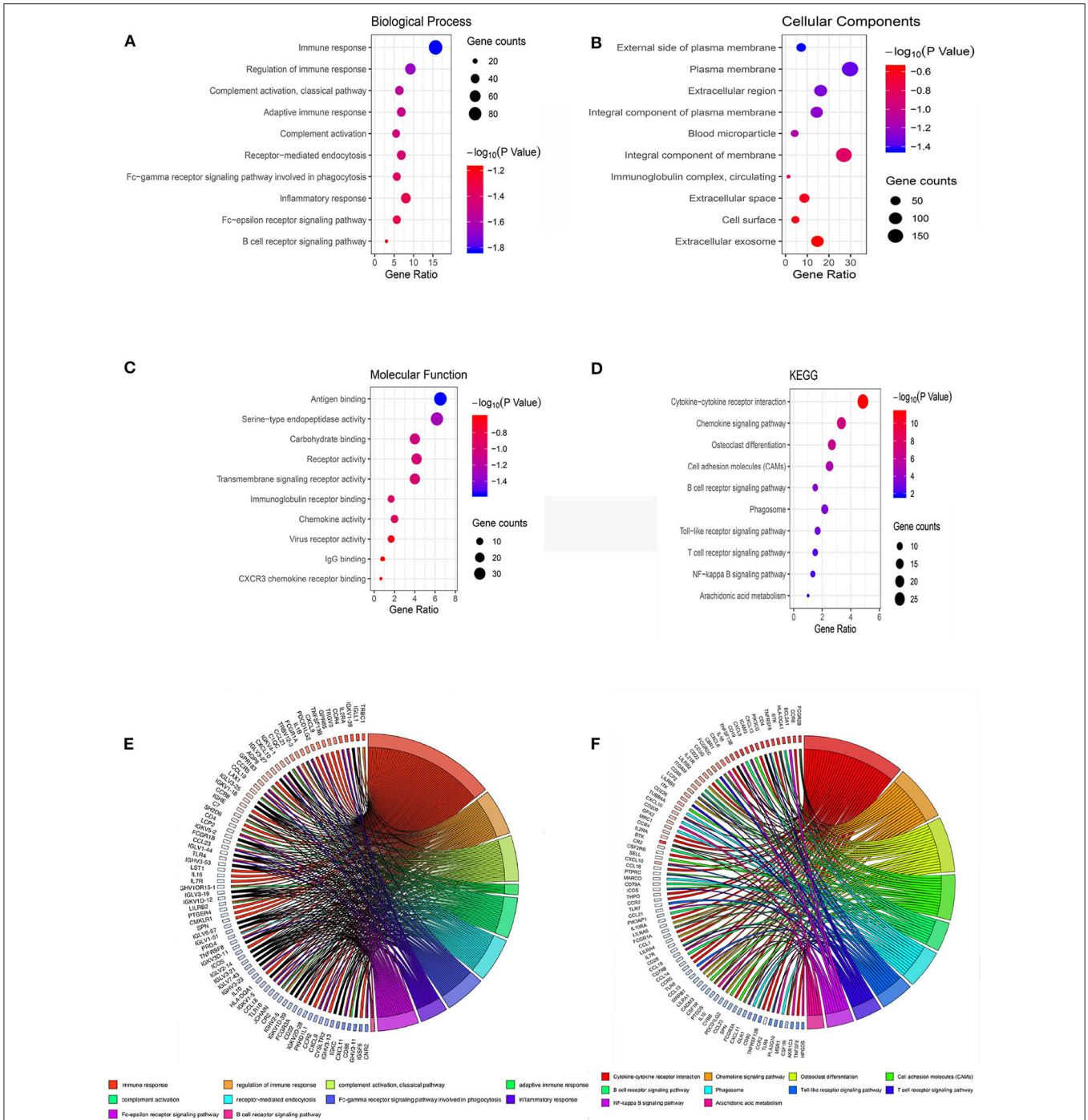

FIGURE 3 | Bubble map of the top 10 GO terms and KEGG pathway enrichment analysis data of differentially expressed IRGs. **(A)** GO analysis of differentially expressed IRGs in biological processes. **(B)** GO analysis of differentially expressed IRGs in cellular components. **(C)** GO analysis of differentially expressed IRGs in terms of molecular function. **(D)** KEGG enrichment analysis of differentially expressed IRGs. A high gene ratio represents a high level of enrichment. The size of the dot indicates the number of target genes in the pathway, and the color of the dot reflects the *p*-value range. **(E)** GO chord plot of differentially expressed IRGs. **(F)** KEGG chord plot of differentially expressed IRGs.

TABLE 2 | Top five GO terms.

Category	ID	Term	Count	%	*p* value	FDR
Upregulated IRGs						
GOTERM_BP_DIRECT	GO:0006955	Immune response	93	17.95366795	4.59E−75	7.47E−72
GOTERM_BP_DIRECT	GO:0050776	Regulation of immune response	55	10.61776062	1.14E−51	1.86E−48
GOTERM_BP_DIRECT	GO:0002250	Adaptive immune response	41	7.915057915	4.73E−36	7.69E−33
GOTERM_BP_DIRECT	GO:0006956	Complement activation	33	6.370656371	6.95E−34	1.13E−30
GOTERM_BP_DIRECT	GO:0006898	Receptor-mediated endocytosis	40	7.722007722	1.48E−30	2.40E−27
GOTERM_CC_DIRECT	GO:0009897	External side of plasma membrane	43	8.301158301	1.52E−31	1.86E−28
GOTERM_CC_DIRECT	GO:0005886	Plasma membrane	167	32.23938224	5.95E−29	7.29E−26
GOTERM_CC_DIRECT	GO:0005887	Integral component of plasma membrane	85	16.40926641	2.95E−23	3.61E−20
GOTERM_CC_DIRECT	GO:0005576	Extracellular region	86	16.6023166	3.11E−20	3.81E−17
GOTERM_CC_DIRECT	GO:0072562	Blood microparticle	24	4.633204633	4.61E−15	5.71E−12
GOTERM_MF_DIRECT	GO:0003823	Antigen binding	39	7.528957529	6.92E−41	9.44E−38
GOTERM_MF_DIRECT	GO:0004252	Serine-type endopeptidase activity	36	6.94980695	1.11E−21	1.52E−18
GOTERM_MF_DIRECT	GO:0004872	Receptor activity	25	4.826254826	4.79E−13	6.53E−10
GOTERM_MF_DIRECT	GO:0004888	Transmembrane signaling receptor activity	24	4.633204633	2.73E−12	3.72E−09
GOTERM_MF_DIRECT	GO:0030246	Carbohydrate binding	23	4.44015444	3.43E−12	4.68E−09
Downregulated IRGs						
GOTERM_BP_DIRECT	GO:0071395	Cellular response to jasmonic acid stimulus	3	3.75	7.50E−05	0.106090023
GOTERM_BP_DIRECT	GO:0044598	Doxorubicin metabolic process	3	3.75	3.47E−04	0.489670004
GOTERM_BP_DIRECT	GO:0044597	Daunorubicin metabolic process	3	3.75	3.47E−04	0.489670004
GOTERM_BP_DIRECT	GO:0030855	Epithelial cell differentiation	4	5	0.002002754	2.797891239
GOTERM_BP_DIRECT	GO:0055114	Oxidation–reduction process	8	10	0.004999641	6.849000785
GOTERM_CC_DIRECT	GO:0043025	Neuronal cell body	6	7.5	0.00495641	5.157580931
GOTERM_CC_DIRECT	GO:0008076	Voltage-gated potassium channel complex	3	3.75	0.038309612	34.05203615
GOTERM_CC_DIRECT	GO:0005576	Extracellular region	11	13.75	0.053136406	44.11602637
GOTERM_CC_DIRECT	GO:0005892	Acetylcholine-gated channel complex	2	2.5	0.081015685	59.35918267
GOTERM_MF_DIRECT	GO:0016655	Oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor	4	5	2.36E−06	0.002915475
GOTERM_MF_DIRECT	GO:0047086	Ketosteroid monooxygenase activity	3	3.75	3.72E−05	0.045918231
GOTERM_MF_DIRECT	GO:0047115	trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity	3	3.75	7.42E−05	0.091606887
GOTERM_MF_DIRECT	GO:0018636	Phenanthrene 9,10-monooxygenase activity	3	3.75	7.42E−05	0.091606887
GOTERM_MF_DIRECT	GO:0004032	Alditol:NADP+ 1-oxidoreductase activity	3	3.75	2.58E−04	0.318095483

showed that upregulated IRGs were mainly involved in the immune response (BP, GO: 0006955), external side of plasma membrane (CC, GO: 0009897), and antigen binding (MF, GO: 0003823). The downregulated IRGs were mainly involved in the cellular response to jasmonic acid stimulus (BP, GO: 0071395), neuronal cell body (CC, GO: 0043025), and oxidoreductase activity, and in acting on NAD(P)H, quinone or similar compounds as acceptors (MF, GO: 0016655) (**Figures 3A–C,E**; **Table 2**). The KEGG pathway enrichment results in the upregulated IRGs were mainly involved in cytokine–cytokine receptor interactions, chemokine signaling pathways, and cell adhesion molecules (CAMs). However, the KEGG pathway enrichment results of downregulated IRGs were mainly involved in arachidonic acid metabolism, metabolic pathways, and tyrosine metabolism (**Figures 3D,F**; **Table 3**).

Development of a Prognostic Model for the Training Cohort

To generate a prognostic model for lung adenocarcinoma, univariate regression analysis was first performed to screen the key prognostic genes in the training cohort. Thereafter, 58 significantly differentially expressed IRGs correlated with prognosis were considered for LASSO regression analysis. Finally, three key IRGs (*CLEC17A*, *INHA*, and *XIRP1*) were selected to generate an immune prognostic model. The results of the multivariate Cox regression analysis are summarized in **Table 4**. The risk score was determined using the following formula:

[Expression level of *CLEC17A* × (−0.13549042)] + [Expression level of *INHA* × (0.01207179)] + [Expression level of *XIRP1* × (0.6263501)].

TABLE 3 | Top five KEGG pathway enrichment results.

Category	Term	Count	%	*p*-value	Genes	FDR
Upregulated IRGs						
KEGG_PATHWAY	Cytokine–cytokine receptor interaction	28	5.405405405	2.94E−13	CCL1, IL21R, CXCL9, TNFRSF8, CXCL6, CXCL11, IL7R, IL10, CXCL10, CCL23, CCL21, IL10RA, CSF2RB, IL1B, CSF1R, IL2RA, TNFRSF13B, CCL19, CCL18, TNFSF8, CCR8, CCL13, CCL14, TNFSF13B, CCR5, CCR4, CXCL13, CCR2	3.53E−10
KEGG_PATHWAY	Chemokine signaling pathway	19	3.667953668	3.51E−08	CCL1, PIK3CG, ITK, CXCL9, CCL19, CXCL6, CXCL11, CCL18, CXCL10, CCR8, DOCK2, CCL13, CCL23, CCL14, CCR5, CCL21, CXCL13, CCR4, CCR2	4.22E−05
KEGG_PATHWAY	Cell adhesion molecules (CAMs)	15	2.895752896	1.03E−06	PTPRC, CADM3, SELL, ICAM3, ITGAM, PDCD1LG2, HLA-DQA1, CD86, CD80, ICOS, CD22, CD4, CD226, SPN, CD28	0.00123743
KEGG_PATHWAY	B cell receptor signaling pathway	9	1.737451737	7.44E−05	PIK3CG, CD19, CR2, FCGR2B, CD22, PIK3AP1, CD79B, CD79A, BTK	0.089383945
KEGG_PATHWAY	Phagosome	12	2.316602317	2.37E−04	MRC1, MARCO, MSR1, FCGR2B, OLR1, FCGR2C, FCGR1A, CD209, TLR4, FCGR3A, ITGAM, HLA-DQA1	0.284714595
Downregulated IRGs						
KEGG_PATHWAY	Arachidonic acid metabolism	4	5	0.002057483	AKR1C3, GPX2, CBR1, PLA2G10	2.102810979
KEGG_PATHWAY	Metabolic pathways	13	16.25	0.002420062	ETNPPL, DDC, ODC1, PLA2G10, OGDHL, HAL, HGD, TAT, AKR1C3, CBR1, HMGCS2, ENO3, NAT8L	2.469211516
KEGG_PATHWAY	Tyrosine metabolism	3	3.75	0.009368241	DDC, HGD, TAT	9.255598588
KEGG_PATHWAY	Metabolism of xenobiotics by cytochrome P450	3	3.75	0.038441569	AKR1C2, CBR1, AKR1C1	33.26837318
KEGG_PATHWAY	Phenylalanine metabolism	2	2.5	0.069378576	DDC, TAT	52.38108893

TABLE 4 | Multivariate Cox regression analysis of key immune-related genes.

Gene	Coef	HR	HR.95L	HR.95H	*p*-value
CLEC17A	−0.13549042	0.192289385	0.071601478	0.516402855	0.001071204
INHA	0.01207179	1.141665832	1.046649718	1.245307623	0.002804616
XIRP1	0.6263501	2.024671234	1.219239069	3.362173761	0.006410729

Evaluation of the Prognostic Model in the Training Cohort

LASSO regression analysis was performed to construct and evaluate the prognostic model (**Figures 4A,B**). Patients were divided into high- and low-risk-score groups in accordance with the best separation of risk scores. The high-risk-score group had significantly worse OS than the low-risk-score group ($p < 0.0001$; **Figure 4C**). The area under the ROC curve for predicting the 1-, 3-, and 5-year survival of lung adenocarcinoma was 0.699, 0.631, and 0.669, respectively (**Figure 4D**).

Additionally, the risk curve indicated that the high-risk-score group had a higher mortality and worse prognosis than the low-risk-score group (cutoff value: 0.889; **Figure 4E**). Further analysis of the relationship between risk score and pathological stage revealed that patients with early-stage lung adenocarcinoma (stages 1 and 2) scored lower than those with advanced stage lung adenocarcinoma ($p = 0.043$; **Figure 4F**).

Univariate Cox analysis showed that pathological staging and risk score had statistical significance, while age and sex had no statistical significance (**Figure 4G**). However, multivariate Cox analysis showed that pathological stage (HR, 1.995; 95% CI, 1.113–3.574; $p = 0.020$) and risk score (HR, 1.120; 95% CI, 1.025–1.223; $p = 0.012$) were independent prognostic factors (**Figure 4H**).

GSEA of the Mechanism Underlying the Prognostic Differences Between the Two Groups

In this study, the possible molecular mechanisms of the prognosis difference between the two groups of patients were analyzed by GSEA analysis. The results showed that the GO and pathway enrichment in the high-risk-score group was mainly involved in metabolism-related pathways (**Figures 5A,C**). However, GO and pathway enrichment in the low-risk-score group was primarily

focused on immunoregulation and immune cell activation (**Figures 5B,D**). The detailed GSEA results are described in **Table 5**.

Validation of the Prediction Model in the Testing Cohort

The Kaplan–Meier results showed that the high-risk group had worse OS than the low-risk group ($p < 0.0001$) (**Figure 6A**). The area under the ROC curve for predicting the 1-, 3-, and 5-year survival of lung adenocarcinoma was 0.725, 0.712, and 0.660, respectively (**Figure 6B**). Additionally, risk curve revealed that the high-risk-score group had a worse prognosis than the low-risk-score group (**Figure 6C**). These results were consistent with the results of the training set.

The Mechanism of Action of Hub IRGs in the TCGA Database

To further analyze the potential function of hub IRGs, our results were verified using the TCGA and GTEx databases. First, the mutation characteristics of these hub IRGs were analyzed among patients with lung adenocarcinoma. The mutation rates of these hub IRGs in patients with lung adenocarcinoma were 0.8, 1, and 2.6% (**Figure 7A**). Moreover, each hub gene had different mutation forms, including mutation, deletion, and amplification, in lung adenocarcinoma. For example, the mutation form of CLEC17A was mainly amplification, the mutation form of INHA was mainly amplification and missense mutation, while the mutation form of XIRP1 was mainly deep deletion and missense mutation (**Figure 7B**).

In addition, analysis of pathways in the GSCALite database revealed that CLEC17A is primarily involved in the activation of the epithelial–mesenchymal transition (EMT) and RAS pathways and cell cycle inhibition. INHA is primarily involved in the activation of the mTOR pathway and inhibition of the apoptosis pathway. XIRP1 was mainly involved in the activation of the apoptosis and the EMT pathways and inhibition of the DNA damage and the PI3K pathways (**Figures 7C,D**).

Finally, the TIMER database was used to analyze the correlation between hub IRGs and immune cells. The results showed that these key genes were significantly correlated with the infiltration of CD4+ T cells, CD8+ T cells, macrophages, B cells, and neutrophils. Assuming that a correlation coefficient >0.3 was considered a strong correlation, further analysis showed that CLEC17A was positively correlated with the infiltration of B cells and CD4+ T cells. However, INHA was negatively correlated with the infiltration of CD8+ T and dendritic cells. However, there was no strong correlation between XIRP1 and the infiltration of immune cells (**Figure 7E**). Moreover, the relationship between copy number variation (CNV) of hub IRGs and immune cell infiltration was further analyzed. The results showed that there were significant differences between the CNV of these hub IRGs and immune cell infiltration. Arm-level deletion of the CLEC17A gene was closely related to the infiltration of B cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells. Arm-level gain of the INHA gene was closely related to the infiltration of CD4+ T cells. Arm-level deletion of the XIRP1 gene was closely related to the infiltration of CD4+ T cells and macrophages (**Figure 7F**).

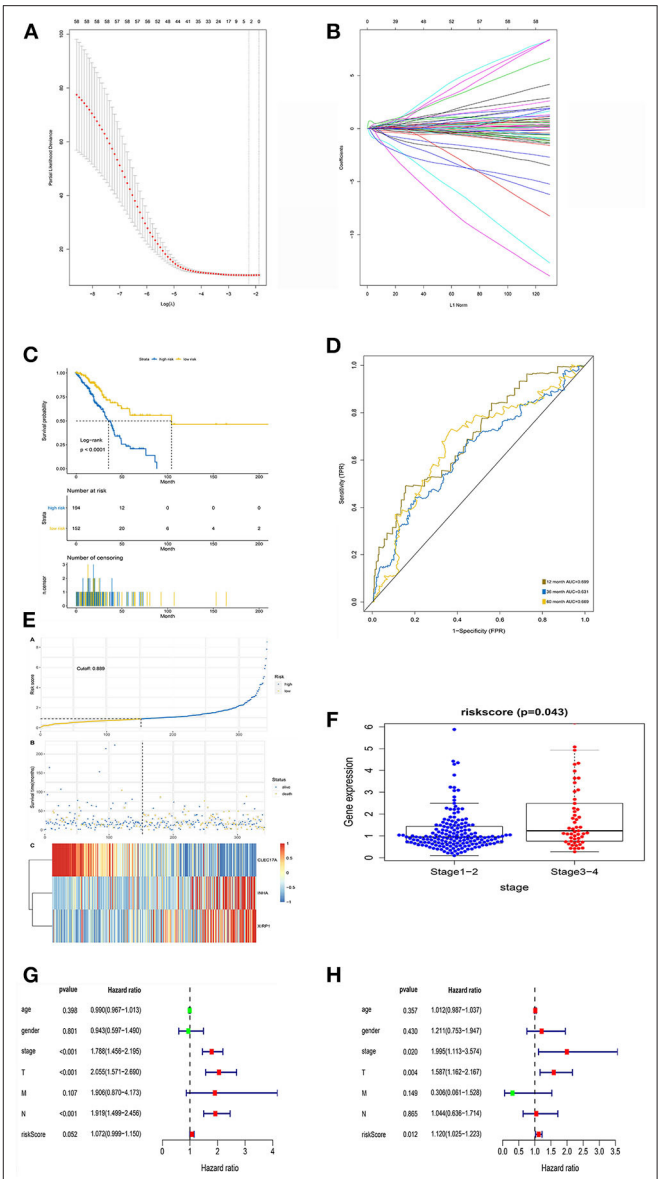

FIGURE 4 | LASSO regression analysis and identification of prognostic signatures in the training set. **(A)** Ten-fold cross-validation for turning parameter selection in the LASSO Cox regression model. **(B)** Coefficient profiles in the LASSO Cox regression model. **(C)** Survival curve of low- and high-risk groups stratified by immune-stromal score signature. **(D)** ROC analysis of the TCGA dataset for prognostic signature. **(E)** LASSO regression analysis of low- and high-risk groups. **(F)** Relationship between risk score and clinical stage. **(G)** The forest plot of the prognostic signature by univariate analysis. **(H)** The forest plot of the prognostic signature by multivariate Cox proportional regression analysis.

DISCUSSION

Tumor-infiltrating immune cells in the TME significantly contribute to the prognosis of lung adenocarcinoma. Therefore, it

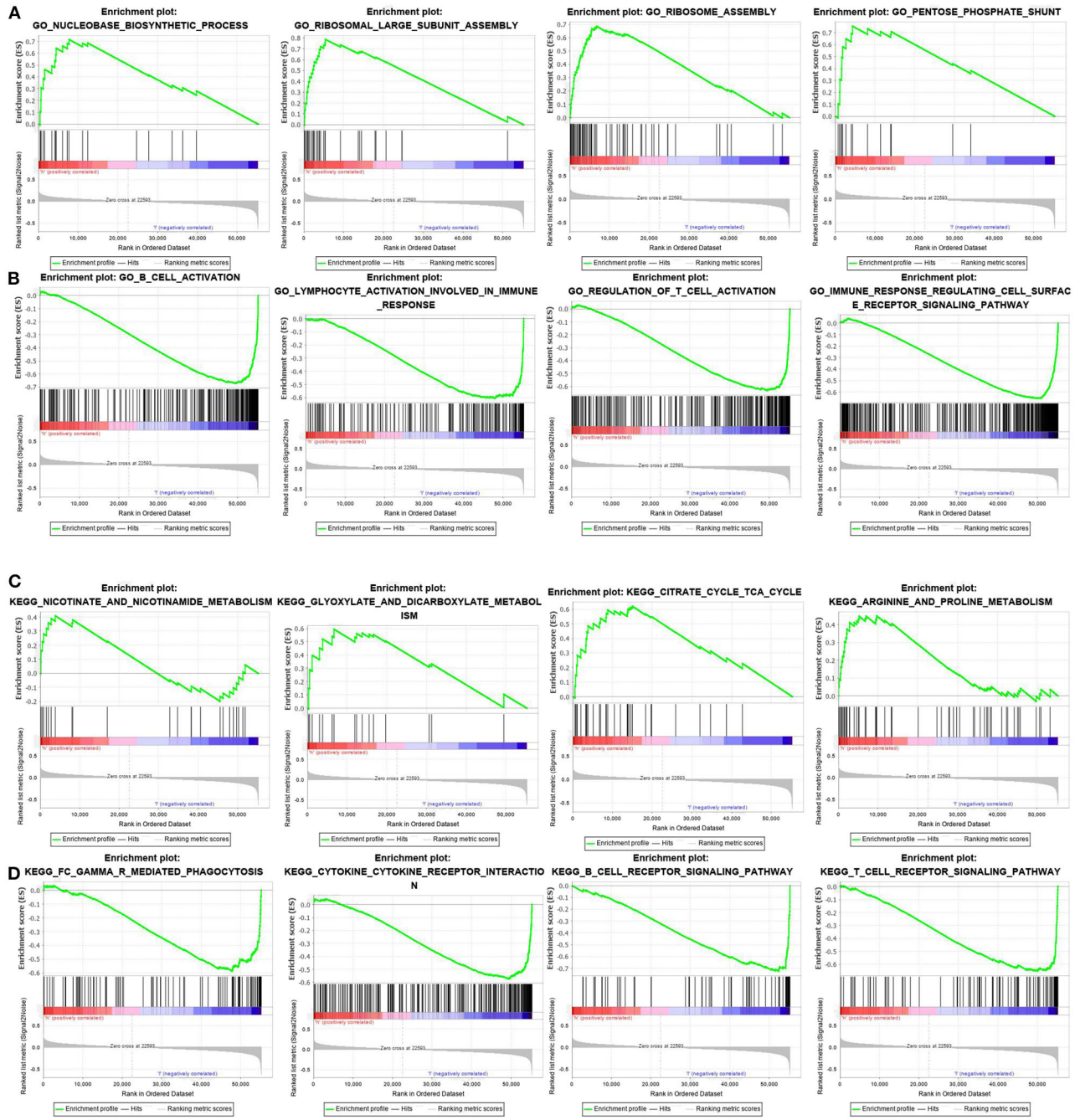

FIGURE 5 | GSEA analysis of differences in pathway enrichment in the training set. **(A,B)** GO terms in the high- and low-risk score groups. **(C,D)** KEGG pathway enrichment in the high- and low-risk score groups.

is essential to develop a TME-related immune prognostic model for appropriate clinical management of lung adenocarcinoma. Accordingly, the aim of this study was to develop a novel TME-related immune prognostic model for lung adenocarcinoma.

Although some studies have explored the prognostic value of TME-related IRGs in lung adenocarcinoma, certain key issues of these models remain to be resolved.

Yang et al. (18) used the CIBERSORT algorithm to analyze a TME-related prognostic immunity-based model for lung adenocarcinoma. This model was developed to evaluate the relative levels of the 22 immune cell phenotypes, primarily including B cells, T cells, macrophages, dendritic cells, plasma cells, natural killer cells, and mast cells. Moreover, this algorithm is primarily used to evaluate immune cells; however, it cannot be

TABLE 5 | Detailed results of gene set enrichment analysis.

NAME	ES	NES	NOM *p* value
GO Terms			
High score group			
GO_RIBOSOMAL_LARGE_SUBUNIT_ASSEMBLY	0.78643316	1.9444792	0.00203252
GO_PENTOSE_PHOSPHATE_SHUNT	0.7535411	1.8539398	0.008298756
GO_RIBOSOME_ASSEMBLY	0.68597955	1.8540714	0.016528925
GO_NUCLEOBASE_BIOSYNTHETIC_PROCESS	0.71662575	1.7974799	0.01980198
Low score group			
GO_B_CELL_ACTIVATION	−0.6719656	−2.3789294	0
GO_IMMUNE_RESPONSE_REGULATING_CELL_SURFACE_RECEPTOR_SIGNALING_PATHWAY	−0.654903	−2.3589978	0
GO_LYMPHOCYTE_ACTIVATION_INVOLVED_IN_IMMUNE_RESPONSE	−0.60444784	−2.3353372	0
GO_T_CELL_ACTIVATION	−0.6102059	−2.343195	0
KEGG Pathway Enrichment			
High score group			
KEGG_CITRATE_CYCLE_TCA_CYCLE	0.62047356	1.6142783	0.046184737
KEGG_GLYOXYLATE_AND_DICARBOXYLATE_METABOLISM	0.5940425	1.5761957	0.060194176
KEGG_ARGININE_AND_PROLINE_METABOLISM	0.45005503	1.4639851	0.055900622
KEGG_NICOTINATE_AND_NICOTINAMIDE_METABOLISM	0.4092993	1.3358487	0.115384616
Low score group			
KEGG_B_CELL_RECEPTOR_SIGNALING_PATHWAY	−0.72458243	−2.4734645	0
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY	−0.6531744	−2.3155243	0
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	−0.5735697	−2.178205	0.001968504
KEGG_FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS	−0.5871511	−2.1173847	0

used to evaluate stromal cells in the TME. Yue et al. (19) used the ESTIMATE algorithm to investigate the TME-related immune prognostic characteristics of lung adenocarcinoma. However, they directly enumerated immune and stromal cells from their expression profiles of all genes expressed in lung adenocarcinoma and normal tissues. Moreover, differentially expressed IRGs were analyzed using Wilcoxon correlation analysis between tumor and normal tissue. However, some limitations are associated with the analysis of multi-dimensional tumor gene expression profiles through the Wilcoxon rank-sum test. These findings indicate that these TME-related prognostic immunity-based models have not been adequately evaluated. These issues can be resolved primarily by improving the algorithm of IRGs in the TME and to identify more specific TME-related IRGs for lung adenocarcinoma. Therefore, it is essential to develop a new TME-related immune prognostic model for lung adenocarcinoma.

To address these aforementioned limitations, in the present study, a new method was developed to identify differentially expressed IRGs. First, TME-related differentially expressed IRGs were identified exclusively from tumor samples by evaluating tumor-infiltrating immune cells and stromal cells via the ESTIMATE algorithm; this probably effectively reflected the TME-related IRGs in tumor tissue. Second, differentially expressed IRGs were analyzed on the basis of significant differences in OS between the high- and low-immune-score groups in terms of lung adenocarcinoma, rather than differences between lung adenocarcinoma and normal tissue using the Wilcoxon rank-sum test. The prognostic model, based on prognosis-associated differentially expressed genes, might more accurately predict the prognosis of lung adenocarcinoma. Finally, intersecting differentially expressed IRGs with significant prognostic characteristics in both immune and stromal scores were used for subsequent analysis. Both immune cells and stromal cells in each tumor sample were assessed, thus better reflecting the characteristics of the TME. Therefore, the TME-related immune prognostic model developed herein was different from those developed previously. In this study, we developed a more robust prognostic model of TME-related IRGs in lung adenocarcinoma.

Furthermore, this study shows that patients with lung adenocarcinoma and high immune scores had a better prognosis than those with low immune scores, which might be due to the involvement of upregulated IRGs in immune cell infiltration factors, such as cytokines and B cell immune pathways. Clinical studies have also shown that lung cancer patients with high immune infiltration of helper T cells have a better prognosis than those with low infiltration (20, 21). These findings were consistent with our results. Previous studies have suggested that IL-2 is involved in antitumor T cell infiltration, increasing the efficacy of immunotherapy (22). IL-33 also promotes myeloid-derived suppressor cells (MDSCs) and interferes with CD8+ T and natural killer (NK) cell infiltration (23). These studies have suggested that certain cytokines are involved in antitumor immune pathways, potentially elucidating the mechanisms associated with prognosis.

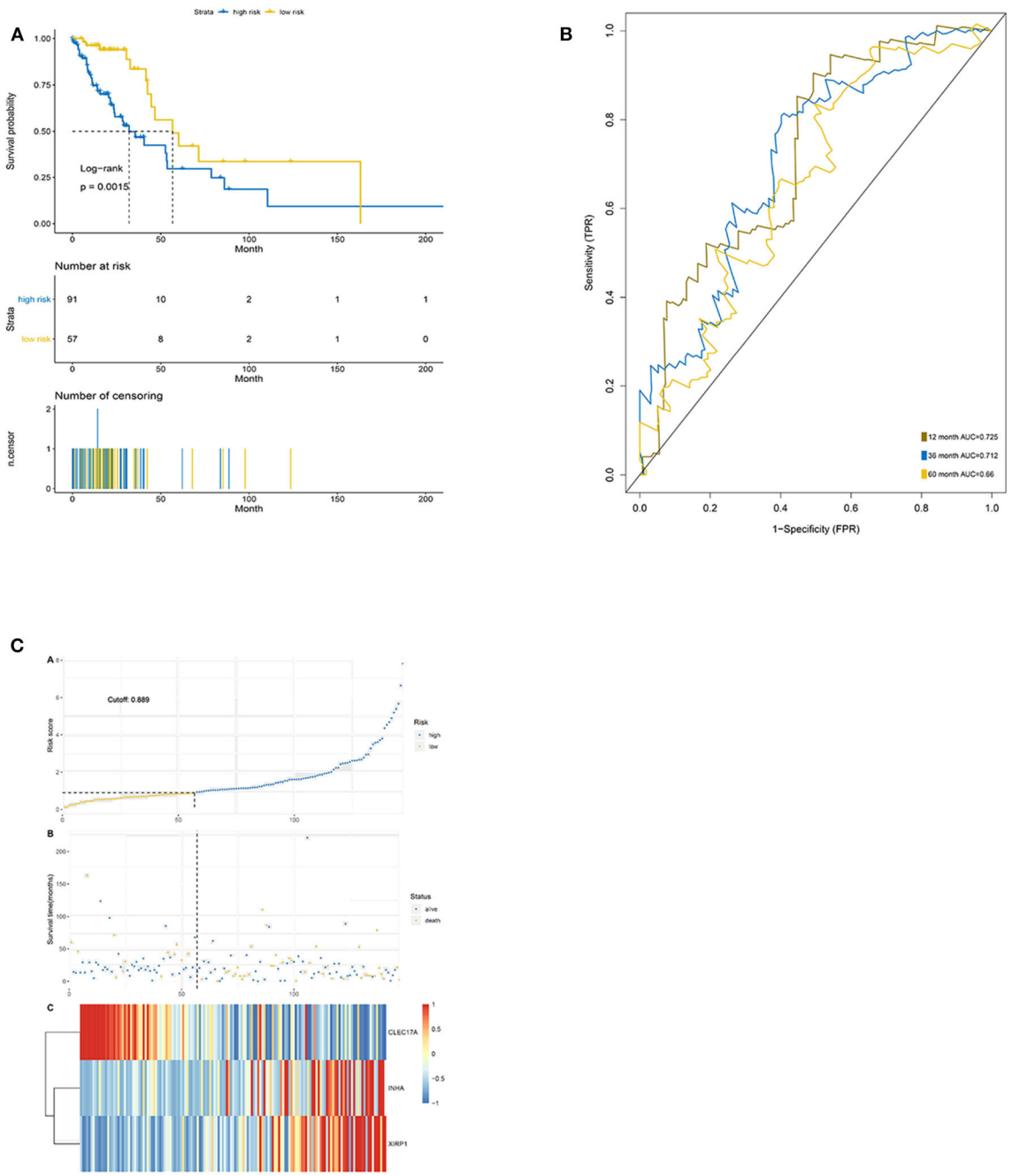

FIGURE 6 | Validation of the prediction features. **(A)** Survival curve of low- and high-risk groups stratified by immune-stromal score signature. **(B)** ROC analysis of the TCGA dataset for prognostic signature. **(C)** LASSO regression analysis of low- and high-risk groups.

Moreover, GSEA was performed to further investigate the potential mechanism underlying the differences in prognosis between the two groups. The present results indicate that the immunoregulation and immune cell activation pathways are potentially associated with a better prognosis. The underlying putative mechanism potentially involves the enrichment of B and T cell immune pathways. Furthermore, the infiltration of these immune cells is associated with an enhanced prognosis among patients with lung adenocarcinoma. Our results are concurrent with those of the aforementioned studies.

Furthermore, this study described the functional prediction of potential hub IRGs. An enhanced understanding of these potential hub genes is essential to elucidate their mechanisms of action in the TME in lung adenocarcinoma. The present results suggest that although these genes were prognosis-related IRGs, they harbored different mutations involved in different

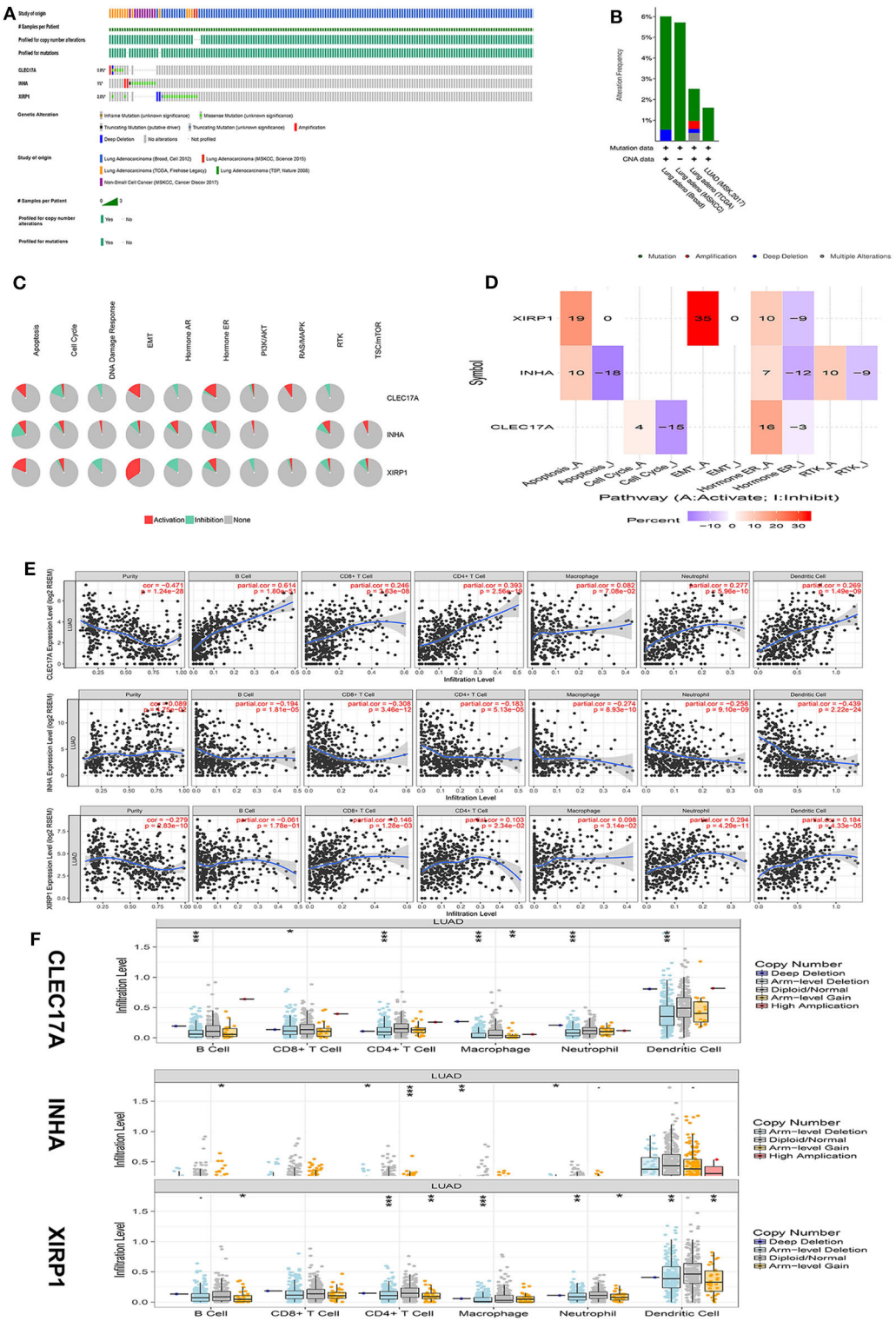

FIGURE 7 | Mechanism analysis of hub genes in the TCGA database. **(A)** Matrix heat map shows genomic alterations of hub genes in five lung datasets. **(B)** The alteration frequencies of hub genes across five studies on lung adenocarcinoma. **(C)** The pathways of hub genes were analyzed by the GSCALite tool. **(D)** Heatmap percentage of hub genes. **(E)** The correlation between the hub gene and immune cells was analyzed in the TIMER database. **(F)** The relationship between copy number variation (CNV) of hub genes and immune cell infiltration was further analyzed.

pathways in lung adenocarcinoma, indicating their potential involvement in different immunoregulatory pathways in lung adenocarcinoma. Further analysis of the function of these genes revealed that these hub genes and their CNVs were different. Moreover, the association between these hub genes and the infiltration of B cells, CD4+ T cells, CD8+ T cells, neutrophils, and other immune cells was also different. These results indicate that different CNVs of these hub genes warrant further differentiation to better understand the association between hub genes and immune cell infiltration. Based on the aforementioned results, our results indicate that *CLEC17A*, *INHA*, and *XIRP1* are potential novel biomarkers for the prognosis of lung adenocarcinoma.

CLEC17A is a human lectin found in lymph node B cells and is involved in a variety of biological processes, including cell adhesion, intercellular interactions, and pathogen recognition (24). Previous studies have shown that CLEC17A is related to the B cell receptor signaling pathway and plays an important role in the pathogenesis of chronic lymphocytic leukemia (25). The present results further indicate that CLEC17A is associated with immune cell infiltration in lung adenocarcinoma, concurrent with previous reports. XIRP1 is a striated muscle protein and belongs to the Xin actin-binding repeat-containing protein (XIRP) family. Previous studies have shown that the XIRP1 gene is related to hypertension and nervous system development (26, 27). The function of the gene has not been reported in tumors. However, our study showed that it was not only related to the TME in lung adenocarcinoma but is also related to the prognosis of lung adenocarcinoma. INHA encodes a member of the transforming growth factor-beta (TGF-beta) superfamily of proteins. The function of the gene has not been reported in lung cancer. Our results showed that INHA was a marker of poor prognosis in lung adenocarcinoma. The possible mechanism was that INHA was involved in tumor angiogenesis, leading to tumor metastasis and poor prognosis (28). Further studies are required to elucidate the roles of these hub genes in the TME in the pathogenesis of lung adenocarcinoma.

This study also had some limitations. First, this study only mined data in the TCGA database and did not combine GEO database analysis. However, in our study, patient data were segregated into training and testing cohorts. In addition, the results were verified and analyzed in comprehensive TCGA and GSCALite datasets. Second, the function of the hub gene in our study was analyzed based on the TCGA database, and the validation function of the hub gene needs to be further confirmed by basic experiments. Constructing an immune-related prognosis model was the focus of our research; hence, there was no basic experiment on hub prognostic genes. Third, our study only analyzed the correlation of differentially expressed IRGs with immune cell infiltration, thus lacking the correlation analysis of the expression of PDL1 and tumor mutational burden.

CONCLUSIONS

The robust TME-related immune prognostic model developed herein effectively predicted the prognosis of patients with lung adenocarcinoma, thus potentially guiding personalized treatment of lung adenocarcinoma in accordance with prognostic stratification. Further studies are required to elucidate the regulatory mechanisms of these IRGs in the TME and develop new treatment strategies.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://portal.gdc.cancer.gov/.

AUTHOR CONTRIBUTIONS

XQ designed the study, analyzed the data, and drafted the paper. CQ critically revised it for important intellectual content. BQ and XK assisted in data acquisition and analysis. YH and WH revised the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.541330/full#supplementary-material

Supplementary Table 1 | Immune-Stromal score of lung adenocarcinoma.

Supplementary Table 2 | Differential IRGs estimated by immune score.

Supplementary Table 3 | Differential IRGs estimated by stromal score.

Supplementary Table 4 | Intersection of differential IRGs.

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A Potential Biomarker of Combination of Tumor Mutation Burden and Copy Number Alteration for Efficacy of Immunotherapy in *KRAS*-Mutant Advanced Lung Adenocarcinoma

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Objectives: The *Kirsten Rat Sarcoma* (*KRAS*) mutation is the commonest oncogenic drive mutation in lung adenocarcinoma (LUAD) and immunotherapy may be quite promising for *KRAS*-mutant LUAD. While the effects of tumor mutation burden (TMB) and copy number alteration (CNA) are poorly understood in this illness, our study aimed to explore the roles TMB and CNA play in the prediction of response to immune checkpoint inhibitor (ICI) therapy in advanced *KRAS*-mutant LUAD.

Methods: Mutation and clinical data were downloaded from cBioPortal. We evaluated *KRAS* mutation status and divided patients into different subgroups based on TMB and CNA cutoffs to investigate the predictive value of these biomarkers on ICI response.

Results: *KRAS* mutation with concurrent *TP53* or *STK11* mutations had higher TMB and CNA compared to *KRAS* mutation alone. The *KRAS* G12C and G > T mutation subgroups, with *TP53* or *STK11* co-mutation, also had higher TMB and CNA. We found that TMB and CNA were independently associated with progression-free survival (PFS) and durable clinical benefits (DCB); TMB was positively correlated with PFS ($P = 0.0074$) and DCB ($P = 0.0008$) while low CNA was associated with prolonged PFS ($P = 0.0060$) and DCB ($P = 0.0018$). However, TMB alone did not distinguish benefits among *KRAS*-mutant patients. Notably, when combining TMB and CNA, low TMB and high CNA revealed worse outcomes of ICI therapy (mPFS: 2.20m, $P = 0.0023$; proportion of DCB: 24%, $P = 0.0001$).

Conclusion: The combination of TMB and CNA provides more sensible and accurate prediction of ICI response than individual factors in *KRAS*-mutant LUAD. Moreover, low TMB and high CNA can be utilized as a potential biomarker to predict adverse outcome in *KRAS*-mutant LUAD.

Keywords: *KRAS* mutation, lung adenocarcinoma, tumor mutation burden, copy number of alteration, biomarker

INTRODUCTION

In lung adenocarcinoma (LUAD), the most frequent oncogene driver mutation is *Kirsten Rat Sarcoma (KRAS)* (1). While patients harboring other driver genes, such as those for *Epidermal Growth Factor Receptor* (*EGFR)* and *Anaplastic Lymphoma Kinase (ALK),* may respond to therapy with tyrosine kinase inhibitors (TKIs), those harboring a *KRAS* mutation lack efficient treatment regimens. Despite decades of research, the KRAS protein remains a challenging therapeutic target due to the lack of an ideal small molecule binding pocket in the protein and its high affinity toward the abundance of guanosine triphosphate (GTP). While several novel inhibitors targeting the mutant protein *KRAS* G12C (missense substitution at codon 12; glycine to cysteine) with covalent bonding to the cysteine amino acid have been used in early phase clinical trials, there are many *KRAS* mutation subtypes, such as G12V (missense substitution at codon 12; glycine to valine) and G12D (missense substitution at codon 12; glycine to aspartic acid) (2). Besides, although the *KRAS-MAPK* pathway is downstream of *EGFR* signaling, patients with a *KRAS* mutation do not respond to *EGFR* TKIs (3). In addition, patients with *KRAS*-mutant advanced non-small cell lung cancer (NSCLC) exhibit inferior responses to cytotoxic chemotherapy as well as decreased progression-free survival (PFS) and overall survival (OS) compared to patients harboring native *KRAS* (4). Recently, immunotherapy has become regarded as most promising for *KRAS*-mutant LUAD (5).

Immune checkpoint inhibitors (ICIs) have revolutionized the management of NSCLC. Treatment with anti-cytotoxic T lymphocyte antigen 4 (CTLA4) antibody and programmed cell death-1 (PD-1) or PD-1 ligand (PD-L1) inhibitors has greatly improved patient survival. Even though ICIs have emerged as epochal milestones in anti-cancer therapy, only a subset of patients exhibits objective responses and while others show disease progression. Patients treated with ICIs may also suffer life-threatening immune-related adverse effects and even suffer hyper progression of the disease (6). A detailed understanding of key predictive factors necessary to identify patients who may potentially benefit from treatment with ICIs is thus urgent.

To date, among patients with PD-L1-positive disease, tumor-infiltrating lymphocytes have proven to be indicators of ICI therapy (7, 8). Importantly, increasing evidence suggests that the diversity and composition of gut microbiota impacts patient response to ICIs (9, 10). Since the advent of next generation sequencing, an increasing number of genetic tumor features have also been detected, including tumor mutation burden (TMB), microsatellite instability and copy number alteration (CNA), which have been correlated with therapeutic response. The number of non-synonymous single nucleotide variants, or TMB, in a tumor was found to strongly positively correlate with response to ICIs in NSCLC (11, 12). However, Merkel cell carcinoma was reported to respond better than TMB alone expects, while colorectal carcinoma was found to have worse outcomes than that predicted by TMB alone (13). Interestingly, a pan-cancer analysis based on The Cancer Genome Atlas revealed a negative relationship between CNA and immune infiltration. Meanwhile, in the setting of anti-CTLA4 therapy, CNA was reported to be a potential predictive factor of survival, independent of TMB (14).

Here, to evaluate the potential utility of TMB and CNA together in identifying distinct patient subgroups of *KRAS*-mutant LUAD, we compared the distribution of TMB and CNA among different *KRAS* mutations and then analyzed efficacy of ICI treatment in subgroups based on TMB and CNA.

MATERIALS AND METHODS

Clinical Cohorts

Data were collected from published articles. Mutation data of 860 advanced LUAD patients were retrieved from cBioPortal[1]. From this website, we obtained DNA sequencing data to analyze TMB and CNA distributions among multiple *KRAS* mutations. Details of samples included were shown as a flowchart in **Supplementary Figure 1**.

Clinical and mutation data of 240 NSCLC patients were also retrieved from cBioPortal[2]. We collected 186 advanced LUAD. All patients were treated with anti-PD-1/PD-L1 monotherapy or in combination with anti-CTLA4 blockade between April 2011 and January 2017. Details of these samples were also shown as a flowchart in **Supplementary Figure 1**. All patients had undergone the MSK-IMPACT assay, a next generation sequencing tumor profile test. Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 was performed to assess efficacy. Efficacy was additionally identified as durable clinical benefit (DCB; complete response (CR) or partial response (PR); or stable disease (SD) that lasted >6 months) or no durable benefit [NDB; progressive disease (PD) or SD that lasted ≤6 months]. Patient PFS was assessed from the date of immunotherapy initiation to the date of disease progression or death for any reason (15).

Tumor Mutation Burden Analysis

Somatic mutation data of advanced LUAD were retrieved from cBioPortal. In the MSK-IMPACT assay, tumor and matched normal data were used to identify somatic variants and optimize mutation calling filters; 100× coverage was needed to defect mutations with true variant frequencies ≥10% with 98% power. All exons and selected introns of custom gene panels of 341 (version 1), 410 (version 2), and 468 (version 3) genes were sequenced and targeted. Patients were classified according to the coding region captured in each panel, thus covering 0.98, 1.06, and 1.22 megabases (Mb) in the 341-, 410-, and 468-gene panels, respectively. The TMB cutoff value was obtained using X-tile, a tool for outcome-based biomarker cut-point optimization (16).

Copy Number Alteration Analysis

Data concerning CNA in the MSKCC database were analyzed by MSK-IMPACT sequencing. Via comparison of sequence coverage

[1]https://www.cbioportal.org/study/summary?id=lung_msk_2017

[2]https://www.cbioportal.org/study/summary?id=nsclc_pd1_msk_2018

of targeted regions in a tumor sample with a standard normal sample, CNA was identified. The Genome Analysis Toolkit (GATK) was used to obtain coverage of targeted regions, and a Loess normalization was applied to adjust guanosine-cytosine content. Log-ratio coverage values were subsequently segmented by circular binary segmentation. Germline cells were removed to ensure somatic final copy number variants. Log_2 copy number gain >0.2 or loss <-0.2 ($P < 0.05$) was used to determine significant whole gene gain or loss events (17).

Statistical Analysis

Statistical analysis was conducted by Graph Prism (version 8.0) and SPSS (version 22.0). The Mann–Whitney U test was performed to compare TMB and CNA values; TMB and CNA were presented using box plots that presented mean, interquartile ranges, and ranges. Hazard ratio was determined via univariate and multivariate Cox proportional hazard regression analyses. Kaplan–Meier curve analysis was applied to evaluate PFS and OS using log-rank analysis. Proportional DCB representation was detailed by a 100% stacked column graph. Pearson's Chi-squared test was applied to evaluate the difference in DCB proportion among different subgroups. All reported *P*-values were two-tailed, and for all analyses, $P \leq 0.05$ was considered statistically significant.

RESULTS

Prognostic Value of *KRAS* Mutation Status in Advanced Lung Adenocarcinoma

Among the 860 metastatic LUAD patients who underwent genomic analysis in the MSKCC-IMPACT study (1), *KRAS* mutation was common (**Figure 1**). As shown in **Supplementary Figure 1**, we deleted 115 patients without matched survival data. A total of 207 patients with *KRAS* mutations had statistically shorter OS as compared with 538 patients with wild-type *KRAS* tumors (HR = 1.515; 95% CI: 1.172–1.960; $P = 0.0015$, **Figure 2A**).

The most common concurrent pathogenic mutations were *TP53* (84 patients, 40.6%) and *STK11* (67 patients, 32.4%), consistent with previous studies (18). We divided *KRAS*-mutant patients into two groups based on concurrent *TP53* and *STK11* mutation status. One group was the *KRAS* co-mutation group (*KRAS*-mutant patients with either *TP53* or *STK11* mutation) and the other was the *KRAS* mutation group (*KRAS*-mutant patients without *TP53* or *STK11* mutation). We found that patients in the *KRAS* co-mutation group had shorter OS than those in the *KRAS* mutation group (HR = 1.618; 95% CI: 1.128–2.505; $P = 0.0108$, **Figure 2B**). Further analysis revealed that *KRAS*-mutant patients with co-occurring *STK11* mutation had shorter OS than those with either co-occurring *TP53* (HR = 1.864; 95% CI: 1.115–3.117; $P = 0.0176$) or both *TP53* and *STK11* (HR = 2.856; 95% CI: 1.645–4.958; $P = 0.0002$) mutations. No significant difference between *KRAS*-mutant patients with and without co-occurring *TP53* and *STK11* mutations was noted (HR = 2.219; 95% CI: 0.886–5.555; $P = 0.0234$), likely because *KRAS*-mutant patients with co-occurring *TP53* and *STK11* mutations only totaled 16 (**Figure 2C**).

The *KRAS* G12C mutation (missense substitution at codon 12; glycine to cysteine) has been previously reported to be oncogenic and potentially targetable; several novel *KRAS* G12C inhibitors, such as AMG150 and MRTX849, are being studied (2). In advanced LUAD, the *KRAS* G12C mutation was the most common, accounting for 45.4% of all *KRAS*-mutant advanced LUAD (G12C: $N = 94$, 45.4%; G12V, missense substitution at codon 12; glycine to valine: $N = 31$, 15.0%; G12D, missense substitution at codon 12; glycine to aspartic acid: $N = 28$, 13.5%). At the same time, G > T substitution (nucleotide substitution in sequences coding for amino acids in protein; G is substituted by T, $N = 129$, 62.3%) was the most common nucleotide substitution in *KRAS*-mutant advanced LUAD. On Kaplan–Meier analysis, the *KRAS* G12C mutation subtype was associated with shorter OS than wild-type *KRAS* (HR = 1.741; 95% CI: 1.209–2.509; $P = 0.0012$, **Figure 2D**), as was the *KRAS* G > T mutation subtype (HR = 1.583; 95% CI: 1.154–2.170; $P = 0.0044$, **Figure 2E**). In further analysis of the effect of concurrent *STK11* mutation, the *KRAS* G12C mutation subtype with or without concurrent *STK11* mutation was not found to have significantly different OS (HR = 1.668; 95% CI: 0.872–3.190; $P = 0.1218$, **Figure 2F**). The *KRAS* G > T mutation subtype with co-occurring *STK11* mutation, however, was found to have a much shorter OS when compared to the co-occurring *STK11* mutation alone (HR = 1.869; 95% CI: 1.063–3.286; $P = 0.0299$, **Figure 2G**).

Correlation Between *KRAS* Mutation and Tumor Mutation Burden in Advanced Lung Adenocarcinoma

Investigation of whether *KRAS* mutation status impacted TMB revealed significant differences in TMB among *KRAS* mutation and wild-type patients ($P < 0.0001$, **Figure 3A**). Moreover, patients with either *TP53* or *STK11* co-mutation had higher TMB than those with *KRAS* mutation alone ($P < 0.0001$, **Figure 3B**). Interestingly, each concurrent mutation was found to have higher TMB than *KRAS* mutation alone (*KRAS*&*TP53*&*STK11* vs. *KRAS*, $P = 0.0023$; *KRAS*&*TP53* vs. *KRAS*, $P < 0.0001$; *KRAS*&*STK11* vs. *KRAS*, $P = 0.0005$; **Figure 3C**).

Next, we sought to confirm the association between *KRAS* mutation subtypes and TMB. Results revealed that both *KRAS* G12C and G > T substitution mutations had higher TMB than did wild-type *KRAS* ($P < 0.0001$, **Figure 3D**; $P < 0.0001$, **Figure 3E**). We further found that *KRAS* G12C with either *TP53* or *STK11* co-mutation had higher TMB (*KRAS* G12C&*TP53* vs. *KRAS* G12C, $P = 0.0005$; *KRAS* G12C&*STK11* vs. *KRAS* G12C, $P = 0.0264$; **Figure 3F**). Similarly, *KRAS* G > T substitution mutation with either *TP53* or *STK11* co-mutation had higher TMB (*KRAS* G > T&*TP53* vs. *KRAS* G > T, $P = 0.0004$; *KRAS* G > T&*STK11* vs. *KRAS* G > T, $P = 0.0129$; **Figure 3G**).

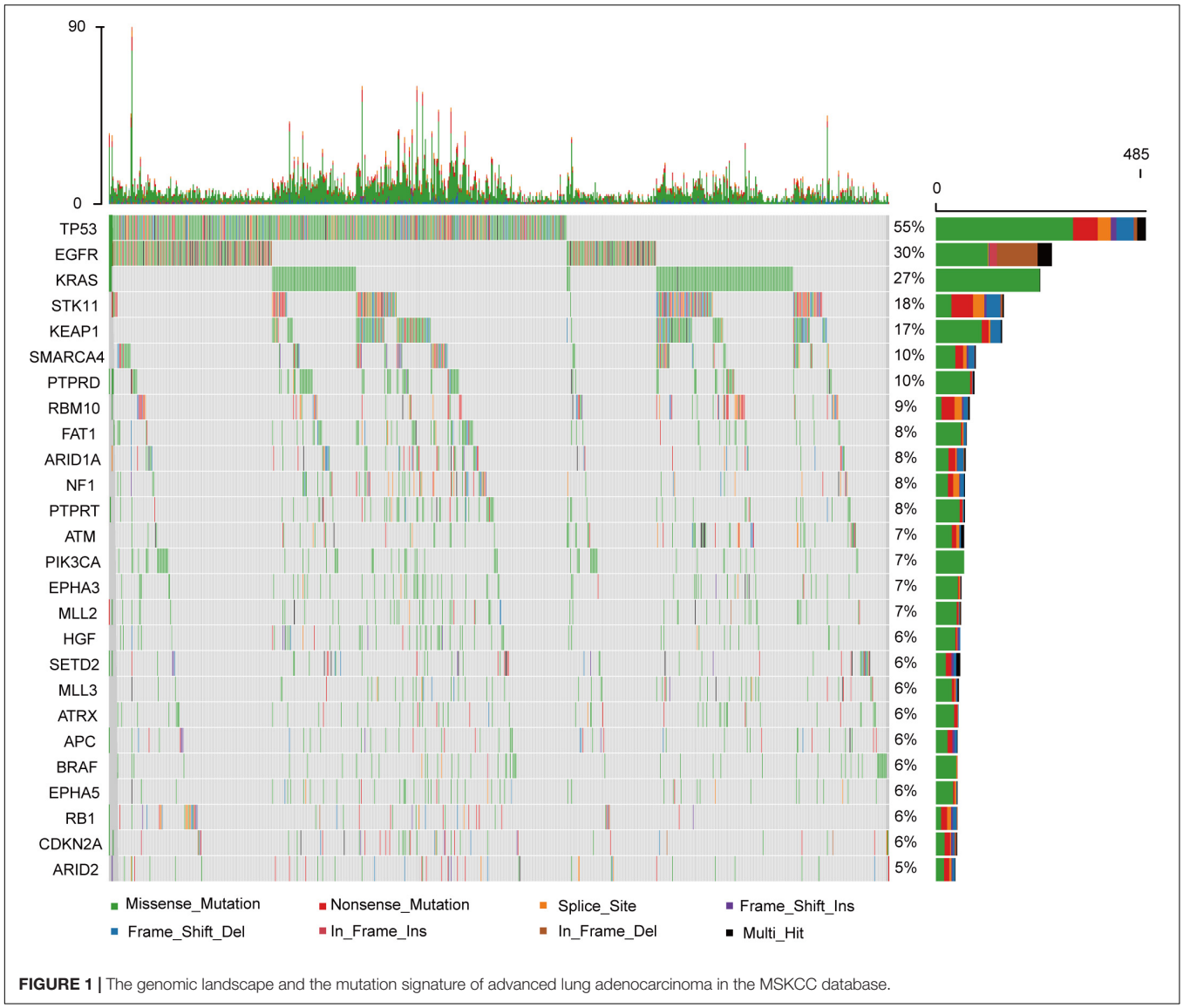

FIGURE 1 | The genomic landscape and the mutation signature of advanced lung adenocarcinoma in the MSKCC database.

KRAS Mutation Status and Copy Number Alteration in Advanced Lung Adenocarcinoma

Recent studies have reported CNA to be useful in the construction of predictive models concerning response to ICI treatment (13, 14). Our analysis revealed that *KRAS* mutation with concurrent mutations had higher CNA compared with *KRAS* mutation alone ($P < 0.0001$, **Figure 4A**). We further found that *KRAS* mutation with either *TP53* or *STK11* co-mutation significantly differed in CNA (*KRAS*&*TP53* vs. *KRAS* mutation, $P = 0.0021$; *KRAS*&*STK11* vs. *KRAS* mutation, $P = 0.0002$; **Figure 4B**). Analysis of the relationship between the common *KRAS* G12C and G > T substitution mutation subtypes and CNA revealed similar findings; both subtypes with either *TP53* or *STK11* co-mutation had significant differences in CNA (*KRAS* G12C&*TP53* vs. *KRAS* G12C, $P = 0.0014$; *KRAS* G12C&*STK11* vs. *KRAS* G12C, $P = 0.0029$; **Figure 4C**; *KRAS* G > T&*TP53* vs. *KRAS* G > T, $P = 0.0022$; *KRAS* G > T&*STK11* vs. *KRAS* G > T, $P = 0.0015$; **Figure 4D**).

Independent Predictive Value of Tumor Mutational Burden and Copy Number Alteration for Immune Checkpoint Inhibitor Response in Advanced Lung Adenocarcinoma

To estimate the predictive value of TMB and CNA in patient response to ICI treatment, available data in the MSKCC database were analyzed. A total of 240 patients with advanced NSCLC who underwent PD-1/PD-L1 inhibitor treatment alone or in combination with anti-CTLA-4 treatment were identified (15). We chose 186 patients with advanced LUAD for further analysis. For this particular population with ICI (PD-1/PD-L1 inhibitor alone or in combination with anti-CTLA-4), optimal cutoff points

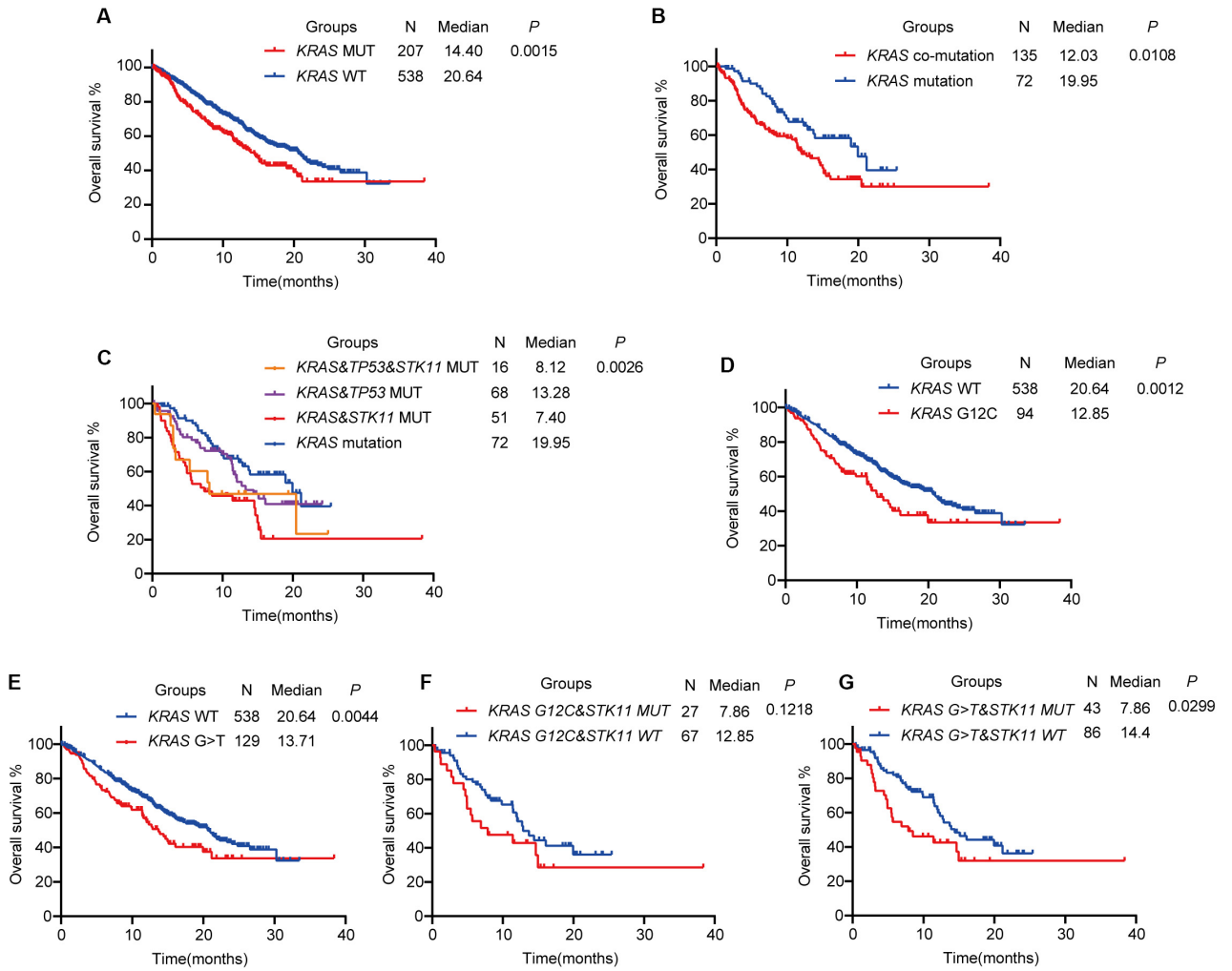

FIGURE 2 | The prognostic value of *KRAS* mutational status in advanced lung adenocarcinoma. **(A)** Kaplan–Meier survival analysis based on *KRAS* mutation status. **(B)** *KRAS*-mutant patients with co-mutations have shorter overall survival than those with *KRAS* mutation alone. **(C)** Kaplan–Meier survival analysis of *KRAS* co-mutation subtypes. **(D,E)** Kaplan–Meier survival analysis of *KRAS* mutation subtypes G12C **(D)** or G > T **(E)** with wild-type. **(F,G)** Kaplan–Meier survival analysis of *KRAS* mutation subtypes G12C **(F)** or G > T **(G)** with concurrent *STK11* mutation. MUT, mutant; WT, wild-type.

for TMB (13.27 mut/Mb) and CNA (0.05) were acquired using X-tile software. This population was subsequently divided into high (TMB ≥ 13.27 mut/Mb) and low (TMB < 13.27 mut/Mb) TMB groups; high TMB group patients were found to have significantly prolonged PFS (HR = 0.596; 95% CI: 0.408–0.870; P = 0.0074, **Figure 5A**) as well as an increased proportion of DCB (50 vs. 27%, P = 0.0008, **Figure 5E**). Analysis of patients classified into high (CNA ≥ 0.05) and low (CNA < 0.05) CNA groups revealed high CNA to be associated with shortened PFS (HR = 1.578; 95% CI: 1.140–2.184; P = 0.0060, **Figure 5B**) and a decreased proportion of DCB (24 vs. 45%, P = 0.0018, **Figure 5F**). Cox proportional hazard regression analysis revealed, after multivariate adjustment, TMB and CNA to be independent biomarkers for ICI response (TMB, HR = 0.46, P = 0.0011; CNA, HR = 1.86, P = 0.0007, **Table 1**).

We evaluated the data of 77 *KRAS*-mutant patients from the population outlined above to further confirm our findings, but no significant differences in PFS (HR = 0.636; 95% CI: 0.319–1.266; P = 0.1975, **Figure 5C**) and proportion of DCB (high vs. low TMB; 33 vs. 33%, **Figure 5G**) were noted in the *KRAS*-mutant population. Significantly prolonged PFS (HR = 0.497; 95% CI: 0.293–0.837; P = 0.0085, **Figure 5D**) and higher proportion of DCB (high vs. low CNA; 26 vs. 52%, P = 0.0002, **Figure 5H**) were observed in *KRAS*-mutant patients of the low CNA group as compared to those in the high CNA group.

Recent studies revealed high TMB to be correlated with combination PD-1 and CTLA-4 inhibitor treatment efficacy in NSCLC (11, 19). However, the predictive value of TMB in PD-1/PD-L1 inhibitor efficacy in patients with advanced NSCLC remains uncertain. We classified 159 advanced LUAD patients treated with anti-PD-1/PD-L1 monotherapy into two (high and low TMB) groups using a TMB cutoff value of 13.27 mut/Mb. Our findings revealed that high TMB was

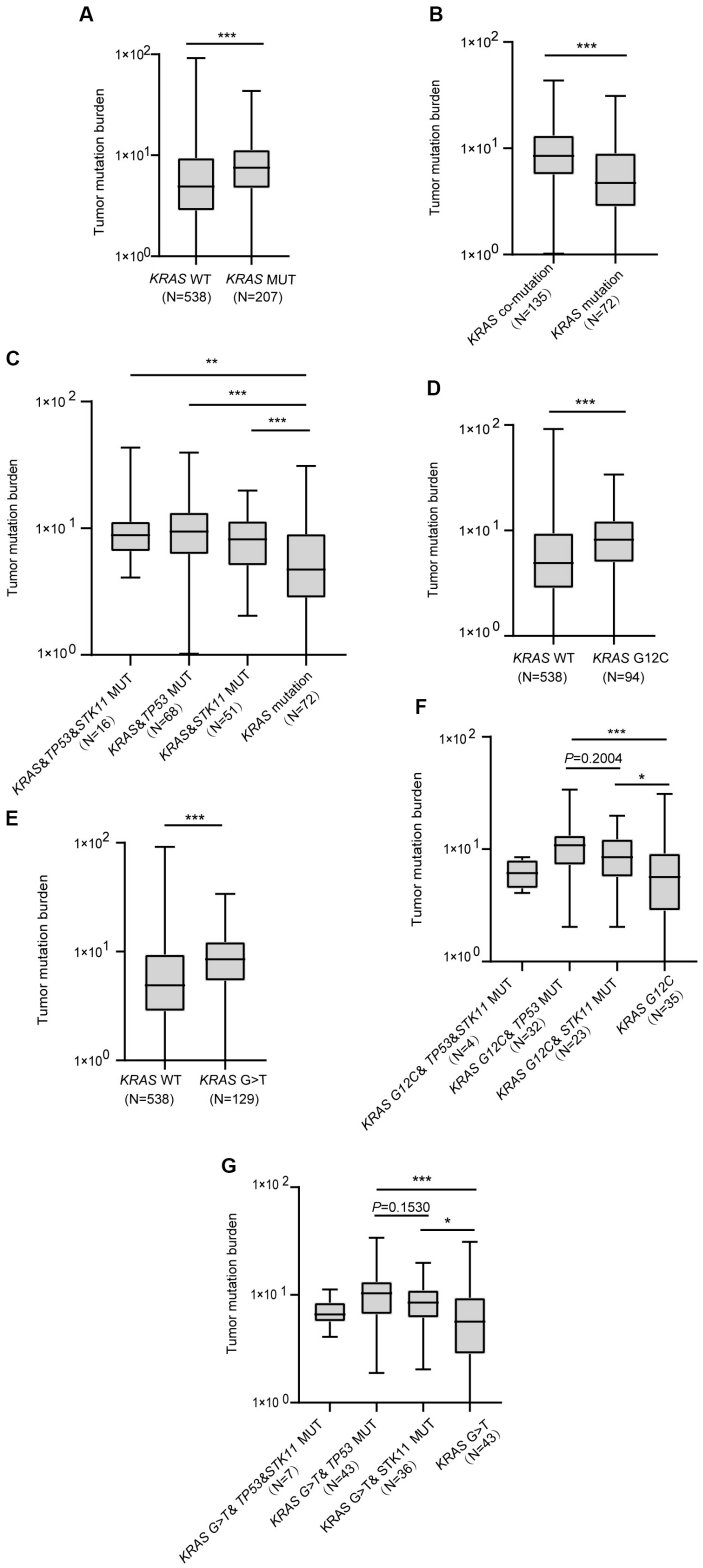

FIGURE 3 | The correlation between *KRAS* mutational status and tumor mutation burden. **(A)** Patients with *KRAS* mutation have greater tumor mutation burden. **(B)** Patients with *KRAS* mutation and concurrent mutations have greater tumor mutation burden than those with *KRAS* mutation alone. **(C)** Comparison of tumor mutation burden in *KRAS* co-mutation subtypes. **(D,E)** Comparison of tumor mutation burden in *KRAS* G12C **(D)** and G > T **(E)** subtypes. **(F,G)** Comparison of tumor mutation burden in G12C **(F)** and G > T **(G)** subtypes with co-mutations. MUT, mutant; WT, wild-type. Box plot data are presented as mean, interquartile ranges, and ranges. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

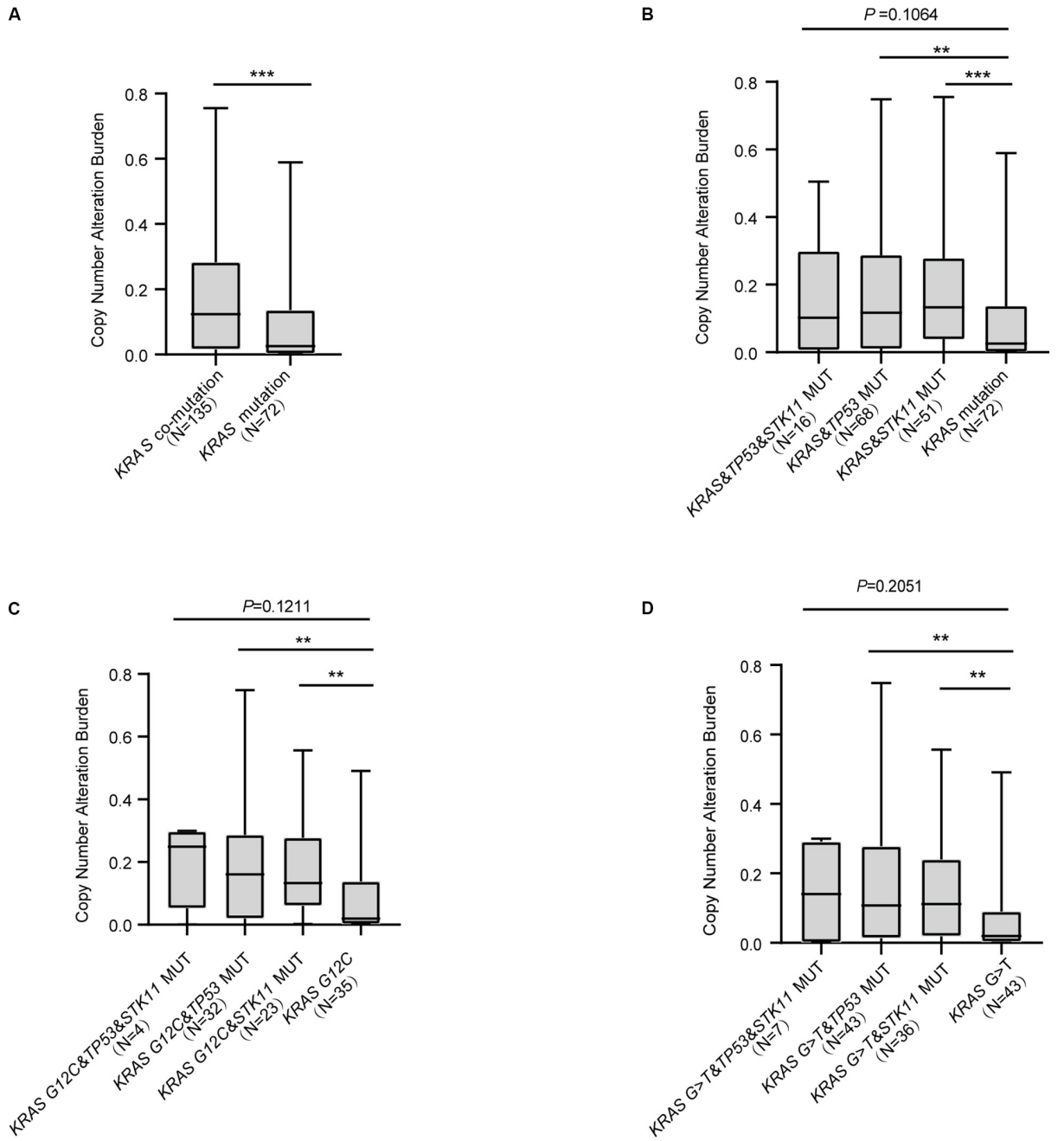

FIGURE 4 | The correlation between *KRAS* mutational status and copy number alteration burden. **(A)** Patients with *KRAS* concurrent mutations have greater copy number alteration burden with only *KRAS* mutation. **(B)** Comparison of copy number alteration burden in *KRAS* co-mutation subtypes. **(C,D)** Comparison of copy number alteration burden in *KRAS* G12C **(C)** or G > T **(D)** subtypes with concurrent mutations. MUT, mutant; WT, wild-type. Box plot data are presented as mean, interquartile ranges, and ranges. ***$P < 0.001$; **$P < 0.01$.

significantly correlated with prolonged PFS and greater DCB (HR = 0.564; 95% CI: 0.382–0.834; P = 0.0041, **Figure 5I**; DCB, 46 vs. 22%, P = 0.0003, **Figure 5K**). We found that low CNA was also associated with prolonged PFS and greater DCB (median PFS in high vs. low CNA group patients, 2.73 vs. 5.40 months, P = 0.0156, **Figure 5J**; DCB, 21 vs. 41%, P = 0.0022, **Figure 5L**).

Low Tumor Mutational Burden and High Copy Number Alteration Together Predict a Poor Response to Immune Checkpoint Inhibitor Therapy

As TMB and CNA were established independent predictive factors of ICI response, we conjectured that combined use of both

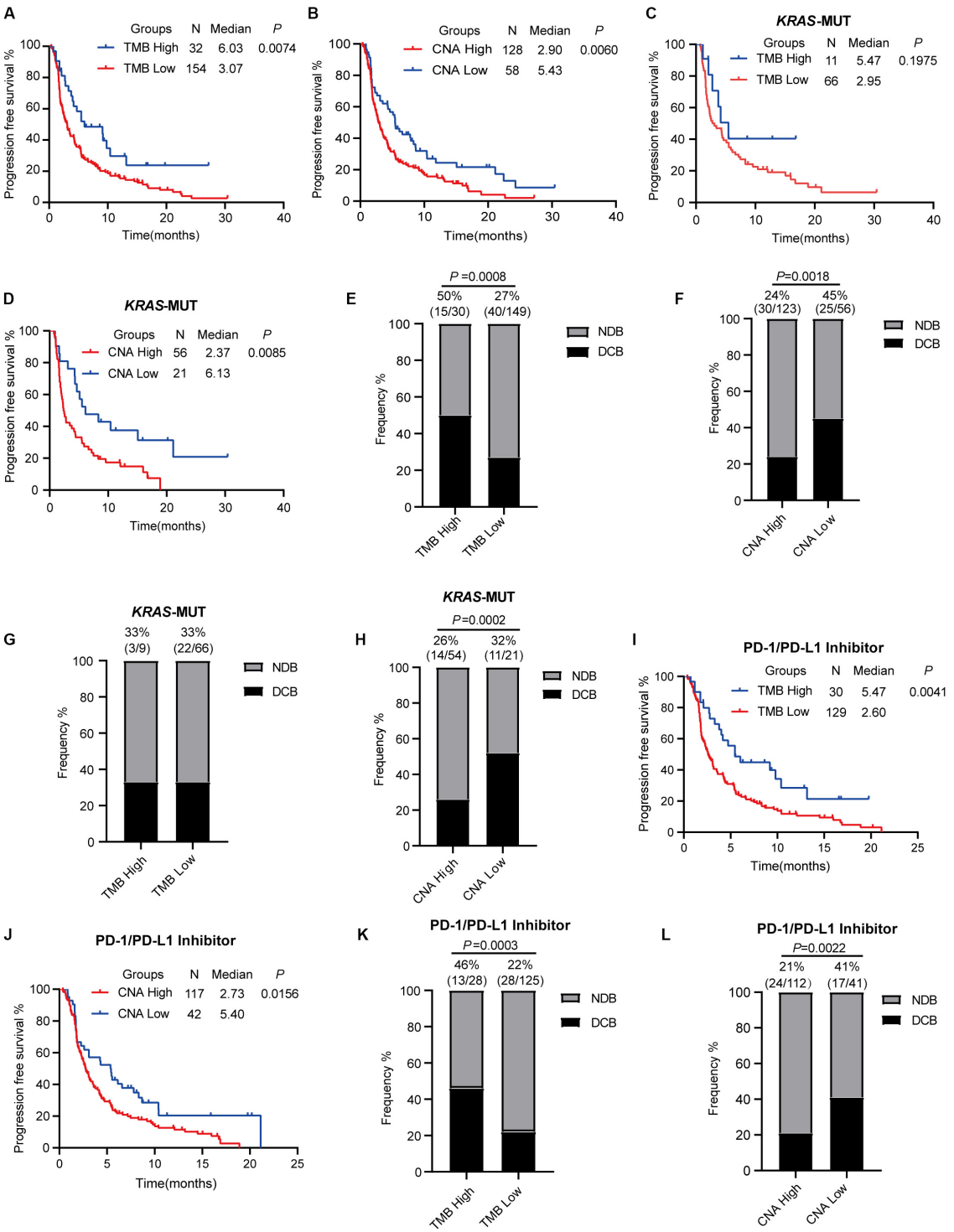

FIGURE 5 | Tumor mutation burden and copy number alteration burden correlated with clinical response to immune checkpoint inhibitor treatment. **(A,B)** Progression-free survival curve for patients receiving ICI (PD-1/L1 inhibitor or in combination with anti-CTLA-4) based on tumor mutation burden **(A)** or copy number alteration burden **(B)**. **(C,D)** Progression-free survival curve for *KRAS*-mutant patients receiving ICI (PD-1/L1 inhibitor or in combination with anti-CTLA-4) based on tumor mutation burden **(C)** and copy number alteration burden **(D)**. **(E,F)** Proportional representation of durable clinical benefits in advanced lung adenocarcinoma patients receiving ICI (PD-1/L1 inhibitor or in combination with anti-CTLA-4). **(G,H)** Proportional representation of durable clinical benefits in advanced *KRAS*-mutant lung adenocarcinoma patients receiving ICI (PD-1/L1 inhibitor or in combination with anti-CTLA-4). **(I,J)** Progression-free survival curve for patients receiving PD-1/PD-L1 inhibitor alone based on tumor mutation burden **(I)** and copy number alteration burden **(J)**. **(K,L)** Proportional representation of durable clinical benefits in advanced *KRAS*-mutant lung adenocarcinoma patients receiving PD-1/PD-L1 inhibitor alone. MUT, mutant; WT, wild-type; DCB, durable clinical benefit; NDB, no durable clinical benefit.

TABLE 1 | Univariable and multivariable Cox proportional hazards regression.

	Univariable analysis				Multivariable analysis			
		95%CI				95%CI		
Variable	**HR**	**Lower**	**Upper**	***P***	**HR**	**Lower**	**Upper**	***P***
Age	1.00	0.99	1.01	0.942				
Gender (male vs. female)	1.04	0.76	1.42	0.809				
Smoker (yes vs. no)	0.74	0.51	1.06	0.103				
TMB (≥13.27 mut/Mb vs. <13.27 mut/Mb)	0.54	0.34	0.85	0.008	0.46	0.29	0.73	0.0011
CNA (≥0.05 vs. <0.05)	1.63	1.15	2.31	0.007	1.86	1.30	2.66	0.0007

HR, hazard ratio; CI, confidence interval.

TMB and CNA would better predict ICI efficacy. In advanced LUAD patients with ICI (PD-1/PD-L1 inhibitor alone or in combination with anti-CTLA-4), low TMB and high CNA were found to have significantly shorter PFS compared to patients with high TMB and high CNA, high TMB and low CNA, and low TMB and low CNA (low TMB and high CNA vs. high TMB and high CNA: HR = 1.803, 95% CI: 1.199–2.712, $P = 0.0047$; low TMB and high CNA vs. high TMB and low CNA: HR = 2.693, 95% CI: 1.276–5.683, $P = 0.0094$; low TMB and high CNA vs. low TMB and low CNA: HR = 1.752, 95% CI: 1.240–2.476, $P = 0.0015$; **Figure 6A**). Patients with low TMB and high CNA had the significantly lowest proportion of DCB as compared to those in the three aforementioned subgroups (low TMB and high CNA vs. high TMB and high CNA vs. high TMB and low CNA vs. low TMB and low CNA; 19 vs. 46 vs. 75 vs. 42%, $P < 0.0001$, **Figure 6C**). Our analysis revealed findings consistent with those above in advanced LUAD patients with PD-1/PD-L1 inhibitor alone; patients with low TMB and high CNA were confirmed to have the significantly shortest PFS (low TMB and high CNA vs. high TMB and high CNA: HR = 1.771, 95% CI: 1.156–2.713, $P = 0.0086$; low TMB and high CNA vs. high TMB and low CNA: HR = 2.851, 95% CI: 1.385–5.872, $P = 0.0045$; low TMB and high CNA vs. low TMB and low CNA: HR = 1.608, 95% CI: 1.095–2.363, $P = 0.0154$, **Figure 6B**) and lowest proportion of DCB (low TMB and high CNA vs. high TMB and high CNA vs. high TMB and low CNA vs. low TMB and low CNA: 16 vs. 42 vs. 75 vs. 38%, $P < 0.0001$, **Figure 6D**).

Next, we further analyzed the predictive value of low TMB and high CNA in *KRAS*-mutant LUAD. In those patients with ICI (PD-1/PD-L1 inhibitor alone or in combination with anti-CTLA-4), although there were no *KRAS*-mutant LUAD patients in the high TMB and low CNA subgroup, patients with low TMB and high CNA were found to have shortened PFS (low TMB and high CNA vs. high TMB and high CNA: HR = 1.977, 95% CI: 1.025–3.814, $P = 0.0420$; low TMB and high CNA vs. low TMB and low CNA: HR = 2.338, 95% CI: 1.368–3.995, $P = 0.0019$, **Figure 6E**) and a smaller proportion of DCB (low TMB and high CNA vs. high TMB and high CNA vs. low TMB and low CNA: 24 vs. 33 vs. 52%, $P = 0.0001$, **Figure 6G**). Significant differences in PFS (low TMB and high CNA vs. high TMB and high CNA: HR = 1.994, 95% CI: 1.021–3.894, $P = 0.0433$; low TMB and high CNA vs. low TMB and low CNA: HR = 2.022, 95% CI: 1.131–3.616, $P = 0.0176$, **Figure 6F**) and DCB (low TMB and high CNA vs. high TMB and high CNA vs. low TMB and low CNA: 25 vs. 33 vs. 50%, $P = 0.0008$, **Figure 6H**) in patients with low TMB and high CNA receiving anti-PD-1/PD-L1 monotherapy were noted compared with those of the other two groups. Thus, the combination of TMB and CNA was confirmed to increase the sensitivity of ICI efficacy prediction in advanced *KRAS*-mutant LUAD. In addition, the combination of low TMB and high CNA was confirmed to predict poor ICI response in advanced *KRAS*-mutant LUAD.

DISCUSSION

Among lung cancer patients, *KRAS* mutation is the commonest mutation and 27% of LUAD patients harbor it (20). Patients suffering *KRAS*-mutant NSCLC continue to have a poor prognosis and lack efficient treatment strategies. Effective pharmacologic targeting of *KRAS* mutations also remains an unprecedented challenge. Recent studies, however, have reported that patients suffering *KRAS*-mutant NSCLC treated with ICI therapy had improved OS and PFS compared to those treated with chemotherapy (21, 22). In addition, TMB and CNA have been reported to be features of the genomic landscape that affect ICI efficacy (13). Here, we found that combined use of TMB and CNA increased the predictive sensitivity for ICI response in patients suffering *KRAS*-mutant advanced LUAD. Importantly, we found that low TMB and high CNA were associated with a poor prognosis, and TMB level positively correlated with response to anti-PD-1/PD-L1 monotherapy.

Recent studies have reported *KRAS*-mutant tumors to show greater PD-L1 expression (23) and T-cell infiltration (24). Here, our analysis of the correlation between KRAS mutation status and TMB revealed TMB to be associated with tumor immunogenicity and greater benefit of ICI therapy (25). We found that *KRAS*–mutant tumors showed higher TMB than did wild-type tumors. In further analysis of mutation subtypes and co-mutations, we demonstrated that *KRAS* with either co-occurring *TP53* or *STK11* mutation had greater TMB as compared to *KRAS* mutation alone. In *KRAS*-mutant LUAD, *KRAS* with *STK11* co-mutation was reported to facilitate immune escape and resistance to anti-PD-1 therapy and to mostly be an "immune desert" phenotype (26, 27). Interestingly, *TP53* inactivation in *KRAS*-mutant LUAD was reported to increase inflammatory marker levels and improve PFS (21, 27).

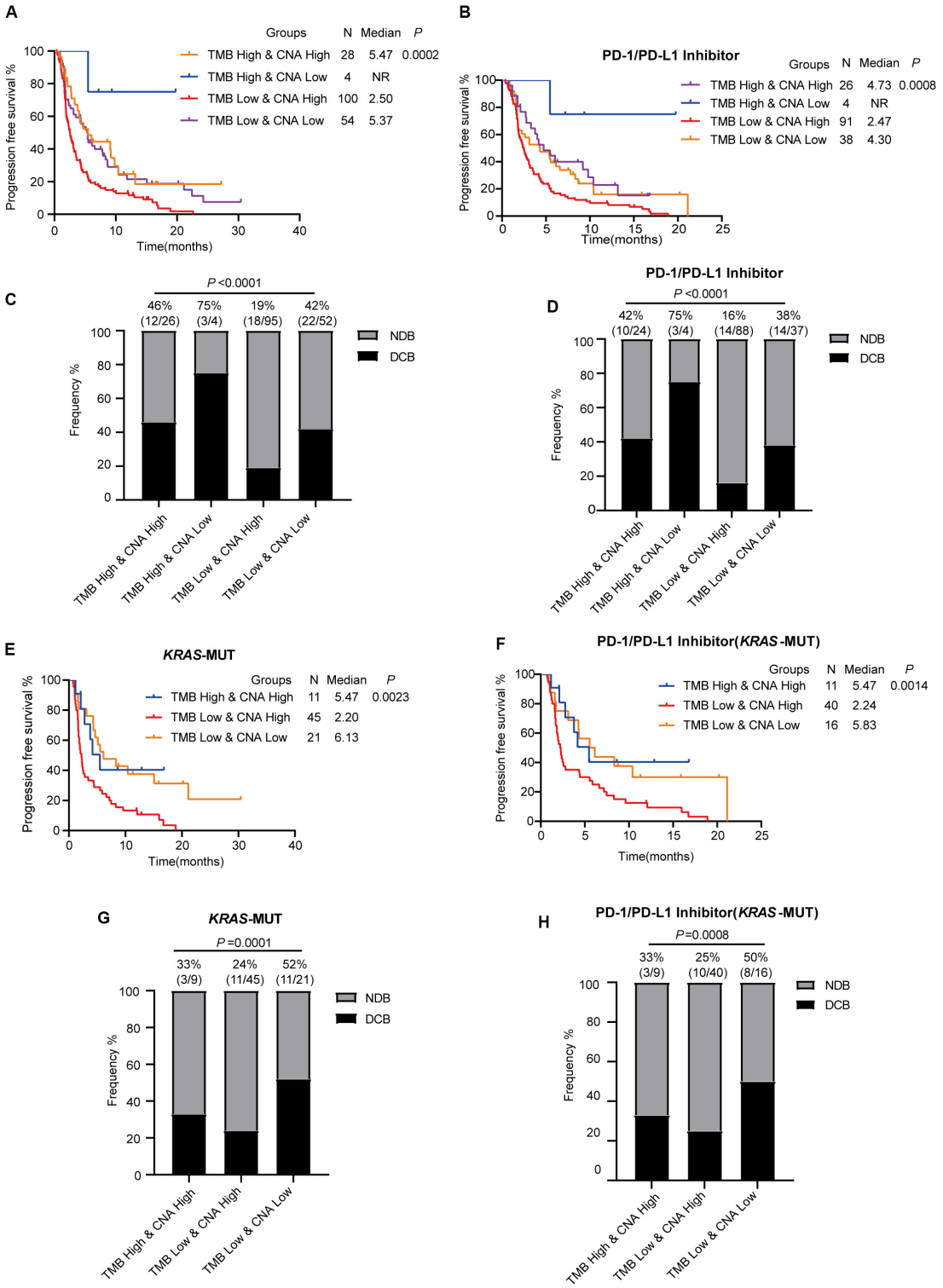

FIGURE 6 | Low tumor mutational burden and high copy number alteration together predict a poor response to immune checkpoint inhibitor therapy. **(A,B)** Low TMB and high CNA show shorter progression-free survival in patients receiving ICI (PD-1/L1 inhibitor or in combination with anti-CTLA-4) **(A)** and patients receiving PD-1/PD-L1 inhibitor alone **(B)**. **(C,D)** Low TMB and high CAN show decreased proportion of DCB in patients receiving ICI (PD-1/L1 inhibitor or in combination with anti-CTLA-4) **(C)** and patients receiving PD-1/PD-L1 inhibitor alone **(D)**. **(E,F)** Low TMB and high CNA show shorter progression-free survival in *KRAS*-mutant patients receiving ICI (PD-1/L1 inhibitor or in combination with anti-CTLA-4) **(E)** and *KRAS*-mutant patients receiving PD-1/L1 inhibitor alone **(F)**. **(G,H)** Low TMB and high CNA show decreased proportion of DCB in *KRAS*-mutant patients receiving ICI (PD-1/L1 inhibitor or in combination with anti-CTLA-4) **(G)** and *KRAS*-mutant patients receiving PD-1/L1 inhibitor alone **(H)**. MUT, mutant; WT, wild-type; DCB, durable clinical benefit; NDB, no durable clinical benefit.

Tumor CNA burden has been reported to be a pan-cancer prognostic factor for recurrence and death (28). Here, we found that *KRAS* with either co-occurring *TP53* or *STK11* mutation had higher CNA. Furthermore, high CNA was a potential predictor of poor ICI efficacy in *KRAS*-mutant advanced LUAD. This finding was in agreement with prior evidence of CNA as a biomarker predictive for ICI response. Recently, CNA was reported to improve cell proliferation, reduce immune infiltration, and at lower levels correlate with poor ICI response (14). Of note, CNA likely is involved in the suppression of antigen presentation in cancer cells (29).

Although TMB and CNA have been reported to impact immune infiltration and predict ICI response, there have been few studies exploring associations among the combined application of TMB and CNA and clinical benefits of ICI. Multivariate Cox proportional hazard regression analysis of TMB and CNA confirmed that these two biomarkers were independent predictive factors for ICI response. Thus, while CNA provides complementary analysis of clinical ICI response, combining TMB and CNA improves the predictive sensitivity and accuracy of ICI response compared to use of these biomarkers independently. We divided patients into subgroups based on the cutoff value of TMB (13.27 mut/Mb) and CNA (0.05) from X-tile software. Previous studies have revealed that a cut-off value for TMB of 14.31 mut/Mb was used to predict survival in patients who underwent immunotherapy for advanced gastric cancer (30), while intermediate CNA was found to discriminate for recurrence in a prostate cancer population (31). Therefore, more researches are needed to speculate the optimal cutoff for clinical practices. We found that patients with low TMB and high CNA suffered significantly worse outcomes in the setting of ICI therapy. In *KRAS*-mutant LUAD, combination of TMB and CNA revealed that patients with low TMB and high CNA suffered a significantly worse prognosis. Thus, combined application of TMB and CNA values can be used to accurately select patients who would benefit from ICI treatment.

Our research had several limitations. First, all of our data were obtained from open databases, and patient characteristics were limited. As such, we were confined to analyzing data that was available. For example, patients receiving ICI treatment had PFS but lacked OS data; thus we could only analyze differences in PFS. In addition, we were only able to obtain genomic and clinical data; as PD-L1 mRNA expression and TPS data were unavailable, we could not compare any difference among them across *KRAS*-mutant LUAD subgroups. Finally, as our analysis was retrospective in nature, prospective and multi-center clinical trials should further be performed prior to utilization of combined TMB and CNA in the prediction of patient outcomes to ICI therapy.

CONCLUSION

In conclusion, we here detailed that combining TMB and CNA provides a potential biomarker that effectively predicts patient response to ICI therapy. We found that TMB and CNA were higher in *KRAS*-mutant tumors as compared to wild-type tumors. Furthermore, *KRAS* with either *TP53* or *STK11* co-mutations had higher TMB and CNA as compared with *KRAS* alone. Our findings highlight that low TMB and high CNA is useful in predicting adverse patient outcomes for ICI therapy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Xi'an Jiaotong University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LX, XF, KN, and TT designed the study and wrote the manuscript. XW, WL, and XZ downloaded and analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.559896/full#supplementary-material

Supplementary Figure 1 | Flowchart of study. TMB, tumor mutation burden; CNA, copy number alteration; MUT, mutant; WT, wild-type; DCB, durable clinical benefit; NDB, no durable clinical benefit.

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Risk of Developing Checkpoint Immune Pneumonitis and Its Effect on Overall Survival in Non-small Cell Lung Cancer Patients Previously Treated With Radiotherapy

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Introduction: Immune checkpoint inhibitor-related pneumonitis (ICIP) is a potentially life threatening immune-related adverse event (irAE), especially in non-small cell lung cancer (NSCLC) patients. Currently, the potential for increased irAE in patients who receive radiotherapy is scarcely known, although a connection between antitumor immune responses and irAEs has been suggested. In this study, we evaluated the development of ICIP in non-small cell lung cancer patients with prior radiotherapy, treated with immunotherapy in the second-line.

Methods: In this retrospective trial, we included patients treated with second-line immunotherapy at the National Cancer Institute in Mexico City from February 2015 to February 2018. Clinical, radiological and treatment variables were evaluated according to the presence of ICIP as defined by the Common Terminology Criteria for Adverse Events (4.0) in patients with or without a previous (≥months) history of radiotherapy.

Results: Among 101 NSCLC patients who received treatment with ICIs, 22 patients (21.8%) were diagnosed with ICIP, of which 73% (16/22) had a history of radiotherapy (OR 6.04, 95% CI 2.03−18.0, $p < 0.001$). Median progression free survival and overall survival were similar in patients who developed ICIP compared with those who did not, however, patients who presented grade ≥ 2 ICIP had an increased risk of mortality (HR 2.54, 95% CI 1.20−5.34, $p = 0.014$).

Conclusion: In this real-world cohort of NSCLC patients treated with ICI, the history of prior radiotherapy was associated with increased risk for ICIP development. Unlike other irAEs, grade ≥ 2 ICIP is an independent prognostic factor for decreased survival in NSCLC patients.

Keywords: checkpoint immune therapy, pneumonitis, radiotherapy, NSCLC, lung cancer, immune related adverse effects

INTRODUCTION

Immune-checkpoint inhibitors anti-PD-1 and PD-L1 have changed the paradigm of treatment in several malignancies, including non-small cell lung cancer (NSCLC). Immunotherapy has demonstrated to improve overall survival (OS) and is now the standard of care for many solid tumors. The inhibition of the PD-1/PD-L1 axis can disrupt normal mechanisms of immune tolerance, resulting in increased immune activation in normal tissues; this in turn, can be associated with a unique set of adverse side effects, also known as immune-related adverse events (irAEs). Among them, immune checkpoint inhibitor-related pneumonitis (ICIP) has been reported as a potentially life-threatening irAE (1, 2).

Currently, a considerable subset of patients with locally advanced unresectable or metastatic NSCLC receive treatment with radiotherapy, alone or in combination with chemotherapy before being candidates to receive immunotherapy (3, 4). Moreover, radiotherapy has an important role in patients with brain metastases, as well as in radical treatment of patients with oligometastatic disease (5, 6).

Radiotherapy offers good local control of tumor growth; however, it also has multiple immune-modulatory effects. Radiation therapy induces DNA and membrane cellular damage, increasing reactive oxygen species (ROS) that activate transcription factors and signaling pathways modulating the immunophenotype and immunogenicity of tumor cells, restoring antitumor T-cell response in the tumor microenvironment, increasing tumor antigen release, while also improving antigen presentation and T-cell infiltration (7, 8). New evidence suggests that immunotherapy enhances antitumor immunogenicity of radiotherapy when used after local control with radiation (9). Fractionated radiotherapy in combination with anti PD-1/PD-L1 monoclonal antibodies was shown to generate effective CD8+ T-cell responses that improve local tumor control and long-term survival (10). Therefore, the risk of treatment-related symptoms might change with the advent of new treatment-combination modalities especially in real-world settings.

Immune checkpoint inhibitor-related pneumonitis is a challenging entity to diagnose, and currently there is no specific diagnostic test or symptoms which can outline ICIP patients. The most common scenarios with ICIP-like symptoms, such as infection and malignant lung infiltration, should always be initially ruled out. The risk of severe ICIP (grade 3 and 4) has been reported in randomized controlled trials more frequently in patients that receive monotherapy with CTLA-4 targeting agents (2−4%), followed by anti-PD-1 (1−3%) and anti-PD-L1 (0.4%) monoclonal antibodies (9, 11–13). Moreover, the rate increases (10%) when combinations with anti-PD-1/PD-L1 plus anti–CTLA-4 monoclonal antibodies are used (9), however, the incidence of ICIP may be underreported, as suggested by several retrospective studies of real-world data, in which the percentage of patients with ICIP is higher (10−20%) (14, 15).

The association with the history of radiotherapy and the risk of developing pneumonitis after treatment with ICIs has been scarcely described. We hypothesized that the risk of pneumonitis in patients that receive treatment with immunotherapy after radiotherapy increases compared with those who do not have a history of radiotherapy.

MATERIALS AND METHODS

Study Design and Patient Selection

We performed a retrospective cohort study to evaluate the incidence of ICIP on patients with advanced NSCLC who had previously undergone radiotherapy and were currently receiving ICIs in the second-line setting, as well as its effect in terms of survival outcomes. Patients who were treated at the National Cancer Institute in Mexico City between February 2013 and February 2018 were screened for inclusion. Patient demographic, clinical and pathological characteristics, immunotherapy treatment type, previous history of radiotherapy, irradiated region and doses (radiation doses were considered additive among different regions), previous chemotherapy and other outcomes were collected from electronic medical records. Every medical charts and image studies were evaluated by a multi-disciplinary team including at least: a medical oncologist, thoracic surgeon and a radiation oncologist. Infectious processes were excluded by utilizing blood cultures or sputum cultures, as needed by each patient; furthermore, every patient received clinical evaluation by an experienced medical oncologist prior to every treatment session.

Patients treated concomitantly with immunotherapy and radiotherapy were excluded.

ICIP Definition and Grading

All CT-scan were retrospectively reviewed. ICIP diagnosis was determined using a combination of clinical, radiological and biological tests to rule out differential diagnoses such as disease progression, infections, other comorbidities as well as radiation recall pneumonitis (pneumonitis limited to the radiation field). Pneumonitis toxicity grade was assessed according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events version 4.0 (CTCAE v4.0). Immune checkpoint inhibitors therapy was suspended in all patients who presented with G2 or higher ICIP and who required treatment with high doses of corticosteroids.

Statistical Analysis

For descriptive purposes, continuous variables were summarized as arithmetic means and standard deviations (SDs), and categorical variables were expressed as frequencies and proportions. The $\chi 2$ and Fisher exact tests were used for evaluating the statistical significance between patient and treatment characteristics and the development of any grade of CIP. Progression free survival (PFS) was determined from the initiation of immunotherapy until progression of disease; OS was determined from initiation of immunotherapy until death by any cause or loss to follow-up. PFS and OS were analyzed by the Kaplan-Meier Method; risk factors for time to development of pneumonitis and univariate survival analysis were modeled by using a Cox proportional hazards model. All statistical tests were two-sided and $p < 0.05$

was deemed to be statistically significant. SPSS software (version 22; SPSS; Chicago, IL, United States) was used for data analysis.

RESULTS

A total of 101 NSCLC patients treated with immune checkpoint inhibitors as second line were included for the analysis. Median age for all population was 61 years old (±12.3). Most patients were female (57.4%) and had a positive smoking history (53.5%). The most common histological subtype was adenocarcinoma (84.1%). PD-L1 status was known in 35.6% of patients (36/101 patients), of whom, 75% (27/36 patients) were positive. Other baseline characteristics of the cohort are presented on **Table 1**.

Regarding the treatment scheme, 41.6% (42/101) of patients were treated with nivolumab and 58.4% (59/101) with pembrolizumab as second-line of treatment. Among the included population, 40 patients (39.6%) received radiotherapy prior to ICI therapy; additionally, among radiotherapy-treated patients 17 (42.5%) received radiotherapy exclusively to the lung, 20 (50%) received radiotherapy to the vertebral column and three (7.5%) to mediastinal lymph nodes. The overall incidence of any-grade ICIP was 21.8% (22/101 patients). Incidence of ICIP in patients with history of radiotherapy was significantly higher compared with radiotherapy-naïve patients [40% vs. 9.8%; OR 6.11; 95% CI 2.13−17.52 ($p < 0.001$)]. In addition, doses greater than 60 Gy of radiation were associated with an increased risk of developing ICIP (OR 7.21; 95% CI 1.83−28.40) compared to patients who received less than 60-Gy (OR 5.35; 95% 1.56−18.42), however, this was not statistically significant.

Median time from ICI initiation to pneumonitis onset was 4.5 months (range 0.72−13.14 months). No association was found between line of treatment and the elapse time to ICIP development. The incidence of ICIP was similar between both ICI drugs (54.5% vs. 45.5% for nivolumab and pembrolizumab, respectively, $p = 0.16$).

Grade ≥ 2 ICIP developed in 12 patients (11.9%); and grade ≥ 3 in four patients (4%). Incidence of grade ≥ 2 ICIP was also higher in patients who received previous radiotherapy (22.5% vs. 4.9%). Remarkably, all patients that developed grade ≥ 3 pneumonitis had been previously treated with radiotherapy (**Table 2**). Despite the fact that tomography patterns can be superimposed, predominantly ground glass opacities, we can classify the damage based on the predominant injury; the tomographic pattern more frequently found was ground glass opacities, which was seen in 50% (12/22 patients), cryptogenic organizing pneumonia-like and pneumonitis not otherwise specified were found in 18.2% (4/22 patients), besides interstitial lung pattern, and hypersensitivity pneumonitis were reported (4.5% in both cases) (**Figure 1**).

Median PFS was 3.6 months (95% CI 2.6−4.6) and median OS was 16.3 months (95% CI 10.9−21.7). There were no significant differences in OS between patients who developed ICIP or those who did not. However, developing grade ≥ 2 pneumonitis conferred the patients a statistically significant increased risk in mortality (HR 2.54, 95% CI 1.20−5.34, $p = 0.014$), **Figure 2**.

Mean follow-up was of 9.22 months (range 0.22 – 44.2). Follow up was performed by scheduling medical visits ever 3-weeks. Patients that did not presented to scheduled visits during 2 months were contacted by a phone call; those patients that were unable to be contacted were censored from analysis.

In addition, a one, three, and six-months landmark analysis confirmed a trend toward a better OS at every point for the patients without ≥ grade 2 pneumonitis; with landmark analysis reaching statistical significance at 6 months [median OS of 23.7 (95% CI NR-NR) months for patients without pneumonitis ≥ grade 2 vs. 16.2 months (95% CI 6.8 – 25.6) for patients with pneumonitis ≥ grade 2; p 0.047]; landmark analysis at 1 month almost reached statistical significance for better OS in patients without pneumonitis ≥ grade 2 when compared with patients that developed pneumonitis ≥ grade 2 [median OS 22.4 months (95% CI 14.9 – 29.8) vs. 12.4 months (95% CI 5.5 – 19.4); $p = 0.057$], (**Supplementary Figure 1**).

A wide univariate analysis was performed to evaluate many base-line and treatment characteristics (**Table 3**). Furthermore, clinically relevant variables, and those that reached statistical significance at the univariate analysis were analyzed and adjusted through a multivariate model which is also presented at **Table 3**.

DISCUSSION

Lung toxicity presents with low frequency, but is often a serious treatment treatment-related complication in patients with NSCLC receiving ICI therapy. We retrospectively analyzed the frequency of ICIP and its correlation with prior radiotherapy in a cohort of patients with advanced NSCLC from a single medical center. Our results show that ICIP incidence in the real world scenario might be higher than reported in clinical trials (5, 7), with an all-grade ICIP incidence of 21.8% and a grade ≥ 3 ICIP of 4%, however, the frequency of ICIP increased when patients had a history of radiotherapy (40%).

Several studies have shown that the presence of irAEs is associated with a higher efficacy and improved overall survival in several solid tumors treated with ICIs, including NSCLC (16–18). Nonetheless, ICIP might be an exemption to this rule. As suggested by the publication of Suresh K. et al., ICIP increases mortality in patients with NSCLC, this observation is reported in patients with adenocarcinoma subtype histology, in whom risk of death increased proportionally with ICIP grade. These previous results support the findings in this study. Overall our data suggests that a history of radiation therapy increases the risk of ICIP, and increases mortality.

We observed an increased ICIP rate among patients with prior thoracic radiation, which supports the immunomodulatory effect of radiotherapy that converts an entirely or partially non-immunogenic tumor into an immunogenic one. Cell-death induced by radiotherapy generates molecular signals and inflammatory cytokines that promote the ability of dendritic cells to release antigens to T cells (19).

Different clinical trials have proven the benefit of using combination therapy. The PACIFIC trial demonstrated the

TABLE 1 | Demographic characteristics.

	Total (*n* = 101)
	n (%)
Sex	
Female	58 (57.4)
Male	43 (42.6)
Age (years)	
Mean (SD)	61.07 (±12.34)
<60 years	45 (44.6)
≥60 years	56 (55.4)
History of smoking	
Never	47 (46.5)
Smoker	54 (53.5)
Woodsmoke exposure	
No	78 (77.2)
Yes	23 (22.8)
ECOG	
0	10 (9.9)
1	88 (87.1)
≥2	3 (3)
Stage	
III	11 (10.9)
IV	90 (89.1)
Histology	
Adenocarcinoma	85 (84.1)
Squamous	11 (10.9)
Adenosquamous	5 (5)
CNS Metastases	
Yes	31 (30.7)
No	70 (69.3)
***EGFR* mutation**	
Positive	16 (15.8)
Negative	76 (75.2)
Undetermined	9 (8.9)
***ALK* mutation**	
Positive	0 (0)
Negative	88 (87.1)
Undetermined	13 (12.9)
***KRAS* mutation**	
Positive	0 (0)
Negative	34 (33.7)
Undetermined	67 (66.3)
PDL-1 status	
Positive	27 (26.7)
Negative	9 (8.9)
Undetermined	65 (64.4)
First-line therapy	
Platinum + Taxane	39 (38.6)
Platinum + Pemetrexed	34 (33.7)
Platinum + Gemcitabine	6 (5.9)
EGFR TKI	14 (13.9)
Other	8 (7.9)
Immunotherapy	
Nivolumab	42 (41.6)
Pembrolizumab	59 (58.4)
Radiotherapy prior to ICI	
Yes	40 (39.6)
No	61 (60.4)
Radiotherapy dosage	
<60 Gy	21 (52.5)
≥60 Gy	19 (47.5)

(Continued)

advantage of using combined treatment modalities with chemo-radiation and anti-PD-L1 (durvalumab) compared with chemo-radiation and placebo in patients with NSCLC. PFS was 16.8 months in the durvalumab group versus 5.6 months in the placebo group also with a higher response rate (28.4% vs. 16% $p < 0.001$). However, the combination treatment leads to an increased risk of any grade of pneumonitis as observed in both groups (33.9% vs. 24.8%), as well as represents the most frequent adverse event leading to treatment discontinuation. A secondary analysis from phase 1 KEYNOTE-001 evaluated disease control and pulmonary toxicity in 97 patients with NSCLC that received pembrolizumab (20). This analysis reported that 8% (2/24) of patients who received prior chest-radiotherapy developed ICIP, compared with 1% (1/73) of patients who had not received prior chest-radiotherapy. Similarly, our results show that the incidence of ICIP was higher in the sample of patients who received prior radiotherapy. The KEYNOTE sub analysis also showed that OS was longer in patients who received pembrolizumab and radiotherapy, and in those who received extracranial radiotherapy, compared with those who did not (HR 0.59 95% CI 0.36−0.96). This supports the possibility of an enhancing the effect of radiotherapy on the immune system by combining this with pembrolizumab. Patients with a history of thoracic radiation had an overall higher frequency of treatment-related pulmonary toxicity in 63% (15/24) compared with no-previous lung radiotherapy with 40% (29/73) $p = 0.052$ (20).

The ongoing PEMBRO-RT trial was randomized phase II study that evaluated the improvement in overall response rate (ORR) at 12 weeks in 76 patients with NSCLC receiving pembrolizumab with or without prior stereotactic body radiotherapy (SBRT). The ORR at 12 weeks was 18% in the control arm vs. 36% in the experimental arm ($P = 0.07$). Median progression-free survival was 1.9 months (95% CI, 1.7−6.9 months) vs. 6.6 months (95% CI, 4.0−14.6 months) (hazard ratio, 0.71; 95% CI, 0.42−1.18; $P = 0.19$), and median overall survival was 7.6 months (95% CI, 6.0−13.9 months) vs. 15.9 months (95% CI, 7.1 months to not reached) (hazard ratio, 0.66; 95% CI, 0.37−1.18; $P = 0.16$), (21).

Furthermore, patients who previously received thoracic radiotherapy were more likely to have any grade of pulmonary toxicity. These data highlight the role of radiotherapy in priming the immune response and thereby potentiating immune-mediated toxicity, also known as radiation recall syndrome. Radiation recall pneumonitis (RRP) is a specific subtype of radiation pneumonitis that occurs after the trigger of cytotoxic agents in a previous radiated lung. While the exact mechanism

TABLE 2 | Characteristics among patients who experienced ICIP.

	Pneumonitis (Any Grade)			Pneumonitis (Grade ≥ 2)			Pneumonitis (Grade ≥ 3)		
	No pneumonitis	**Pneumonitis**	***p***	**No pneumonitis**	**Pneumonitis**	***p***	**No pneumonitis**	**Pneumonitis**	***p***
All patients (%)	79 (78.2)	22 (21.8)		89 (88.1)	12 (11.9)		97 (96)	4 (4)	
Sex									
Female	45 (77.6)	13 (22.4)	**0.85**	52 (89.7)	6 (10.3)	**0.57**	57 (98.3)	1 (1.7)	**0.20**
Male	34 (79.1)	9 (20.9)		37 (86)	6 (14)		40 (93)	3 (7)	
Age (years)									
<60 years	38 (84.4)	7 (15.6)	**0.17**	42 (93.3)	3 (6.7)	**0.14**	44 (97.8)	1 (2.2)	**0.39**
≥60 years	41 (73.2)	15 (26.8)		47 (83.9)	9 (16.1)		53 (94.6)	3 (5.4)	
History of smoking									
Never	34 (72.3)	13 (27.7)	**0.18**	40 (85.1)	7 (14.9)	**0.38**	45 (95.7)	2 (4.3)	**0.63**
Smoker	45 (83.3)	9 (16.7)		49 (90.7)	5 (9.3)		52 (96.3)	2 (3.7)	
Woodsmoke Exposure									
No	66 (86.8)	10 (13.2)	**0.02**	71 (93.4)	5 (6.6)	**<0.01**	74 (97.4)	2 (2.6)	**0.19**
Yes	13 (56.5)	10 (43.5)		17 (73.9)	6 (26.1)		21 (91.3)	2 (8.7)	
ECOG									
<2	79 (80.6)	19 (19.4)	**<0.01**	88 (89.8)	10 (10.2)	**<0.01**	94 (95.9)	4 (4.1)	**0.88**
≥2	0 (0)	3 (100)		1 (33.3)	2 (66.7)		3 (100)	0 (0)	
Stage									
III	7 (63.6)	4 (36.4)	**0.21**	9 (81.8)	2 (18.2)	**0.49**	11 (100)	0 (0)	**0.62**
IV	72 (80)	18 (20)		80 (88.9)	10 (11.1)		86 (95.6)	4 (4.4)	
Histology									
Adenocarcinoma	69 (81.2)	16 (18.8)	**0.24**	76 (89.4)	9 (10.6)	**0.64**	82 (96.5)	3 (3.5)	**0.6**
Squamous	7 (63.6)	4 (36.4)		9 (81.8)	2 (18.2)		10 (90.9)	1 (9.1)	
Adenosquamous	3 (60)	2 (40)		4 (80)	1 (20)		5 (100)	0 (0)	
***EGFR* mutation**									
Positive	13 (81.3)	3 (18.8)	**0.66**	13 (81.3)	3 (18.8)	**0.65**	16 (100)	0 (0)	**0.39**
Negative	58 (76.3)	18 (23.7)		68 (89.5)	8 (10.5)		73 (96.1)	3 (3.9)	
Undetermined	8 (80)	1 (20)		8 (88.9)	1 (21.1)		8 (88.9)	1 (11.1)	
PDL-1 status									
Positive	19 (70.4)	8 (29.6)	**0.17**	22 (81.5)	5 (18.5)	**0.29**	26 (92.9)	2 (7.1)	**0.53**
Negative	9 (100)	0 (0)		9 (100)	0 (0)		10 (100)	0 (0)	
Undetermined	51 (78.5)	14 (21.5)		58 (89.2)	7 (10.8)		61 (96.8)	2 (3.2)	
Chemotherapy									
Platinum + Taxane	29 (85.3)	10 (25.6)	**0.36**	34 (87.2)	5 (12.8)	**0.82**	38 (97.4)	1 (2.6)	**0.77**
Platinum + Pemetrexed	29 (85.3)	5 (14.7)		31 (91.2)	3 (8.8)		33 (97.1)	1 (2.9)	
Platinum + Gemcitabine	4 (66.7)	2 (33.3)		5 (83.3)	1 (16.7)		6 (100)	0 (0)	
Prior TKI treatment									
No	65 (76.5)	20 (23.5)	**0.44**	75 (88.2)	10 (11.8)	**0.79**	81 (95.3)	4 (4.7)	**0.40**
Yes	12 (85.7)	2 (14.3)		12 (85.7)	2 (14.3)		14 (100)	0 (0)	
Immunotherapy Drug									
Nivolumab	30 (71.4)	12 (28.6)	**0.16**	34 (81)	8 (19)	**0.6**	40 (95.2)	2 (4.8)	**0.72**
Pembrolizumab	49 (83.1)	10 (21.8)		55 (93.2)	4 (6.8)		57 (96.6)	2 (3.4)	
Radiotherapy									
Yes	24 (60)	16 (40)	**<0.01**	31 (77.5)	9 (22.5)	**<0.01**	36 (90)	4 (10)	**<0.01**
No	55 (90.2)	6 (9.8)		58 (95.1)	3 (4.9)		61 (100)	0 (0)	

Bold text indicate analyzed characteristics and corresponding p values.

of RRP development is not yet well understood, the clinical, radiological and functional characteristics of the patients are similar as in radiation pneumonitis and the diagnosis is based on the premise of a previously radiated lung and the exposure to a triggering agent. The time range of RRP appearance since the triggering agent completion is wide (22−169 days) with a mean of 47 days (22). A diverse range of chemotherapeutic agents have been related to RRP, while only a few associations with molecular-targeted therapy have been reported (23).

As such, in this study we build on the previous evidence regarding the incidence of ICIP in NSCLC patients treated with ICIs and a history of radiation. The strength of this association is

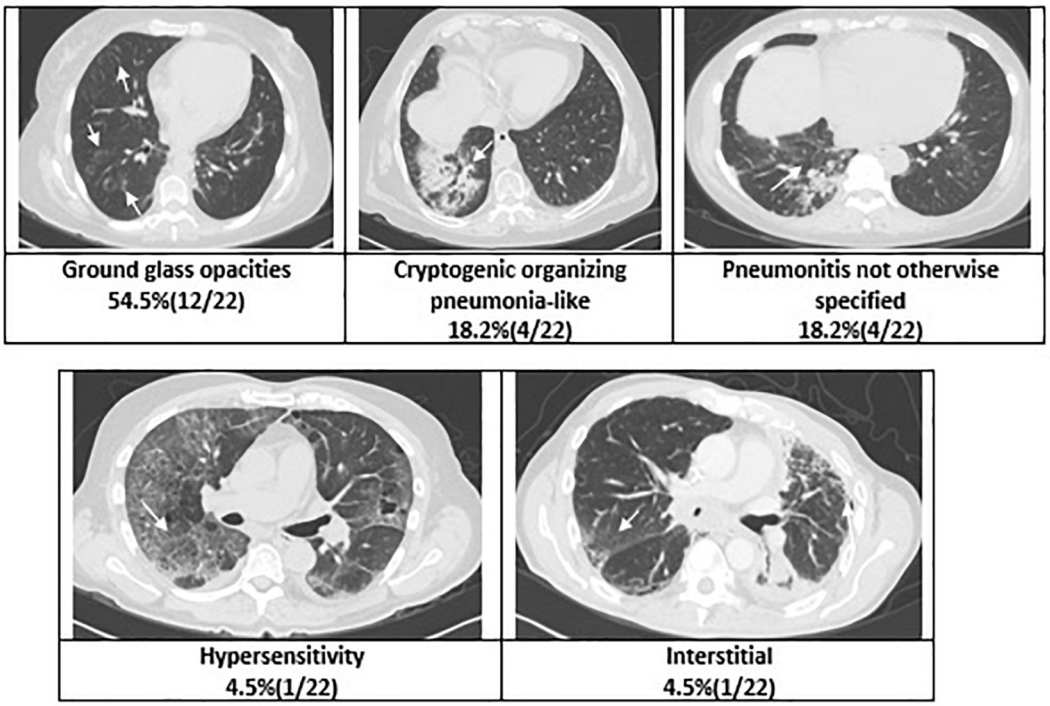

FIGURE 1 | Radiographic patterns of pneumonitis.

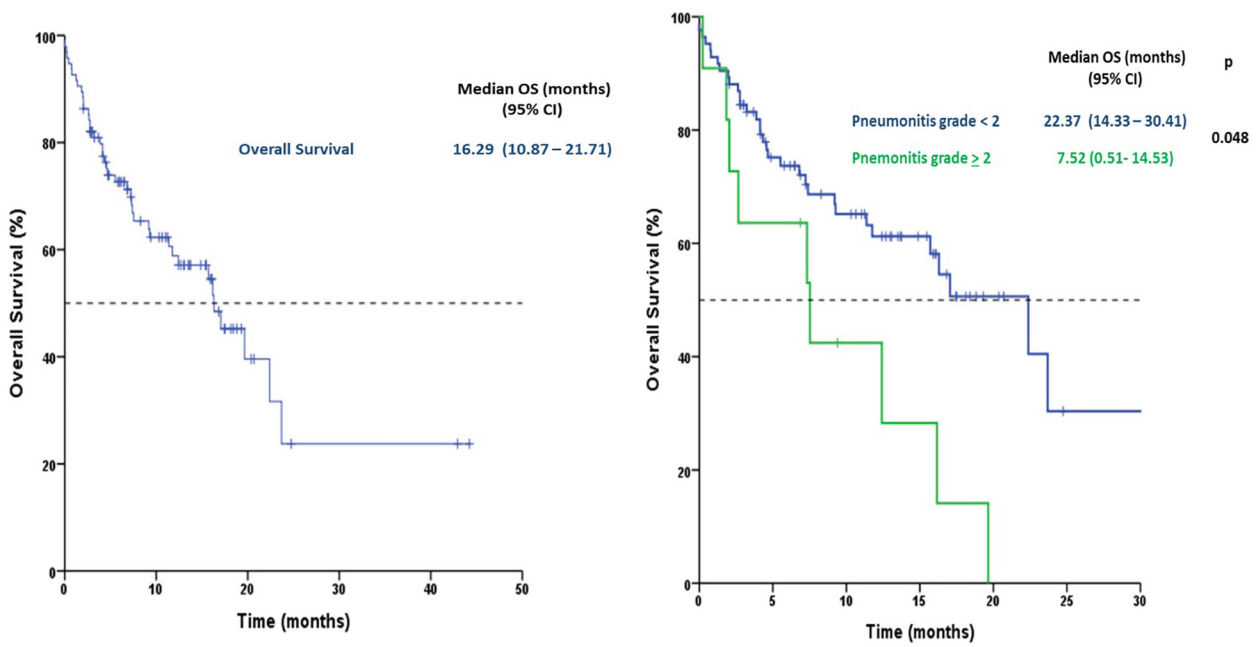

FIGURE 2 | Kaplan-Meyer curves for the association between checkpoint-immune pneumonitis and mortality.

considerable (OR 6.04), however, another previous study which reported 15 cases of ICIP among ICI treated patients had also identified a strong relationship between ICIP incidence and previous radiotherapy history. In this study, the authors report that 67% of patients with ICIP had undergone radiotherapy, nonetheless, the study included patients with a wide variety of

TABLE 3 | Factors associated with overall survival.

				Unadjusted		Adjusted	
	No. (Events)	**Median (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Overall	95 (42)	16.29 (10.8–21.7)					
Sex							
Female	55 (20)	19.6 (15.3–23.9)					
Male	40 (22)	12.4 (10.6–21.4)	0.18	0.66 (0.36–1.22)	0.18	1.56 (0.34–1.18)	0.15
Age (years)							
<60	42 (21)	16.2 (10.6–21.9)					
≥60	53 (21)	19.6 (5.3–33.9)	0.62	0.85 (0.46–1.57)	0.62	0.81 (0.44–1.50)	0.51
Histology							
Adenocarcinoma	81 (35)	16.3 (12.9–19.7)					
Other	14 (7)	7.3 (NA-NA)	0.64	1.21 (0.53–2.74)	0.64		
Tobacco exposure							
No	46 (19)	16.2 (10.0–22.5)					
Yes	49 (23)	17.0 (5.5–28.5)	0.72	0.94 (0.69–1.28)	0.72		
Wood-smoke exposure							
No	44 (27)	16.1 (11.1–21.1)					
Yes	40 (21)	19.9 (2.68–36)	0.46	0.75 (0.36–1.59)	0.45		
ECOG PS							
<2	92 (39)	17.0 (10.9–23.1)					
≥2	3 (3)	16.1 (2.01–30.30)	0.67	1.11 (0.47–2.64)	0.80		
CNS Metastases							
No	65 (29)	16.2 (10.7–21.6)					
Yes	30 (13)	18.2 (10.9–21.7)	0.99	0.99 (0.52–1.92)	0.98		
***EGFR* mutation**							
Absent (wild-type)	71 (29)	17.0 (13.2–20.8)					
Present (*EGFR* mutated)	15 (8)	12.4 (5.8–1.2)	0.21	1.24 (0.18–1.89)	0.3		
Radiotherapy							
Yes	40 (19)	17.5 (10.4–23.7)					
No	55 (23)	16.2 (8.4–24.0)	0.930	0.97 (0.52–1.80)	0.93		
Pneumonitis							
No	74 (31)	16.2 (9.5–23.0)					
Yes	22 (11)	16.1 (10.3–22.0)	0.71	1.13 (0.57–2.26)	0.71		
Pneumonitis ≥ 2							
No	84 (33)	22.3 (14.3–30.41)					
Yes	12 (9)	7.5 (0.51–14.5)	**0.048**	2.48 (1.18–5.23)	**0.028**	2.54 (1.20–5.34)	**0.014**

Bold text indicate analyzed characteristics and corresponding p values.

neoplasms, including melanoma, esophageal cancer and lung cancer, encompassing thus a heterogeneous sample. Despite this observation, the authors highlight that lung regions affected by the primary tumor, metastasis or radiotherapy had a significantly higher probability of ICIP compared with others, with an OR of 10.8 (24).

The relative high risk of pneumonitis among NSCLC patients may be explained because they are prone to develop drug-related lung toxic effects associated with several factors inherently related to the patient demographics, including their exposure to tobacco and underlying lung conditions (chronic obstructive pulmonary disease and pulmonary fibrosis). Existing tumor burden in the lung may also limit the lung tolerance to exogenous stress and injury. These underlying conditions may contribute to more serious clinical consequences from lung injury during pneumonitis. Drug-induced pneumonitis remains an exclusion diagnosis, and requires consideration of competing diagnoses, including infection and disease progression.

As it has been noted, there are huge differences among reported incidence of ICIP in patients with NSCLC that receive immunotherapy; the exact reason of such huge variations is not known, however, it might be related to ethnicity of studied population, awareness and recognition of ICPI, and study design. Lack of and specific diagnostic test for ICPI further complicates its diagnosis and reporting system. Therefore, we consider that it is important to evaluate the incidence and risk factors for ICIP in a real-world population with a future prospective study. In the immunotherapy era, the anti-cancer therapy has been extended the reach of the immune system and many findings are extremely encouraging. However, the adverse effects of the novel combination therapies should be considered. Improvements in the treatment and understanding of the biology of pneumonitis

are needed to optimize and maximize the therapeutic effect of checkpoint inhibitors in NSCLC patients.

CONCLUSION

Results from this study show that ICIP incidence is higher in real-world settings compared with clinical trials, with up to 21.8% of patients treated with ICIs diagnosed with ICIP. Our data might suggest that a prior history of radiotherapy could increase the risk of developing ICIP, which in turn might increase the mortality. However, prospective studies are needed to corroborate our results as well as to appropriately identify the true incidence and risk factors for developing ICIP. Beside this, the appropriate dose and modality of radiotherapy, and the concomitant or sequential use of immunotherapy should be explored in clinical trials.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

FB, RS, and OA: conception or design of the work. FB, RS, MA-H, CB, MR-R, and DF-E: data collection. FB, RS, and RC: data analysis and interpretation. RS, ZZ-B, and RC: drafting the article. FB, AC, and OA: critical revision of the article. All authors approved the final version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.570233/full#supplementary-material

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Harmonization of Molecular Testing for Non-Small Cell Lung Cancer: Emphasis on PD-L1

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Comprehensive molecular testing plays a critical role in the choice of treatment for non-small lung cell cancer (NSCLC). The analysis of druggable alterations in EGFR, BRAF, MET, KRAS, ALK, ROS1, RET and NTRK1/2/3 genes is more or less standardized and can be achieved using a single diagnostic platform, e.g., next generation sequencing (NGS) or polymerase chain reaction (PCR). In contrast to above targets, PD-L1 testing requires the use of immunohistochemistry (IHC). There are multiple PD-L1 IHC assays, which utilize distinct antibodies and detection systems. These PD-L1 tests are tailored to distinct drugs, often rely on different thresholds and scoring guidelines, and are characterized by incomplete inter-laboratory and inter-observer reproducibility. Several studies evaluated the performance of PD-L1 RNA expression tests, as PCR-based RNA analysis is compatible with other NSCLC molecular testing platforms, can be performed in a semi-automated manner, and has a potential for proper standardization. These investigations revealed a correlation between PD-L1 protein and RNA expression; however, there were NSCLCs demonstrating decent amounts of PD-L1 transcript in the absence of PD-L1 IHC staining. Clinical studies are required to evaluate, which of the two PD-L1 testing approaches, i.e., RNA or protein expression measurement, has a better predictive value.

Keywords: non-small cell lung cancer, molecular testing, PD-L1, PCR, review

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While only a decade ago the laboratory diagnosis of non-small cell lung cancer (NSCLC) required mainly conventional morphological analysis, the process of examination of NSCLC tissues is getting increasingly complex nowadays, thanks to the invention of new targeted drugs. EGFR tyrosine kinase inhibitors (TKIs) were the first to trigger the molecular profiling of lung cancer, as they demonstrated high response rates in tumors with EGFR exon 19 and 21 drug-sensitizing mutations. Subsequent advances were based on the discovery of ALK and ROS1 rearrangements, which also turned to be linked to the pronounced tumor sensitivity to corresponding TKIs. Interestingly, the development of gefitinib, erlotinib and crizotinib actually preceded the identification of their genuine molecular targets, so the incorporation of these drugs into the NSCLC management was somehow attributed to some chance discoveries. This is in stark contrast with the history of the invention of inhibitors of the mutated BRAF, which is clearly an output of a pre-planned research, starting from the systematic search for kinase activating mutations and eventually resulting in the intentional development of specific antagonists of the BRAF V600E protein. There is a multitude of new NSCLC drug targets, e.g., NTRK1-3

and RET gene fusions, MET exon 14 skipping mutations, KRAS G12C substitutions, etc. In addition, administration of several inhibitors of immune checkpoints involves testing for PD-L1 expression (1–3).

NSCLC diagnostic pipeline includes a spectrum of molecular assays which usually rely on distinct laboratory platforms. The analysis of EGFR, BRAF, and KRAS mutations usually requires allele-specific PCR and/or gene sequencing. The detection of ALK, ROS, RET, and NTRK1-3 rearrangements may be based on the immunohistochemistry (IHC) guided detection of the overexpression of the kinase portion of the corresponding protein or on the break-apart FISH assay (3). The methodology of MET testing remains to be standardized (4). PD-L1 expression analysis is apparently the most complicated assay for the time being. There are several approved antibodies for the PD-L1 status evaluation. These antibodies are tailored to particular diagnostic platforms, linked to the use of distinct therapeutic modulators of the PD-L1/PD1 pathway, have different scoring guidelines and utilize varying thresholds between "positive" and "negative" samples. The detailed listing of PD-L1 antibodies, detection systems, associated therapeutic compounds and staining patterns is provided in several reviews (5–7). Most importantly, while the majority of clinical trials involving PD-L1/PD1 pathway inhibitors generally demonstrate an association between PD-L1 expression and clinical benefit from the drug, there is a great variability across the NSCLC studies with regard to medical applicability of observed findings (**Table 1**).

Many NSCLCs are diagnosed as a metastatic disease, therefore tumor tissue material is represented by a single tiny biopsy sample. These samples must be divided for mutational analysis (PCR, sequencing) and visualization-based tests (IHC, FISH). There is a great need for a "one-for-all" approach, which would allow for a comprehensive NSCLC examination performed on a single platform. Next generation sequencing (NGS) provides a viable diagnostic opportunity, as it is capable of detecting all relevant genetic alterations within a single run. At the present time, NGS has significant limitations, such as relatively high cost, need for significant turn-around time, and requirement for sophisticated equipment (3, 26, 27). Furthermore, NGS is not yet fully compatible with a high-precision analysis of gene expression. There are ongoing efforts to utilize PCR for all types of NSCLC molecular analysis. These assays include simultaneous isolation of DNA and RNA in a single tube, synthesis of complementary DNA (cDNA) on the RNA template, conventional analysis of mutations and the test for 5′/3′-end unbalanced expression of rearranged kinases. The latter approach allows for identification of all druggable gene fusions irrespective of the translocation variant (28). PCR analysis is relatively non-expensive and is more flexible for the incorporation of new predictive tests, as exemplified by the development of the assay for detection of MET exon 14 skipping mutations (4).

While many NSCLC tests can be performed by a number of interchangeable approaches, PD-L1 analysis remains restricted to IHC technology. Use of IHC scoring is time-consuming and may be a subject of significant interobserver variability (5–7). Explicit analysis of the comparability of the existing IHC assays has been recently published by Koomen et al. (29). PD-L1 IHC comparative studies generally demonstrate acceptable results with regard to assays' interchangeability, inter-observer variability, and inter-laboratory agreement. However, it is necessary to keep in mind that the pathologists involved in research activities and scientific publishing are likely to have somewhat better standards of the laboratory practice, so the real-world inconsistencies in the IHC performance may be substantially higher when compared to pre-planned investigations. In addition, while the numerical comparisons of PD-L1 scores show good correlation, there is an alarming rate of discordance when clinically accepted thresholds are utilized (29). Consider a situation in which one pathologist determines the proportion of PD-L1 tumor cells slightly below 1%, while another pathologist determines this proportion to be slightly over 1%. When formal correlation coefficients for continuous numerical variables are calculated, these two results will be considered concordant; however, in clinical practice this difference may critically affect the access to immune therapy, as PD-L1 score of 1% is a commonly accepted threshold for consideration of immune therapies in several clinical scenarios.

Measurement of RNA expression of the gene of interest can offer advantages over IHC. In particular, PCR-based RNA expression analysis offers better reproducibility, as it evaluates not the quantity of the gene-specific transcript *per se*, but the ratio between the RNA messages of the gene-target and gene-referee. Furthermore, PCR tests are usually performed in a semi-automated manner, so they are less labor-consuming as compared to morphology-based analyses (30–32). However, RNA testing has several limitations. First, gene transcription is not always an equivalent of gene translation, as the production and decay of gene-specific RNAs and proteins involves different layers of regulation. Second, IHC analysis is capable to assess intracellular localization of the protein, while RNA assays evaluate only the bulk amount of gene product. Third, some analytical solutions, such as PD-L1 IHC assays, offer individual scoring for various cell types, for example, tumor cells and immune cells (6, 7). This advantage is not compatible with currently established PCR procedures. Several studies attempted to investigate in parallel the expression of PD-L1 on the level of RNA and protein in cell cultures and tumor tissues. These small-scale studies provided generally encouraging results indicating that the correlation between PD-L1 RNA and protein level does exist (30–36).

Recently published CLOVER study represents the first systematic attempt to evaluate the feasibility of PCR-based PD-L1 testing in comparison with IHC (37). The authors analyzed 437 NSCLC samples by three PD-L1 IHC assays (Ventana SP142, Ventana SP263, Dako 22C3) and by the laboratory-developed real-time PCR test for PD-L1 RNA expression. In agreement with other investigations, the CLOVER study showed significant concordance between the SP263 and the 22C3 IHC, while the SP142 produced lower rate of PD-L1 positive tumors. Indeed, the Blueprint Phase 1 study, which included 39 lung tumors stained with four different antibodies, showed that SP263 and 22C3 assays demonstrated similar IHC patterns in the majority of cases, while SP142 stained fewer number of tumor cells with generally lower intensity (38). The Blueprint Phase 2 study

TABLE 1 | Selected clinical studies on immune checkpoint inhibitors, which evaluated associations between clinical outcomes and the level of PD-L1 expression analysis.

References	Brief description of the study	Survival	Predictive role of PD-L1
Pembrolizumab (IHC: 22C3)			
Herbst et al. (8) (KEYNOTE-010)	Assessment of long-term outcomes of pembrolizumab vs. docetaxel monotherapy in previously treated NSCLC with PD-L1 expression in >/=1% tumor cells	OS PD-L1 1-49%: pembrolizumab: 11.8 months; docetaxel: 8.4 months PD-L1 >/=50%: pembrolizumab: 16.9 months; docetaxel: 8.2 months	Dramatic improvement of OS for pembrolizumab in patients with PD-L1 expression in >/=50% tumor cells; moderate improvement of OS in patients with PD-L1 expression score 1–49%
Gadgeel et al. (9) (KEYNOTE-189)	First-line therapy, non-squamous NSCLC: pembrolizumab or placebo plus pemetrexed and platinum	OS PD-L1 <1%: pembrolizumab plus chemotherapy: 17.2 months; pembrolizumab plus placebo: 10.2 months PD-L1 1-49%: pembrolizumab plus chemotherapy: 21.8 months; pembrolizumab plus placebo: 12.1 months PD-L1 >/=50%: pembrolizumab plus chemotherapy: not reached; pembrolizumab plus placebo: 10.1 months PFS PD-L1 <1%: pembrolizumab plus chemotherapy: 6.2 months; pembrolizumab plus placebo: 5.1 months PD-L1 1-49%: pembrolizumab plus chemotherapy: 9.2 months; pembrolizumab plus placebo: 4.9 months PD-L1 >/=50%: pembrolizumab plus chemotherapy: 11.1 months; pembrolizumab plus placebo: 4.8 months	Pembrolizumab plus chemotherapy outperformed pembrolizumab plus placebo regardless of the PD-L1 status, however the magnitude of the effect was higher in tumors with high PD-L1 expression
Garon et al. (10) (KEYNOTE-001)	Assessment of long-term outcomes of pembrolizumab monotherapy in treatment-naïve and previously treated patients	OS in treatment-naïve patients: PD-L1 <1%: not evaluated (low number of patients) PD-L1 1–49%: 19.5 months PD-L1 >/=50%: 35.4 months OS in previously treated patients: PD-L1 <1%: 8.6 months PD-L1 1–49%: 8.5 months PD-L1 >/=50%: 15.4 months	Pembrolizumab treatment was associated with improved OS in patients with PD-L1 expression in >/=50% tumor cells
Mok et al. (11) (KEYNOTE-042)	First-line therapy: pembrolizumab vs. chemotherapy for NSCLC with PD-L1 expression in >/=1% tumor cells	OS: PD-L1 >/=1%: pembrolizumab: 16.7 months; chemotherapy: 12.1 months PD-L1 >/=20%: pembrolizumab: 17.7 months; chemotherapy: 13.0 months PD-L1 >/=50%: pembrolizumab: 20.0 months; chemotherapy: 12.2 months PFS: PD-L1 >/=1%: pembrolizumab: 5.4 months; chemotherapy: 6.5 months PD-L1 >/=20%: pembrolizumab: 6.2 months; chemotherapy: 6.6 months PD-L1 >/=50%: pembrolizumab: 7.1 months; chemotherapy: 6.4 months	Improvement of OS for pembrolizumab was observed both for PD-L1 >/=50% and >/=1% expression thresholds, however the magnitude of the effect was greater for the high expressors
Paz-Ares et al. (12) (KEYNOTE-407)	First-line therapy, squamous NSCLC: pembrolizumab or placebo plus chemotherapy	OS: PD-L1 <1%: pembrolizumab plus chemotherapy: 15.9 months; pembrolizumab plus placebo: 10.2 months PD-L1 1–49%: pembrolizumab plus chemotherapy: 14.0 months; pembrolizumab plus placebo: 11.6 months PD-L1 >/=50%: pembrolizumab plus chemotherapy: not reached; pembrolizumab plus placebo: not reached PFS: PD-L1 <1%: pembrolizumab plus chemotherapy: 6.3 months; pembrolizumab plus placebo: 5.3 months PD-L1 1–49%: pembrolizumab plus chemotherapy: 7.5 months; pembrolizumab plus placebo: 5.2 months PD-L1 >/=50%: pembrolizumab plus chemotherapy: 8.0 months; pembrolizumab plus placebo: 4.2 months	Pembrolizumab plus chemotherapy outperformed pembrolizumab plus placebo regardless of the PD-L1 status

(Continued)

TABLE 1 | Continued

References	Brief description of the study	Survival	Predictive role of PD-L1
Nivolumab (IHC: 28-8)			
Hellmann et al. (13) (CHECKMATE 227)	First-line therapy: nivolumab plus ipilimumab vs. nivolumab alone vs. chemotherapy for NSCLC with PD-L1 expression in >/=1% tumor cells; nivolumab plus ipilimumab vs. nivolumab plus chemotherapy vs. chemotherapy for NSCLC with PD-L1 expression in <1% tumor cells	OS: PD-L1 <1%: nivolumab plus ipilimumab: 17.2 months; nivolumab plus chemotherapy: 15.2 months; chemotherapy: 12.2 months PD-L1 >/=1%: nivolumab plus ipilimumab: 17.1 months; nivolumab alone: 15.7 months; chemotherapy: 14.9 months PD-L1 >/=50%: nivolumab plus ipilimumab: 21.2 months; nivolumab alone: 18.1 months; chemotherapy: 14.0 months PFS: PD-L1 <1%: nivolumab plus ipilimumab: 5.1 months; nivolumab alone: 5.6 months; chemotherapy: 4.7 months PD-L1 >/=1%: nivolumab plus ipilimumab: 5.1 months; nivolumab alone: 4.2 months; chemotherapy: 5.6 months PD-L1 >/=50%: nivolumab plus ipilimumab: 6.7 months; nivolumab alone: 5.6 months; chemotherapy: 5.6 months	Nivolumab plus ipilimumab outperformed chemotherapy regardless of the PD-L1 status, however the difference was more pronounced in patients with PD-L1 expression in >/=50% tumor cells
Ready et al. (14) (CHECKMATE 568)	First-line therapy: nivolumab plus ipilimumab	PFS: PD-L1 <1%: 2.8 months PD-L1 >/=1%: 6.8 months PD-L1 >/=50%: 6.8 months	PD-L1 expression was associated with higher rate of objective responses and longer PFS
Carbone et al. (15) (CHECKMATE 026)	First-line therapy: nivolumab vs. chemotherapy for NSCLC with PD-L1 expression in >/=1% tumor cells	OS: PD-L1 >/=1%: nivolumab: 13.7 months; chemotherapy: 13.8 months PD-L1 >/=5%: nivolumab: 14.4 months; chemotherapy: 13.2 months PD-L1 >/=50%: nivolumab: 15.9 months; chemotherapy: 13.9 months PFS: PD-L1 >/=1%: nivolumab: 4.2 months; chemotherapy: 5.8 months PD-L1 >/=5%: nivolumab: 4.2 months; chemotherapy: 5.9 months PD-L1 >/=50%: nivolumab: 5.4 months; chemotherapy: 5.8 months	No predictive value for PD-L1 expression
Borghaei et al. (16) (CHECKMATE 057)	Nivolumab vs. docetaxel monotherapy in previously treated patients with non-squamous NSCLC	OS: PD-L1 <1%: nivolumab: 10.5 months; docetaxel: 10.1 months PD-L1 >/=1%: nivolumab: 17.7 months; docetaxel: 9.0 months PD-L1 >/=5%: nivolumab: 19.4 months; docetaxel: 8.1 months PD-L1 >/=10%: nivolumab: 19.9 months; docetaxel: 8.0 months PFS: PD-L1 <1%: nivolumab: 2.1 months; docetaxel: 3.6 months PD-L1 >/=1%: nivolumab: 4.2 months; docetaxel: 4.5 months PD-L1 >/=5%: nivolumab: 5.0 months; docetaxel: 3.8 months PD-L1 >/=10%: nivolumab: 5.0 months; docetaxel: 3.7 months	Nivolumab outperformed docetaxel only in patients with PD-L1 expression in >/=1% tumor cells
Brahmer et al. (17) (CHECKMATE 017)	Nivolumab vs. docetaxel monotherapy in previously treated patients with squamous NSCLC	OS: PD-L1 <1%: nivolumab: 8.7 months; docetaxel: 5.9 months PD-L1 >/=1%: nivolumab: 9.3 months; docetaxel: 7.2 months PD-L1 >/=5%: nivolumab: 10.0 months; docetaxel: 6.4 months PD-L1 >/=10%: nivolumab: 11.0 months; docetaxel: 7.1 months	No predictive role of the PD-L1 status

(Continued)

TABLE 1 | Continued

References	Brief description of the study	Survival	Predictive role of PD-L1
		PFS: PD-L1 <1%: nivolumab: 3.1 months; docetaxel: 3.0 months PD-L1 >/=1%: nivolumab: 3.3 months; docetaxel: 2.8 months PD-L1 >/=5%: nivolumab: 4.8 months; docetaxel: 3.1 months PD-L1 >/=10%: nivolumab: 3.7 months; docetaxel: 3.3 months	
Atezolizumab (IHC: SP142)			
Socinski et al. (18) (IMpower 150)	First-line therapy: atezolizumab plus carboplatin plus paclitaxel (ACP) vs. bevacizumab plus carboplatin plus paclitaxel (BCP) vs. atezolizumab plus BCP (ABCP) for non-squamous NSCLC	ABCP vs. BCP comparison, PFS: TC3 or IC3: 12.6 months vs. 6.8 months TC1/2/3 or IC1/2/3: 11.0 months vs. 6.8 months TC1/2 or IC1/2: 8.3 months vs. 6.6 months TC0/1/2 and IC0/1/2: 8.0 months vs. 6.8 months TC0 and IC0: 7.1 months vs. 6.9 months	Addition of atezolizumab to carboplatin, paclitaxel and bevacizumab improved PFS regardless of the PD-L1 status, however the magnitude of the effect was higher for tumors with high PD-L1 expression
Rittmeyer et al. (19) (OAK)	Atezolizumab vs. docetaxel monotherapy in previously treated NSCLC patients	OS: TC3 or IC3: 20.5 months vs. 8.9 months TC2/3 or IC2/3: 16.3 months vs. 10.8 months TC1/2/3 or IC1/2/3: 15.7 months vs. 10.3 months TC0 and IC0: 12.6 months vs. 8.9 months	Atezolizumab outperformed docetaxel regardless of the PD-L1 status, however the magnitude of the effect was higher for tumors with high PD-L1 expression
Fehrenbacher et al. (20) (POPLAR)	Atezolizumab vs. docetaxel monotherapy in previously treated NSCLC patients	OS: TC3 or IC3: 15.5 months vs. 11.1 months TC2/3 or IC2/3: 15.1 months vs. 7.4 months TC1/2/3 or IC1/2/3: 15.5 months vs. 9.2 months TC0 and IC0: 9.7 months vs. 9.7 months	Atezolizumab outperformed docetaxel only in patients with PD-L1 expression in >/=1% tumor cells or >/=1% tumor-infiltrating immune cells
Avelumab: (IHC: 73-10)			
Barlesi et al. (21) (JAVELIN Lung 200)	Avelumab vs. docetaxel monotherapy in previously treated NSCLC patients	OS: PD-L1 >/=1%: avelumab: 11.4 months; docetaxel: 10.3 months PD-L1 >/=50%: avelumab: 13.6 months; docetaxel: 9.2 months PD-L1 >/=80%: avelumab: 17.1 months; docetaxel: 9.3 months	Improved outcomes for avelumab were observed only in patients with high PD-L1 expression
Gulley et al. (22) (JAVELIN Solid Tumor)	Avelumab in previously treated NSCLC patients	OS: PD-L1 <1% tumor cells: 4.6 months PD-L1 >/=1% tumor cells: 8.9 months PD-L1 >/=5% tumor cells: 10.6 months PD-L1 >/=25% tumor cells: 8.4 months PD-L1</=10% immune cells in hot-spots: 8.5 months PD-L1 >/=10% immune cells in hot-spots: 8.9 months PFS: PD-L1 <1% tumor cells: 5.9 weeks PD-L1 >/=1% tumor cells: 12.0 weeks PD-L1 >/=5% tumor cells: 11.9 weeks PD-L1 >/=25% tumor cells: 11.9 weeks PD-L1</=10% immune cells in hot-spots: 11.3 weeks PD-L1 >/=10% immune cells in hot-spots: 8.4 weeks	Improved outcomes for avelumab were observed when PD-L1 expression in >/=1% tumor cells was used as a threshold
Durvalumab (IHC: SP263)			
Rizvi et al. (23) (MYSTIC)	First-line therapy: durvalumab with or without tremelimumab vs. standard chemotherapy	OS: PD-L1 <1%: durvalumab plus tremelimumab: 11.9 months; durvalumab alone: 10.1 months; chemotherapy: 10.3 months PD-L1 >/=1%: durvalumab plus tremelimumab: 10.9 months; durvalumab alone: 14.6 months; chemotherapy: 12.3 months PD-L1 25–49%: durvalumab plus tremelimumab: 10.5 months; durvalumab alone: 11.1 months; chemotherapy: 13.3 months PD-L1 >/=50%: durvalumab plus tremelimumab: 15.2 months; durvalumab alone: 18.3 months; chemotherapy: 12.7 months	Improved outcomes for durvalumab were observed only in patients with high PD-L1 expression

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TABLE 1 | Continued

References	Brief description of the study	Survival	Predictive role of PD-L1
Paz-Ares et al. (24) (PACIFIC)	Durvalumab vs. placebo after chemoradiotherapy in unresectable stage III NSCLC	OS: PD-L1 <1%: durvalumab 33.1 months; placebo: 45.6 months PD-L1 1–24%: durvalumab 43.3 months; placebo: 30.5 months PD-L1 >/=25%: durvalumab: not reached; placebo: 21.1 months PFS: PD-L1 <1%: durvalumab 10.7 months; placebo: 5.6 months PD-L1 1–24%: durvalumab: not reached; placebo: 9.0 months PD-L1 >/=25%: durvalumab 17.8 months; placebo: 3.7 months	Improved PFS for durvalumab was observed across all subgroups; improved OS for durvalumab was seen for patients with PD-L1 expression in >/=1% tumor cells
Garassino et al. (25) (ATLANTIC)	Durvalumab as a third-line or later treatment in NSCLC patients	OS: Cohort EGFR+/ALK+: PD-L1 <25%: 9.9 months PD-L1 >/=25%: 13.3 months Cohort EGFR-/ALK-: PD-L1 <25%: 9.3 months PD-L1 >/=25%: 10.9 months Cohort PD-L1>/=90%: not reached PFS: Cohort EGFR+/ALK+: PD-L1 <25%: 1.9 months PD-L1 >/=25%: 1.9 months Cohort EGFR-/ALK-: PD-L1 <25%: 1.9 months PD-L1 >/=25%: 3.3 months Cohort PD-L1>/=90%: 2.4 months	PD-L1 expression was associated with improved outcomes

OS, overall survival; PFS, progression-free survival; IHC, immunohistochemistiry; TC, tumor cells; IC, immune cells; the details for TC and IC scoring are explained in (20).

essentially replicated these results using a "real-world" series of 81 lung cancer specimens (39). The CLOVER study compared the performance of PCR-based PD-L1 expression measurement against conventional IHC assays. Strikingly, negative PCR tests appeared to be a reliable predictor for the lack of PD-L1 expression as determined by immunohistochemistry. This is an expected observation, given that the PCR is considered to be an ultrasensitive method for detection of biological molecules; so if the gene product cannot be detected by PCR it is unlikely to be seen by other methods. However, positive predictive value of PCR was low, as many PD-L1 RNA expressing tumors turned out to be PD-L1-negative by IHC analysis.

The results of the CLOVER study may potentially be relevant to already existing PCR diagnostic pipelines. It is relatively easy to add one more gene-specific assay to established PCR procedures, so if PCR is indeed helpful to identify PD-L1 non-expressors, its use may avoid unnecessary IHC tests. The reliability of this approach remains to be determined in subsequent studies. Overall, the CLOVER investigation calls for further efforts related to the harmonization of PD-L1 testing. Contrary to many studies, the CLOVER considered PD-L1 RNA measurement as a categorical variable by grouping tumors as "positive" and "negative" (37). It is essential to consider RNA expression as a continuous variable. Furthermore, the estimation of meaningful thresholds requires tedious consideration of various clinical and laboratory endpoints. The CLOVER study utilized a conditional threshold for the PCR test and did not adjust its value; so the additional efforts are needed to define the categories of PD-L1 expressors using biologically or clinically relevant criteria. Most importantly, the CLOVER investigation used IHC tests as a comparator for PCR assays. Ideally, studies of this type should consider treatment outcomes instead of surrogate markers; this is particularly true for PD-L1 testing, given that many PD-L1/PD1 targeted drugs show activity irrespective of the results of PD-L1 analysis (6, 7).

The instances of discordant results of PD-L1 expression measurement deserve a more systematic investigation on a case-by-case basis, given that the outcome of PD-L1 testing may dramatically influence clinical treatment decisions. There are examples of surprising discordance, when the same specimen is strongly positive by one antibody but clearly negative by another IHC assay (38, 39). Several factors may contribute to these discrepancies. Human error may be one of the factors when large series of tumors are analyzed. The Blueprint project revealed that the inter-observer variability may play a role in the interpretation of the results of PD-L1 staining (38). PD-L1 IHC assays calculate only the proportion of stained cells, while the intensity of the staining is not considered; therefore, the cut-off point between weak and absent staining may be defined differently. Intratumoral heterogeneity of PD-L1 expression may also contribute to these discrepancies, given that even serial sections may differ from each other with regard to the percentage of stained cells. The process of industrial development of distinct PD-L1 antibodies by definition

involves distinct protein epitopes and distinct animals, so the individual antibody clones may differ in their ability to recognize various PD-L1 isoforms. The incorporation of the RNA testing adds complexity to this issue. It is not impossible that some tumor specimens lose their ability to interact with diagnostic antibodies during the archiving process; these samples may retain detectable PD-L1 RNA expression but show PD-L1 negativity by IHC.

There is a growing enthusiasm towards the use of PCR-based expression assays as a substitute or complement for IHC analysis. For example, Oncotype Dx breast cancer panel includes estrogen receptor, progesterone receptor and HER2 measurement to aid conventional IHC testing (40). Some studies demonstrate that Ki-67 RNA-based expression analysis has non-inferior or even better clinical performance as compared to conventional IHC tests (41, 42). There are several reported PCR-based biomarkers, which could assist the administration of immune checkpoint inhibitors (18, 43). It is highly likely that PD-L1 testing will undergo significant modification in a very near future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Neoadjuvant and Adjuvant Immunotherapy: Opening New Horizons for Patients With Early-Stage Non-small Cell Lung Cancer

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Lung cancer is the most common malignant tumor with the highest mortality, and about 84% are non-small cell lung cancer (NSCLC). However, only a small proportion of patients with newly diagnosed lung tumors can receive curative surgery and have a high risk of postoperative recurrence. At present, there are many perioperative treatment methods being continuously explored, such as chemotherapy and targeted therapy, continuously enriching the content of neoadjuvant and adjuvant therapy in early-stage NSCLC. But disappointingly, for patients with driver gene mutation, the significant disease-free survival (DFS) benefit of targeted drugs failed to translate into overall survival (OS) benefit, and for negative patients, chemotherapy has reached a plateau in improving efficacy and survival. Immunotherapy represented by immune checkpoint inhibitors (ICIs) has been researched in more and more clinical trials in patients with early-stage operable disease, gradually enriching the existing treatments. This review focuses on the research progress of clinical trials of neoadjuvant and adjuvant therapy with ICIs in early-stage NSCLC, the exploration of response evaluation and predictive biomarkers, and the urgent problems to be solved in the future.

Keywords: non-small cell lung cancer, neoadjuvant therapy, response evaluation, immune checkpoint inhibitor, predictive biomarker

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Abbreviations: ACSO, American Society of Clinical Oncology; ALK, anaplastic lymphoma kinase; CTCs, circulating tumor cells; ctDNA, circulating tumor DNA; DC, dendritic cell; DDR, DNA damage response; DDR, DNA damage response; DFS, disease-free survival; dMMR, DNA mismatch repair deficiency; EFS, event-free survival; EGFR, epidermal growth factor receptor; FDA, Food and Drug Administration; HLA, human leukocyte antigen; ICIs, immune checkpoint inhibitors; ICOS, inducible T cell co-stimulator; irAEs, immune-related adverse events; irPRC, immune-related pathological response criterion; MPR, major pathologic response; MSI, microsatellite instability; NCCN, National Comprehensive Cancer Network; NK cells, natural killer cells; NSCLC, non-small cell lung cancer; ORR, objective response rate; OS, overall survival; pCR, pathologic complete response; PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; PFS, progression-free survival; PR, partial response; PsPD, Pseudoprogression; QSP, quantitative system pharmacology; RATS, robot-assisted thoracic surgery; RFS, relapse-free survival; SD, stable disease; SLD, sum of lesion diameters; TCR, T cell receptor; TILs, tumor-infiltrating lymphocytes; TIM, tumor-infiltrating myeloid cells; TMB, tumor mutation burden; TME, tumor microenvironment; TMU, tumor metabolism uptake; TRAEs, treatment-related adverse events; Treg, T regular cell; VATS, video-assisted thoracic surgery.

INTRODUCTION

According to global cancer statistics in 2018, lung cancer is the most common (11.6% of all cases) malignant tumor with the highest mortality (18.4% of total cancer deaths) (1), of which about 84% is non-small cell lung cancer (NSCLC). However, only about 20–30% of newly diagnosed lung tumors can receive radical surgery, and many of them have a high risk of recurrence (25–70%) due to the presence of preoperative micrometastases (2). At present, there are many perioperative treatment methods being continuously explored to reduce the risk of recurrence and improve long-term survival, such as targeted therapy and chemotherapy, which are continuously enriching the content of neoadjuvant and adjuvant therapy in early-stage NSCLC, making the treatment lines continuously advanced and bringing a brand-new different era for operable patients. For patients with driver gene-positive early-stage lung cancer, a number of studies have been tried and made breakthrough results. The phase III ADJUVANT study (3), phase II EVAN study (4), and EMERGING study (CTONG 1103) (5) all achieved positive results for disease-free survival (DFS) or progression-free-survival (PFS) in perioperative treatment with targeted drugs, suggesting that targeted therapy can change the treatment pattern of early-stage lung cancer. However, the latest long-term follow-up results showed that significant DFS benefit failed to translate into overall survival (OS) benefit. For patients with negative driver gene, the National Comprehensive Cancer Network (NCCN) guidelines recommend conventional adjuvant chemotherapy for patients with postoperative pathological stage IIB or higher and adjuvant chemotherapy for high-risk patients with stage IB/IIA (6). Several meta-analyses suggested that the survival benefit of neoadjuvant chemotherapy is comparable to that of postoperative adjuvant chemotherapy, with the 5-year survival rate increased by about 4–8% (7, 8). It can be seen that the benefit is unsatisfactory, and despite surgery and adjuvant therapy, about 20–30% of patients with stage I, 50% with stage II, and 60% with stage IIIA die within 5 years (9), which led researchers to focus on exploring new drugs of neoadjuvant and adjuvant therapies. Immunotherapy represented by immune checkpoint inhibitors (ICIs) has been researched in more and more clinical trials in patients with early-stage operable disease, gradually enriching the existing treatments, and these trials found that it has more advantages in killing tumor, preventing postoperative recurrence, and improving survival. This review focuses on the research progress of clinical trials of neoadjuvant and adjuvant therapy with ICIs in early-stage NSCLC, the exploration of response evaluation and predictive biomarkers, and the urgent problems to be solved in the future.

ADVANTAGES AND DISADVANTAGES OF NEOADJUVANT IMMUNOTHERAPY

The goals of neoadjuvant therapy include decreasing tumor TNM stage, increasing R0 resection rate, controlling micrometastases, improving DFS and overall OS, and assessing drug efficacy or conducting drug susceptibility studies. Recent studies have found that the immunosuppressive tumor microenvironment (TME) already exists in tumor tissues of stage I NSCLC. The immune cell composition and phenotype in the early TME have changed significantly, including T cells, natural killer (NK) cells, and tumor-infiltrating myeloid cells (TIM). The researchers showed that the lesions are enriched in a variety of inhibitory cells, such as programmed cell death-ligand 1 (PD-L1) ^{hi}CD64^{hi}CD14^{hi}PPARγ^{hi} IL-6^{hi} macrophages, CD1c^{+}DC, CD39^{hi}CD38^{hi}PD1^{hi}CTLA^{hi} T regular cell (Treg), and exhausted T cells, and depleted of cells that can effectively exert anti-tumor effector functions, such as CD141^{+} dendritic cell (DC), CD16^{+} monocytes, NK cells, and granzyme B^{+} effector cells. These differences may synergistically promote the immunosuppressive microenvironment. Therefore, immunotherapy is essential for patients with early-stage tumor. Neoadjuvant therapy with ICIs given before surgical resection of early-stage NSCLC can induce a more sustained anti-tumor T cell immune response, thereby more effectively preventing tumor recurrence (10). (i) Neoadjuvant immunotherapy can increase the number of activated tumor-specific CD8^{+} T cells, which can release more new tumor antigens while killing tumors, and then these antigens are presented to specific effector T cells of tumors at different sites (primary tumor, metastases, circulation); (ii) activated T cells can reach micrometastases through blood vessels and lymphatic vessels, triggering a range of specific anti-tumor immune responses; (iii) in addition, compared with postoperative adjuvant therapy, the structure of the lymphatic system around the lung cancer before resection is relatively intact, providing a greater chance of interaction between tumor cells and immune cells. Moreover, the presence of a wider repertoire of tumor neoantigens can enhance immune recognition and produce a strong anti-tumor immune response and early immune memory. Preclinical studies and early clinical trials seem to support the neoadjuvant approach. Nevertheless, the exploration of immunotherapy in the treatment of early-stage lung cancer also has some risks: delaying surgery and making the disease progress; increasing the difficulty and risk of surgery, such as increased pleural adhesions; and increasing intraoperative and postoperative complications and overtreatment. Therefore, it is necessary to deeply explore the efficacy and safety of neoadjuvant immunotherapy to weigh the benefit/risk ratio to maximize the clinical benefit of the patients.

However, neoadjuvant immunotherapy also has some disadvantages. Firstly, it remains unknown whether it can effectively improve the long-term survival of the patient. Secondly, neoadjuvant immunotherapy may have an impact on the feasibility of surgery, such as delaying surgery or risk of progression before surgical treatment, and may increase the possibility of surgical complications and overtreatment. In addition, there are challenges for optimal response assessment and biomarker exploration of neoadjuvant immunotherapy, which may limit the application and development of neoadjuvant immunotherapy to some extent.

REVIEW AND PERSPECTIVE ON NEOADJUVANT THERAPY WITH IMMUNE CHECKPOINT INHIBITORS FOR EAR*LY-STAGE* NON-SMALL CELL LUNG CANCER

Neoadjuvant Monotherapy With Immune Checkpoint Inhibitors

The CheckMate 159 study (11) was the first research to prospectively explore the feasibility and safety of neoadjuvant therapy with ICIs in 22 patients with treatment-naive and resectable stage I–IIIA NSCLC, with 20 patients [2 partial response (PR) and 18 stable disease (SD)] undergoing curative surgery after neoadjuvant nivolumab and 45% achieving major pathologic response (MPR). At follow-up, the recurrence rate within 18 months was 73%, the OS rate was 95%, and the 24-month relapse-free survival (RFS) estimated by the Kaplan–Meier curve was 69%. Although the sample size was small, this trial confirmed the safety of neoadjuvant immunotherapy for NSCLC, laying the foundation for subsequent studies (11–13). The phase II LCMC3 study (14) evaluated the safety and efficacy of neoadjuvant atezolizumab in 101 patients with resectable stage IB–IIIA NSCLC with 7% being PR, 89% being SD, 18% being MPR, and 5% being pathologic complete response (pCR), and the therapy was well tolerated by patients with 6% of immune-related adverse event (irAE) of grade ≥3. The phase IB ChiCTR-OIC-17013726 study (15) treated 22 patients with resectable IB–IIIA stage squamous NSCLC with neoadjuvant sintilimab. Postoperative pathological results showed that 45.5% achieved MPR and 18.2% achieved pCR, and the objective response rate (ORR) was 13.6%. Comparison of PET–CT before and after treatment showed that 8 of 9 patients with 30% decrease in tumor metabolism uptake (TMU) achieved MPR, while no MPR was found in 11 patients with less than 30% decrease or increase in TMU, suggesting that changes in TMU on PET–CT before and after treatment may predict postoperative MPR status. As a whole, sintilimab has shown good safety profiles in neoadjuvant therapy for resectable NCSLC. Another study by Li et al. (16) also showed that neoadjuvant sintilimab treatment in NSCLC patients was well tolerated, with an MPR of 40.5% and a pCR of 16.2%. A decrease in TMU on PET–CT rather than a change in the sum of lesion diameters (SLD) was also identified as a predictor of pathological response to anti-programmed cell death-1 (PD-1) therapy in early-stage NSCLC in another study. In addition, it was found that primary lesions and metastatic lymph nodes may have a large heterogeneity in response to neoadjuvant sintilimab treatment. The indications of sintilimab in the treatment of early-stage lung cancer need to be intensively studied in the future, and key factors to overcome heterogeneous responses and delay disease progression need to be explored.

Immune Checkpoint Inhibitor-Based Neoadjuvant Combination Therapy

Given the limited efficacy of neoadjuvant immune monotherapy and the synergistic effect of chemotherapy and immunotherapy in cancer therapy, several trials have been designed to assess the efficacy and safety of immunotherapy combined with chemotherapy in the neoadjuvant treatment of early-stage NSCLC. A phase II study exploring the use of neoadjuvant atezolizumab in combination with chemotherapy (nab-paclitaxel and carboplatin) in resectable stage IB–IIIA NSCLC, with preliminary results in 14 patients, has reported radiographic PR in 57% of patients and MPR in 7 of 14 patients (50%), including 3 pCR, and is ongoing (17). The phase II NADIM study (18) is the first study to explore the efficacy and safety of nivolumab in combination with paclitaxel and carboplatin in neoadjuvant/adjuvant therapy in patients with resectable stage IIIA NSCLC. After neoadjuvant combination therapy, 93% patients had downstaging, and R0 resection was performed in 41/46 patients; MPR was 83% and pCR reached 71% after operation; PR was 72% and CR was 6.5%; survival data showed that in the ITT population, 12-month PFS was 96%, 18-month PFS rate was 81%, and 18-month OS rate was 91%. In summary, the MPR and survival data of the study reached unprecedented new breakthroughs. The CheckMate 77T study further expanded the sample size on the basis of the NADIM study to demonstrate the exact efficacy of this neoadjuvant modality in the context of a phase III study. In this study, II–IIIA or IIIB (T3N2) epidermal growth factor receptor (EGFR)/anaplastic lymphoma kinase (ALK)-negative NSCLC, the primary study endpoint was event-free survival (EFS) assessed by an independent review. Preliminary results from a small clinical trial (NCT03366766) at the 2020 American Society of Clinical Oncology (ACSO) meeting showed that nivolumab combined with platinum doublet was well tolerated in 13 patients with stage IB–IIIA resectable NSCLC; postoperative MPR appeared in 11/13 patients (85%) and pCR in 5/13 (38%); imaging response rate was 46% (PR 5, CR 1), and no recurrence was observed after 10 months of follow-up.

In addition, neoadjuvant strategies for the combination of dual ICIs are also being explored. The phase II NEOSTAR study (19) assessed the efficacy of neoadjuvant nivolumab (group N) and nivolumab in combination with ipilimumab (group NI) in 44 patients with stage I–IIIA resectable NSCLC. Overall MPR was 24%, overall MPR + pCR was 25% (N vs. NI = 17% vs. 33%), pCR was 18% (N vs. NI = 9% vs. 29%), ORR was 20% (N vs. NI = 22% vs. 19%), and ORR was positively correlated with MPR ($p < 0.001$). In 37 patients with surgical resection, MPR was 30% (N vs. NI = 19% vs. 44%), and the group NI had a significantly lower percentage of viable tumor cells than the group N (20% vs. 70%, $p = 0.077$). Moreover, markers analysis showed that $CD3^+CD103^+$ memory cells (81.2% vs. 54.4%, $p = 0.021$) and the proportion of $CD8^+$T cells (56.2% vs. 38.3%, $p = 0.057$) significantly increased in combination immunotherapy. In terms of safety, treatment-related adverse events (TRAEs) of grade 3 to 5 included death from bronchopleural fistula caused by pneumonia associated with steroid therapy (one case, grade 5, group N); grade 3 pneumonia, hypoxia, and hypermagnesemia (each one case, group N); and grade 3 diarrhea (one case, group NI). Thus, neoadjuvant combination

therapy seems safe and more effective compared with immune monotherapy. Overall, the NEOSTAR study showed that the complexity of surgery and lung function of patient were not affected by neoadjuvant immunotherapy, and the overall resection rate was comparable to the effect of neoadjuvant chemotherapy, as well as there was no increase in unacceptable toxicity or perioperative morbidity and mortality. However, five patients who failed to undergo surgical resection and one patient who died during perioperative period suggested that neoadjuvant immune monotherapy or combination therapy for patients with resectable NSCLC should be carefully selected after balancing the factors of treatment efficacy, surgical difficulty, and risk.

The corresponding results of completed clinical trials of neoadjuvant therapy with ICIs for resectable NSCLC are detailed in **Table 1**.

The Safety and Efficacy Analysis of Neoadjuvant Therapy With Immune Checkpoint Inhibitors in Early-Stage Non-small Cell Lung Cancer

Although neoadjuvant immunotherapy has attracted much attention in the surgical treatment of tumors, it still deserves attention for the possible technical challenges during surgery and drug side effects during or after treatment (such as pneumonia and endocrinopathy). To solve the problems, the surgical conditions, perioperative safety, and complications after neoadjuvant immunotherapy were comprehensively analyzed in multiple studies. In the NA00092076 study, the proportion of thoracotomy was 70% (14/20), and the incidence of postoperative complications was 50%, of which the most common was atrial arrhythmia (30%), but no surgery-related death occurred (20). In the NEOSTAR study, thoracotomy accounted for 73% (27/37), and the combination therapy significantly reduced the probability of subsequent surgical treatment (two cases in group N, five cases in group NI). The incidence of postoperative complications was 21.6%, of which the most common was persistent air leak, with a surgery-related mortality rate of 3%. A prospective study by Yang et al. (21) showed that compared with induction with standard neoadjuvant chemotherapy, the morbidity and mortality were not increased in 13 patients with stage II–IIIA NSCLC who received neoadjuvant treatment with ipilimumab. A recent study confirmed that neoadjuvant immunotherapy of nivolumab in NSCLC patients did not increase the difficulty of surgery, blood loss was usually low, and there was no unexpected morbidity with atrial fibrillation in six patients (30%); postoperative pneumonia, empyema, and persistent air leak in one patient each (5% each); and low incidence of serious irAEs. However, it should be noted that 54% of the cases were converted to thoracotomy from robot-assisted thoracic surgery (RATS) or video-assisted thoracic surgery (VATS), mostly due to pleural adhesions, perihilar inflammation, and fibrosis (20). For the immunotherapy cycles, the CheckMate 159 study, NEOSTAR study, and NADIM study administered 1–2, 3, and 3 cycles before operation, respectively. The final results did not affect the timing of operation, and relevant studies are still being explored.

The current studies of neoadjuvant therapy with ICIs in NSCLC are all phase I/II exploratory clinical studies, most of which are single-arm designs with a small sample size (10 to 101 cases). Preliminary results showed that the safety of immunotherapy was good, but the MPR was low. Although the MPR of neoadjuvant therapy with nivolumab reached 45% in the CheckMate 159 study, the subsequent LCMC3 study and NEOSTAR study failed to replicate the results. The NADIM study of immunotherapy combined with chemotherapy achieved the highest MPR and pCR so far and all performed surgery as scheduled. But the sample size of this study was small and the incidence of specific adverse events was not published. The MPR of dual immunotherapy regimen could improve to some extent, but it significantly reduced the chance of patients receiving subsequent surgical treatment, and the CheckMate 617 study of dual immunotherapy was terminated early. Therefore, the selection of immunotherapy regimen needs to be carefully selected after balancing factors of treatment efficacy, safety, and surgery rate. Overall, RECIST criteria and MPR assessment showed good anti-tumor activity and safety of neoadjuvant therapy with ICIs, which prompted an important step toward longer-term survival in early-stage NSCLC although the reliability and reproducibility of the results have yet to be further confirmed.

TABLE 1 | The results of completed clinical trials of neoadjuvant therapy with ICIs for resectable NSCLC.

Clinical trial	Phase	Stage	Intervention used	Sample size	Primary endpoint	Primary outcomes
CheckMate 159	I	I–IIIA	Nivolumab	22	Safety and feasibility	MPR: 45%, pCR: 10%
LCMC3	II	IB–IIIA	Atezolizumab	101	MPR	MPR: 18%, pCR: 5%
Li et al. (13)	II	IA–IIIB	Sintilimab	40	Safety	MPR: 40.5%, pCR: 16.2%
Li et al. ChiCTR-OIC-17013726	IB	IA–IIIA	Sintilimab	22	Drug-related adverse event; surgery complications; no-delay surgery rate	MPR: 45.5%, pCR: 18.2%
NADIM	II	IIIA	Nivolumab + chemotherapy	46	PFS at 24 months	MPR: 83%, pCR: 71%
NEOSTAR	II	I–IIIA	Nivolumab vs. nivolumab + ipilimumab	44	MPR	MPR: 24%, pCR: 18%

NSCLC, non-small cell lung cancer; MPR, major pathologic response; pCR, pathologic complete response; ICIs, immune checkpoint inhibitors; PFS, progression-free survival.

Ongoing Trials of Neoadjuvant Therapy With Immune Checkpoint Inhibitors in Patients With Resectable Non-small Cell Lung Cancer

Given the current breakthrough, multiple studies of neoadjuvant therapy with ICIs for stage II/III NSCLC are planned or ongoing, which will provide more data on safety and efficacy and contribute to the development of more effective treatment strategies. The primary endpoints of most studies are MPR, EFS, or DFS, while a few studies set to OS. The trials explored different neoadjuvant immunotherapy regimens. For example, the MK3475-223 (NCT02938624), TOP 1501 (NCT02818920), IONESCO (NCT03030131), Columbia University (NCT02716038), PRICNEPS (NCT0299457), and a phase II study (NCT02927301) are studying treatment using ICIs alone, the last study of which explored drug efficacy and preliminary results of 54 patients showed that the MPR rate was 20% and the tolerability was good with only one patient having delayed surgery due to pneumonia. Six trials are exploring the efficacy and safety of ICIs combined with chemotherapy in early-stage NSCLC, including phase II SAKK 16/14 trial (NCT02572843) and NADIM-II clinical trial (NCT03838159), phase III IMpower030 study (NCT03456063), CheckMate 816 study (NCT02998528), Keynote-671 study (NCT03425643), and AEGEAN study (NCT03800134). There are some other treatment options. Preliminary recent results of SAKK 16/14 showed an ORR of 44.8% in the chemotherapy stage compared with 59.7% in the immune neoadjuvant stage; 81% of patients underwent surgery (the most important reason for not undergoing surgery was disease progression, accounting for 33.3%). Encouragingly, the study showed that the 1-year EFS rate was 73.3%, surpassing the previous rate of about 50% in patients with stage IIIA disease. Thus, the treatment mode of chemotherapy with sequential immunotherapy is worthy of further expanding the sample size to demonstrate its exact benefit and looks forward to the publication of subsequent results. In addition to neoadjuvant chemotherapy combined with immunotherapy, the efficacy and safety of other regimens have also been explored. The phase II randomized study NeoCOAST is underway to compare the clinical activity and feasibility of durvalumab ± oleclumab (MEDI9447) or monalizumab (IPH2201) or danvatirsen in patients with resectable stage I–IIIA NSCLC. Unfortunately, a third arm of the CheckMate 816 study in patients receiving nivolumab plus ipilimumab has been discontinued due to intolerance in patients. Besides, radiotherapy can enhance the therapeutic effect of local lesions, reduce micrometastatic lesions, increase the immunogenicity of tumors, and also may lead to the upregulation of PD-L1 expression in tumors. Therefore, several trials (NCT03110978, NCT03237377, and NCT02904954) are evaluating the synergistic anti-tumor effect of radiotherapy with ICIs in early-stage NSCLC.

In addition to the different treatment regimens, several studies with neoadjuvant therapy continue ICI therapy in the adjuvant setting for 1 year, such as the SAKK 16/14 trial, IMpower030, Keynote-671, and NADIM-II, or perform consolidation therapy with ICI after adjuvant therapy, such as the TOP 1501 trial. But they may affect the evaluation of the efficacy of neoadjuvant therapy, needing more well-designed studies to confirm the results. The details of ongoing clinical trials of neoadjuvant ICIs and ICI-based combination therapy for earlier-stage NSCLC are listed in **Table 2**.

THE RESEARCH PROGRESS OF ADJUVANT THERAPY WITH IMMUNE CHECKPOINT INHIBITORS IN PATIENTS WITH RESECTABLE NON-SMALL CELL LUNG CANCER

At present, the study of adjuvant therapy for NSCLC is in the exploratory stage with no mature research result. There are two main ongoing trials of adjuvant therapy with anti-PD-1 agents, ANVIL and PEARLS, and three main trials of that with anti-PD-L1 agents (22–24) for earlier-stage NSCLC, all of which are ICIs with or without chemotherapy. The primary endpoint of most studies is DFS. The specific details of these trials are exhaustively described in **Table 3**. All clinical trials of adjuvant immunotherapy are expected to be completed during 2024–2027; thus, the role of anti-PD-1/PD-L1 drugs in adjuvant therapy may remain unclear over a period of time. In addition, there are no ongoing clinical trials that compare the efficacy and safety data of neoadjuvant immunotherapy against the adjuvant immunotherapy strategies, and some trials have followed adjuvant therapy after neoadjuvant therapy, all of which still limit the judgment of the effectiveness of the treatments to some extent. Adjuvant therapy, neoadjuvant therapy, or the combination of both, which is the best treatment strategy, remains unknown. The results of these studies may have a substantial impact on the clinical practice of patients with locally resected NSCLC, and the development of more large-scale prospective clinical trials is expected in the future.

RESPONSE EVALUATION TO NEOADJUVANT IMMUNOTHERAPY

Neoadjuvant clinical trial endpoints include pCR, DFS, and OS. Pathological response is considered to improve the efficiency of the study and predict survival, which is approved by the United States Food and Drug Administration (FDA) and European Medicines Agency for survival surrogate endpoints in neoadjuvant breast cancer studies. However, the pCR of neoadjuvant chemotherapy for lung cancer in 15 studies is only 4%, which greatly limits its application (25). In addition, OS is considered the most widely accepted study endpoint and the "gold standard" for demonstrating the clinical benefit of any cancer treatment; DFS, a composite endpoint combining time to disease recurrence and OS, is commonly used as a surrogate for OS. However, the use of these endpoints to predict clinical benefit in early-stage NSCLC is problematic and may slow down the process of drug development. First, in the neoadjuvant setting

TABLE 2 | Ongoing clinical trials of neoadjuvant therapy with ICIs for earlier-stage NSCLC.

Clinical trial	Phase	Stage	Intervention used	Estimated sample size	Primary endpoints
MK3475-223 (NCT02938624)	I	I–II	Pembrolizumab	28	Toxicity, MPR
TOP 1501 (NCT02818920)	II	IB–IIIA	Pembrolizumab	32	Surgical feasibility
IONESCO (NCT03030131)	II	IB-II	Durvalumab	81	R0 resection
Columbia University (NCT02716038)	II	IB–IIIA	Atezolizumab	30	MPR
PRICNEPS (NCT0299457)	II	IB–IIIA	Atezolizumab	60	Toxicity
NCT02927301	II	IB–IIIA	Atezolizumab	180	MPR
NeoCOAST	II	I–IIIA	Durvalumab ± oleclumab (MEDI9447) or monalizumab (IPH2201) or danvatirsen	160	–
SAKK 16/14 (NCT02572843)	II	IIIA (N2)	Durvalumab + chemotherapy	68	EFS
NADIM-II (NCT03838159)	II	IIIA/IIIB with T3N2	Chemotherapy + nivolumab vs. chemotherapy	90	pCR
IMPower030 (NCT03456063)	III	II–IIIB	Chemotherapy + atezolizumab vs. chemotherapy + placebo	374	MPR, EFS
CheckMate 816 (NCT02998528)	III	IB–IIIA	Chemotherapy vs. chemotherapy + nivolumab vs. nivolumab + ipilimumab	350	EFS, pCR
Keynote-671 (NCT03425643)	III	II–IIIB	Chemotherapy + pembrolizumab vs. chemotherapy	786	EFS, OS
AEGEAN (NCT03800134)	III	IIA–IIIB	Chemotherapy + durvalumab vs. chemotherapy + placebo	300	MPR
NCT03110978	I/II	I–IIA	Radiotherapy + nivolumab vs. radiotherapy	140	EFS, secondary malignancy, and death
NCT03237377	II	IIIA	Durvalumab + radiation or durvalumab + tremelimumab + radiation	32	Safety, feasibility
NCT02904954	III	II–III	Durvalumab with or without radiotherapy	-	DFS

NSCLC, non-small cell lung cancer; MPR, major pathologic response; pCR, pathologic complete response; ICIs, immune checkpoint inhibitors; EFS, event-free survival; OS, overall survival; DFS, disease-free survival.

TABLE 3 | Ongoing clinical trials of adjuvant therapy with ICIs for earlier-stage NSCLC.

Clinical trial	Phase	Stage	Intervention used	Estimated sample size	Primary endpoints
ANVIL (NCT02595944)	III	IB–IIIA	Nivolumab vs. observation with or without adjuvant chemotherapy	903	OS, DFS
PEARLS/Keynote-091 (NCT02504372)	III	IB–IIIA	Pembrolizumab vs. placebo with or without adjuvant chemotherapy	1,380	DFS
IMpower010 (NCT02486718)	III	IB–IIIA	Atezolizumab + chemotherapy vs. best supportive care + chemotherapy	1,280	DFS
BR31 (NCT02273375)	III	IB–IIIB	Durvalumab vs. placebo with or without adjuvant chemotherapy	1,100	DFS in PD-L1-positive patients and in all randomized patients

NSCLC, non-small cell lung cancer; ICIs, immune checkpoint inhibitors; OS, overall survival; DFS, disease-free survival; PD-L1, programmed cell death-ligand 1.

for localized NSCLC, it is difficult to include a large number of patients to have sufficient power to identify a difference in survival. Second, it may take many years to reliably establish improvements in OS and DFS. Therefore, identifying surrogate endpoints that do not require long-term follow-up and can accurately predict OS is important. Recently, researchers have proposed MPR, which refers to neoadjuvant therapy-induced tumor response with pathological residual tumor less than 10%, and have verified its effectiveness in neoadjuvant chemotherapy. Residual tumor cells were positively associated with the risk of death after neoadjuvant chemotherapy, but not with the risk of death from surgery alone. A follow-up report also showed that MPR was associated with OS (residual tumor cells >10%, HR 2.39, $p = 0.05$). The study of neoadjuvant chemotherapy for

NSCLC conducted by Pataer et al. (26) also identified that the OS rate and DFS of patients in the MPR group were significantly greater than those in the non-MPR group, and MPR was still associated with survival when controlling for pathological stage. Therefore, MPR is considered a surrogate endpoint measure for studies of neoadjuvant therapy in patients with NSCLC, and multiple studies of neoadjuvant immunotherapy have selected MPR as a primary or secondary study endpoint (11, 27). In the NEOSTAR study, RECIST-assessed disease response was positively correlated with MPR ($p < 0.001$); the results of the CheckMate 159 study at 34.6 months of follow-up showed that neoadjuvant MPR with nivolumab was associated with recurrence rate. However, the association between MPR response and DFS or OS is not clear, still needing longer follow-up verification; and when comparing different clinical trials, how to accurately measure and evaluate risk based on different HR values is not uniform.

Besides, in the clinical efficacy evaluation, considering their peculiar mechanisms of action different from chemotherapy, immunotherapies make the inconsistency between pathological and imaging assessment and would develop atypical response patterns that extend beyond those of cytotoxic agents, such as pseudoprogression (PsPD), delayed responses, etc., which makes it difficult to accurately grasp the response rate of immunotherapy by traditional imaging assessment alone. Although the situation may be improved with the development of techniques such as PET–CT, many difficulties are still faced. A study of neoadjuvant nivolumab in NSCLC showed that ORR by radiographic assessment at surgery was only 10% (2/20), while MPR by pathological assessment reached 45% (9/20) (11). Therefore, precise evaluation of neoadjuvant immunotherapy response is particularly important for surgical treatment. Attempts have been made to develop a new quantitative immune-related pathological response criterion (irPRC) to standardize the assessment of pathological response after neoadjuvant treatment with ICIs for NSCLC. This standard added the area of the regression lesions to the areas of residual active tumor and necrosis and detailed terms "stroma," "fibrosis," and "inflammation" that specifically describe tumor-infiltrating lymphocytes and regenerating lymphatic structures, as well as confirmed their utility in standardizing the pathological assessment of the efficacy of immunotherapy (28). However, an abstract from the ASCO meeting in 2020 indicated that in 24 NSCLC patients with stage I–IIIA treated with neoadjuvant nivolumab or nivolumab + ipilimumab, the heterogeneity of CT images was significantly increased in patients who achieved MPR, possibly reflecting increased T cell infiltration or tumor necrosis (29) and suggesting that imaging features are associated with treatment MPR. Therefore, further studies are needed to determine the effectiveness and feasibility of neoadjuvant therapy for patients with early-stage lung cancer based on non-invasive markers of imaging characteristics combined with pathological markers in a larger cohort of patients. In addition, neoadjuvant immunotherapy has diverse pathological changes and is complex and cumbersome to evaluate, which requires data from multiple large randomized clinical trials and long-term follow-up to verify the reliability of MPR and irPRC as surrogate markers of RFS and OS. With this background to establish a valid surrogate endpoint, there are multiple problems to be addressed in neoadjuvant studies: (i) The lack of a uniform endpoint in ongoing studies makes the situation complicated; (ii) nearly all trials of neoadjuvant immunotherapy are multiple small non-randomized "exploratory" phase II studies; (iii) confounding regimen incorporating single immunotherapy as well as chemoimmunotherapy makes the interpretation and comparison of study results difficult; and (iv) given the large ongoing phase III adjuvant immunotherapy studies, it is challenging to enroll sufficient patients into large neoadjuvant studies.

PREDICTIVE BIOMARKERS OF NEOADJUVANT IMMUNOTHERAPY

At present, the overall efficacy of neoadjuvant immunotherapy fluctuates widely, ranging from 10 to 90%, and is limited by different therapeutic means. Therefore, it is difficult to correctly predict which populations could benefit more from neoadjuvant immunotherapy. Effectively predictive biomarkers will be specific for the selection of patients in clinical trials of ICI neoadjuvant therapy. Currently, markers that are being collected in phase III clinical trials related to the efficacy of immunotherapy include the following four major categories: (i) tumor cell-associated biomarkers, including PD-L1 expression, tumor mutation burden (TMB), DNA damage response (DDR) pathways [e.g., DNA mismatch repair deficiency (dMMR)/microsatellite instability (MSI)], specific mutant gene pathways (e.g., IFN-γ pathway, KRAS and STK11 mutation), and neoantigen load; (ii) TME-related biomarkers, including PD-L1 expression, and tumor-infiltrating immune cells, including immune cells with specific phenotypes (e.g., $CD39^+CD8^+$T, $CD4^+$T cells, $FOXP3^+$T cells, $NKp46^+$ cells), diversity of immune repertoires [e.g., richness and clonality of tumor-infiltrating lymphocytes (TILs), T cell receptor (TCR) repertoire], and immune status score; (iii) liquid biopsy-related biomarkers, including peripheral blood cells [e.g., $CD45RO^+/CD8^+$T cells, $CD4^+$ICOS (inducible T cell co-stimulator)$^+$T cells, circulating tumor cells (CTCs)], circulating tumor DNA (ctDNA), and other circulating molecular biomarkers (e.g., exosomes, cytokines, and inflammatory factors); and (iv) host-related markers, involving general characteristics (e.g., gender, age, and body fat distribution), intestinal commensals, and host germline genetics [e.g., human leukocyte antigen (HLA) diversity and other specific mutations].

In early lung cancer, some widely studied markers, such as PD-L1, TMB, and immune status of the TME, were first explored preliminarily. First, as a proposed test by the U.S. FDA, PD-L1 on tumor cells is considered a biomarker of anti-PD-1 inhibitors, especially for NSCLC patients to receive pembrolizumab. Markers analysis of the NEOSTAR study (19) showed that pretreatment PD-L1 expression was higher in responders than in non-responders (80% vs. 1%, $p = 0.015$). The percentage of viable tumor cells was lower in patients with PD-L1 > 1%. However, several studies hold different

views that MPR was not associated with PD-L1 expression, such as the CheckMate 159 study (11) and LCMC3 study (14), highlighting the limitations of the PD-L1 assay as an effective predictor for neoadjuvant immunotherapy. Besides, high TMB is an emerging potential predictive biomarker for MPR after adjuvant and neoadjuvant immunotherapy, meaning the total number of mutations present in tumor specimens. In the study of Forde et al. (11), anti-PD-1 therapy increased the number of neoantigen-specific T cell clones in tumor and peripheral blood in resectable NSCLC, suggesting that TMB may be used as a predictor of treatment response. Nevertheless, TMB alone is not effective in predicting treatment response/survival in patients, which needs to be further explored. The immune status of the TME also needs to be analyzed. In the LCMC3 study (14), compared with patients without MPR, patients with MPR had lower baseline levels of T cells and NK cells, but after neoadjuvant therapy, these patients experienced expansion of NK cells and granulocytes and increased abundance of dendritic cells and B cells in lymph nodes, as well as decreased abundance of monocytes, suggesting that ICIs play a key role in preoperative activation of tumor-specific immune killing. A study in patients with stage III melanoma treated with neoadjuvant ICIs found that expansion of tumor-resident T cell clones and a favorable IFN-γ gene signature were associated with RFS (30). In addition, liquid biopsy is a promising tool to non-invasively monitor response to neoadjuvant or adjuvant immunotherapy. The CheckMate 159 study explored the relationship between efficacy and specific expansion of tumor-specific T cells in peripheral blood and found that the clonal subtype of tumor-specific T cells increased continuously with treatment in patients with MPR and persistent disease-free status, but it gradually decreased in patients with non-MPR and recurrence (31). Therefore, dynamic remodeling of tumor-specific T cells in peripheral blood can serve as a predictive biomarker for neoadjuvant immunotherapy. ctDNA appears to be present in 50–95% of stage I to III patients (32, 33), suggesting that changes in ctDNA before and after neoadjuvant immunotherapy may be another more broadly applicable biomarker. The clearance of ctDNA and the expansion of tumor-specific T cells in peripheral blood may early monitor the treatment response and recurrence (34). However, whether ctDNA and tumor-specific T cells in peripheral blood are associated with MPR or even OS or DFS is not clear. The NADIM study is performing an immune repertoire profiling of peripheral blood TCR in patients with stage IIIA NSCLC receiving immunotherapy in combination with chemotherapy. In addition, the blood collection process, blood sample storage conditions, and centrifugation speed of separated plasma are all limiting factors associated with clinical practice (35, 36). To explore the clinical utility of these tests in patients receiving adjuvant and neoadjuvant immunotherapy, future trials should include serial collection of liquid biopsies.

The above results of biomarker studies in early-stage tumors are approximately similar to those in advanced tumors. However, other studies have also shown that the exploratory results in early-stage tumors are inconsistent. For example, in advanced lung cancer, driver gene mutations like EGFR and ALK have been shown to be associated with reduced response rates to ICIs and low TMB; therefore, the FDA does not recommend first-line ICI treatment in patients with EGRF or ALK-positive tumors (37, 38). But in the LCMC3 study of neoadjuvant immunotherapy, 37.5% (3/8) of EGFR/ALK-positive patients had pathological response (14), suggesting that NSCLC with specific gene mutations is not necessarily a limitation and contraindication for neoadjuvant immunotherapy in early-stage tumors. However, since the results were observed only in a small number of patients, several neoadjuvant trials have excluded EGFR/ALK-positive patients. In advanced disease, the role of ICIs in populations with driver mutations is also not clear. Therefore, further studies need to be conducted to benefit more patients.

A study developed a quantitative system pharmacology (QSP) model to predict response to neoadjuvant and adjuvant anti-tumor immunotherapy of human NSCLC (39). This model integrates knowledge of tumor growth, antigen processing and presentation, T cell activation and distribution, antibody kinetics, and immune checkpoint kinetics. The results showed that, in addition to TMB, the number of effector T cells and regulatory T cells in the tumor and blood was a predictor of responders. This suggests that it may be promising to obtain the most effectively comprehensive predictive markers by extracting features with large samples and multiple dimensions and constructing multivariate models using machine learning. Given the availability of preoperative and postoperative specimens during the study, neoadjuvant therapy has some advantages in the discovery and exploration of predictive markers. Although multiple studies have explored predictive markers of efficacy to neoadjuvant immunotherapy, the results have not been consistent, and considering that they are only preliminary exploratory analyses, the credibility of results still deserves further scrutiny. At present, there is no standard predictive marker for efficacy to neoadjuvant immunotherapy, and prospective large-scale studies are still needed to identify the most effective duration of neoadjuvant therapy and the best predictive biomarker of response.

CONCLUSION AND FUTURE PROSPECTS

In view of the high risk of postoperative recurrence of resectable NSCLC, many perioperative treatment methods are being continuously explored to prevent postoperative recurrence and obtain long-term survival benefit, such as chemotherapy, targeted therapy, and immunotherapy, continuously enriching the content of neoadjuvant and adjuvant therapy and bringing a brand-new different era for operable patients. ICIs are currently a hot topic and breakthrough point in cancer therapy and are gradually applied in earlier NSCLC. Through continuous conduction of relevant clinical trials, ICIs have made many breakthroughs with significant improvement in efficacy and in the exploration of response evaluation and predictive biomarkers. Although important clinical trials are still ongoing, exciting preliminary results have been obtained from the completed trials, in which the MPR of immune monotherapy reached 22–45%, the MPR of immunotherapy combined with chemotherapy reached

50–83%, and the safety was good, indicating that neoadjuvant immunotherapy is a promising treatment strategy for patients with resectable lung cancer. Studies currently ongoing include (i) phase III adjuvant chemoimmunotherapy studies, (ii) multiple small phase II neoadjuvant immunotherapy studies, (iii) small phase II chemoimmunotherapy studies, and (iv) phase III neoadjuvant chemotherapy plus immunotherapy followed by different lengths of postoperative adjuvant immunotherapy.

Nevertheless, neoadjuvant and adjuvant therapy with ICIs for NSCLC are still in the initial stage of exploration, and there are still many challenges for clinical adaptability and feasibility. First, as we have seen in some studies, neoadjuvant immunotherapy may affect the timing of surgery and increase the difficulty and risk of surgery due to its side effects or disease progression, suggesting that it is particularly important for screening treated patients. irAEs may still occur during and after treatment, especially in combination with dual immunotherapy. Also, the assessment of pseudoprogression and hyperprogression problems of immunotherapy still needs more exploration and evidence. Second, the study design of optimal treatment mode of neoadjuvant immunotherapy, including the choice of immunotherapeutic drugs, application cycle, and time point, is still challenging. On the one hand, most studies on neoadjuvant therapy will continue using adjuvant chemotherapy, adjuvant immunotherapy, or consolidation immunotherapy after surgery, which may affect the accurate observation of the efficacy of neoadjuvant therapy; on the other hand, most of them are preliminary exploratory studies in phases I–II, while there are few prospective phase III studies. The existing phase III studies lack a conventional chemotherapy control trial, so it is unknown whether the efficacy of neoadjuvant therapy could have a significant impact on existing study results. In addition, whether alternative endpoints (such as pCR and MPR) can predict the survival rate and have a positive impact on DFS or OS, as well as the development and validation of reliable evaluation criteria for response to neoadjuvant immunotherapy, is not clear. The treatment decision needs to be carefully selected after balancing the factors, such as treatment efficacy, safety, and surgery rate, to maximize patient outcomes. Finally, there is no standardized biomarker to identify the patient population who can benefit or develop irAEs. Although some available markers have been explored, including PD-L1, TMB, and liquid biopsies proposed recently, none have sufficient evidence to directly correlate with MPR or OS. In future studies, it is most important to develop biomarkers that reflect both tumor–immune system and immune system–host interactions based on the characteristics of immunotherapy itself to aid clinicians identify the patient population that will benefit the most from neoadjuvant immunotherapy. Of note, other treatment modalities are also being continuously explored; for example, the ADAURA study (ASCO abstract #LBA5) suggests that adjuvant chemotherapy followed by EGFR-TKIs is expected to be an effective treatment regimen. A multidisciplinary collaborative model for early-stage lung cancer is constantly being explored and developed, all of which have prompted better application of immunotherapy in the surgical treatment of resectable NSCLC, and more and larger prospective clinical studies are expected in the future to develop the best treatment strategy.

AUTHOR CONTRIBUTIONS

RB carried out the primary literature search, drafted and revised the manuscript, and participated in discussions. LL, XC, NC, and WS helped modify the manuscript. JC carried out the literature analysis, drafted and revised the manuscript, and participated in discussions. All authors read and approved the final manuscript.

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REVIEW
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Obesity, Sarcopenia, and Outcomes in Non-Small Cell Lung Cancer Patients Treated With Immune Checkpoint Inhibitors and Tyrosine Kinase Inhibitors

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Body composition refers to the proportional content of body fat mass and lean body mass that can lead to a continuum of different phenotypes ranging from cachectic/sarcopenic state to obesity. The heterogenetic phenotypes of body composition can contribute to formation of some cancer types and can sometimes lead to disparate outcomes. Both of these extremes of the spectrum exist in patients with non-small cell lung carcinoma (NSCLC). The discovery of new pathways that drive tumorigenesis contributing to cancer progression and resistance have expanded our understanding of cancer biology leading to development of new targeted therapies including tyrosine kinase inhibitors (TKI) and immune checkpoint inhibitors (ICI) that have changed the landscape of NSCLC treatment. However, in the new era of precision medicine, the impact of body composition phenotypes on treatment outcomes and survival is now being elucidated. In this review, we will discuss the emerging evidence of a link between body composition and outcomes in patients with NSCLC treated with TKI and ICI. We will also discuss suggested mechanisms by which body composition can impact tumor behavior and anti-tumor immunological response.

Keywords: non-small cell lung cancer, body composition, obesity, sarcopenia, tyrosine kinase inhibitor, immune checkpoint inhibitor, overall survival

Abbreviations: ALK, anaplastic lymphoma kinase gene; BMI, body mass index; CI, confidence interval; cm, centimeter; CT, computed tomography; CTLA-4, cytotoxic associated lymphocyte antigen-4; DEXA, dual-energy x-ray absorptiometry; EGFR, epidermal growth factor receptor; HR, hazard ratio; HU, Hounsfield unit; IARC, international agency for research on cancer; ICI, immune checkpoint inhibitor; IL-6, interleukin-6; IL-10, interleukin-10; IL-15, interleukin-15; IMAC, Intermuscular adipose content; irAEs, immune related adverse events; kg, kilogram; L3, third lumbar; m, meter; NA, not available; NK, natural killer; NR, not reached; PD-1, programmed death 1; PD-L1, programmed death-ligand-1; PFS, progression free survival; PMI, psoas muscle index; N, number; NSCLC, non-small cell lung cancer; ORR, objective response rate; OS, overall survival; SMI, skeletal muscle index; SCFM, sub-cutaneous fat mass; STAT3, signal transduction and activator of transcription 3; TGF-β, transforming growth factor-β; TKI, tyrosine kinase inhibitor; Treg, T-regulatory; TTF, time to treatment failure; VSR, visceral to subcutaneous adipose tissue area ratio; WHO, world health organization.

INTRODUCTION

Body composition refers to the proportional distribution of different body mass contents amongst various compartments including adipose tissue and lean body mass. The most clinically distinct body phenotypes are obesity and sarcopenia. Obesity plays a significant role in tumorigenesis. It is believed that some cancers develop in obese individuals because of the chronic meta-inflammation associated with obesity in which an abundance of hormones and cytokines can potentiate epithelial cell proliferation and cancer formation (1). The link between obesity and cancer development in multiple tumor types has long been recognized in multiple epidemiological studies including a landmark report by the International Agency for Research on Cancer (IARC) showing an increased incidence of several solid and hematological malignancies in obese patients compared to the general population (2). Interestingly, this link is weak in the case of lung cancer (2).

In addition to its role in cancer development, obesity has now emerged as a prognostic factor that may predict cancer mortality (3–5). Obesity can be associated with either inferior or superior outcomes, depending on the cancer type. For example, systematic reviews and meta-analyses of population-based follow-up studies of obese patients with non-metastatic prostate and breast cancer across all clinical stages found a higher mortality in obese compared to normal weight patients regardless of treatment modality (4, 5). Surprisingly, obese and overweight patients with lung cancer seem to have better survival rates compared to normal weight subjects with a relative risk (RR) of 0.78 (95% CI: 0.75–0.82) in overweight and 0.79 (95% CI: 0.73–0.86) in obese patients respectively, however, this only applies to smokers (3). Moreover, a more recent Chinese study confirmed a strong inverse relationship between body mass index (BMI) and lung cancer mortality, which was again seen primarily in smokers (6).

The favorable effect of obesity on mortality in patients with non-small cell lung cancer (NSCLC) which is the most common type of lung cancer has been demonstrated in patients with local and metastatic disease who received different treatment modalities including surgery, radiation, and chemotherapy (7, 8). For example, Yang et al. demonstrated that obesity was associated with longer survival in lung cancer patients (N= 14,751) which was consistent among all stages (local, regional and distant) (7) Furthermore, a large retrospective study showed a trend for better survival in obese patients with NSCLC treated with combination doublet chemotherapies (N= 2,585) (9).

The other clinically distinct phenotype of body composition is sarcopenia which is defined by severe reduction of lean body mass and wasting of skeletal muscle. Sarcopenia has been identified as an independent prognostic factor for mortality in some cancer types including NSCLC (10, 11). In addition, a distinct overlap syndrome of increased adipose tissue (obesity) and loss of lean body mass (sarcopenia) has been recognized as an important factor contributing to worse prognosis in some cancers including NSCLC (12). These effects of obesity on mortality and worse prognosis in the presence of sarcopenia in cancer patients have generated an unprecedented interest in the field of oncology to study the interconnection between body composition phenotypes and cancer behavior including NSCLC (which will be the focus of this review) in an attempt to solve this conundrum. What adds to the importance of analyzing this association is that lung cancer has the highest prevalence, annual incidence and mortality rates worldwide compared to all cancer types in men and women (13). Likewise, body composition phenotypes such as obesity and sarcopenia are prevalent in lung cancer patients. As an example, up to 43% of patients with NSCLC cancer can develop sarcopenia and cachectic syndrome (14) and approximately about half of NSCLC patients are considered to have a BMI >25 mg/m^2 (considered overweight or obese) (15).

Recently, there has been a revolution in our understanding of lung cancer biology with the detection of driver mutations such as epidermal growth factor receptor (EGFR) mutation as well as the exploration of the role of immune system dysfunction in cancer progression (16–18). These discoveries have transformed the outcomes of advanced lung cancer with the development of tyrosine kinase inhibitors (TKI) targeting driver mutations such as EGFR directed TKIs and immune checkpoint inhibitors (ICI) which unleash the host immune system against tumor cells (19–24). This recent development of therapeutics in NSCLC was accompanied by a huge effort to identify patients who benefit the most from these medications given that a significant proportion of patients do not respond (25). Given the established link between different body composition and outcomes in NSCLC that was outlined by prior research, it was plausible for researchers to analyze whether different body phenotypes could be a predictive factor for response and outcomes with novel therapies.

With an evolving landscape of treatment options in advanced and metastatic lung cancer, it is imperative to further understand if body composition phenotypes can predict response and outcomes to these new classes of medications and whether there is a mechanistic effect of proportionate body components and tumor microenvironment. In this review, we first introduce the common approaches used in clinical research to estimate body composition including obesity and sarcopenia. Next, we discuss the available evidence of a biological crosstalk between different body composition phenotypes and tumor microenvironment in NSCLC. Lastly, we highlight the available studies conducted to analyze the implications of body composition phenotypes on survival outcomes in patients with NSCLC who were treated with either EGFR TKI or ICI; we also provide our recommendations on the conceptual utility of incorporating body composition calculations into prospective trials.

DISCUSSION

Methods Used To Estimate Body Composition in Patients With Non-Small Cell Lung Cancer

Many measures have been used in recent and ongoing research investigating the effect of body composition phenotypes on

cancer outcomes including NSCLC. The most conventional and easiest method of estimating body composition to identify obesity is through calculation of BMI which is defined by weight divided by the square of body height [kg/m^2]. Based on the world health organization (WHO) classification, individuals can be divided into six groups to estimate the degree of obesity (**Table 1**) (26, 27). However, the complexity and heterogeneity of body composition and nutritional status might not be reflected accurately with the use of BMI alone due to its low sensitivity as indicted by discrepancies between BMI and central obesity (15, 28). In addition, calculation of BMI does not offer an accurate depiction of lean body mass which, when reduced, is considered an independent prognostic factor for high mortality in patients with NSCLC (10, 11). It has also been recognized that a subset of obese patients (defined by BMI > 30 kg/m^2) are considered to be metabolically healthy whereby they are considered to have a favorable distribution of fat mass with a normal inflammatory profile which potentially reduces the risk incurred by diseases related to obesity such as cancer and cardiovascular disease (29). Moreover, the calculation of BMI cannot distinguish between different patterns of body fat distribution (subcutaneous versus visceral) which could lead to multiple heterogeneous obesity phenotypes that could be associated with different biology and are not accurately reflected by BMI (30). Lastly, definition of obesity based on WHO classification can vary depending on ethnicity (26). This has led to the utilization of other indicators and calculations of adipose tissue content to study their relationship with outcomes in NSCLC patients such as visceral fat mass, subcutaneous fat mass, visceral to subcutaneous ratio, and fat mass index among others (31).

The measurement of sarcopenia is based on calculation of skeletal muscle index (SMI) (32, 33). Calculation of SMI is defined as total cross-sectional skeletal muscle mass (cm^2) normalized by height (m^2). Skeletal muscle mass also referred to as skeletal muscle area is derived from the total skeletal muscle mass of the eight abdominal muscles (psoas, erector spinae, quadratus lumborum, transversus abdominis, latissimus dorsi, external, and internal obliques, and rectus abdominis) measured by surface area (cm^2) at the third lumbar (L3) landmark using a single cross-sectional computed tomography (CT) image (32, 34). The L3 landmark is visible in CT scan protocols routinely performed for diagnostic and monitoring reasons in most cancer populations: abdomen (T10-L4), chest- abdomen (T1-L4), or chest-abdomen-pelvis (T1-L5). The use of a single CT image for regional body composition analysis at L3 has been described and validated in great detail in several seminal papers (32, 34, 35). The most valuable feature of this L3 landmark is that it is linearly related to whole-body fat free mass, appendicular skeletal muscle mass and whole-body fat mass as measured by dual-energy x-ray absorptiometry (DXA) in non-cancer and cancer populations (34). In brief, the CT scanner differentiates between adipose, skeletal muscle, and other compartments like bone based on specific attenuation thresholds according to the CT unit of measurement, the Hounsfield unit (HU) scale (e.g., skeletal muscle attenuation threshold is -29 to 150 HU). Cross-sectional tissue surface areas (cm^2) are semi-automatically determined by a medical imaging software such as SliceOmatic v 5.0 (Tomovision Montreal, Quebec, Canada) based on HU tissue-specific thresholds (34, 35) (**Figure 1**). Then, tissue boundaries are manually corrected as needed by a trained investigator following the semi-automatic analysis. Cutoff points for sarcopenia using the L3 SMI according to ethnicity, gender and BMI (sarcopenic obesity) have been validated with adverse cancer-related outcomes in several studies (32, 33).

Sarcopenic obesity is defined as low skeletal muscle mass (i.e., sarcopenia) according to a SMI cut-off such as <38.5 cm^2/m^2 for women and <52.4 cm^2/m^2 for men in the context of a BMI > 30 kg/m^2 as published by Prado et al. (32). Similarly, sarcopenia and sarcopenic obesity can also be determined using BMI and SMI specific cut-offs for men (SMI < 43 cm^2/m^2 for underweight and normal weight men, SMI < 53 cm^2/m^2 for overweight and obese men) and for women (<41 cm^2/m^2 across all BMI categories) as published by Martin et al. (33). Although the Martin et al. and Prado et al. SMI cut-off values have been used extensively by other investigators, there is currently no consensus on CT-derived SMI reference cut-off values to identify sarcopenia or sarcopenic obesity in healthy and clinical populations and is an active area of research. In addition, the L3 landmark may not be ideal for assessing SMI in patients with lung cancer, including NSCLC, who often only undergo chest CT scans (i.e., L3 vertebral landmark often not visible, T1-L1). Several research studies have examined alternative chest CT scan landmarks including lumber one (L1) and two (L2) and have provided potential SMI cut-offs for sarcopenia in healthy and in lung cancer populations, however these landmarks have not been adequately tested or validated particularly in large racially diverse cancer populations (35–38). Thus, identifying an appropriate and valid single vertebral landmark from a chest CT scan and derivation of specific cut-offs at these newer landmarks to identify sarcopenia also remains an emerging area of research.

Various researchers have used single muscle groups to determine sarcopenia such as the psoas muscle index (PMI) also usually at the L3 region (39) (see **Figure 1**). The major advantage of using a single muscle group is the speed by which this analysis can be conducted in comparison to having to capture all the muscle groups at this landmark. A major criticism for the use of single muscle groups is that it does not correlate with total lumbar skeletal muscle area and thus not representative of the entirety of the lumbar muscle groups and more importantly appears to be a poor indicator of clinical

TABLE 1 | World health organization classification of obesity based on body mass index.

Body Mass Index (BMI) (kg/m^2)*	Definition
<18.5	Underweight
18.5 – 24.9	Normal weight
25.0 – 29.9	Overweight (Pre-obesity)
30.0 – 34.9	Obesity class 1
35.0 – 39.9	Obesity class 2
≥40	Obesity class 3

BMI cut off points used to define obesity can vary depending on ethnicity (26).

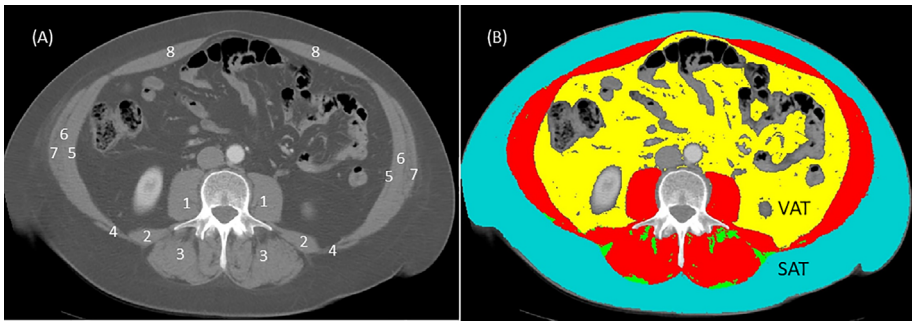

FIGURE 1 | Computed tomography (CT) image at third lumbar (L3) before **(A)** and after **(B)** analysis using sliceOmatic software (Tomovision, Montreal, Quebec, Canada). In the image without coloring **(A)**, individual muscle groups are represented by numbers and these are: 1 = psoas; 2 = quadratus lumborum; 3 = erector spinae; 4 = latissimus dorsi; 5 = transversus abdominis; 6 = internal obliques; 7 = external obliques; 8 = rectus abdominis. In the image with coloring **(B)**, analysis is based on Hounsfield unit thresholds for each tissue: skeletal muscles (red) -29 to 150 HU; subcutaneous adipose tissue (SAT) in teal and intermuscular adipose tissue (IMAT) in green -30 to -190 HU; visceral adipose tissue (VAT) in yellow -50 to -150 HU; air in black -1,000 HU; bone (L3 vertebra) 400 to 4,000 HU). Skeletal muscle index (SMI) is calculated from the total surface area (cm^2) of skeletal muscles (in red, image **(B)** normalized (divided) for height (m^2). For example, the total L3 skeletal muscle (in red, image **(B)** for this image is 166 cm^2 assuming that this person is female with a height of 164 cm or 2.68 m^2 the SMI for this person = 166 cm^2/2.68 m^2 or 61.4 cm^2/m^2. Using the Prado et al. (32) cut off of SMI < 38 cm^2/m^2 for women, this individual would not have sarcopenia. Some researchers use single muscle groups such as the psoas muscles (1 in image **(A)** normalized for height to determine sarcopenia. Although using single muscle groups to determine sarcopenia is debatable, only the surface area for both psoas muscles (1 in image **(A)** would be used to calculate psoas muscle index (PMI). Thus, PMI = psoas muscle surface areas (cm^2) divided by height (m^2).

outcomes in cancer populations (40, 41). Given these limitations, the use of PMI is less favorable than the well-established and validated technique using total lumbar skeletal muscle area for the calculation of SMI as previously described.

The importance of identifying sarcopenia comes from the fact that it has high prevalence across all BMI subtypes as well as having a detrimental prognostic effect on patients with NSCLC (42, 43).

As previously mentioned, an overlap syndrome of sarcopenia and obesity (sarcopenic obesity) has been identified and associated with adverse clinical implications in different cancer types, including NSCLC. For example, an observational study of patients with NSCLC who were treated with chemotherapy found that patients with sarcopenic obesity had shorter overall survival (OS) compared to obese patients without sarcopenia (44). The variable methodologies used to estimate body composition from obesity to sarcopenia and their effect on cancer progression and outcomes pose a challenge on how to compare and derive conclusions from studies in NSCLC. Nevertheless, the effort to analyze the effect of different elements of body composition and outcomes in NSCLC is of much importance, as each phenotype could have distinct biological implications on the host and the tumor.

What Is the Effect of Obesity on Non-Small Cell Lung Cancer and Anti-Tumor Immune Response?

Obesity is considered a protective factor in both early stage and advanced NSCLC patients who are treated with surgery or chemotherapy (7, 45). This effect can be explained partially by the fact that obese patients who receive chemotherapy tend to develop less medication related toxicities leading to lower discontinuation rates of cancer treatment (46). However, the biological landscape associated with obesity seems to be more complex and is believed to play a role in cancer behavior and progression. As an example, some hormonal factors in obese patients such as leptin plasma levels can affect prognosis in NSCLC, as low leptin levels correlate with shortened OS (47). Inversely, adiponectin, which is another hormone secreted from the adipose tissue has been suggested as a factor contributing to tumor progression in NSCLC, but biological mechanisms explaining the action of this hormone are not well understood (48). Different body composition phenotypes in obese patients and their variable hormonal and inflammatory profiles have led to distinguishing obesity as "metabolically unhealthy obesity" versus "metabolically healthy obesity" whereby patients can have a high BMI consistent with obesity definition but have a favorable fat distribution with decreased systemic inflammation leading to low disease morbidity (29).

The biological and immunological aspects of the inverse relationship between obesity and prognosis in NSCLC, termed "obesity paradox" can be explained through several mechanisms (49). Obesity can lead to exhaustion of T-cells resulting in increased tumor growth and it can upregulate programmed death-1 (PD-1) expression on CD8+ T-cells in tumor mice models (50). PD-1 receptors are checkpoint protein receptors present on immune cells that when bound to their respective ligand receptor can decrease anti-tumor efficacy of the host immune system against tumor cells (51). Although, the mechanism by which obesity can increase PD-1 expression has not been fully elucidated, increased levels of leptin secreted from adipose tissues has been suggested to boost cascade signaling indirectly through signal transduction and activator of transcription 3 (STAT3) which leads to upregulation of PD-1 receptors on T-cells (**Figure 2**). This can, in part, explain the improved response rates and survival noted in patients with

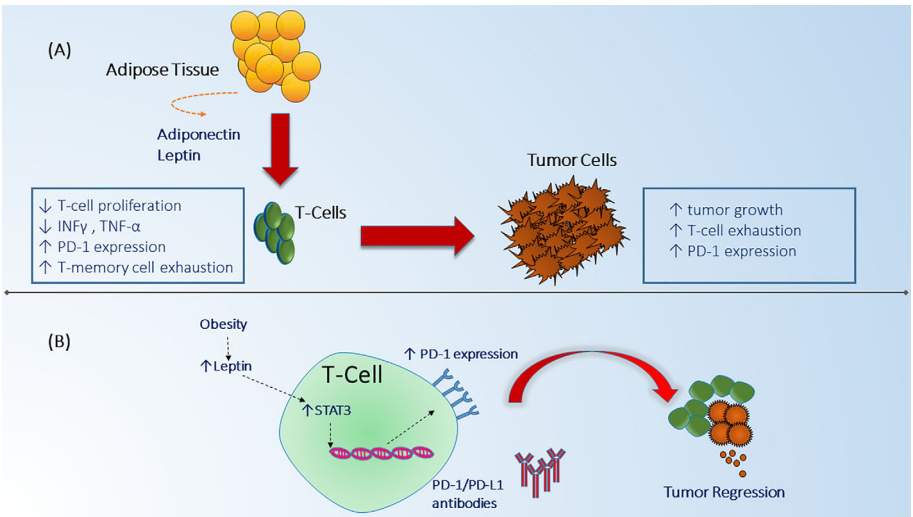

FIGURE 2 | Obesity effect on immune system function and anti-tumor mechanisms. **(A)** Adipose cells in the fat tissue secrete different adipokines including adiponectine and leptin that can alter immune system function by suppressing T-cell proliferation, decreasing INF-y, TNF-a, and increasing T-cell memory dysfunction which in turn can lead to enhanced tumor escape from immune surveillance leading to tumor growth and progression (48). Obesity related tumors can as well be associated with increased expression of checkpoint proteins which have a negative regulatory effect on immune cell proliferation (50). **(B)** The mechanism by which obesity can interact with immune checkpoint receptors in the tumor microenvironment is believed to be through increased secretion of leptin which in turn increases PD-1 expression on CD8+ T-cells through STAT3 signaling (50). Increased expression of PD-1 receptors can lead to enhanced response to immune checkpoint monoclonal antibodies and immune cells mediated tumor regression (50).

obesity across different tumor types who are treated with ICI including PD-1/PD-L1 (programmed death ligand-1) inhibitors that target the interaction of these checkpoint receptors (52–54). Moreover, obesity can modulate other T-cell subsets such as T-regulatory (Treg) cells which function as immunosuppressant cells and when down regulated in obese patients, they can reduce production of interleukin-10 (IL-10) which leads to exacerbation of the chronic inflammatory state (55). Similarly, Treg cells have been found in the tumor microenvironment of NSCLC and lead to an inhibitory effect on effector T-cell proliferation (56). Therefore, the effect of obesity on Treg cells can play a role in modulating Treg response in NSCLC although this has not been studied yet. Another type of immune cell that is important in obesity and NSCLC are the Natural Killer (NK) cells which are responsible for innate immunity and anti-cancer function. NK cells have been shown to be impaired in patients with obesity (57). Likewise, NSCLC patients can lack the cytotoxic effect of NK cells and have defective granulation leading to decreased innate anti-tumor response (57–59). Finally, obesity can affect the balance between macrophage subtypes (M1- M2) favoring the M1 subtype (pro-inflammatory cells) over M2 (immunosuppressive and pro-tumorigenic) (60). An increase in M2 macrophages can lead to increased host immunosuppression and more aggressive tumor behavior which could theoretically explain the improved outcome profile noted in patients with NSCLC who are obese (61) (**Table 2**). This collective evidence suggests an alteration of anti-tumor immune function and the favorable outcomes in obese patients with NSCLC which can partially explain the improved outcomes noted recently with the use of ICI since this class of medications primarily affects the T-cell but also has been found to have partial mechanisms of action through other immune cells such as NK cells and macrophages (65, 66).

What is the Effect of Sarcopenia on Non-Small Cell Lung Cancer and Anti-Tumor Immune Response?

Sarcopenia is usually considered to be a consequence of the changes accompanying malignancy such as malnutrition and alterations in the hormonal milieu including the surge of cytokines due to the presence of the tumor (67). However, the presence of sarcopenia which is manifested by reduced lean body mass including decreased skeletal muscle mass is believed to influence host immune system leading to immune senescence (68). Hence, sarcopenia can have a deleterious effect on the anti-tumor response mediated by the immune system. Also, altered cytokines levels such as elevated IL-6 and decreased IL-15 can affect immune cell function. For example, skeletal muscle cells are essential producers of IL-15 which have a positive effect on NK cell expansion, proliferation and cytotoxic effects (63, 64, 69). As such, subclasses of NK cells express PD-1 receptors and their downregulation in sarcopenia can partially explain the decreased response and worse outcomes in NSCLC patients treated with ICI (70, 71) (**Figure 3**). It should be noted that although the role of NK cells in prognosis of NSCLC patients has been suggested, the mechanism behind their role in the tumor microenvironment in lung cancer is still not well-defined (75). Similarly, it has been found that in IL-15 deficient mice models there is a reduction in the presence of CD8+ which suggests that lack of IL-15 can lead to less targetable cytotoxic T-cells by ICI (62, 76). In addition, the levels of IL-6 are increased in sarcopenic patients which can

TABLE 2 | Immune cell modulation in obesity, sarcopenia and non-small cell lung cancer.

Immune Cell Type	Modulation of immune cells, cytokines in Obesity	Modulation of immune cells, cytokines in Sarcopenia	Modulation of immune cells, cytokines in NSCLC	Reference
T-Cell				
CD8+	↑ CD8+/CD4+ ratio ↑ expression of PD-1 on CD8+	↓IL-15 ↓CD8+	↑ PD-1 expression on CD8+	50, 62
Treg	Dysregulated Treg ↓IL-10 ↑ Immunosuppression ↑ Inflammation	NA	↑ Treg (immunosuppression) ↑ CTLA-4	55, 56
NK Cells	↓ Cytotoxic NK cells	↓ IL-15 ↓ NK cell activity	↓NK Degranulation ↓ Lytic activity	55–59, 63 64
Macrophage	↓ M2 Macrophages (pro-tumorigenic)	NA	Some NSCLC ↑ M2 Macrophages which leads to immune suppression	60, 61

CTLA-4, cytotoxic lymphocyte associated antigen-4; IL-10, interleukin-10; IL-15, interleukin-15; NA, not available; NK, natural killer cells; NSCLC, non-small cell lung cancer; PD-1, programmed death-1; Treg, T- regulatory cells.

contribute to tumor growth and alters the function of immune cells including T-cell subsets (77). The possible role the pleiotropic effect of IL-6 on the immune system has tempted researchers to study the effect of targeting this proinflammatory pathway in combination with PD-1 blockade and preclinical results have shown a synergistic effect on T-cell trafficking and antitumor immunity (78). Likewise, the levels of transforming growth factor-β (TGF-β) are altered in association with sarcopenia and have been found to have a negative effect on the regulation of immune system leading to T-cell exhaustion and dysfunctional NK cells (72–74) (**Table 2**). This cumulative interconnection between sarcopenia and immune dysregulation has also been examined as in obesity to determine whether lower lean body mass impacts the response and prognosis in patients with NSCLC.

The evidence of an existing molecular and pathological relationships between different components of body composition and improved outcomes in obese and non-sarcopenic patients has been described in several retrospective studies and meta-analyses (39, 79, 80). In the following sections, we will summarize the current evidence of the impact of body composition phenotypes and cancer-related outcomes in patients with NSCLC treated with TKI-EGFR or ICI.

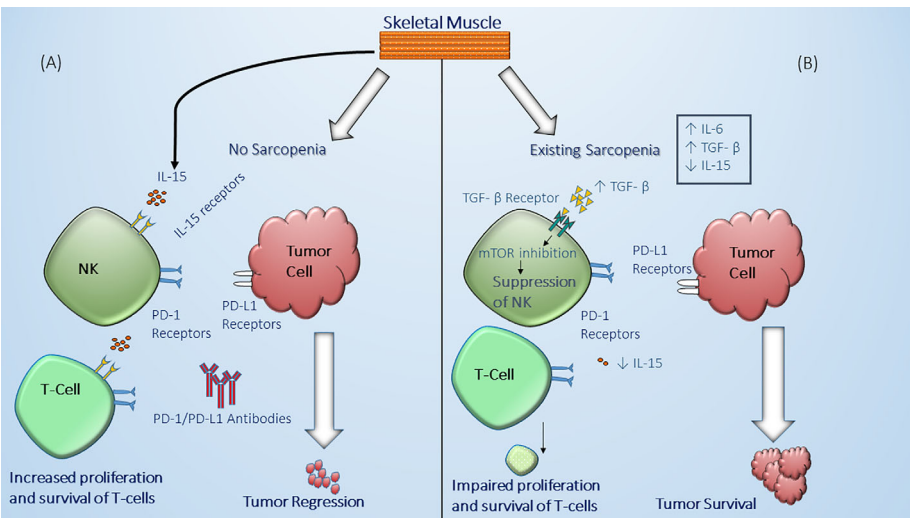

FIGURE 3 | Effect of sarcopenia on immune system function and anti-tumor mechanisms. **(A)** in individuals without skeletal muscle wasting (no sarcopenia), there is sufficient production and secretion of IL-15 by skeletal muscle cells which in turn can bind to IL-15 receptors on the natural killer (NK) cell surface and T-cells leading to enhanced functional natural killer cell and proliferation and maintenance of T-cells including CD8+ T-cells against tumor (69). **(B)** In the presence of significant muscle wasting (sarcopenia), there is decreased production and secretion of IL-15 by skeletal muscle cells (69), as well as an increased chronic inflammatory status in the body associated with high levels of IL-6 and TGF-β (72–74). The latter can lead to NK suppression through mTOR inhibition leading to dysfunctional NK cell which cannot effectively eliminate malignant cells (63, 64, 75). Decreased IL-15 production can lead as well to impaired maintenance, proliferation, and survival of T-cells which are considered potential targets for immune checkpoint inhibitors.

Tyrosine Kinase Inhibitors, Body Composition, and Outcomes in Non-Small Cell Lung Cancer

Targeted therapy has changed the landscape of the management and prognosis in NSCLC. Targetable alterations in NSCLC include mutations in the epidermal growth factor receptor (EGFR) (accounting for up to 15% NSCLC in Europe and United States, and up to 45% in Southeast Asia) (81, 82), translocation in the anaplastic lymphoma kinase gene (ALK) (accounting for approximately 5% of cases), with a lower frequency of other mutations (ROS1, BRAF, NTRAK, and HER2) (83). Most of the phase 3 randomized controlled trials testing tyrosine kinase inhibitors (TKI) that led to approval of these targeted therapies in patients with NSCLC did not conduct subgroup analyses according to body composition (19, 20, 84). However, evidence suggests that body composition can affect outcomes in patients with NSCLC who harbor EGFR mutation and are treated with EGFR TKIs (85). A retrospective study of 630 patients with metastatic EGFR-mutant NSCLC who received either gefitinib or erlotinib (as first or later line therapy) found a relationship between higher BMI and improved progression free survival (PFS) in patients with BMI ≥ 25 kg/m^2 compared to BMI < 18.5 kg/m^2 (15.6 months versus 8.5 months, respectively) and OS (28.8 months versus 26.7 months, respectively) (85). Multivariate analysis in this study showed BMI as an independent risk factor in terms of PFS and OS (85). Another study used body weight (kg) to estimate PFS and OS in patients with stage IV NSCLC who were treated with gefitinib (cut off point 53 kg) (N = 138) and found a trend towards improved PFS and OS in patients with higher body weight (> 53 kg), however, it was statistically non-significant (86). Lin et al. examined the impact of weight loss prior to starting gefitinib (defined as loss of more than 5% of body weight in a 3 months period before diagnosis) on objective response rate (ORR), PFS and OS. This retrospective analysis included 75 patients and found no difference in ORR but improved PFS in patients with weight loss <5% compared to patients >5% of weight loss (12.4 months versus 7.6 months; hazard ratio [HR] 0.356, 95% confidence interval [CI] 0.212–0.596, p <0.001). This study also reported an improved OS in patients with <5% weight loss compared to those with >5% weight loss (28.5 months vs. 20.7 months, respectively; HR 0.408, 95% CI 0.215–0.776, p = 0.006) (87). In contrast, another study in patients with NSCLC who received osimertinib (N= 47) did not show any significant relationship between BMI, PFS, and OS (88). The only study that examined the association of both measures (sarcopenia and BMI) with outcomes in patients with NSCLC and EGFR mutation was a retrospective study of 167 patients who received gefitinib, erlotinib, or afatinib as a first or later line therapy (89). This study showed a BMI < 18.5 kg/m^2 to be an independent prognostic factor for worse PFS (HR 1.70 [1.03–2.81], p= 0.04) and OS (HR 1.72 [1.11–2.67], p = 0.02). However, sarcopenia defined by measurements of psoas muscle index (PMI), intermuscular adipose tissue content (IMAC), and visceral to subcutaneous adipose tissue area ratio (VSR) failed to show any effect on different outcomes (89).

Previous discrepancies in the findings of different studies challenge the theory of the effect of body composition on outcomes when NSCLC is treated with EGFR TKI. However, most studies were retrospective and observational in nature and included small sample sizes which could have undermined the relationship between body composition and efficacy of TKIs. The lack of a proposed mechanism supporting a link between body composition and signaling pathways using targetable mutations such as in EGFR mutations poses a question on whether body composition should be examined further as a marker of treatment response in this patient population. However, given the impact of body composition as an independent prognostic factor in NSCLC that have already been established with surgery and chemotherapy and its relationship with altered outcomes in patients treated with different modalities supports the need for further investigation examining these associations. Future studies examining the link between body composition and cancer-related outcomes should consider including the following parameters: better patient selection (i.e., appropriate inclusion and exclusion criteria), larger sample sizes, inclusion of newer medications like osimertinib, as well as accounting for confounding factors such as performance status and metastatic sites (90, 91).

Immune Checkpoint Inhibitors, Body Composition, and Outcomes in Non-Small Cell Lung Cancer

The discovery of immune checkpoint molecules has revolutionized our understanding of tumor biology and resistance mechanisms (92, 93). Immune checkpoints are receptor proteins that are expressed by various immune cells that when bound to their ligands lead to suppression of effector immune cell function (94). Some inhibitory checkpoints can be related to decreased anti-tumor effect against malignant cells which usually use checkpoint ligation to escape immune surveillance (17–19). The two first targetable checkpoint receptors in tumor microenvironment discovered were cytotoxic associated lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) (92, 93). Their discovery led to the development of monoclonal antibodies directed against checkpoint receptors which entered the clinical realm in NSCLC and changed the standard of care after demonstrating improved PFS and OS as first or later line therapy (21–24, 95, 96). However, not all patients retain a good response to these medications with only some patients deriving benefits with a sustained response. This has led to an effort to discover and develop predictive biomarkers to identify appropriate patient selection. Many biomarkers have emerged as predictor markers such as programmed death- ligand 1 expression (PD-L1), tumor mutational burden, and lymphocyte infiltration in tumor bed (97–99). However, adoption of these biomarkers as a conventional method to predict response and survival can be challenging given the lack of standardized definition and methodology used to quantify some of these biomarkers. Thus, the utilization of simpler available patient characteristics such as

gender or body habitus seemed plausible to understand if there is any association with response to immune checkpoint inhibitors (ICI) (98–100).

The prognostic implication of body composition and survival in cancer patients has long been established in different tumor types regardless of stage or treatment approach (33, 101, 102). This concept was later adopted by researchers to examine body composition as a predictive rather than a prognostic marker for response to ICI. Perhaps the earliest link between body composition and survival when using ICI was established in melanoma patients. The work by Daly et al. demonstrated that loss of muscle mass (sarcopenia) can be associated with worse OS in melanoma patients treated with CTLA-4 inhibitors (103). This was followed by other studies that corroborated the findings as a proof of concept and were soon tested in different malignancies including NSCLC (53, 79, 104). There are two different approaches that were used to study the association between body composition and survival. The first was considering the effect of adipose tissue and obesity on survival in patients treated with ICI; while the other approach used skeletal muscle indices as a surrogate for sarcopenia. The feasibility of using such indicators to understand the interconnection was convenient given the simple methodology used to obtain these variables and soon led to several observational and comparative retrospective studies that are summarized in **Table 3** (31, 39, 52, 105–113). The findings from retrospective studies seem to be consistent with a trend towards improved PFS and OS in patients with NSCLC treated with PD-1/PD-L1 inhibitors and have been verified with meta-analyses (79, 114). Both high BMI (overweight and obese) and normal SMI (absence of sarcopenia) were associated with improved survival. It should be noted that different cut points were used to identify overweight, obese and sarcopenic patients as these indicators have different established cut points depending on variable factors such as ethnicity and health status (cancer versus no cancer) (26, 33). The use of predictive biomarkers combined with body composition status has proved a stronger correlation than using body composition alone in predicting improved PFS and OS in NSCLC patients treated with ICI in a recent large retrospective study (52). In this important paper, stronger OS/PFS benefit was observed in overweight and obese patients with PD-L1 positive tumors (defined by PD-L1 expression of > 5% of tumor cells or tumor infiltrating immune cells) compared to normal weight patients, which implies that in NSCLC, PD-L1 expressed by either tumor cells or by immune cells is critical for OS prediction in obese patients, and obesity is secondary to PD-L1 tumor status (52). Interestingly, evidence suggests that occurrence of immune related adverse events (irAEs) of any grade in different cancer types is higher in overweight and obese patients, while irAEs themselves are associated with improved PFS and OS (114, 115). Therefore, in analyzing the effect of obesity/sarcopenia on survival in NSCLC patients treated with ICI, future studies should consider analyzing the effect of body composition in different subpopulations such as patients with PD-L1 positive tumors, high tumor mutational burden, and also occurrence of irAEs. Limitations that can hinder the robustness of the previously mentioned findings in **Table 3** include the retrospective nature of the studies and small sample sizes which are both prone to sampling error and inability to detect a significant difference in specific sub-groups (such as high PD-L1 expression, irAEs).

How to Implement Body Composition Phenotypes in Designing Future Clinical Trials for Patients With Advanced Non-Small Cell Lung Cancer?

Current effort in the management of NSCLC is focused on targeting pathways involved in immune surveillance against tumor cells as well as developing novel drugs against resistant mutations that emerge after exposure to specific targeted therapy. Alongside this effort, it appears to be important to further identify subpopulations who will derive the best benefit. Given the emerging evidence of a crosstalk between different body compositions and cancer biology it will be important to incorporate subgroup analysis in prospective clinical trials when testing current available medication or novel therapies in NSCLC. This would help determine if body composition phenotypes could serve as predictive indicators for the implemented therapies or whether they serve as prognostic factors in NSCLC.

Another area of interest for future research is the changing landscape of cytokine production in obese and sarcopenic NSCLC patient categories and their effect on cancer biology and whether supplementary targeting of specific inflammatory or cytokine pathways could augment the response to immunotherapy. For instance, administration of IL-15 which has been shown to boost anti-tumor immunity *in vitro* (116). Lastly, it would be intriguing to analyze life style modification such as modulation of nutritional status and exercise or medical interventions to stabilize components of body composition such as lean body mass or adipose tissue and their effect on body composition balance and the outcomes in NSCLC when treated with ICI and other novel therapies. The impact of exercise in improving outcomes has already been established although no focus was put on weight changes as a response to therapy (117). Another example, is the use of anamorelin a ghrelin receptor agonist which maintains lean body mass and has been tested previously for the treatment of cachexia-sarcopenia syndrome in NSCLC (118).

CONCLUSION

In conclusion, the study of body composition as a predictive marker in NSCLC patients treated with novel immune and targeted therapies is an area of compelling interest. Future studies should focus on incorporating subgroup analysis in large prospective trials to better analyze this association. Given that in several studies, obesity plays predictive role among smokers or primarily in PD-L1 positive NSCLC tumors, further studies focusing on BMI among these subsets are warranted. Inclusion of newer promising biomarkers such as

TABLE 3 | Studies on effect of body composition on tumor response and survival in patients with stage IV non-small cell lung cancer treated with immune checkpoint inhibitors.

Publication	Sample Size	Male, %	Number of PD-L1 Positive Patients	Immune Checkpoint Inhibitor	Surrogate for Body Composition	Cut-off for Surrogate	End Point	Results*	P-Value
Kichenadasse et al. (52)	1434	890 (62)	938 **	Atezolizumab	BMI	Per WHO Class	OS	Obesity vs. normal weight. HR 0.64 [CI 95%, 0.51-0.81]	P < 0.001
							PFS	Overweight and obese vs. normal weight HR 0.88 [CI 95%, 0.78-0.99]	P = 0.03
Cortellini et al. (105)	976 total with 635 NSCLC cases	663 (67.9)	NA	Pembrolizumab, Nivolumab, Atezolizumab	BMI	Overweight/ obese >= 25 vs. non-overweight <25	ORR	41.3 % vs 20.9%	P < 0.0001
							TTF	9.3 [95% CI: 8.1-11.6] vs 3.6 [95% CI: 3.2 - 4.1] months HR= 0.51 [95% CI: 0.44 – 0.60]	P < 0.0001
							PFS	11.7 [95% CI: 9.4 – 15] vs 3.7 [95% CI: 3.2 – 4.1] months HR= 0.46 [95%CI: 0.39 – 0.54]	P < 0.0001
							OS	26.6 [95% CI: 21.4 – 36.8] vs 6.6 [95% CI: 5.8 – 8.5] months HR= 0.33 [95%CI: 0.28 – 0.41]	P < 0.0001
Ichihara et al. (106)	Cohort 1: 84	68 (80.9)	84 ***	Pembrolizumab	BMI	22	ORR	(evaluated in 74 pts.) 0% complete response 44.6% -partial response, 32.4%- stable disease, 23%-progressive disease	
							PFS	7.3 vs. 4.7 months (HR): 0.94; 95 % CI: 0.53–1.65	P = 0.84
							OS	NR vs. 17 months HR: 0.67; 95 % CI: 0.32–1.40	P = 0.29
	Cohort 2: 429	338 (78.7)	45	Pembrolizumab, Nivolumab, Atezolizumab			ORR	(evaluated 403 pts.) 1.5% complete response, 23.3% partial response, 36.2% stable disease, 3% progressive disease	
							PFS	3.7 vs 2.8 months HR: 0.79; 95 % CI: 0.64–0.98	P = 0.036
							OS	15.4 vs 13.5 months HR: 0.73; 95 % CI: 0.57–0.95	P = 0.021
							High PDL-1 and High BMI vs Low PDL-1 and Low BMI	PFS: 17 vs 3.5 months OS: NR vs 16.1 months	P = 0.007 P = 0.031
Magri et al. (107)	46	28 (60.87)	NA	Nivolumab	Weight loss	Weight loss > 5% prior to therapy vs weight loss <5%	OS	2 vs 10 months	P = 0.0076
Popinat et al. (31)	55	41 (75)	13 ****	Nivolumab	SCFM	5 kg/m^2	1-year OS	HR: 0.75	P = 0.006
Minami et al. (108)	74	48 (64.8)	28 *****	Nivolumab, Pembrolizumab, Atezolizumab	BMI,	BMI cutoff point 18.5 Higher BMI vs lower BMI	OS	15.8 vs. 3.3 months HR = 1.83 (0.79 - 4.21)	P < 0.01
							PFS	No significant difference	-
					IMAC	Men: 0.358 Women: 0.229	OS	Low IMAC favorable for OS (HR 0.43, 95% CI 0.18 - 0.998)	P = 0.0496
							PFS	No significant difference	-

(Continued)

TABLE 3 | Continued

Publication	Sample Size	Male, %	Number of PD-L1 Positive Patients	Immune Checkpoint Inhibitor	Surrogate for Body Composition	Cut-off for Surrogate	End Point	Results*	P-Value
Shiroyama et al. (39)	42	26 (61.9)	NA	Nivolumab, Pembrolizumab	PMI Sarcopenia vs non-sarcopenia	Male: 6.36 cm^2/m^2 Female: 3.92 cm2/m2	PFS Overall response rate	2.1 vs 6.8 months 9.1 % vs. 40%	P= 0.004 P = 0.025
Nishioka et al. (109)	38	26 (68.4)	16 ****	Nivolumab, Pembrolizumab	Psoas Muscle Major Area change Sarcopenia vs non-sarcopenia	Change of equal or more than 10%	ORR PFS	0 % versus 41% 47 vs. 204 days [CI 23-76] vs [CI 59-NA]	P = 0.0154 P = 0.00186
Katayama et al. (110)	35	24 (68.6)	22****	Pembrolizumab, Nivolumab, Atezolizumab	BMI	>20	PFS OS	HR 0.43 [CI 95%, 0.19-0.95] No significant findings	P = 0.036 -
Tsukagoshi etr al. (111)	30	23 (76.7)	NA	Nivolumab	SMI	Male: 6.36 cm2/m2. Female 3.92 cm2/m2	PFS OS Partial response	7.5 vs 2.8 months 25 vs. 10 months 35.3% vs 0%	P = 0.008 P = 0.03 P = n/a
Roch et al. (112)	142	93 (65.5)	56 *** This cut off was only for those with pembrolizumab as first line	Pembrolizumab, Nivolumab	SMI Sarcopenia vs no-sarcopenia Evolving Sarcopenia	Male: 52.4 cm^2/m^2 Female: 38.5 cm2/m2 (SMI) loss of ≥ 5%. Similar to definition of cachexia	PFS OS PFS OS	2.3 vs 4.1 months 7.6 vs. 12.6 months 2.3 vs 5.1 months 11.2 vs 15.2 months	P = 0.56 P = 0.08 P = 0.04 P = 0.07
Takada et al. (113)	103	84 (81.6)	25***	Nivolumab, pembrolizumab	SMI Low SMI vs. high SMI BMI (univariate analysis)	Male: 25.63 cm2/m2 Female: 21.73 cm2/m2 Male: 21.9 Female 19.8	PFS OS PFS OS RR	HR 1.6 [CI 95%, 1.02- 2.50] HR 2.04 [CI 95%, 1.14- 3.63] Not significant HR 1.20 (0.78–1.86) HR 1.88 (1.09–3.27) No effect of SMI or BMI on response rate	P = 0.0399 P = 0.0155 P = 0.4047 P = 0.0243 P = 0.0117

** Results reported comparing the higher than cut point group to the lower than cut point group; results are reported as either median PFS, OS or hazard ratios with confidence intervals.*

*** PD-L1 positivity identified by ≥5%*

**** PD-L1 positivity identified by ≥ 50%*

***** PD-L1 >1%*

****** Tumor proportion score > 1%*

Results are reported across different tumor types of which the majority were non-small cell lung cancer.

BMI, body mass index; CI, confidence interval; HR, hazard ratio; IMAC, Intermuscular adipose content; NA, not available; NR, not reached; PD-L1, programmed death-ligand-1; PFS, progression free survival; PMI, psoas muscle index; NSCLC, non-small cell lung cancer; ORR, objective response rate; OS, overall survival; SMI, skeletal muscle index; SCFM, sub-cutaneous fat mass; TTF, time to treatment failure; WHO, world health organization.

type of EGFR mutations, PD-L1 expression and tumor mutational burden (TMB) in combination with body composition seems plausible. Unifying the definitions and cut points of different surrogate indicators of obesity or sarcopenia can be challenging but would improve our understanding of the effect of obesity and sarcopenia on survival in non-small cell lung cancer patients in the era of precision medicine.

AUTHOR CONTRIBUTIONS

KK conceptualized the idea of the manuscript with supervision from YB. KK performed literature search and wrote the manuscript in consultation with YB. KK provided **Figures 2**, **3** and constructed **Tables 1**–**3**. SG-P wrote the section *Methods Used in Estimating Body Composition* and provided **Figure 1**. NJ verified the information mentioned in the manuscript and contributed to **Tables 2**, **3**. JP and YB supervised the project. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Resistance Mechanisms of Lung Cancer Immunotherapy

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Immunotherapy has revolutionized lung cancer treatment in the past decade. By reactivating the host's immune system, immunotherapy significantly prolongs survival in some advanced lung cancer patients. However, resistance to immunotherapy is frequent, which manifests as a lack of initial response or clinical benefit to therapy (primary resistance) or tumor progression after the initial period of response (acquired resistance). Overcoming immunotherapy resistance is challenging owing to the complex and dynamic interplay among malignant cells and the defense system. This review aims to discuss the mechanisms that drive immunotherapy resistance and the innovative strategies implemented to overcome it in lung cancer.

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INTRODUCTION

The discovery of the immune checkpoint inhibitors (ICIs), represented by the monoclonal antibodies that block cytotoxic T−lymphocyte−associated protein 4 (CTLA-4), programmed death protein 1 (PD-1), and programmed death protein ligand 1 (PD-L1), has revolutionized the therapeutic landscape of lung cancer. The significant survival benefit derived from ICI-containing treatment has established it as the mainstay first-line therapy in patients with advanced or locally advanced non-small cell lung cancer (NSCLC) and extensive small-cell lung cancer (SCLC). Unprecedented long-term clinical benefit or even, in some cases, a complete recovery has been witnessed in lung cancer, particularly in patients with high PD-L1-expressing tumors (1–3). Currently, investigations are under way aimed at integrating immunotherapy in the treatment of early-stage lung cancer.

However, most patients with NSCLC develop primary resistance during ICI monotherapy and only 15 to 20% achieve partial or complete response (3). Acquired resistance also occurs in initially responding patients with advanced NSCLC treated with ICIs, after a median progression-free survival (PFS) of 4–10 months (4–9). The mechanisms of resistance to immunotherapy are not yet fully understood, and methods to overcome them must be developed. Herein, we discuss the pathways driving resistance to immunotherapy in lung cancer to help clinicians in their current practice, as well as identify future research priorities and treatment strategies.

DIFFERENT SCHEMAS OF RESISTANCE TO IMMUNOTHERAPY

Unlike molecular targeted therapy and chemotherapy targeting tumor cells, immunotherapy targets the immune system of the host by mobilizing the immune cells to recognize and eventually

eliminate tumor cells. This mechanism of action determines the complexity of the resistance mechanisms in immunotherapy. Different mechanisms of immunotherapy resistance are listed in **Table 1**.

In accordance with the timing of development, resistance can be considered as either primary, when no initial response or clinical benefit to the therapy is observed, or acquired, as disease progression occurs after an initial period of clinical benefit (10). Clinically, 6-month treatment duration is adopted as a cutoff value (11). This classification schema correlates with real-time observations by clinicians and contributes to the clinical decision-making process in the absence of other information such as immune characteristics and tumor genetics.

Resistance is additionally classified as intrinsic or extrinsic to cancer cells. The former occurs in the tumor cell itself and encompasses the inherent characteristics related to gene expression, cell signaling, immune recognition, and DNA damage response, whereas the latter is seen in the microenvironment or systemic circulation throughout the T-cell bioactivation process (12, 13).

The cancer−immunity cycle is linked to immunotherapy resistance in another related schema (14). This classification divides resistance from an immunological perspective into immune desert (tumor fails to evoke an immune reaction), immune inflamed (tumor inhibits immune activities notwithstanding abundant immune cells infiltration), or excluded (tumor prevents immune cells infiltration in spite of adequate immunogenicity) (13).

It is noteworthy that the immune response is a continuous and dynamic process rather than categorical (binary). Multiple complex interactions, including immunologic, genomic, and host characteristics and treatment interventions, rather than a single, dominant determinant are involved in the resistance to immunotherapy. The fs can be overlapping or parallel in some cases despite the different timing of occurrence (11).

TABLE 1 | Different schemas of resistance to immunotherapy.

Schemas	Classifications	Description
Temporal perspective	Primary	Lack of initial response or clinical benefit to therapy
	Acquired	Disease progression after an initial period (6 months) of clinical benefit
Spatial perspective	Intrinsic	Tumor-related resistance
	Extrinsic	Factors involved in microenvironment or tumor-immunity cycle
Immunological perspective	Immune inflamed	Tumor inhibits immune activities notwithstanding abundant immune cells infiltration
	Immune desert	Tumor fails to evoke an immunoreaction
	Immune excluded	Tumor prevents immune cells infiltration in spite of adequate immunogenicity

RESISTANCE MECHANISMS TO IMMUNOTHERAPY

Underlying mechanisms of primary resistance span an extensive range from tumor factors including genomic features, transcriptomic signatures, and immune landscape, to host factors. The potential mechanisms of acquired resistance at least partly overlap with those involved in primary resistance and mainly include loss of neoantigen and deficiency in presentation, loss of T-cell effector function, and up-regulation of alternate immune checkpoint receptors (10). Here, we will discuss the mechanisms of resistance to immunotherapy from tumor aspects (intrinsic and extrinsic mechanisms) and host-related characteristics in order to avoid confusion and repetition (**Figure 1**).

Tumor Cell-Intrinsic Mechanisms

Genomic Features

Low tumor mutation burden and neoantigen load

Tumor-specific antigens are the key to activate T cells to recognize tumor as foreign, which is the first step of tumor-induced adaptive immune responses and immune-mediated tumor killing (15). These neoantigens, interestingly, are derived from somatic mutations and contain new epitopes, and subsequently lead to tumor immunogenicity. Preclinical and clinical studies have revealed that the response of neoantigen-specific effector T cell (Teff) paralleled tumor shrinkage (16–20).

With the improvement of sequencing techniques, it was found that nonsynonymous mutations can generate neoantigens that trigger cytotoxic responses against tumors (21, 22). Nonsynonymous mutation burden, rather than total mutation burden of exons, was demonstrated to be more closely associated with the clinical advantage of anti-PD-1 treatment, validating the importance of neoantigens in dictating response (23). Tumor mutation burden (TMB) is calculated as the total number of nonsynonymous mutations per DNA Megabase (Mb) (21, 24, 25). Low TMB, or low numbers of clonal neoantigens, presenting reduced tumor immunogenicity, is considered as a primary resistance marker to immunotherapy (15, 26).

Clinically, low TMB or neoantigen load has correlated with inferior response and poor PFS to monotherapy of anti−PD1/PD-L1 antibodies in NSCLC (25, 27–30). However, it fails to predict the clinical outcomes, in regard to overall survival (OS) and combination regimens (31, 32). The influence on the OS by subsequent treatments and the additional complexities to the study of immunotherapy resistance added by combinations may partly explain these controversial findings. Recently, a corrected TMB (cTMB) approach based on the adjustment of tumor purity was developed by Anagnostou and colleagues, which was identified on abundant tumor samples mined from The Cancer Genome Atlas (TCGA) and then confirmed in a patient cohort received ICIs therapy. This cTMB more accurately predicted the outcomes of immunotherapy, suggesting that the TMB in samples with low tumor purity was mistakenly underestimated, which was especially important for metastatic NSCLC, because

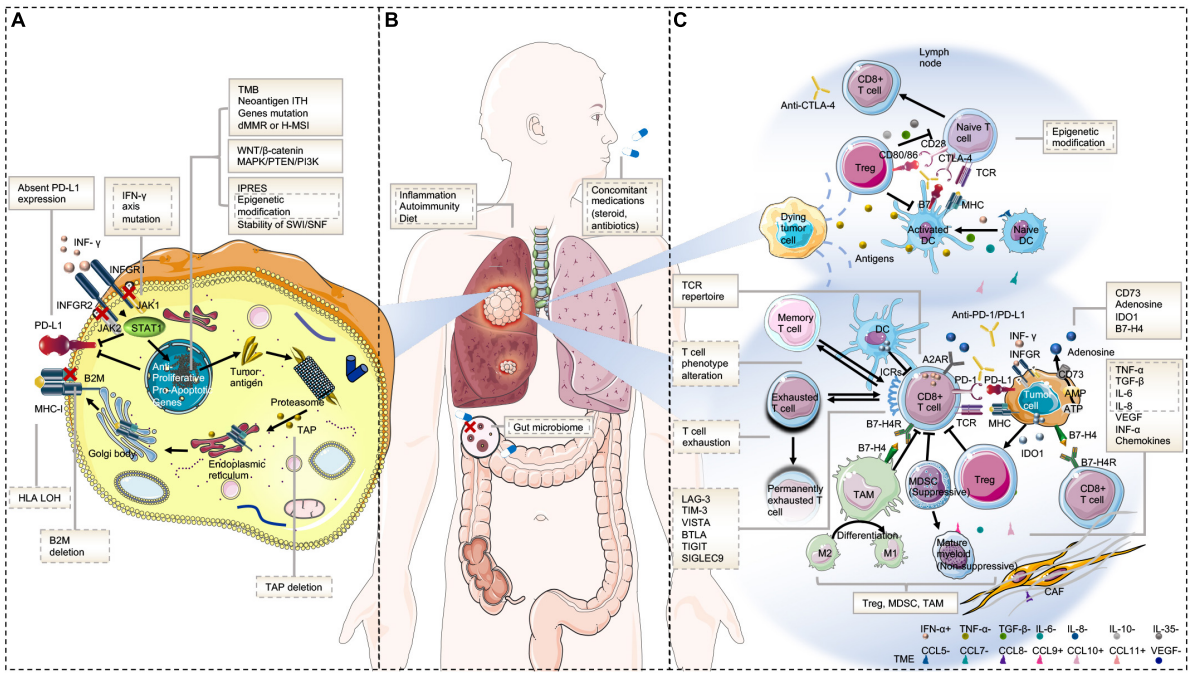

FIGURE 1 | Mechanisms of resistance to immunotherapy. **(A)** Tumor intrinsic mechanisms that are associated with resistance to immunotherapy include lack of tumor immunogenicity (low TMB, heterogenous antigens, mutation of certain genes, and IPRES transcriptional signatures), deficiency in antigen presentation (alterations in INF-γ signaling pathway, HLA LOH, B2M, and TAP deletion), aberrations in several signaling pathways (MAPK, PI3K, WNT, and IFN), and absent PD-L1 expression. **(B)** Host-related characteristics that lead to primary or secondary resistance include the gut microbiome, diet, concomitant medications, inflammation state, and autoimmunity. **(C)** Tumor extrinsic mechanisms involved in resistance to immunotherapy include T cell-related factors (alternative immune checkpoints, T cell exhaustion and phenotype alteration, TCR repertoire, and epigenetic modification), immunosuppressive cells (Treg, MDSC, and M2-TAM), and cytokines and metabolites (e.g., TGF-β, adenosine) released into the tumor microenvironment. Factors in the solid text boxes are involved in primary resistance, whereas those in the dotted text boxes are involved in secondary or acquired resistance. Factors with solid and dotted dual text boxes are involved in both. Cytokines with "+" and "-" represent positive and negative modulators to antitumor immune response, respectively. Abbreviations: TME, tumor microenvironment; MHC, major histocompatibility complex; TCR, T cell receptor; Treg, regulatory T cell; MDSC, myeloid-derived suppressor cell; M2-TAM, type II tumor-associated macrophage; ICR, immune checkpoint receptor; CAF, cancer-associated fibroblast; and IPRES, innate anti-PD-1 resistance.

the tumor purity of tissue samples obtained by bronchoscopy or puncture biopsy was often limited (33).

The dilemma of insufficient tissue sample for TMB assessment in a considerable number of patients with NSCLC has given rise to the employment of peripheral blood TMB (bTMB) as a substitute predictor of response or resistance to ICIs in NSCLC (34). In keeping with what was previously reported in tissue, low bTMB evaluated by different plasma sequencing assays was significantly correlated with poor survival or response to immunotherapy in several retrospective and prospective studies (35–37).

Increased neoantigen intratumor heterogeneity

In addition to the TMB or the numbers of clonal neoantigens, increased neoantigen intratumor heterogeneity (ITH, defined as relative fraction of subclonal neoantigens) can also impair the sensitivity to ICIs by elevating the likelihood of selection of subclones with poor immunogenicity (25, 38). The considerable variation of neoantigen heterogeneity was demonstrated by McGranahan and colleagues in seven primary NSCLCs (25). On average, 44% of heterogeneous neoantigens were reported only in a subset of tumor regions. They conducted neoantigen and clonality analysis in lung cancer data from TCGA and then validated the approach in a cohort of NSCLC patients treated with ICIs. Compared with high TMB alone, the combination of high TMB with low ITH seems to have a stronger association with clinical benefit to ICIs in this population.

Aberrations in certain oncogene/tumor suppressor genes

Aberrations in oncogenes and tumor suppressor genes can regulate immune response by amending cytokine profile and immune cell composition and thus render tumor cells resistant or sensitive to ICIs.

Generally, alterations in oncogenic driver genes are characterized as resistant markers to immunotherapy. Although epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) rearrangement tend to have high PD-L1 expression due to the activation of signaling pathways (39, 40), the low mutation or neoantigen load (41), along with the following mechanisms, impairs the immunotherapy sensitivity in this group of patients with lung cancer. First, EGFR mutations have the potential to shape an inert immune environment by up-modulating a series of immune suppressors including inhibitory immune checkpoints

(e.g., PD-1 and CTLA-4), immunosuppressive cells (macrophages and regulatory T cells), and cytokines (like TGF-β, IL-6, and IL-10) (42, 43). It has been reported that activated EGFR cascade was associated with elevated T-cell exhaustion and reduced cytotoxic T lymphocytes (CTLs) in a lung adenocarcinoma model (40). Second, downstream pathways of EGFR mutation, such as MAPK, PI3K/AKT, and Janus kinase (JAK)/STAT pathway, negatively affect immunoregulation. Other oncogenic driver-genes that frequently have high PD-L1 expression in lung cancer include ROS1 rearrangements (44), MET exon 14 skipping mutations (45), and BRAF mutations (44, 46). In contrast, RET rearrangements (47) and HER2 mutations (44) have been reported recently to exhibit low PD-L1 expression. None of these oncogenotypes demonstrated favorable clinical responses to ICIs monotherapy except for BRAF mutations, either V600E or non-V600E.

STK11 gene inactivation either by mutational or non-mutational machinery is linked to an indolent immune microenvironment with lower Tumor-infiltrating lymphocyte (TILs; CD3+, CD4+, and CD8+ cells) and PD-L1 expression in spite of the existence of moderate to high TMB (48). Inactivated STK11 gene was recently reported to weaken the innate immune responses by epigenetic inhibition of stimulator of IFN genes (STING), suggesting epigenetic silencing is likely to mediate the promotion of T-cell exclusion by the loss of STK11 (49). In line with these findings, it has been observed in several studies that compared with the wild-type gene, STK11 mutation predicted poorer clinical outcomes of immunotherapy in advanced NSLCL (50, 51). The tumor suppressor TP53 mutation, a well-known negative prognostic factor in lung cancer, is found to be associated with increased PD-L1 expression and higher TMB in non-squamous NSCLC (30) and KRAS-mutated lung adenocarcinoma (51).

KRAS-mutated lung cancer presents distinct immune profiles, biology, and therapeutic vulnerabilities in different subsets classified by co-occurring genetic events (50). Generally, KRAS/TP53 co-mutation predicts sensitivity while KRAS/STK11 co-mutation predicts resistance to immunotherapy in NSCLC. Dong et al. identified TP53/KRAS co-mutated subclass exhibited the highest percentage of PD-L1+/CD8A+ and particular increased PD-L1 expression. They further confirmed a remarkable clinical benefit from pembrolizumab in this population (52). Co-mutation of STK11 was shown to cause the accrual of neutrophils with T-cell-suppressive effects, accompanied with an analogous elevation in the production of T-cell depletion biosignatures and tumor-promoting cytokines (50, 53). TIL numbers and the expression of PD-L1 were also decreased (53). Consistent with these preclinical predictions, patients with KRAS/STK11 co-mutation or single mutation of STK11 had poor response and survival compared with those with wild-type when treated with ICIs (51, 54).

The Kelch-like ECG-associated protein 1 (KEAP1) gene, regulating the cellular antioxidant and cytoprotective transcriptional programs, plays a key role in mediating immune evasion in NSCLC. Depletion of KEAP1 is associated with reduced leukocyte infiltration, increased PD-L1 expression and might also influence other immune cells such as NK cell recruitment and function (55, 56). Co-occurring KEAP1 and phosphatase and tensin homolog (PTEN) inactivation represent an immunologically "cold" tumor while concurrent mutations in KEAP1 and STK11 leads to absence of pro-cancerogenic M2 macrophages (57). However, there are conflicting data on the role of KEAP1 mutation and its co-mutation with STK11 in immunotherapy resistance in NSCLC. KEAP1/STK11 co-mutations were verified to correlate with resistance to ICIs in patients with NSCLC despite high TMB (58). Similarly, STK11, and/or KEAP1 genomic variations posited lack of clinical advantages from combination of immunotherapy with chemotherapy in patients with NSCLC (59). However, inconsistent results were reported recently that clinical benefit from pembrolizumab compared to chemotherapy was poorer in the patients with STK11 and KEAP1 mutation compared with those in wild type in Keynote 042 trial, but the response and survival to immunotherapy were not significantly different between mutant and wild subgroups (60).

The WNT/β-catenin pathway is an additional immunotherapy resistance mechanism. A negative relationship was demonstrated between the level of β-catenin and TILs, which was modulated by deficiency in the recruitment of CD103+ dendritic cells (DCs) essential to T-cell priming and reduced expression of the cytokine CCL4, suggesting WNT/β-catenin signaling pathway is likely to mediate ICIs resistance through T-cell exclusion (61).

Similarly, the MAPK/PTEN/PI3K signaling pathway has been identified to be involved in immunotherapy resistance. Loss of PTEN and the bioactivation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway in tumors decrease the activity of CTLs through the recruitment of inhibitory cells to the microenvironment and Vascular endothelial growth factor (VEGF) expression (62, 63), so that they promote resistance to ICIs (63, 64). The association of PTEN deletions or *PIK3CA/AKT* mutations with increased PD-L1 expression and immune resistance was also found in glioma (65). It was shown in preclinical models that a PI3K-γ inhibitor decreased myeloid-derived suppressor cells (MDSCs) and improved response to ICIs (66).

DNA repair and replication gene alterations

Genetic instability caused by alterations in DNA replication and repair genes can augment immunogenicity via a high-TMB neoantigen load (67–69). Correspondingly, deficient DNA mismatch repair (dMMR) or high microsatellite instability (H-MSI) are suggested as sensitive predictors to ICI immunotherapy in many tumor types. Beyond high TMB, increased CD8+ TILs were also reported to be associated with alterations in mismatch repair genes (70), *BRCA2* (71), and *POLE* (72) in different tumors. However, the role of these genes in immunoregulation in NSCLC remains to be elucidated.

Interferon-gamma signaling mutation

The interferon-gamma (INF-γ) signaling cascade is a crucial component of immunotherapy and tends to serve a critical function in primary, adaptive, and acquired resistance to ICI treatment (73–75). IFN-γ is a critical cytokine secreted by activated T cells, natural killer (NK) T cells, in the cancer

microenvironment, and it moderates the immune reaction via the downstream enzymes JAK 1/2 and the signal transducer and activators of transcription (STATs) (76). The INF-γ axis exerts both positive and negative impacts on antitumor immune reactions (77). On one hand, it activates an functional antitumor immune reactive via (1) intensifying antigen presentation by up-modulated secretion of MHC-I; (2) recruiting other immune cells by up-regulation of the expression of chemokines (CXCL9, CXCL10, and CXCL11) with effective chemoattractant impacts on T cells (78); and (3) exerting direct anti-proliferative and pro-apoptotic impacts on cancer cells (79). On the other hand, IFN-γ acts in a negative-feedback axis to elevate PD-L1 expression as well as other crucial immune inhibitory components, including IDO1, down-modulating the cytotoxic reaction and adaptive resistance to cancer cells (80, 81) (**Figure 1A**).

Additionally, copy-number alterations (CNAs) linked to DNA damage response and regulation of DNA editing/repair gene expression were shown to emanate from the malignant exposure to IFN-γ-secreting antigen-specific CTLs *in vivo*, implying that intensified genetic instability could be among the mechanisms through which CTLs and IFN-γ immunoedits cancers, changing their immune resistance due to genetic evolution (82).

Tumors neutralize the impact of IFN-γ by mutating or down-regulating the molecules involved in the IFN-γ signaling pathway, including IFN-γ receptor chains, regulatory factors, JAK1/2, and STATs upon continuous IFN-γ exposure (73, 83). Multiple studies have demonstrated that mutations of IFN-γ axis and consequent loss of JAK/STAT contribute to immune escape of tumor cells and by that leads to primary or acquired resistance to ICI therapy via incapacity of up-regulating the expression of PD-L1 and MHC-I (73, 78, 84). Any deficiencies in IFN-γ, JAK1/2, or STATs including gene mutations, loss of protein expression, negative regulator presence, or epigenetic silencing would prevent signaling in response to IFN-γ and thereby end up to the up-regulated tumor growth and apoptosis inhibition and down-regulated T-cell infiltration and expression of PD-L1 and MHC-I (74, 78, 85, 86). Correspondingly, genomic changes disturbing IFN-γ pathway genes, including the amplification of suppressor genes PIAS4 and SOCS1 and the deletion of IFNGR1, IFNGR2, IFIT1, IFIT2, IFIT3, IRF1, MTAP, and miR31, have been described as possible machinery of primary resistance to various ICI therapies (73). An IFN-γ-related mRNA profile that contains 10 genes (CCR5, CXCL9, CXCL10, CXCL11, GZMA, HLA-DRA, IDO1, IFNG, PRF1, and STAT1) was additionally identified to predict the response to anti-PD-1 therapy in melanoma (87).

Transcriptomic Signatures

In a recent publication, transcriptional signatures, referred to as innate anti-PD-1 resistance (IPRES) with inflammatory and mesenchymal tumor phenotypes, were shown to manifest poor response to anti-PD-1 therapy in metastatic melanoma (88). Approximately 700 differentially expressed genes (DEGs) were identified between the responsive and non-responsive pretreated tumors. Compared with those of responsive tumors, the transcriptomes of non-responsive tumors were dominated by gene up-regulation events. The up-regulated DEGs in non-responsive tumors, considered as T-cell-suppressive, are involved in mesenchymal transition (*TWIST2, TAGLN, FAP, AXL, ROR2, WNT5A,* and *LOXL2*), monocyte/macrophage chemotaxis (*CCL2, CCL7, CCL8*, and *CCL13*), immunosuppression (*IL10, VEGFA*, and *VEGFC*), and angiogenesis and wound healing (89–91). By contrast, down-regulated gene *CDH1* (which is typically down-regulated by mesenchymal tumor cells) was also detected in non-responsive pretreated tumors. Interestingly, there was no difference in the expression of INF-γ pathway signatures, other T-cell-related genes (e.g., *CD8A/B*, *PD-L1*, and *LAG3*), and the genes that presumably modulate immune checkpoint sensitivity between responsive and non-responsive groups, suggesting that T-cell-suppressive inflammatory and mesenchymal phenotypes of tumor are associated with primary resistance to anti-PD-1 therapy.

Epigenetic Modification

Emerging evidence has suggested that epigenetic modification may mediate primary resistance and contribute to acquired resistance during ICI therapy through the profound effect on many aspects of antitumor immunity: neoantigen presentation and processing; T-cell functions, differentiation, and proliferation; memory T-cell phenotype acquisition; interfering with T-cell migration; and mediating T-cell exhaustion (10, 92–94).

Epigenetic targeting agents, including those targeting histone deacetylation or methylation as well as targeting DNA methylation, have exhibited encouraging antitumor activity either as monotherapy or in combination with immunotherapy in preclinical studies (94, 95). Clinical trials investigating the performance of these agents combined with adaptive T-cell transfer (ACT) in patients with acquired resistance to prior immunotherapy are ongoing (96).

Stability of Chromatin Remodeling Complexes

Stability of chromatin remodeling complexes within tumor cells can also contribute to immunotherapy resistance by multiple mechanisms. It was found that tumor cells were more sensitive to CTL killing, which leads to increased response to anti-PD-1/PD-L1 therapy, due to the deficiency in chromatin remodeling complex SWI/SNF (97, 98). BRG1-associated factor (BAF) and polybromo-associated BAF (PBAF), as the mammalian analogs of the SWI/SNF complex, are essential tumor suppressors and loss of function (LOF) mutations of them were shown to sensitize tumor cells to ICI therapy (98). The inactivated PBAF subunits exhibited elevated CXCL9/CXCL10 expression and TILs recruitment as a result of increase of chromatin accessibility to transcriptional regulators of IFN-γ-inducible genes (97). ARID1A/B subunits are unique to BAF, while other subunits (ARID2, BRD7, and PBRM1) are exclusively contained by PBAF, despite the high similarity of these complexes (99). In another study, loss of ARID1A was found to elevate MSI by defective recruitment of mismatch repair genes and thus increase TMB, which eventual sensitize tumor cells to PD-L1 inhibitor (100).

Absent Tumor PD-L1 Expression

The PD-1/PD-L1 axis represents one of the foremost mechanisms of modulation of peripheral immune tolerance as well as T-cell

activation. Up-regulation of PD-L1 by cancer cells and antigen-presenting cells (APCs) is one approach through which tumors avoid immunosurveillance and constitutes the principle behind PD-1/PD-L1 blockade therapies (101). Absent PD-L expression of tumors has been found to be generally associated with less responses and inferior survival benefits to anti-PD-1/PD-L1 therapies compared with higher expression (102) and may serve as a resistant marker. However, up to 20% of PD−L1−negative malignancies showed responses to PD−1 inhibitors in some cohorts (103), as PD-L1 expression can be up-regulated by other factors including activated IFN-γ cascade (will be discussed in a separated part), suggesting tumor PD-L1 expression alone is not dependable at predicting outcomes of PD-1/PD-L1 inhibitors.

Any factors that affect the PD-L1 expression of tumor cells may lead to resistance to immunotherapy. Beyond encoding genes, PD-L1 expression can be affected by the mutational features of tumor although it is not paralleled with TMB in most of the tumors (104–106). The inherent mechanisms, which have been shown to result in constitutive expression of PD-L1 by tumor cells, consist of alterations in the PTEN/PI3K/AKT pathway (65, 107), MYC overexpression (108), EGFR mutations (40), CDK5 truncation (109), and elevated PD-L1 transcripts stabilized by disruption of the 3-untranslated region (UTR) of this gene (110). Tumor-specific immune response may also be affected by constitutive expression of PD-L1 caused by these oncogenic signaling processes on tumor cell surface. Although it is still unclear whether it causes an increased or decreased possibility of responding to anti-PD-1/PD-L1 therapies, the constitutive PD-L1 expression could result into inadequate response to other immunotherapeutic approaches by suppressing antitumor effect of T cells. The other transcriptional factors constituting HIF1, NFkB, and STAT3, as well as epigenetic factors, additionally participate in the modulation of PD-L1 expression (111).

Inflammatory and hypoxic tumor microenvironment (TME) can also lead to PD-L1 expression on many cell types including tumor cells by Toll-like receptor (TLR) ligands. The recruitment of activated T cells can increase the inflammatory mediators and successively induce the PD-L1 expression on the surface of tumor cells. These tumor cells specifically locate at the invasive periphery where T cells are often abundant (112, 113).

Besides, PD-L1 is stabilized through N-glycosylation and palmitoylation (114, 115). This is crucial for its interaction with PD-1. The resistance to anti-PD-1/PD-L1 treatment could moreover be attributed to the degree of generation and secretion of soluble forms of PD-L1. These variants without the transmembrane domain because of alternative splicing have been reported in recurrent NSCLC incidences that re-occurred after anti-PD-L1 antibody therapy with the ability to act as soluble imitates for anti-PD-L1 antibodies (116).

Deficiency in Antigen Presentation

Loss of neoantigen

Loss of neoantigens in the context of immune-mediated pressure is postulated to be another mechanism leading to resistance. In the concept of immunoediting, the constant interactions between tumor cells and the immune system trigger the production of subclones that do not express neoantigens, consequently conferring poor immunogenicity and resistance to ICIs (117). It was demonstrated by Anagnostou and colleagues that seven to eight putative neoantigens were lost in the recurrent NSCLC after ICI treatment, suggesting that immunoediting plays a role in acquired resistance to immunotherapy (118). T-cell-mediated neoantigen immunoediting can be induced by the dynamic interactions between T cells and tumor cells, consequently causing partial or total loss of neoantigen (119). Consistently, deficiency in genes that encode target tumor antigens was demonstrated to be associated with acquired resistance in a murine model treated with adoptive T-cell therapy (ACT) in melanoma (120). However, this relationship between acquired resistance and loss of target neoantigens was not observed in a single patient case who achieved a complete response to ACT in a separate study (121), suggesting that down-regulation/loss of neoantigens may occur during immunotherapy, but should be taken as a canonical mechanism of acquired immune resistance.

Proinflammatory cytokines are likely to contribute to immune escape by inducing loss of antigen expression, resulting in acquired resistance too. The process of Tumor necrosis factor-α (TNF-α)-induced epithelial-to-mesenchymal de-differentiation was shown to lead to a loss of neoantigens causing transformation to a tumor phenotype that is less immunogenic and can more readily evade immune surveillance in the ACT-treated mouse model in melanoma (122). Other TIL generated cytokines, such as IL-6 or TGF-β, are also shown to be involved in the induction of epithelial-to-mesenchymal transition in mouse models across numerous types of tumors, indicating that acquired resistance can be promoted by inflammation.

Defective neoantigen presentation

Defective neoantigen presentation serves a crucial function in ICI acquired resistance. The alterations in this process could happen in beta-2-microglobulin (B2M), transporters associated with antigen processing (TAP), or MHC itself (123, 124) (**Figure 1A**).

As part of the MHC class I (MHC-I), B2M is crucial during antigen presentation and its genetic deficiency, including loss of heterozygosity (LOH) and deletions or point mutations, was identified to be an important route for primary and acquired resistance to ICIs (125, 126). Other defects that would affect neoantigen presentation include T-cell receptor (TCR) binding domain mutations of MHC-I (127), loss of tapasin (a MHC-I antigen processing molecule), selective epigenetic silencing of the human leukocyte antigen (HLA) A3 antigen, loss of one HLA haplotype (128, 129), and LOH in HLA (130). Homozygosity in one or more of the three highly variable genes (HLA-A, HLA-B, and HLA-C) that encode MHC-I, which are likely to restrict neoantigen presentation to CTLs, was identified to have a significant association between resistance to ICI therapy in a large cohort of cancer patients (131). In contrast to anti-CTLA-4, the expression of MHC-II (but not MHC-I) proteins by tumor and the presence of IFN-γ-mediated gene signatures were found to be associated with the positive responses to anti-PD-1 therapy in melanoma (132).

Defective neoantigen presentation may be mediated by IFN-γ signaling pathway through JAK1/2 and the STATs, by

down-regulating the expression of MHC-I (133). Actually, the IFN-γ pathway has both unfavorable and favorable impacts on antitumor immune responses and plays a key role in acquired and primary resistance to ICI therapy (as discussed above).

Tumor Cell-Extrinsic Immune Landscape

T-Cell-Related Factors Involved in Tumor-Cancer Immune Cycle

Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes constitute a complex group of immune cells with distinct functions and different clinical impacts. Among them, tumor-specific CD8+ T cells can execute anti-cancer activities by killing tumor cells directly and has a strong prognostic effect in NSCLC (134, 135). CD4+ cells are composed of a group of lymphocytes (Tregs CD4+, Th1, Th2, and Th17) secreting diverse cytokine to activate and suppress CD8+ cells. Th1 secretes IFN-γ and IL2, while Th2 secretes IL-4, IL-5, IL-9, IL-10, IL-13, and IL-25 (136, 137). CD45RO+ T cells, also known as memory T lymphocytes, are another subclass of TILs. Regulator and memory T lymphocytes will be discussed in *section* "Suppressive tumor microenvironment."

Low CD8+ TIL density was correlated with impaired efficacy and survival in NSCLC patients treated with ICIs (138), suggesting that immunotherapy resistance was mediated by low TILs but was then positively modulated by PD-L1. TILs can be assessed by immunohistochemistry or standard hematoxylin and eosin (H&E) staining; however, no consensus has been reached hitherto in the various scoring models using H&E staining in NSCLC (139–142). A radiomic fingerprint of CD8+ TIL derived via computerized tomography was developed recently and showed promising efficacy in predicting response to ICI therapies but requires further validation (143).

Thus, tumors can be described as three main immune organization profiles (hot, altered, and cold) as per the presence of TILs and correlated proinflammatory cytokines (144). The "cold" immune tumor is characterized as absence of TIL within and at the edges of tumor, manifesting resistance to immunotherapy either due to absent immune stimulation (as with low neoantigen cancer's poor antigen presentation) or because of failed T-cell priming (as with intrinsic insensitivity to T-cell killing). The "altered" immune tumor is characterized as low TIL within the tumor ("immunosuppressed") or high TIL at the edges of the tumor ("excluded"), whereas "hot" is high degree of TIL (144). Recently, intratumorally geospatial heterogeneity of TIL was revealed in NSCLC. Tumor subclones from "cold" immune regions were related to mutation space more closely and diversifying more recently compared with those from "hot" immune regions. Higher risk of recurrence was observed in tumors with more than one "cold" immune region (145).

Impaired T-cell priming and infiltration

Reduced proliferation and inadequate diversification of T cells possibly contribute to ICI resistance. Impeded priming of naive T cells by blocked DCs recruitment was demonstrated in melanoma to be correlated to the lack of TILs and ICIs resistance (146). The function of DCs can be potentially influenced by the cytokines in the TME through (1) impaired migratory capacity as well as decreased synthesis of costimulatory components (CD86/80) by TGF-β (147, 148); (2) prevented DCs maturation by IL-6-gp130-STAT3 axis; and (3) inhibited activity by Indoleamine 2,3-dioxygenase 1 (IDO, will be discussed in *Section* "Suppressive tumor microenvironment"). IFN-α signaling pathway is important to the priming of T cells by DCs. It was found that TME with remarkable insufficient IFN-α-producing DCs naturally led to lessened antitumor T-cell priming and thus resistance to ICIs (149, 150). Activated IFN-α stimulated production of the chemokine CXCL10 to recruit TILs to tumor beds and in turn initiate spontaneous antitumor T-cell response (149–151). Preliminary trials combining IFN-α 2b therapy with anti-CTLA-4 inhibitors have indicated clinical activity, which could be caused by diminished populations of MDSC (152, 153). Combinations of other ICIs and IFN-α 2b are currently investigated (154).

Immune resistance also occurs if the tumors evolve the ability to prevent infiltration even if tumor-specific Teffs are formed. Mechanisms that lead to impaired T-cell infiltration involve components in the epigenetic silencing of immune cells (155) and the modification of secreted chemokines (156, 157). Transcriptional program that is associated with T-cell exclusion and thereby predictive resistance to anti-PD-1 therapy was identified in melanoma (158). Stromal cells surrounding tumors within TME can develop the capacity to obstruct effector T-cell entry, and the TGF-β cascade appears to serve a crucial role in promoting T-cell exclusion features in peritumoral fibroblasts (123, 159).

T-cell receptor clonality

T-cell receptor clonality is emerging as a new biomarker to predict the resistance and immune-related adverse events to ICIs therapy. Since baseline CD8+ T-cell density was found to overlap between respondents and non-respondents to ICI therapy (160–162), it led to the speculation that a constrict TCR arsenal possessed by the baseline T cells concentrated on the antitumor immune reaction and is associated with response to ICI therapies. T-cell clones can be identified by detecting TCR rearrangements constituting genes in the variable (V)-diversity (D)-joining (J) region, which generate the antigen-specific complementarity-determining region 3 (CDR3). The responsivity of TCRs generated by TILs determines their potential to interplay with tumor antigens that are presented on APCs. Thus, the assessment of T-cell clonality divulges the extent of T-cell expansions caused by tumor antigens and contributes to explore the mechanisms underlying T-cell toleration to tumor antigens.

A lower baseline clonal T-cell arsenal has been shown to be linked to worse clinical benefits to ICIs and survival in cancer patients (162, 163). Besides, a remarkable increase in T-cell clones was reported in responders during anti-PD-1 therapy compared to non-responders, implying a cancer-specific reaction to immunotherapy for these patients. Moreover, baseline TCR clonality did not strongly associate with TIL density, implying that low-TIL density tumors could still respond to anti-PD-1 treatment if TIL has a narrow TCR clonality specific to the

tumor antigen (164). Inconsistently, it was recently found that T-cell clonality had a positive relationship with T-cell density, PD-L1 expression, and TMB, and a negative relationship with EGFR mutation in NSCLC (165). A corresponding relationship was found between the number of TCR sequences and the number of nonsynonymous mutations, spatial heterogeneity in expanded TCR repertoire, and spatial mutational heterogeneity within tumors in NSCLC, respectively. This intratumorally spatial heterogeneity of TCR repertoire maps the neoantigen landscape, sculptured by focal antigen processing defects or HLA loss (166). Thereby, further investigations to identify the role of TCR clonality in immunotherapy are required.

Alternate immune checkpoint receptor up-regulation

Compensatory up-regulation of numerous alternate immune checkpoint receptors during ICI therapy as a result of the activation of diverse cellular signals and IFN-γ signaling pathway were observed across multiple studies and have been characterized to be linked to ICI adaptive resistance in NSCLC (84, 167, 168). The expression of CD8+ T cells harboring receptors showed serious flaws in proliferation, migration, and cytokine secretion, indicating their immunosuppressive capacity. In addition, progressive T-cell exhaustion was found in the tumors with highly expressive or co-expressive receptors and different receptor displayed different exhausted phenotype (167).

Among these receptors, lymphocyte activation gene 3 (LAG-3) has great potentiality in cancer immunotherapy because co-expression of PD-1 and LAG-3 was often found in T-cell-depleted immune microenvironment, and PD-1 inhibitors combined with LAG-3 blockades showed strong synergic antitumor responses in preliminary models (169). LAG-3 is a co-inhibitory receptor extensively expressed in TILs in various tumors and serves a crucial function in mediating immune escape by suppressing T-cell antitumor functions. It exerts immunosuppression via binding to MHC-II molecules and other ligands such as galectin-3 and fibrinogen-like protein 1 (FGL1) (170). Thus, blocking LAG-3 can restore antitumor immunity and the combined LAG-3 inhibitors therapy may accordingly overcome immunotherapy resistance. In addition, the expression of these ligands, on the basis of the receptor–ligand interactions, may serve as important biomarkers to predict the efficacy of LAG-3 blockades in lung cancer (167).

The other alternative immune checkpoint receptors, e.g., T-cell immunoglobulin and mucin-3 (TIM-3), V-domain immunoglobulin-containing suppressor of T-cell activation (VISTA), B and T lymphocyte attenuator (BTLA; also referred to as CD272), T-cell immunoreceptor tyrosine-based inhibition motif domain (TIGIT), and sialic acid-binding Ig-like lectin 9 (SIGLEC9), have been discovered (144). Thus, these alternate immune checkpoints are likely to be combined with existing ICI therapy to conquer the resistance. Increased efficacy of PD-1 inhibitors combined with anti-TIM-3 or anti-LAG-3 regimens has been observed in either pre-clinical models or phase I clinical trials (171, 172). Currently, numerous clinical trials evaluating the therapeutic impact of alternate immune checkpoint blockade applied on its own or in combination with PD-1/PD-L1 inhibitors in multiple malignances are ongoing.

T-cell exhaustion and phenotype alteration

T-cell exhaustion is another factor involved in the primary and acquired resistance to ICI therapy (**Figure 1C**). Exhausted T cells exhibit impaired activity with progressive LOF and antigen persistence compared with Teffs and can be induced by the PD-1/PD-L1 interactions (173). Chronic exposure to cognate antigen triggers increased expression of PD-1, which results in the accumulation of T-cell exhaustion and thus T-cell dysfunction (174). The presence of PD-1 high expression can either exist prior to PD-1 inhibitors, which is associated with primary resistance partially depending on tumor-associated regulatory T cells (Tregs), or develop after the anti-PD-1 therapy, which leads to acquired resistance by severe T-cell exhaustion. In contrast, studies showed that the exhausted T cells with PD-1 low to intermediate phenotype retain the capacity to be reinvigorated by ICIs (158, 175). Epigenetic alterations were found to be associated with T-cell exhaustion too recently. Exhausted T cell displayed a unique chromatin landscape, which alters the transcriptional state, limits its effect function, and determines its capacity to be reprogrammed after therapeutic intervention (176–178).

The formation of memory T cells is crucial to the avoidance of tumor relapse and therapy resistance following drug withdrawal, especially in the long-lasting duration of responses to ICI therapy. Research evidence shows that patients with resistance to anti-PD-1 treatment have fewer tumor-correlated memory T cells relative to sensitive patients (179). Memory T cells remain dormant until antigen re-challenge (180, 181) and if precursor memory T cells are exhausted under chronic antigen exposure, it will lead to memory T-cell deletion and lack of formation (173, 177).

Acquired resistance can be mediated by the alteration from cytotoxic activity to inactivity phenotype of antitumor T cells during TCR-engineered ACT. The original highly cytolytic profile when administrated to patients, which showed strong efficacy initially, was reported to change to a phenotype with impaired cytotoxic functions and Th2-related cytokine release when tumor relapses within months (182, 183).

Suppressive Tumor Microenvironment

Increased immunosuppressive cells

The TME is a complex net consisting of a variety of immune and stromal cells, cytokines, extracellular matrix, and vasculature, which affect response to immunotherapy. Immune-suppressive cells, including Tregs, MDSCs, M2 macrophages, along with inhibitory cytokines in the TME, can contribute to the inhibition to immune responses (136, 184) (**Figure 1C**).

Tregs can inhibit Teff reactions by secreting certain inhibitory cytokines (IL-10, IL-35, and TGF-β) or by direct cell contact (185–187). The cytokine IL-10 influences antigen presentation by down-regulating the expression of MHC-II and co-stimulatory components on DCs, thus intercepting the Teff activation (187). The ratio of Teffs to Tregs was shown to be related to the responses to ICIs in mouse models, in that incapacity of either increasing Teffs or decreasing Tregs may cause resistance to immunotherapy (188, 189). Factors that affect Tregs activity, at the same time, are putative biomarkers of resistance. For instance, soluble CD25, an IL-2 receptor whose binding is assumed to stimulate Treg proliferation, was established as a

negative predictor of OS for patients treated with anti-CTLA−4 (190). However, tumor-infiltrating Tregs might likely coexist with multiple immune cells, insinuating a potential immunoreactivity. It was reported that a high baseline expression of FoxP3+ Tregs in the tumor is positively associated with better survival in a retrospective study involving patients under the treatment of anti-CTLA-4 antibodies (161).

Myeloid-derived suppressor cells promote immune evasion and tumor growth and have emerged as critical modulators of immune responses in cancer. Studies have suggested the existence of MDSCs in TME correlates with reduced efficacy of immunotherapies, including ICIs therapy (191), ACT (192), and DC vaccination (193). Therefore, reprogramming or eradicating MDSCs might improve clinical response to immunotherapy.

Tumor-associated macrophages (TAMs) can be classified into M1 and M2 macrophages according to disparities in surface molecules, expression of transcription factors, cytokine profiles, and metabolism (194, 195). They promote antitumor immunity effects (mediated by M1) and pro-tumorigenic properties (mediated by M2) that modify the TME (196). The role of TAMs in mediating immunotherapeutic resistance in tumor has been discussed in several reports (197, 198). It was indicated to directly inhibit T-cell responses through PD-L1 in preclinical studies of liver (199) and ovarian cancer (200). The inhibitor of CSF-1R, a receptor for macrophage colony-stimulating growth factor, was investigated in mouse models of pancreatic cancer where it decreased the frequencies of TAMs, while increasing IFN production and delaying tumor progression (201, 202). Similarly, CSF-1R inhibitor was found to synergize ACT therapy in a melanoma model (203). These data suggest that CSF-1R inhibitor may overcome the resistance to immunotherapy.

Specific chemokines, such as CCL5, CCL7, and CXCL8, play an important role in the recruitment of Tregs and MDSCs to the TME, consequently boosting an immunosuppressive climate (204). Alternately, chemokines CXCL9 and CXCL10 recruit CTLs to the TME (205) and the epigenetic silencing of the genes encoding them can reduce TILs and consequently promote resistance to ICIs (205). Epigenetic modulators of these chemokine receptors relieved the suppression of these Th1-cell-type chemokines and increased TILs leading to improved therapeutic efficacy of PD-L1 inhibitor in a model for ovarian cancer (155).

Elevated immunosuppressive cytokines

The cytokine milieu is critical to the recruitment, activation, and proliferation of immune cell, performing both immune stimulatory and suppressive effects (206). Transforming growth factor-β (TGF-β) is a cytokine playing key roles in angiogenesis and immunosuppression by stimulating Tregs (207) and excluding T cell in peritumoral fibroblasts (123, 159). Up-regulated TGF-β signaling was correlated with poorly immunogenic tumors and restrained response to ICIs in a colorectal cancer model, indicating resistance to therapy (159). Consequently, enhanced antitumor response to ICIs was observed following application of TGF-β inhibitor either alone or in combination with anti-CTLA-4 or radiation therapy (208, 209). Bintrafusp alfa, a bifunctional fusion protein composed of the extracellular domain of TGF-β receptor II (a TGF-β "trap") fused to a human immunoglobulin G1 antibody blocking PD-L1, demonstrated favorable efficacy in patients with advanced NSCLC. Ongoing phase III trial is expected to validate the efficacy of bintrafusp alfa vs. pembrolizumab in the first-line setting in advanced NSCLC (NCT03631706).

Tumor necrosis factor-α pathway is postulated to be another immune evasion machinery conferring resistance to PD1 blockade. The expression of TNFα in an inflamed TME positively correlates with the expression of PD-L1 and TIM-3, along with impaired accumulation and increased activation-induced death of CD8+ TILs in melanoma models treated with anti-PD1 therapy. Accordingly, inhibition of TNF-α prevents the expression of PD-L1 and TIM3 and hampers anti-PD1-induced TIL death (210). Therefore, this study offers a rationale for the combination of PD-1/PD-L1 inhibitors with TNFα blockade as a novel immunotherapeutic strategy to overcome resistance in lung cancer, and the phase I clinical trial testing the combination is ongoing (NCT03293784).

Vascular endothelial growth factor has been linked to both decreased T−cell infiltration and immunosuppressive effects in addition to promoting angiogenesis and thus is associated with resistance to ICIs (211). Multiple mechanisms are involved in the interaction of VEGF with antitumor immunity: (1) VEGF prevented the commitment of lymphoid progenitors, decreasing progression to the T-cell lineage (212); (2) VEGF signaling promotes the infiltration of Tregs through a selective endothelium and reduces trafficking and extravasation of CTLs into the TME (213); and (3) VEGF increases expression of inhibitory receptors, contributing to CTL exhaustion (214). Higher levels of VEGF were found in anti-PD-1-resistant patients than sensitive ones (160). Based on these findings and the synergy between angiogenesis blockade and ICI therapies observed in preliminary studies, multiple trials of combination therapy are underway, including bevacizumab and VEGFR-TKI with anti-PD-1 therapy.

Higher levels of interleukin 6 (IL-6) and interleukin 8 (IL-8) have been found recently to be linked to reduced responses and worse clinical outcomes to ICI therapies across multiple types of cancers (215–217). IL-6 is a proinflammatory cytokine generated by T cells and macrophages and is usually involved in the immunoregulation connected to the IFN-γ signaling pathway. It can reduce the expression of PD-L1 and MHC-I, and result in tumor escape and resistance to ICI therapy (218). IL-8 is a proinflammatory chemokine and a chemoattractant for myeloid leukocytes expressed in multiple cancers (219, 220). It potently regulates the chemotaxis of neutrophils (221, 222) and exerts direct pro-tumorigenic effects (223). High levels of IL-8 are regarded to be associated with more neutrophil and monocyte infiltration, defective T-cell functions, and impaired antigen presentation, which subsequently result in resistance to ICI therapy (216, 217).

Additional immunoregulative molecules

Contributions from inflammatory processes could participate in quashing the desired impacts of ICIs (**Figure 1C**). Adenosine can be produced under the condition of hypoxia and ischemia

caused by tumor inflammation. It was reported to inhibit the cytotoxic function and proliferation of T cells via the A2A receptor on T cells (224). CD73, which mediates the generation of adenosine through dephosphorylation of adenosine monophosphate, was also demonstrated to suppress immune function (225). CD73 overexpression promotes T-cell exhaustion and is linked to the resistance to ICIs (226, 227).

IDO1 expressed in myeloid cells and cancer cells is a rate-limiting enzyme that converts tryptophan to its immunosuppressive metabolite kynurenine. This enzyme can induce T-cell anergy and apoptosis by gathering kynurenines and consuming the essential amino acid tryptophan and prevent the T-cell clonal expansion (228). It is particularly activated in DCs after binding with CTLA−4 and can be unregulated by CTLA−4 during adaptive immune resistance (229). Reduced expression of IDO at baseline was noted to be associated with poor response to ipilimumab in a phase II study in melanoma (161). Low level of IDO is likely to manifest insufficiency of suppressed TILs feasible to be reactivated by immunotherapy. Correspondingly, IDO-knockout mice exhibited improved OS with ICI compared with wild-type mice, and ICI therapy combined with IDO inhibitors showed both increased numbers and functions of TILs in the TME in an experimental setting (230, 231). However, despite encouraging results observed in preclinical and early-phase clinical studies in different types of tumors, no difference was shown in pembrolizumab combined with IDO1-selective inhibitor vs with placebo in a phase III study in metastatic melanoma (232).

B7-H4 has been proposed as another resistance marker to ICIs recently due to its negative modulation of T cells. B7-H4 constitutes a type I transmembrane protein of the B7 immunoglobulin superfamily and is encoded by the V-set domain containing T-cell activation inhibitor 1 (VTCN 1) gene. It is induced by activated T lymphocytes and down-regulates T-cell function by inhibiting proliferation, cytotoxicity activity, and interleukin secretion, after binding with T cells (233–235). Positive B7-H4 protein expression in patients with advanced NSCLC treated with nivolumab was recently reported to have an enhanced risk of tumor progression and tumor-related death compared with negative expression (236).

Host-Related Characteristics

Host-related characteristics, including gut microbiome, diet, and antibiotic exposure that adversely affect the gut microbiome, diet, steroid use, vaccine exposure, inflammation state, and autoimmunity, have been shown to relate to primary and acquired resistance to ICI therapy in lung cancer (237, 238) (**Figure 1B**).

Gut Microbiome

Evidence is arising to support the vigorous impact of the gut microbiome on immunotherapy resistance. Multiple studies have demonstrated that less bacterial diversity and lack of enrichment of specific species showed a significant correlation with resistance to ICI therapies. Relative abundance of *Bacteroidales* was found in non-responders, while responders were more likely to have *Faecalibacterium* and *Ruminococcaceae* (239, 240). Furthermore, transplanting the responder's feces into aseptic mice exhibited improvement in the treatment of PD-L1 inhibitor (241). Certain species can be altered by the antibiotic's exposure, which partly explained modified response to ICI therapy either in a good or in a bad way (242–244).

The alteration tendency of gut microbiome structure or dominant bacteria may differentially affect the T-cell immune response. It may be due to the cross-reactivity between intestinal microbiota and tumor-associated antigens, enhancing the inflammatory cytokine production, activation of DCs, and antigen presentation (245, 246). IFN-γ-producing CD8+ T cells were successfully induced from a consortium of 11 bacteria in the intestine, and colonization with this 11-bacterial mixture resulted in enhanced efficacy of ICIs in mouse models (247). Consistently, "good" bacteria introduction was reported to significantly increase IFN-γ production in spleen and tumor-draining lymph nodes (TDLN) (246) and induce DCs to secrete IL-12, resulting in increased recruitment of CCR9+CXCR3+CD4+ T cells into tumor beds (244). The activation of DCs was reported to be modulated by the gut microbiome in animal models and cancer patients. The resistance to anti-CTLA-4 therapy can be reversed by oral administration of *Bacteroides fragilis*, which induced Th1 immune response in TDLN and promoted DCs maturation (243). *Bifidobacterium*-feeding mouse presented higher expression of MHC-II in DCs within tumors (248). It remains controversial whether intestinal microbes lead to immunotherapy resistance by affecting the production of Tregs. A higher level of peripheral Tregs was found in patients with "bad" bacteria and was associated with poor response to ipilimumab in metastatic melanoma patients (249). *B. fragilis* can produce a microbial molecule, polysaccharide A, which can promote the formation of the inducible population of CD4+Foxp3+Tregs (a subset of Tregs), thereby negatively regulating the immune system (250), whereas the other two studies reported no differences in Treg differentiation between pancreatic duct adenosarcoma-bearing mice and control, and the number of Foxp3+ T cells between *Bifidobacterium*- and PBS-feeding mice (251, 252). The majority of chemokine genes were reported to be up-regulated by specific species of gut microbiome including *Fusobacterium nucleatum*, *B. fragilis*, and *Escherichia coli* in colorectal cancer cells. Additionally, the gut microbiota-derived microbial load was associated with increased chemokine production (251).

The gut microbiome also acts as an instructive modulator of mutant TP53, which ultimately affects tumor proliferation and the immune system. Kadoshi et al. recently found that mutant TP53 presented contrasting effects in different segments of the gut in a mouse model: a remarkable tumor-suppressive effect in proximal gut and the expected oncogenic effect in distal gut (253). The gut microbiome and its single metabolite gallic acid turned mutant TP53 from a tumor-suppressive effect to an oncogenic one, suggesting that the function of mutant TP53 is plastic and under the control of microbiome and microbiota-derived metabolites.

Concomitant Medications

Antibiotics exposure has been reported to be associated with inferior clinical outcomes during ICIs therapy in NSCLC (244, 254). However, it remains debated if antibiotics exposure represents an independent predictive biomarker of ICIs therapy or it is a surrogate for patients with worse prognosis (e.g., poorer performance status, higher comorbidities). The use of antibiotics has an unfavorable impact on the recolonization and subsequent alterations in microbiota composition, which eventually leads to a decline in microbial symbiotic diversity (255). The antibiotics-induced dysbiosis destroys the gut homeostasis and extends from childhood to adulthood, with long-lasting adverse effects on the immune system as well as body metabolism (256–258). The antitumor immune response-induced cyclophosphamide was impaired by antibiotics exposure in the fibrosarcoma model, which was associated with an improvement in the Teff-to-Treg ratio and the loss of *Enterococcus hirae*-elicited helper T cell in tumor immune infiltrate (259, 260). Correspondingly, the absolute numbers of neutrophils and monocytes decreased after oral administration of imipenem, vancomycin, and neomycin before tumor inoculation in lymphoma and colon models treated with oxaliplatin or IL-10 inhibitor (261). On the contrary, the growth of *Fusobacterium* spp.-containing tumor was slowed down after the administration of metronidazole in the mouse model of colon cancer, suggesting such bacteria promote tumor progression (262).

Long-term use of steroids adversely impacts the efficacy of ICIs because of their supposed anti-inflammatory and immunosuppressive effects that potentially hamper the mode of ICI action (263), whereas a transient use of steroids aimed at management of ir-AEs did not negatively affect patients' survival outcomes (264, 265). Beyond affecting gut microbiome, steroids are known to prevent the activation of T lymphocytes, inhibit the amplification of T helper subsets, recruit Tregs, and promote M2 macrophage polarization (266, 267). Hence, early use of steroids on ICI treatment may prevent this phase of T-cell recruitment and thus impair effective antitumor immune response. The increment of neutrophil-to-lymphocyte ratio (NLR), derived NLR (dNLR), and absolute neutrophil count (ANC) after steroids has been shown to be associated with unfavorable clinical outcome in NSCLC patients treated with ICIs (268). Steroids-induced imbalance of immune cells in TME, especially increased MDSCs resulting in elevated ANC and NLR, is the intermediate of immunotherapy resistance in NSCLC (269).

Diet

Diet can affect tumor growth within TME through systemic or local effects in multiple ways. First, it may alter the composition and diversity of gut microbiome, which in turn exerts drastically different effects on host immune function. Second, specific ingredients (e.g., vitamins) may be regulated by dietary patterns and then have an impact on immune status. It is well-known that the general metabolic status determining deviations from ideal body weight, as well as the metabolic factors (e.g., low-level arginine and tryptophan, high-level lactate, and the adenosine pathway induced by increased glucose metabolism), highly influences the immune activity (270–272). An appropriate diet can maintain the homeostatic equilibrium between the inflammatory cascade triggered by Th17 cells and the anti-inflammatory pathway mainly based on the activity of Treg (273).

Additional Factors

Chronic accumulation and production of inflammatory molecules in the chronic inflammatory status lead to an immunosuppressant state, which is linked to immunotherapy resistance. Proinflammatory and carcinogenic mediators such as IL-6, TNF-α, and chemokines are released in the TME and tend to trigger a variety of molecular signaling cascades including PI3K/MAPK, JAK/STAT, and WNT/B-catenin, which are involved in the resistance to immunotherapy as mentioned previously. The components of immune cells are also altered to be more immunosuppressive with more TAMs, Tregs, and tumor-associated neutrophils within TME (274). Therefore, blocking inflammation might be an effective strategy to improve the outcome of immunotherapy in NSCLC (275).

Tumor development and autoimmunity are two opposite results of imbalanced immune homeostasis in controlling tumor cell growth (low immune responses) and regulating autoreactive responses (immune overreaction). The host autoimmunity affects the efficacy of immunotherapy in bringing more ir-AEs when too strong or incapacity to prime and activate immune cells when too weak (276). In addition, autoimmunity has an inextricable link with host gut microbiome and anti-microbial immunity, as effector responses that lead to inflammatory tissue damage are the same as those that mediate effective host defense (277).

The relationship between smoking and the efficacy of ICIs remains controversial (278–281). Smoking is associated with high TMB, especially nonsynonymous mutations, subsequently enhances the immunogenicity of tumor, and improves the outcome of ICIs therapy (23, 282). Additionally, PD-L1 expression can be up-regulated by smoking through oxidative stress-dependent mechanism (283) and induced by cigarette smoke and the carcinogen benzopyrene (BaP) via aryl hydrocarbon receptor (AhR) (284). Moreover, smoking may also have an impact on the status of TILs (285) and other immune modulators such as B7-H3 (CD276) (286) and in turn affect the efficacy of ICIs therapy.

THERAPEUTIC APPROACHES TO CONQUER IMMUNOTHERAPY RESISTANCE

Research and design of therapies to conquer immunotherapy resistance has been advancing along with mechanistic investigations. Combinatory treatments, either via combinations of diverse immunotherapeutic agents or through combinations with traditional treatments, developed to revitalize the defense system with complementing/synergetic mechanisms, have been introduced to serve as alternative approaches for NSCLC therapy. Diverse targets discussed herein have the potential to serve as both biomarkers of resistance and combination therapy targets. In view of the different resistance mechanisms, the combinatory therapy strategies are mainly manifested in the following aspects

(the examples of ongoing studies trying to reverse resistance are summarized in **Supplementary Table 1**):

Enhance Tumor Immunogenicity

1. Emerging evidence has indicated the positive immunologic effects of chemotherapy (287). On one hand, it regulates the composition and function of immune cells such as CTLs (288), MDSCs, and Tregs (289) in the TME and the molecules expressed on tumor cells; on the other hand, it restores the recognition of immune system to tumor through enhancing tumor antigen presentation via up-regulating the expression of MHC-I and through boosting antitumor immune responses via chemo-induced tumor cell apoptosis (290–292). Multiple randomized phase III clinical trials have compared the combination of chemotherapy and ICIs with chemotherapy alone in treatment-naïve advanced lung cancer (4, 9, 293). The results consistently showed that the combination strategies are superior to chemotherapy alone in the first-line setting, regardless of PD-L1 expression, suggesting that the synergistic activity between chemotherapy and ICIs may offset the insensitivity due to low PD-L1 expression.
2. Similar to the effects induced by chemotherapy, radiation therapy combined with ICIs leads to long-lasting tumor regression through escalating antigen exposure secondary to cancer cell apoptosis, enhancing an inflamed TME (294), raising DCs activation and up-regulating proinflammatory cytokines, causing elevated TILs (295), and facilitating cancer relapsing by non-redundant immune mechanisms (296, 297). Consolidative PD-L1 inhibition after concurrent chemo-radiation significantly improved survival in unresectable stage III NSCLC in the PACIFIC study, and this approach has become the standard care of locally advanced NSCLC (298). Clinical trials evaluating the concurrent administration of radiation therapy with ICIs are ongoing.
3. Vaccines using cancer-specific peptides or DCs (237, 299, 300) and oncolytic virus therapy (301, 302) escalate the antigen presentation and priming of T cells.

Target Oncogenic Genes

1. Blocking the MAPK/PTEN/PI3K axis such as BRAF, MEK, and PI3K inhibitors contributes to Teff expansion, avoiding T-cell exhaustion and apoptosis, activating an immune-stimulatory transcriptional program, and promoting the production of proinflammatory cytokines and T-cell cytotoxicity (303, 304). BRAF and MEK inhibitors in combination with PD-1 blockade therapy showed a 73% overall response rate (ORR) and 93% stable disease in BRAF V600-mutated metastatic melanoma (305). However, cobimetinib, a MEK1/2 inhibitor, combined with atezolizumab was evaluated in a phase Ib umbrella platform MORPHEUS. The combination did not show better efficacy compared with the control arm in the NSCLC cohort of this study (306).
2. PARP inhibitors, as synergistic activating CD8+T-cell-mediated antitumor response despite up-regulating PD-L1 expression, which can be complementally inhibited by anti-PD-L1 therapy (307).
3. The combinations of nivolumab with veliparib and pembrolizumab with olaparib were tested in advanced solid tumors (308, 309). No response was observed and PFS and OS were 9.0 and 26.8 weeks, respectively, in the former trial while no results are available in the latter one.

Promote T-Cell Priming and Enhance TILs

(1) Agonists of TLRs, as contributing to the DC mutations and T-cell priming; (2) STING, as activating inflammatory reactions via IFN-α cascade upon recognition of foreign DNA; (3) dual block CTLA-4 and PD-1, as CTLA-4 inhibitor enhances the T-cell priming, Treg exhaustion, and CTL-mediated immune responses via more antigen recognition (310), while PD-1 inhibitor participates in later reactivation of Teff response; (4) adoptive T-cell transfer either alone or in combination with ICIs therapy, as increasing TILs and T-cell cytotoxicity (311, 312); and (5) bispecific monoclonal antibodies, as redirecting cytotoxic effector cells to the TME, depleting suppressive cells, and activating effector cells by targeting a cancer-specific antigen and either CD3 on CTLs or CD16A on NK cells; or targeting cancer-specific antigen and immune regulators, or targeting dual immunomodulators (313).

Reshape Immunosuppressive TME

(1) Colony-stimulating factor 1 receptor (CSF1R) blockades, as reducing tumor invasion via the MDSCs and M2 macrophages; (2) inhibition of CD73, A2A receptor, as improving TME by targeting suppressive factors; (3) dual blockade of the TGF-β and checkpoint inhibitory receptors, as facilitating tumor penetration with T cells and reversing the immune suppressive TME (208); (4) anti-CXCR2/CXCR4 antibodies, as voiding immune evasion (314); (5) VEGF inhibitors, as normalizing the immune suppressive TME and reversing ICIs resistance (315); and (6) IL-1β inhibitor canakinumab, as targeting tumor inflammatory response and reducing immunosuppression. To date, there are four clinical trials of canakinumab in various settings in the treatment of NSCLC under way, and preliminary results from two of them were released in AACR this year. Pembrolizumab plus chemotherapy combined with canakinumab was safe and well tolerated in the first-line treatment in locally advanced or advanced NSCLC, and the recommended phase III dose of canakinumab was 200 mg s.c. Q3W (316). The efficacy of canakinumab or pembrolizumab monotherapy or in combination as neoadjuvant treatment in resectable NSCLC was assessed in the CANOPY-N study and the results are not reported (317).

Target Alternate Immune Checkpoints and Immune-Stimulatory Receptors

(1) Blockade of alternate coinhibitory immune checkpoint receptors, such as LAG-3, TIM-3, TIGIT, BTLA, VISTA, and SIGLEC9; (2) costimulatory agonists, including 4-1BB, OX40, CD40, GITR, and ICOS, as enhancing T-cell expansion

and effector functions while controlling Treg cell-suppressive functions (318, 319).

Although not being widely used in clinical practice, the antibodies targeting these immune checkpoints have exhibited promising antitumor activity in early clinical trials in various malignancies. LAG-3 blockades, Relatlimab [humanized anti-LAG-3 monoclonal antibody (mAb)], and Eftilagimod alpha (a soluble LAG-3 protein) combined with PD-1 inhibitors achieved an ORR of 15% in previously treated melanoma (320) and 52.9% in treatment-naïve advanced NSCLC (321), respectively. Based on the positive preclinical results, TIGIT blockade, especially in combination with anti-PD-1/PD-L1 mAb, has been explored in various clinical settings of advanced tumors. MK-7684 (an anti-TIGIT mAb) alone or combined with pembrolizumab showed a disease control rate of 35% and 47%, respectively, in a phase I study (322). Etigilimab (a humanized anti-TIGIT mAb) also presented early signs of efficacy as a monotherapy, with a 0% ORR but 22% stabilized disease in advanced malignancies (322). Tiragolumab is a fully human IgG1/kappa TIGIT monoclonal antibody with an intact Fc region that blocks TIGIT from binding to its PVR ligand and to the co-activating receptor CD226. It improved ORR either alone or combined with atezolizumab compared to historical data in a phase I study (323). The clinically meaningful improvement in ORR and PFS was confirmed recently in the CITYSCAPE study (a phase II study of tiragolumab plus atezolizumab vs placebo plus atezolizumab as first-line treatment in patients with PD-L1-selected NSCLC) (324). Cobolimab is a novel IgG4 anti-TIM-3 mAb and showed clinical benefit with an ORR of 15% and 40% stable disease in combination with anti-PD-1 mAb in a phase I clinical trial (325). Other anti-TIM-3 mAbs including MBG453, Sym023, INCAGN2390, LY3321367, BMS-986258, and SHR1702, as well as a bispecific antibody targeting PD-1 and TIM-3 (RO7121661), have also being evaluated in phase I trials with no clinical results available.

Apart from blocking coinhibitory immune checkpoint receptors mentioned above, several costimulatory agonists are also attractive targets, a few of which have stepped into clinical studies. ATOR-1015, a CTLA-4 × OX40 bispecific antibody, was tested in a phase I study for safety and tolerability in advanced solid tumors (326). GSK998, a humanized IgG1 agonistic OX40 mAb, combined with or without pembrolizumab was also evaluated in a phase I trial of advanced solid tumors including NSCLC (327). A lipid nanoparticle encapsulated mRNA encoding human OX40L, mRNA-2416, showed good tolerability when intratumorally injected as monotherapy in advanced malignancies in a phase I/II trial, and the combination with durvalumab is ongoing (328).

Epigenetic Modulation

(1) DNA methyltransferase inhibitors, e.g., sensitizing tumors to PD-L1 blockade and elevating the secretion of the immunostimulatory chemokines CXCL10 and CXCL9 (155); (2) histone deacetylase inhibitors, as down-regulating MDSCs, increasing the expression of MHC-I and antigen presentation, and increasing tumor-infiltrating $CD8^+$ T cells (95); and (3) histone methyltransferase Ezh2 inhibitor, as reversing the effects of loss of immunogenicity and antigen presentation (94).

Gut Microbiota Modulation

Modifying the composition of gut microbiome might eliminate resistance to ICIs. Dietary modification, probiotics, and fecal microbiota transplantation have been emerging as an adjunct treatment to ICIs.

Of note, combination strategies that have been successful in preclinical models do not necessarily pass safety and performance assessments in clinical trials. In addition to considering the complementarity of immunotherapy resistance mechanisms, the timing and sequence are also important when formulating combination treatment strategies. Therefore, the preclinical model, translational study, and pharmacokinetic study of each of these agents in combination and in isolation are indispensable for the clinical success of combination strategies. Furthermore, multimodal approaches, for example, local therapy for oligo-progression after response to ICIs, should be implemented on therapeutic combinations for better clinical benefits.

CONCLUSION

It remains challenging to clarify the resistance mechanisms of immunotherapy since they are complex and dynamic, and certain mechanisms alternately overlap. Further understanding of the primary and acquired resistance mechanisms of immunotherapy will help clinicians to make reasonable combination treatment decisions to bring superior survival and avoid additional toxicity for patients with lung cancer.

AUTHOR CONTRIBUTIONS

FW conceptualized and drafted the review. QZ and SW contributed to the significant portions of the manuscript. All authors listed approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.568059/full#supplementary-material

Supplementary Table 1 | Combination therapeutic strategies and examples of ongoing clinical trials to overcome ICIs resistance in advanced lung cancer.

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Overall Survival in Heart Disease–Related Death in Non-Small Cell Lung Cancer Patients: Nonimmunotherapy Versus Immunotherapy Era: Population-Based Study

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The cardiotoxicity during immunotherapy administration leads to mortality by more than 42% and heart disease–related mortality among immunotherapy-linked cancers is still considered to be underestimated. In this study, the advanced stage of non-small cell lung cancer (NSCLC) with heart disease–related death was selected in accordance with immunotherapy approval time. NSCLC was searched on the Surveillance, Epidemiology, and End Results (SEER) program. Results show that 538 advanced NSCLC cases, those dominated by men and elderly people aged more than 70 years, had a high percentage of heart disease–related death in both eras. The difference between contemporary groups was fairly nonsignificant (P = > 0.05). The overall survival (OS) of all-cause mortality difference showed improved survival in the immunotherapy group (P = 0.0001). In the study of heart disease–related death survival with adjusted data, the NSCLC patients show significant lower survival in the immunotherapy era compared with the nonimmunotherapy era (P = 0.003; hazard ratio [HR] = 1.31; 95% CI = 1.099–1.57). In the multivariate analysis of NSCLC-related immunotherapy, histology revealed that the non-squamous cell type had an independent risk for lower OS than the squamous cell type (P = 0.04; HR= 0.74; CI = 0, 55- 0.99). The results demonstrate the survival benefits for NSCLC in immunotherapy; however, in heart disease–related death, immunotherapy in patients with NSCLC shows decreased OS. This study highlights that NSCLC patients should be highly monitored during immunotherapy administration, and further assessment is needed.

Keywords: non-small cell lung cancer, immunotherapy, SEER database, cardiotoxicity, heart diseases

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INTRODUCTION

The U.S. Food and Drug Administration (FDA) has approved several immunotherapeutic drugs for cancer since 2010, and many more are still being evaluated in other clinical studies to remarkably increase the response and survival rates of patients with advanced cancer (1). Unfortunately, cancer immunotherapies possess potential toxicity that is distinctive from other types of care, mostly due to their etiology. The occurrence of cardiovascular adverse events is particularly challenging to cancer management and has led to various clinical outcomes, ranging from cardiogenic shock to death (2).

Improving clinical effectiveness should be weighed against potentially dangerous adverse events when selecting immunotherapy plans and comparatively evaluating each related adverse event separately. Even though the incidence of cardiotoxicity-linked immunotherapy is rare, recent studies have implied its underestimation and needs to be reconsidered. In addition, an urgent intervention must be planned to reduce the mortality rate associated with adverse effects (3, 4). More studies that aim to create thorough risk and etiological stratification models and identify pathways that specify this toxicity clause are needed to improve the early prevention, detection, and treatment approach (5).

Recent studies suggest a particular increased incidence of cardiovascular toxicity in lung cancer patients among all based immunotherapies cancers (6), and the evidence of OS of heart disease–related death for NSCLC patients is still lacking. In addition to the nonimmunotherapy era, this study explains the survival variability of the differences in cardiac-related death among NSCLC patients by using the SEER database.

PATIENT AND METHODS

Study Cohorts

Patient data were collected from the latest 2018 registry with additional treatment fields on SEER Stat software (version 8.3.6). Using the sixth edition of AJCC, the appropriate codes for advanced lung cancer (IIIB, IV) were selected as labeled site codes (C34/1, 2, 3, and C61.6, respectively). In addition to the period's equality, the same era of the major targeted therapy of tyrosine kinase inhibitors, 2007, was selected as a year of insurance availability in the database when studying the effects of variables in patients with advanced NSCLC compared with 2015 (7). All patients were designated based on the type of follow-up (active follow-up), and only microscopically confirmed cases (positive histology and positive exfoliative cytology, positive histology and immunophenotyping and/or postgenetic studies, and positive microscopic nonspecified method) were included. The following variables were selected: age (20 years or more), COD to site rec KM, year of diagnosis according to contemporary intervals, ICD-0-3 hist/behav, all survival months, grade (I–II, III–IV, or others), sex (male or female), race (white, black, or others), radiation (radiation or others), chemotherapy (yes or no), vital status record, laterality (right, left, or others), ID patients, marital and insurance statuses (yes or others). The detailed inclusion and exclusion criteria are summarized in **Figure 1**.

In addition to excluding small cell types in advanced lung cancer, patients with heart diseases as the cause of death were included with known survival of 20 months as the cutoff value. The baseline demographics of patients were compared using the x^2 test, and the follow-up cutoff value was limited to 20 months,

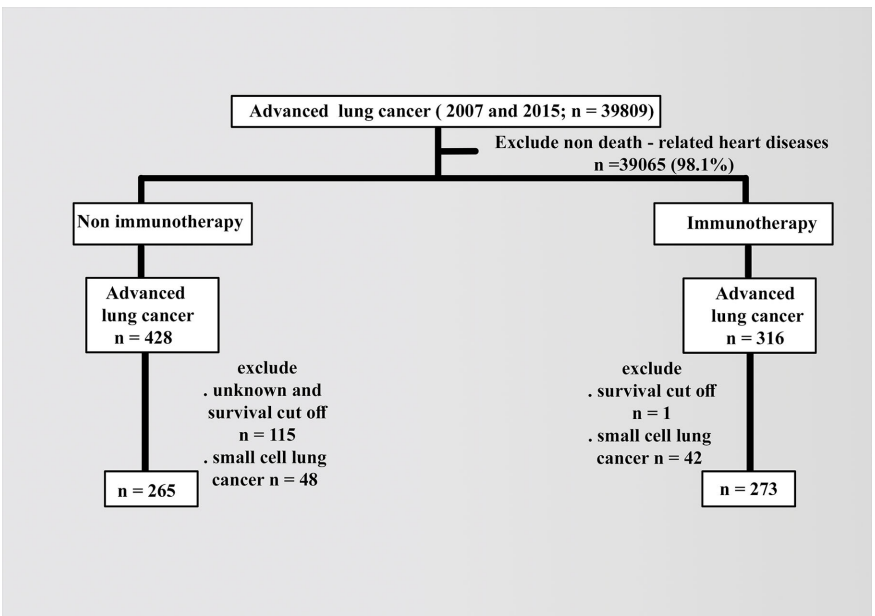

FIGURE 1 | Diagram of exclusion and inclusion criteria.

TABLE 1 | Characteristics of heart disease–related death NSCLC patients.

Parameters	Nonimmunotherapy n = 265(%)	Immunotherapy n = 273(%)	P value
Age			0.6
20-69	98 (37)	107(39.2)	
>70	167(63)	166(60.8)	
Sex			0.7
Male	170(64.5)	179(65.6)	
Female	95(35.8)	94(34.4)	
Race			0.3
White	213(80.4)	205(75.1)	
Black	37(14)	47(17.2)	
Others	15(5.7)	21(7.7)	
Marital status			0.3
Yes	130(49.1)	122(44.7)	
Others	135(50.9)	151(55.3)	
Grade			0.3
I-II	59(22.3)	45(16.5)	
III - IV	67(25.3)	94(34.4)	
Unknown	139(52.5)	134(49.1	
Origin			0.5
Left	116(43.8)	113(41.4)	
Right	149(56.2)	160(58.6)	
Others		1	
Histology			0.002
Adenocarcinoma	96(36.2)	134(49.1)	
Squamous cell cancer	79(29.8)	80((29.3)	
others	90(34)	59(21.6)	
Radiation status			0.8
Yes	97(36.6)	98(35.9)	
No	168(63.4)	175(64.1)	
Chemotherapy			0.8
Yes	105(39.6)	106(38.8)	
No	160(60.4)	167(61.2)	
Insurance			0.8
yes	161(60.8)	168(61.5)	
others	104(39.2)	105(38.5)	

depending on the time of advanced NSCLC. Median OS was analyzed using the Kaplan–Meier (KM) method *via* the log-rank test and Cox proportional hazard model for multivariate analysis. Statistical significance was considered at a p value less than 0.05 and limits of 0.0001.

RESULTS

The database contained 538 advanced NSCLC (265 in nonimmunotherapy era vs. 273 in immunotherapy era). The age of over 70 years (e.g., 52% between 75 and 79 years in lung cancer) and male predominance were high in all cases of heart disease–related death in advanced NSCLC, and the distribution of variables was reasonably uniform in both eras ($p > 0.05$). The details of all characteristics are explained in **Table 1**.

The OS difference between the nonimmunotherapy era and the immunotherapy era for advanced NSCLC was statistically significant with the improvement in median survival of the immunotherapy groups (P = 0.0001; median survival: 4 vs. 6 months) (**Figure 2A**). By contrast, when considering only heart disease–related death, the OS in the nonimmunotherapy era was significantly better in advanced NSCLC than that in the immunotherapy era (5 months OS 42% vs. 0.33%; 10 months OS months 21% vs. 13% and median survival 4 vs. 3 months; P = 0.0001), indicating the negative effect of immunotherapy (**Figure 2B**).

In the KM study of NSCLC difference in OS between the variables of nonimmunotherapy and immunotherapy eras, older age and III–IV grades showed a significant difference in median OS (4 vs. 3 months, 4 vs. 2 months, respectively). In addition, a difference was observed among most other variables with survival

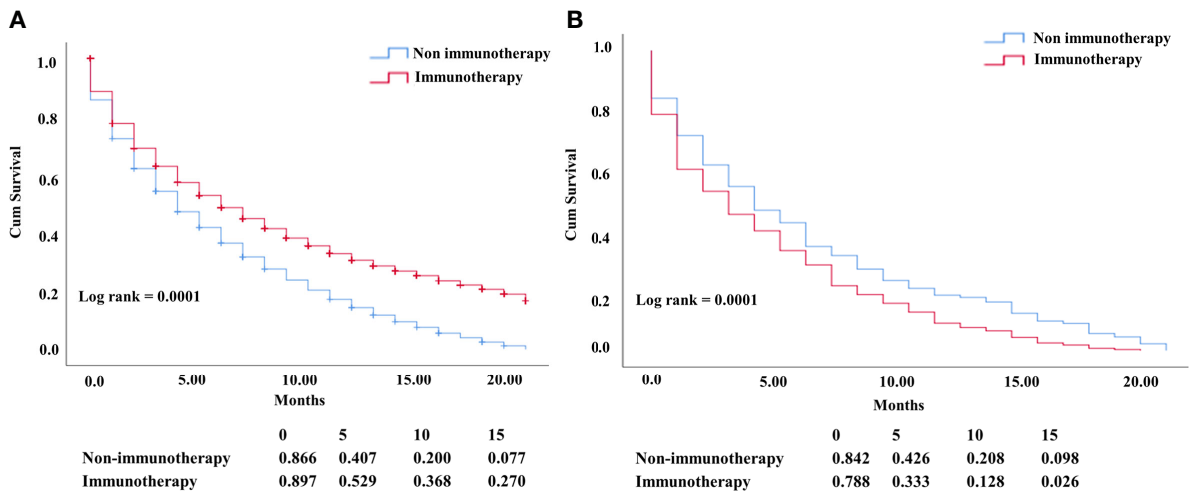

FIGURE 2 | **(A)** KM NSCLC curve comparing OS of all-cause mortality (P= 0.0001). **(B)** OS difference in patients who died from heart diseases with negative OS in the NSCLC immunotherapy era (P = 0.0001).

benefits restricted to patients in the nonimmunotherapy era (e.g., male, female, white, black, laterality, chemotherapy, and radiotherapy status) (**Figure 3**, **Table 2**). The OS in the immunotherapy era was studied, and the results reveal that chemotherapy and radiotherapy use had beneficial significance to OS (*P*= 0.0001, *P* = 0.001, respectively) with no preferences of each on other (*P* = 0.392). In addition, evidence of OS difference was not found in grade, histology, or laterality (*P* = 0.39, 0.08, 0.49, respectively) (**Figure 4A**). The negative OS of heart disease–related death in the immunotherapy era was still significantly evident after adjusting the data for age, sex, marital status, race, chemotherapy and radiation status, grade, laterality, histology, and insurance status by using Cox regression multivariate analysis (*P* = 0.003; hazard ratio [HR] = 1.31; 95% CI = 1.099–1.57) (**Figure 4B**).

The multivariate analysis of advanced lung cancer in the immunotherapy era revealed that the nonsquamous type was significant and showed worse survival of cardiac disease–related death patients than the squamous type (HR = 0.74; 95% CI = 0.55–0.99; *P* = 0.04). Chemotherapy use was not associated with poor OS in cardiac disease–related death (*P* = < 0.0001; HR= 1.812; CI = 1.37-2.38). Radiotherapy use in advanced lung cancer improved the OS in the nonimmunotherapy era but did not predict the OS in the immunotherapy era, mostly because of the large effect of chemotherapy use in this group, as shown in a separate model (*P* = 0.005; HR = 1.47; 95% CI = 1.12–1.93 vs. *P* = 0.06; HR = 1.29; 95% CI = 0.98–1.70) (**Table 3**).

DISCUSSION

The presence of baseline organ dysfunction in patients on immune check inhibitors demonstrates general immune

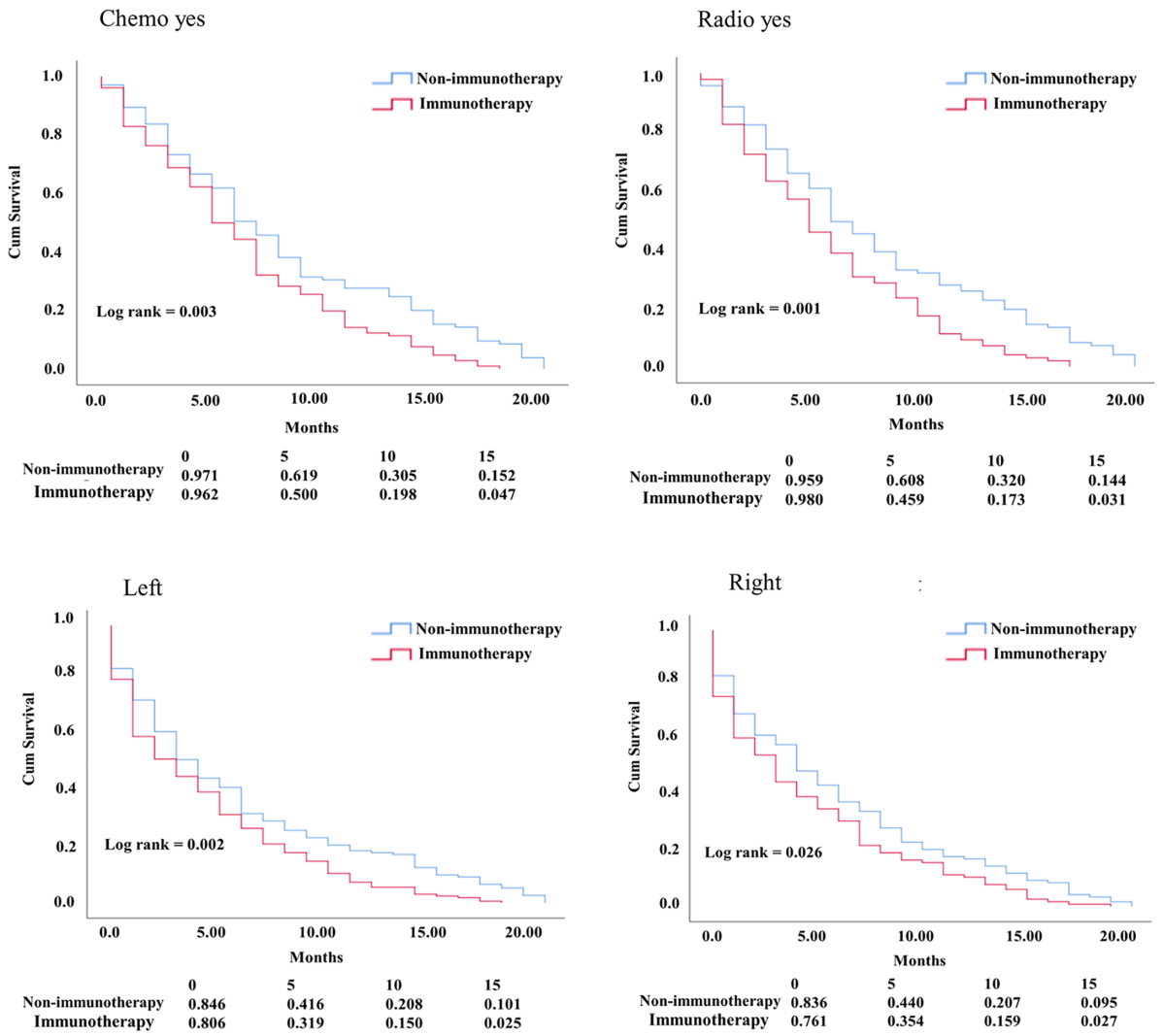

FIGURE 3 | KM curve comparing OS. Factors difference between NSCLC nonimmunotherapy versus immunotherapy era.

TABLE 2 | Survival pattern in heart disease–related death NSCLC patients.

Parameters	Nonimmunotherapy	Immunotherapy	*P* value bylog rank test
Age	median OS (months)	median OS (months)	
20-69	4	4	0.08
>70	4	3	0.001
Sex			
Male	4	3	0.007
Female	5	3	0.007
Race			
White	4	3	0.004
Black	5	3	0.03
Others	4	3	0.1
Marital status			
Yes	4	3	0.002
Others	4	3	0.02
Grade			
I-II	5	3	0.06
III-IV	4	2	0.006
Others	4	3	0.07
Unknown			
Left	4	3	0.02
Right	4	3	0.002
Missed 1			
Histology/ behave			
Adenocarcinoma	3	2	0.2
Squamous cell cancer	4	5	0.1
Others	6	3	0.0002
Radiation status			
Yes	6	5	0.001
No	2	1	0.01
Chemotherapy			
Yes	7	5	0.003
No	2	1	0.006
Insurance status			
Yes	5	3	0.003
No/unknown/ others	4	2	0.01

adverse events similar to those in previous clinical trials that included patients without organ dysfunction (8). Although it is representative of less than 1% of adverse immune events, immune inhibitor-caused myocarditis is a potentially fatal condition associated with 42% mortality (9). However, in general immunotherapy-related cardiotoxicity symptoms, several studies suggest an increase in patients with end organ failure compared with those without organ dysfunction (in both cases of myocarditis or controls without myocarditis) (10). The median time to the presentation of cardiotoxicity-related immunotherapy ranged from 2 to 454 days (23 months), and the majority occurred within the first four cycles of immunotherapy (1st month) (11). To the best knowledge of the authors, the present study was a large unique study that detected the OS of patients who died from heart diseases in the contemporary eras of immunotherapy and nonimmunotherapy NSCLC.

In this decade, many reported clinical trials for immunotherapy in various solid cancer types, including earlier challenging cancers, revealed an increase in OS and considerable strength in the treatment of NSCLC, in either combination or monotherapy (12, 13). The present study, which is a population-based study that used the SEER database, reveals good survival benefits in advanced NSCLC, which were largely attributable to the introduction of immunotherapeutic drugs to therapeutic regimens. Where the median OS increased from 4 to 6 months, this improvement was distinctly lower than those reported by related approved studies (14), However, the latter was predominantly related to the cutoff value for survival with only 20 months in all age groups regardless of their performance status and other comorbidities.

In the study of OS in patients with heart disease–related death, the OS was significantly decreased in the NSCLC immunotherapy era compared with the nonimmunotherapy era. The remaining patients in the first 5 months of survival from NSCLC were markedly lower in the treatment-based immunity era than in the nonimmunity-based era (33% vs. 42%). This finding could be explained by the following: 1) An association between high cardiotoxicity-related mortality rate and immunotherapy exists (15). 2) Regarding the relationship of cardiotoxicity and immunotherapy, most patients present their symptoms shortly after treatment (16). 3) The risk of incidence of major cardiac adverse events is increased in thoracic tumors (17). Except for adenocarcinomas and squamous cell histology of lung cancer, most variables demonstrate significant negative OS in the NSCLC immunotherapy era. In addition, aging is significantly linked to negative OS in patients with NSCLC who died from heart diseases; this finding was also recently shown in a SEER-Medicare study by Bora et al., who report that comorbidities and negative OS are related to old age in NSCLC patients who were started with immune checkpoint inhibitors (18). Recently, several reports indicate that the combining of chemoradiotherapy with immunotherapy has superior efficacy in producing improved anticancer activity (19). In the immunotherapy group of heart disease–related death, chemotherapy and radiotherapy use as monotherapy in advanced NSCLC exhibit improved OS compared with nonuse. In the study difference between radiotherapy and chemotherapy use, no survival significance has been seen. Thereby, the risk of decreased OS in this group was more independently associated with the use of immunotherapy than the synergetic negative effect of other interventions.

Cox multivariate analysis confirms that immunotherapy is a risk predictor for OS in patients who died from heart diseases in the NSCLC immunotherapy era (HR= 1.314; P = 0.003). Even though it was significant in older age based on KM, the difference in age groups demonstrated by the Cox model in the new era of NSCLC did not provide any benefits of survival. In a multicenter retrospective study of the association between age with immune-related events and OS in NSCLC, age was not an independent risk factor of survival (20). Chemotherapy and radiotherapy use was significantly observed in the nonimmunotherapy era as a high predictor of increased OS. By contrast, the use of chemotherapy significantly decreased the risk of heart disease–related death. Radiotherapy use was not a risk factor for survival

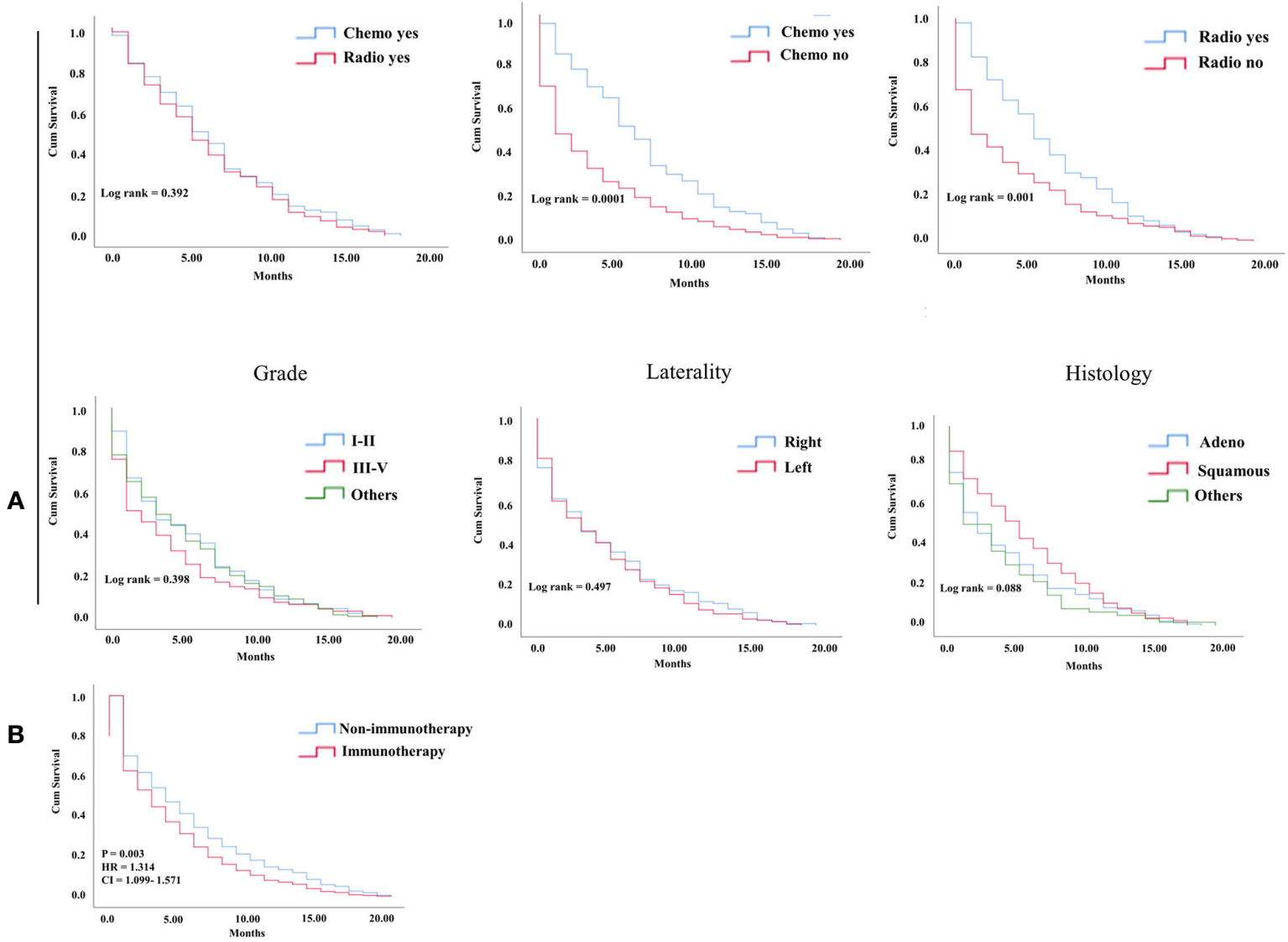

FIGURE 4 | **(A)** KM curve showing OS difference among factors in the NSCLC immunotherapy group of hear related- death patients. **(B)** Cox multivariate analysis difference survival of heart related death patients between NSCLC nonimmunotherapy group and immunotherapy group.

TABLE 3 | Univariate and multivariate analysis of factors affecting the survival in heart disease–related death NSCLC patients.

Parameters	Nonimmunotherapy				Immunotherapy			
	Univariate HR/CI	P value	Multivariate HR/CI	P value	Univariate HR/CI	P value	Multivariate HR(95% CI)	P value
Age (20-69 vs. >70)	1.06(0.82-1.36)	0.6	0.76(0.58-1.00)	0.05	0.92(0.72-1.17)	0.5	1.00(0.77-1.29	0.9
Sex Male vs. Female	1.07(0.83-1.38)	0.5	0.87(0.57-1.22)	0.3	1.03(0.80-1.33)	0.7	0.88 (0.67-1.16	0.3
Marital Yes - Others	0.84(0.66-1.07)	0.1	1.06(0.81-1.38)	0.6	0.93(0.73-1.18)	0.5	0.96(0.75-1.26)	0.8
Race								
White	Reference		Reference		Reference		Reference	
Black	0.81(0.57-1.15)	0.2	0.83(0.57-1.22)	0.3	0.97(0.70-1.34)	0.8	0.85(0.60-1.19)	0.3
Others	0.56(0.32-0.98)	0.04	0.51(0.28-0.91)	0.02	0.99(0.63-1.55)	0.9	0.82(0.51-1.30)	0.4
Chemotherapy yes vs. no	0.57(45-74)	<0.0001	1.72(1.31-2.25)	0.0001	1.76(1.38-2.26)	0.0001	1.81(1.37-2.38)	0.0001
Radiotherapy yes vs. no	0.61(48-0.79)	0.0001	1.47(1.12-1.93	0.005	1.46(1.13-1.87	0.003	1.29(0.98-1.70)	0.06
Grade								
I-II	Reference		Reference				Reference	
III-IV	1.14(0.80-1.62)	0.4	1.28(0.89-1.84	0.1	1.20(0.84-1.71)	0.3	1.24(0.86-1.79)	0.2
Unknown	1.30(0.95-1.77		1.23(0.88-1.72)	0.2	1.04(0.74-1.46)	0.8	1.05(0.73-1.49)	0.7
Laterality	0.99(0.77-1.26)	0.9	1.02(0.79-1.32)	0.8	0.93(0.73-1.18)	0.5	1.21(0.93-1.57)	0.1
Left vs. Right								
Histology/Behav								
Adenocarcinoma	Reference						Reference	
Squamous	0.77(0.57-1.04)	0.094	0.78(0.56-108)	0.1	0.80 (0.61-1.06)	0.133	0.74(0.55-0.99)	0.04
Others	0.71(0.053-0.09)	0.022	0.70(0.52-0.95)	0.02	1.12 (0.82-1.52)	0.474	0.96 (0.69-1.35)	0.8
Insurance yes vs. others	0.85(0.66-1.10)	0.228	1.07(0.81-1.41	0,6	1.21 (0.95-1.55)	0.114	1.25(0.96-1.61)	0.08

differences in the immunotherapy era, mostly due to the substantial effect of newly developed targeted therapies in recent years. The histology of squamous cancers demonstrated a prominent significance with positive survival benefits compared with nonsquamous cancers, indicating that histology could be a protective factor. Another explanation considers it could be the mortality from cancer and its poor prognosis compared with other types of histology (21, 22).

This study has several limitations. First, the SEER database did not provide an explanation about heart disease as the cause of death in patients as either real incidents before or newly related immunotherapeutic events. Second was the short median OS associated with the 20-month follow-up, the comorbidities, and the lack of performance status information. Third, the study lacked a detailed description of immunotherapy for patients.

CONCLUSION

This study demonstrates the OS benefits for NSCLC patients in the immunotherapy era compared with that in the nonimmunotherapy era that was primarily attributed to the immunotherapy. In heart disease–related death, immunotherapy in patients with NSCLC demonstrated decreased OS. Chemotherapy use increased the OS in patients with lung cancer who died from cardiac diseases, whereas no OS difference was found in radiotherapy use. Also, recognition of histology during immunotherapy, especially for nonsquamous types, could be considered as another predictor of OS reduction in patients who died from heart disease during immunotherapy. Although the incidence of cardiac toxicity is less than 1%, the risks must be assessed in all elderly patients with NSCLC. This study strongly highlighted effective clinical and preclinical studies to enhance the results.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ supplementary material.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Conceptualization: MS. Validation: MS and XS. Formal Analysis: MS, JL, MA, and AA-D. Investigation, writing, original draft preparation: MS. Visualization: FP, MA, AA-D and NA-S. Supervision: JL, and RK. Project administration: MS and JL. All authors contributed to the article and approved the submitted version.

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Lung Cancer Immunotherapy in Transplant Patients and in Patients With Autoimmune Diseases

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The use of immune checkpoint inhibitors (ICIs) delivered great and new possibilities in modern treatment of many types of cancers. This therapy based on blockade of such molecules as CTLA-4 (cytotoxic T lymphocyte-associated antigen), PD-1 (programmed cell death receptor type 1), or PD-1 ligand (PD-L1) brings a new hope for patients with non-small cell lung cancer (NSCLC), melanoma, or head and neck squamous carcinoma. Efficacy of immunotherapy was proven in many clinical trials. Unfortunately, ICIs treatment was not addressed to the patients with preexisting allogeneic transplants or autoimmune diseases mainly due to high risk of transplant rejection, exacerbation of autoimmune diseases, and risk of serious toxicity. However, it is possible to receive anti-tumor response to ICIs treatment avoiding graft rejection by adjusting the immunosuppression. Obviously, it depends on the type of transplants: the use of immunotherapy is usually possible in kidney or corneal recipients, but it could be difficult in patients with liver and heart transplant. Therefore, the development of biomarkers for tumor response and transplant rejection in ICIs treated patients is essential. Data coming from published literature support the possibilities of using ICIs in patients with preexisting autoimmune diseases who undergoing proper management of side effects of immunotherapy or when the potential benefits of such treatment outweigh the potential risks. This depends on the type of autoimmune disease and may be difficult or not feasible in patients with systemic lupus erythematosus or systemic sclerosis. Therefore, it may be appropriate to include cancer patients with preexisting autoimmune disease or with allogeneic transplants in clinical trials using immunotherapy when no other effective cancer treatment options exist.

Keywords: programmed cell death receptor type 1, PD-1 ligand, cytotoxic T lymphocyte-associated antigen, preexisting autoimmune disease, transplant recipients

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INTRODUCTION

The treatment of non-small cell lung cancer (NSCLC) is one of the most serious challenges facing modern oncology. It is due to continuous increase in morbidity and mortality caused by this type of cancer. According to the epidemiological data available on the World Health Organization (WHO) websites, in 2040 the estimated increase in the number of lung cancer cases in the world and the

number of deaths resulting from this disease will be 72.5% and 76.3%, respectively. These data clearly indicate the need for continuous development of modern therapies that can improve the treatment outcomes and reduce the number of deaths (1). Enormous expectations are associated with the introduction of immune checkpoint-blocking antibodies into routine clinical practice; mainly programmed death receptor 1 (PD-1) or its ligand (PD-L1) (2–5). The published research results have clearly demonstrated the therapeutic value of this form of immunotherapy. It should be kept in mind that clinical studies constituting the basis for the registration of PD-1 or PD-L1 inhibitors usually did not include indications for use in patients with a history of lung cancer concurrent with autoimmune disease or patients after organ transplants in whom the use of immunosuppressive therapy could lead to immune system dysfunction significantly affecting the detection and elimination of cancer cells (1–6). Understanding the mechanisms determining the occurrence of immune toxicity symptoms and concern for the safety of patients during therapy raised a number of questions regarding the possibility of using this form of treatment in patients with concomitant autoimmune diseases. This is all the more important since, according to the literature data, concomitant autoimmune diseases occur in 14%–25% of patients diagnosed with NSCLC (1). The inability to use immunotherapy in this clinical situation would determine the need for the application of chemotherapy or radiation therapy, which would be a suboptimal treatment for many patients.

A beneficial therapeutic effect resulting from the use of antibodies against PD-1 (nivolumab, pembrolizumab) or PD-L1 (atezolizumab, durvalumab), manifested in longer disease-free or overall survival, was initially demonstrated in patients experiencing cancer progression after the first and subsequent courses of platinum-based chemotherapy. As demonstrated in the CheckMate 017 study, the use of nivolumab in patients diagnosed with squamous cell carcinoma led to longer time to disease progression (3.5 vs. 2.8 months) and overall survival (OS) (9.2 vs. 6.0 months) compared to standard docetaxel-based treatment (2). The use of nivolumab in patients diagnosed with non-squamous cell lung cancer was associated with an increase in OS (12.2 vs. 9.4 months) without affecting progression-free survival (PFS) (2.3 vs. 4.2 months) (3). Slightly different results were obtained in the Keynote-010 study analyzing the efficacy and safety of pembrolizumab at a dose of 2 mg/kg body weight or 10 mg/kg body weight in patients diagnosed with NSCLC experiencing progression after chemotherapy. In this study, the inclusion criteria were PD-L1 protein expression on at least 1% of cancer cells. As demonstrated in the analyzes, the use of pembrolizumab, regardless of dose (2 or 10 mg/kg), was associated with a significant increase in median OS compared to docetaxel (10.4 and 12.7 months for pembrolizumab 2 and 10 mg/kg, respectively, and 8.5 months for docetaxel). The effect of pembrolizumab on the median PFS was observed only in the group of patients with PD-L1 expression on the surface of at least 50% of cancer cells (4).

Understanding the role of PD-1 and PD-L1 interactions in the regulation of anti-tumor response has also initiated a number of studies to determine the effect of using anti–PD-L1 antibodies in the treatment of patients with NSCLC. One of them was the POPLAR study analyzing the efficacy and safety of atezolizumab in NSCLC patients experiencing progression after previous treatment. It has been demonstrated that the use of this antibody, compared to docetaxel, was associated with a significant increase in OS (12.6 vs. 9.7 months), with its therapeutic effect being especially apparent in patients with high percentage of tumor cells with PD-L1 expression (5). The POPLAR study was extended to the OAK phase III study. Compared to docetaxel, atezolizumab was associated with an increase in median OS (13.8 vs. 9.6 months, respectively), the effect being particularly evident in patients with high percentage of tumor cells with PD-L1 expression (mOS 15.7 vs. 10.3 months) and independent of the histological subtype of lung cancer. The benefit of using atezolizumab was also observed in patients without PD-L1 expression on cancer cells (12.6 vs. 8.9 months) (6).

The results of the above studies constitutes the basis for registration of PD-1 or PD-L1 inhibitors for the treatment of NSCLC after the failure of previous therapies based on platinum derivatives, and also inspired a number of studies analyzing the effect of their use in previously untreated patients. One of them was Keynote-024, a randomized Phase III study comparing the benefits of first line therapy with pembrolizumab to platinum-based chemotherapy in patients with advanced NSCLC expressing PD-L1 on the surface of at least 50% of cancer cells. It demonstrated that the use of pembrolizumab as first-line treatment, compared to standard chemotherapy, led to an increase in median PFS (10.4 vs. 6.0 months, HR = 0.50, $p < 0.001$) and OS (30.0 vs. 14.2 months, HR = 0.63, $p = 0.002$) (7).

Statistically significant improvement in median PFS or OS following the use of immune checkpoint inhibitors (ICIs) in monotherapy as the first-line or subsequent therapy and striving for the improvement of treatment outcomes gave birth to a series of concepts involving the combination of PD-1 or PD-L1 inhibitor-based immunotherapy and chemotherapy or radiation therapy (8–11). These concepts were based on a few assumptions. The first, that the increase in the expression of tumor antigens released from cancer cells destroyed by chemotherapy should lead to increased activation of the immune response directed against the tumor cells. Another resulted from the observations demonstrating the increase in tumor immunogenicity due to the induction of tumor cell apoptosis as a result of activation of immunogenic cell death resulting from the use of cytostatics. As a result, damage-associated molecular patterns (DAMPs), including calreticulin, occur on the surface of cancer cells, providing a signal for the activation of immune response directed against cancer cells and, as a consequence, the maturation of dendritic cells and activation of cytotoxic T lymphocytes (8).

This concept formed the basis for a number of studies analyzing the effect of combining chemotherapy and immunotherapy based on anti–PD-1 antibodies in the first-line treatment of patients with NSCLC. The use of pembrolizumab in combination with platinum-based chemotherapy as first-line

treatment of NSCLC patients with non-squamous cell histology (Keynote-189) led to an increase in the median PFS (9.0 vs. 4.9 months, HR = 0.48) and median OS (22.0 vs. 10.7 months, HR = 0.56), and the observed clinical benefit was independent of PD-L1 expression on tumor cells (9).

The Keynote-407 study analyzed the effect of the combined use of pembrolizumab and chemotherapy in the first-line treatment of patients with advanced squamous-cell NSCLC. The use of combination therapy resulted in prolonged median time to disease progression compared to chemotherapy (6.4 vs. 4.8 months, HR = 0.56) and median OS (15.9 vs. 11.3 months, HR = 0.64), with the therapeutic effect being independent of PD-L1 expression, similarly to Keynote-189 study results (10).

A remarkably interesting concept was the use of immunotherapy in consolidation treatment in patients with locally advanced NSCLC. As demonstrated in the PACIFIC study, the use of durvalumab following radical radio-chemotherapy led to the increase in the median time to distant metastasis (11). Updated results from the study were presented at ASCO 2019 annual meeting. Median OS was not reached (NR; 95% CI, 38.4 months–NR) with durvalumab versus 29.1 months (95% CI, 22.1–35.1) with placebo. The 12-, 24-, and 36-month OS rates with durvalumab and placebo were 83.1% versus 74.6%, 66.3% versus 55.3%, and 57.0% versus 43.5%, respectively (7).

The presented beneficial therapeutic effects resulting from the use of immunotherapy based on checkpoint inhibitors are inextricably linked to the risk of complications following the activation of the immune system. The mechanism of their occurrence is not fully understood. As presented in the study of Postow et al., they may be the result of incorrect recognition of host cell antigens by the activated T cells, an increase in the concentration of antibodies recognizing autoantigens or an increase in the concentration of pro-inflammatory cytokines in tissues (12). The occurrence of immunological side effects in the course of therapy applies to almost all patients receiving PD-1, PD-L1 or CTLA-4 inhibitors, with approximately 15%–30% of patients reaching CTCAE (Common Terminology Criteria for Adverse Events) grade 3 or 4. In most patients, temporary cessation of therapy or the use of immunosuppressive treatment leads to amelioration or complete resolution of immunological toxicity enabling drug re-administration. In some patients, despite the use of steroids or other immunosuppressive medications, it is necessary to discontinue the therapy. This is more common in patients receiving anti–CTLA-4 antibodies than in patients treated with anti–PD-1 or PD-L1 (16% vs. <12%, respectively) (2, 13–18). The combined use of PD-1 and CTLA-4 inhibitors leads to a significantly higher percentage of grade 3 or 4 immune complications (46%–59% of patients) (19). The problem of toxicity of the applied immunotherapy is of particular importance in patients with concomitant autoimmune diseases.

In general, the emergence of autoimmune diseases is the result of disorders in the mechanisms that determine the tolerance of own antigens (8, 12, 19). They may be the consequence of incorrect elimination of autoreactive T lymphocytes during their maturation in the thymus, induction of antigen-specific regulatory T lymphocytes (Tregs), or they may stem from the disturbance in the mechanisms of peripheral tolerance, including antigen sequestration, determining varied immunogenicity of different autoantigen epitopes. Higher expression of proinflammatory cytokines in body tissues leads to increased expression of MHC (major histocompatibility complex) particles, co-stimulatory proteins, proteases, and subsequent host cell presentation of low immunogenicity epitopes recognized by the activated T cells (8). In the context of immunotherapy, the particularly important phenomenon is the lymphocyte anergy resulting from virgin lymphocyte stimulation only by a signal coming from the T cell receptor (TCR) in the absence of co-stimulatory signal coming from the CD28 receptor protein, which in turn can lead to lymphocyte death.

An additional factor inducing the state of anergy is the activation of immune checkpoints (e.g., CTLA-4, PD-1). This is important because stimulation of the PD-1 receptor inhibits the effector function of T cells in tissues, which is crucial for preventing the activation of autoreactive T cells in response to the autoantigen present in the body (8, 12, 19). In addition to maintaining peripheral tolerance, the interaction of PD-1 and PD-L1 plays a role in the selection of lymphocytes in the thymus and in immunologically privileged sites. Therefore, the use of therapy based on PD-1 blocking antibodies allows cytotoxic T lymphocytes to effectively destroy cancer cells at the cost of disrupting the process of self-antigen tolerance (20). Comprehension of these correlations helps explain a number of questions regarding the possibility of using immunotherapy in patients with concomitant autoimmune diseases.

POSSIBILITIES OF USING IMMUNOTHERAPY IN PATIENTS WITH CONCURRENT NEOPLASTIC AND AUTOIMMUNE DISEASES

ESMO (European Society for Medical Oncology) recommendations published in 2018 allow the use of immunotherapy based on PD-1-, PD-L1-, or CTLA-4-blocking antibodies in selected patients, noting that it may lead to exacerbation of the symptoms of autoimmune disease, requiring the use of immunosuppressive treatment (21). However, there are no study results that directly compare the toxicity resulting from the use of anti–PD-1 and anti–PD-L1 antibodies. However, it is commonly believed that side effects observed in the course of therapy with anti–PD-1 or anti–PD-L1 antibodies are less pronounced than those observed in the group of patients receiving anti–CTLA-4 antibodies or treated with a combination of anti–CTLA-4 and anti–PD-1 antibodies (22). Available literature data come from retrospective analyzes and include heterogeneous groups of patients both in terms of diagnosed cancers, applied immunotherapy, as well as the type of autoimmune disease.

Based on the literature analysis, Abdel-Wahab et al. presented the results of the use of immunotherapy in cancer treatment in

123 patients with concomitant autoimmune diseases presented in 49 publications (23). The dominant diagnosed types of cancer were cutaneous melanoma (83.7%), followed by NSCLC (13%), renal cell carcinoma (2.4%), and Merkel cell carcinoma (0.8%). In addition, 83.5% of patients received treatment for autoimmune disease prior to the introduction of immunotherapy, 46.2% had symptoms of active disease at the start of immunotherapy, and 43.6% required treatment due to symptoms of active autoimmune disease. Most of the analyzed patients received PD-1 inhibitors (52%) as part of their immunological treatment. Ipilimumab was used in 44% of patients included in the analyzes. Symptoms of toxicity related to immunotherapy were found in 75% of patients, with recurrence or exacerbation of concomitant autoimmune disease symptoms in 41% of patients, while 25% of patients had previously unobserved clinical symptoms, the most common of which were colitis (14%) and hypopituitarism (5%). Importantly, there were no differences in the incidence of adverse events associated with immunotherapy in patients with active and inactive autoimmune disease (67% vs. 75%). Exacerbation of autoimmune disease symptoms has been more frequently observed in patients receiving PD-1 or PD-L1 inhibitors than CTLA-4 inhibitors (62% vs. 36%). Whereas, the use of ipilimumab, compared to nivolumab or pembrolizumab, was associated with more frequent occurrence of immune toxicity symptoms, which had not been observed in patients with autoimmune diseases until the start of immune treatment (42% vs. 26%). The occurrence of adverse effects implied the need for high-dose corticosteroids in 62% of patients, which led to clinically significant improvement in the condition of 90% of these patients. In 17% of patients, despite the use of immunosuppressive therapy, it was necessary to discontinue treatment with ICIs. Five patients with autoimmune disease receiving immunotherapy died due to serious adverse events related to the treatment or progression of cancer (23–26). The occurrence and intensification of the toxicity associated with the applied immunotherapy was correlated with the recorded response to treatment. Partial or complete remission of the neoplastic lesions was found in 50% of patients experiencing adverse effects and 35.7% of patients in whom no complications of the applied treatment were reported.

The above observations are consistent with the results presented by Tison et al., who showed exacerbation of autoimmune disease symptoms in 47% of patients receiving immunotherapy, while in 84% of patients these symptoms did not differ from those observed before the introduction of treatment. On the other hand, intensification of the flare phenomenon was observed mainly in patients whose immunosuppressive therapy was completed less than 3 months before the start of immunotherapy (27).

Interesting observations were also provided by Leonardi et al. (1). The authors analyzed the available literature and identified 56 patients with advanced NSCLC receiving PD-1 or PD-L1 inhibitors who had a history of autoimmune diseases associated with inflammatory changes in the joints, skin and subcutaneous tissue as well as endocrine glands or autoimmune colitis. Seven patients had more than one autoimmune disease. At the start of immunotherapy, 10 of the analyzed patients had symptoms of active autoimmune disease and 11 patients were receiving immunosuppressive or immunomodulatory treatment. The exacerbation of the symptoms of autoimmune disease in the course of immunotherapy concerned only some patients and was characterized with low clinical severity, occasionally requiring intravenous corticosteroid use and withholding the cancer treatment. In most patients experiencing the exacerbation of autoimmune disease, no new symptoms resulting from stimulation of the immune system were observed, and none of the patients included in the study needed complete withdrawal of the immunological treatment. Exacerbation of disease symptoms was more frequently observed in patients whose cancer treatment was initiated in the active phase of the autoimmune disease, while the use or absence of immunosuppressive therapy did not significantly affect the severity of autoimmune symptoms after starting anti–PD-1 or anti–PD-L1 therapy (36 % vs. 20%, $p = 0.43$). Immunological adverse effects resulting from the antineoplastic therapy were present in 38% of patients and were usually of low severity. Grade 3 or 4 was found in 11% of patients, which is a percentage comparable to that observed in clinical studies excluding patients with concomitant autoimmune diseases at the recruitment stage (7%–15%) (1, 13). Only 4 patients required intravenous corticosteroids due to complications. The severity of adverse effects associated with the conducted immunotherapy was the reason for its premature termination in 8 patients (14%), which is a slightly higher percentage than that observed in patients without immune-related diseases (1, 13). Importantly, the use of ICIs in the first or subsequent lines of treatment had no effect on the risk of immune-related complications associated with the therapy or exacerbation of concomitant autoimmune disease. Analyzing the obtained responses, the overall response rate (ORR) of 22% was found in patients with concomitant autoimmune diseases treated with PD-1 or PD-L1 inhibitors, and the frequency of the observed exacerbations of the disease did not correlate with the noted response to immunological treatment (1, 28).

Another analysis, presented by Danlos et al. included 45 patients mainly with either cutaneous melanoma (36 patients) or NSCLC (6 patients), in whom immunotherapy was used to treat cancer despite the presence of autoimmune disease (29). The results of the analyzes were compared with data from 352 patients, with no history of autoimmune diseases, receiving PD-1 or PD-L1 inhibitors for cancer treatment. In result of the applied immunotherapy, the symptoms of immunological toxicity, mainly grade 2 and 3, occurred in 20 patients, whereby in 11 patients these complications were the result of exacerbation of the symptoms of autoimmune disease. The use of corticosteroids led to complete resolution of symptoms in 9 patients and allowed to continue the therapy with anti–PD-1 antibodies in 15 out of 20 patients. Interestingly, in 16 patients with concomitant autoimmune diseases, neither exacerbation of the autoimmune disease symptoms nor the occurrence of toxicity associated with the conducted immunotherapy was observed during the follow-up period (median 5.1 months). In the case of treatment with

anti–PD-1 antibodies, the history of autoimmune diseases determined a higher percentage of observed complications (44.4% vs. 29%) and a reduction in median time to treatment-related side effects (5.4 months vs. 13.0 months). Similarly to other analyses, the results obtained by Danlos et al. did not demonstrate impact of autoimmune diseases on OS and ORR in patients receiving treatment with ICIs.

Slightly different results were presented by Cortellini et al. (30). Based on clinical practice data, they showed a significantly higher frequency of complications of any CTCAE (Common Terminology Criteria for Adverse Events) grade associated with immunotherapy in patients whose cancer coexisted with autoimmune disease compared to that observed in the general population (65.9% vs. 39.9%) (30). The rates of grade 3 or 4 toxicities associated with the immunotherapy were not significantly different in both groups. More importantly, the presence of autoimmune disease was also unaffected by the median PFS and OS in result of the use of anti–PD-1 or anti–PD-L1 antibodies.

Due to the retrospective nature and relatively small groups of patients, the above analyzes should be interpreted with great caution. With one possible exception constituted by the analyzes of Weinstock et al., which included a total of 837 patients diagnosed with autoimmune disease receiving PD-1 or PD-L1 inhibitors to treat cancer (31). As shown, only 9% of patients experienced exacerbation of autoimmune disease in result of therapy, while treatment-related symptoms of toxicity occurred in 17% patients, including 3% with grade 3 or higher (31, 32).

Another "limitation" of the presented analyzes is the fact that they were based on data not derived from clinical trials, which may affect the quality of reporting adverse effects associated with immunological treatment or the symptoms of autoimmune disease exacerbation. The duration of immunotherapy and the extent of active follow-up of the patient after the completion of treatment are also important, as they determine the proper identification of distant toxicities associated with the therapy. Effectiveness of immunotherapy is also determined by treatment protocol and line of treatment in which was applied. The use of anti–PD-1, anti–PD-L1, or anti–CTLA-4 antibodies may be a beneficial and relatively safe therapeutic option in selected patients. The decision to start immunotherapy should be made within a multidisciplinary team and should take the dynamics of autoimmune disease and the need for immunosuppressive therapy into consideration. It seems that cancer treatment based on ICIs may be considered in patients, in whom the exacerbation of symptoms associated with the presence of autoimmune disease does not lead to conditions directly threatening the patient's life and does not require the use of high doses of corticosteroids and other drugs with immunosuppressive effects. As demonstrated by Martinez-Bernal et al., the use of immunotherapy in patients requiring high doses of steroids to control autoimmune symptoms is associated with a worse therapeutic outcome (33). The treatment should be based on PD-1 or PD-L1 inhibitors rather than CTLA-4-blocking antibodies. The combination of PD-1 inhibitors with CTLA-4 inhibitors is not recommended because numerous clinical studies have demonstrated its association with a higher percentage of CTCAE grade 3 and 4 complications (19, 26). Patients diagnosed with autoimmune disease of the nervous system should not be qualified for immunotherapy. The need for high doses of immunosuppressive drugs would imply reduced effectiveness of immunological therapy and may also be associated with greater difficulty in controlling potential immunological toxicity (19). The use of anti–PD-1, anti–PD-L1, or anti–CTLA-4 antibodies in patients with concomitant autoimmune disease is a procedure with a high risk of complications, therefore, it is not recommended to choose this type of treatment as adjuvant therapy.

CANCER IMMUNOTHERAPY IN PATIENTS AFTER ORGAN TRANSPLANTS

Another extremely interesting issue is the possibility of using immunotherapy in the treatment of cancer in patients after organ transplants. The use of immunosuppressive therapy is associated with a significantly increased risk of cancer (34, 35), which is the second most common cause of death in these patients (36). The risk of death in this group of patients is additionally dependent on the severity of the symptoms of immunosuppression toxicity, as well as on the selection of cancer therapy which, due to the risk of transplant rejection, may be suboptimal for a particular cancer treatment. It is also not entirely clear whether rejection of transplanted organs is a direct result of the use of immunotherapy based on PD-1, PD-L1, and CTLA-4 inhibitors or impaired immune response following treatment (37, 38). Moreover, no reliable risk factors for the rejection of the transplanted organ as a result of the applied immunotherapy have been established, nor has the immunotherapy scheme leading to a specific balance between therapeutic benefit and the risk of transplant rejection been described (28).

Despite the significance of the issue, the available literature data are limited and include mostly case reports (39–41) or results of retrospective analysis carried out in small groups of patients. Very interesting data come from analyzes performed by Abdel-Wahab et al. (36). The authors of the study analyzed the result of the use of immunotherapy administered as life-saving treatment in 39 patients with melanoma (62%), cutaneous squamous cell carcinoma (15%), hepatocellular carcinoma (10%), or NSCLC (8%) diagnosed in the course of immunosuppression after kidney (59%), liver (28%), or heart (13%) transplantation. The treatment mainly involved the use of PD-1 inhibitors (77%), while the combination of anti–PD-1 and anti–CTLA-4 antibodies was used in only 3% of patients. The time from transplantation to the introduction of immunological treatment ranged from 1 to 32 years (median 9.0 years). In result of the applied treatment, 16 patients (41%) experienced immunological reactions promoting the rejection of the transplanted organ; the median time from the start of immunotherapy to the onset of the said reactions was 21 days. Despite the reintroduction of immunosuppressive therapy, definitive transplant rejection occurred in 13 of these patients

(81%). The percentage of patients with transplant rejection was not correlated with the time since organ transplantation, the type of antibodies used to block the immune checkpoints or the transplanted organ. In 15 (38%) patients included in the analyzes, there were no signs of transplanted organ dysfunction or of toxicity associated with the conducted immunotherapy. Objective responses to the applied treatment were more frequently observed in patients without the initiation of transplant rejection reaction, as well as in those receiving steroids in a dose smaller than equivalent to 10-mg prednisone at the time of the introduction of immunotherapy (36, 39, 40, 42, 43).

Similar results are presented in the work of de Bruyn et al., who analyzed the effect of immunotherapy in patients with liver (19 patients) or kidney (29 patients) transplantation (42). Response to treatment was more frequently observed in patients after kidney transplantation than the ones after liver transplantation (45% and 21%, respectively), and this effect was also associated with slightly more frequent rejection of the transplanted organ (45% vs. 37%). In 21% of patients, the obtained immune response was not accompanied by the activation of immune responses associated with the rejection of the transplanted organ. The safety of ICIs in kidney transplant recipients was assessed by Monohar et al. (43). Based on a review of the literature, they identified 44 kidney transplant recipients diagnosed with melanoma (68%), lung cancer (11%), and metastatic squamous cell carcinoma of the skin (11%), or other malignant neoplasm (9%) treated mainly with nivolumab (24%), pembrolizumab (25%), or ipilimumab (20%). Acute renal allograft rejection was reported in 18 patients (41%). The median time from the initiation of immunotherapy to the diagnosis of acute rejection was 24 days. Twenty-five (59%) patients had no organ rejection. Complete response, partial response or disease stabilization were seen in 4, 5, and 3 patients, respectively. Progressive disease was diagnosed in 14 patients treated with ICI.

Another systemic review was done by the D'Izarny-Gargas group (44). They identified 48 original case reports or short series of 83 solid organ recipients, among whom kidney transplants were performed in 53 patients, liver in 24 patients, and heart transplantation in 6 of them. Skin melanoma was the predominant maligancy (46 patients). The next ones were hepatocellular carcinoma (12 patients) and squamous cell carcinoma of the skin (10 patients). The median time from completing organ transplantation to initiating immunotherapy was 9.3 years. Most patients received anti–PD-1 antibodies (60 patients) in immunological treatment. 13 patients were treated with ipilimumab, and combined anti–PD-1/anti–CTLA-4 therapy was used in 9 cases. Allograft rejection due to immunotherapy was observed in 33 patients. The median time from the initiation of ICIs to the onset of rejection was 5.6 weeks, but in the majority of patients, allograft rejection was noticed within the first 2 weeks of treatment. Median OS was significantly shorter in liver transplant recipients compared to those with kidney or heart transplant (29.0 vs. 36.0 vs. 46.0 weeks, respectively).

As presented above, the rejection of transplanted organs is one of the most frequently occurring immune complications, however, as the literature data show, it can significantly precede the occurrence of other "classic" adverse effects associated with immunotherapy (28).

The introduction of immunotherapy into routine clinical practice has brought a significant breakthrough in the treatment of many cancers, including NSCLC. The development of transplantology and immunosuppressive therapies results in longer survival of patients after organ transplantation and is the reason for the increase in cancer incidence in this group of patients. In every patient, the use of immunotherapy should be preceded by a detailed discussion of all risks associated with the therapy, including those resulting from transplant rejection, and the final decision should be made in consultation with the patient by a multidisciplinary medical team working closely with specialists in the field of transplantation or clinical immunology. Patients with pre-existing autoimmune disease always should be offered with clinical trials. According to clinicalTrials web page there are two trials dedicated for patients with pre-existing autoimmune disease: NCT03656627 (Nivolumab in Treating Patients With Autoimmune Disorders or Advanced, Metastatic, or Unresectable Cancer) and NCT03816345 [A Phase Ib Study of Nivolumab in Patients With Autoimmune Disorders and Advanced Malignancies (AIM-NIVO)].

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work. TK wrote the first draft of the manuscript. MN and PK contributed to preparation of the submitted and revised versions of the manuscript. All authors contributed to the article and approved the submitted version.

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Imperfect Predictors for Lung Cancer Immunotherapy—A Field for Further Research

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The armamentarium for lung cancer immunotherapy has been strengthened using two groups of monoclonal antibodies: 1) anti-PD-1 antibodies, including pembrolizumab and nivolumab, which block the programmed death 1 receptor on the lymphocyte surface, resulting in increasing activity of these cells, and 2) anti-PD-L1 antibodies, including atezolizumab, durvalumab, and avelumab, which block the ligand for the PD-1 molecule on tumor cells and on tumor-infiltrating immune cells. The effectiveness of both groups of antibodies has been proven in many clinical trials, which translates into positive immunotherapeutic registrations for cancer patients. Regarding the predictive factor, PD-L1 expression on cancer cells is the only biomarker validated in prospective clinical trials used for qualification to immunotherapy in advanced non-small cell lung cancer (NSCLC) patients. However, it is not an ideal one. Unfortunately, no clinical benefits could be noted in patients with high PD-L1 expression on tumor cells against the effectiveness of immunotherapy that may be observed in patients without PD-L1 expression. Furthermore, the mechanism of antitumor immune response is extremely complex, multistage, and depends on many factors. Cancer cells could be recognized by the immune system, provided tumor-specific antigen presentation, and these arise as a result of somatic mutations in tumor cells. Based on novel immunotherapy registration, high tumor mutation burden (TMB) has become an important predictive factor. The intensity of lymphocyte infiltration in tumor tissue may be another predictive factor. The effectiveness of anti-PD-L1 immunotherapy is observed in patients with high expression of genes associated with the effector function of T lymphocytes (i.e., their ability to produce IFN-gamma). This does not end the list of potential factors that become useful in qualification of cancer patients for immunotherapy. There remains a need to search for new and perfect predictive factors for immunotherapy.

Keywords: predictive factors, PD-L1, PD-1, immunotherapy, non-small cell lung cancer

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INTRODUCTION

The immune system is a key player in the efficient monitoring and destruction of cancer cells (1). Let us follow briefly how it works. The immune response cycle begins with the recognition of tumor antigens by antigen-presenting cells and presents them to T lymphocytes in the lymph nodes. Activated cytotoxic T lymphocytes (CTLs) migrate to peripheral tissues, actively seeking the antigen (2, 3). At the site of recognition, the intracellular cytotoxic proteins are released from the CTLs, and together with the nonspecific mechanisms provided by macrophages or NK cells, the cancer cells are eliminated (1–4). Why does this system fail in some cases? It seems that the reasons lie both on the side of insufficient immune system activation and the growing tumor tissue (2, 4). First, the immune checkpoints expressed on immune system cells [CTLA-4 on T regulatory cells, PD-1 on T lymphocytes, PD-L1 on tumor-infiltrating immune cells (IC), e.g., macrophages] play a crucial role in maintaining a balance between immune system overactivation and extinguishing its action (5, 6). Whereas tumor cells (TC) that expressed the PD-L1 molecule could very effectively block the activity of PD-1-positive T lymphocytes. Keeping this in mind, how could we suppress the inhibiting activity of cancer cells and restore the cytotoxic activity of T lymphocytes? It is time for immunotherapy.

From the moment that Professor James Allison and Professor Tasuko Honjo discovered the checkpoint molecules, the next step was to create specific monoclonal antibodies that, by blocking these molecules, restore immune system activity (7, 8). Since this moment, immunotherapy with immunological checkpoint inhibitors (ICIs) has revolutionized cancer treatment, especially for patients without actionable driver mutations (8, 9). In 2016, the American Society of Clinical Oncology named immunotherapy as a top cancer advance of the year.

In the field of lung cancer immunotherapy, two groups of ICIs are widely used. Anti-PD-1 antibodies include pembrolizumab and nivolumab and block the PD-1 receptor on the lymphocyte surface. Anti-PD-L1 antibodies include atezolizumab, durvalumab, and avelumab and block the ligand for the PD-1 molecule on TC and on tumor-infiltrating IC (8–11). Now, one of the most important questions tormenting oncologists is how to choose patients with lung cancer who will benefit the most from immunotherapy? The solution to this problem is to choose the right and most sensitive biomarker (12, 13). At present, the only validated biomarker with a qualification for cancer patients for ICIs is the percentage of TC and/or IC with PD-L1 expression (12–14). Moreover, tumor mutational burden (TMB) and microsatellite instability assay also have a predictive value in qualification for immunotherapy (14–16). However, one should remember the availability of tissue material from cancer patients. What should we do if we cannot collect the cancer cells, or if the tumor is heterogeneous and we only have a small biopsy of tumor tissue? Does the determination of selected markers in the blood serum or plasma of cancer patients give reliable results and indicate those who should be treated with ICIs? Should we make every effort to reobtain tissue material?

In this article, we focus on the advantages and disadvantages of all biomarkers that are approved or were tested in clinical trials and that could be used in qualification of cancer patients for immunotherapy.

BIOMARKERS RELATED TO TUMOR TISSUE

PD-L1 Expression

Immunohistochemical (IHC) testing for PD-L1 expression has become standard in the diagnosis of predictive factors in lung cancer (15–17). IHC is a relatively simple technique that is not related to any major problems. In many prospective trials, the efficacy of ICIs over standard chemotherapy as first-line therapy is demonstrated in patients with TC positive for PD-L1 (15–17). The KEYNOTE-024 study led to pembrolizumab registration in patients with metastatic non-small cell lung cancer (NSCLC) with expression of PD-L1 on ≥50% of TC (18). In this study, a significant increase in overall survival (OS) was observed in patients receiving pembrolizumab compared to patients treated with chemotherapy (18).

Moreover, based on the risk–benefit profile depicted in the KEYNOTE-042 study, pembrolizumab monotherapy can be extended by FDA registration (but not by the European Medicines Agency registration) as first-line therapy to patients with locally advanced or metastatic NSCLC without sensitizing *EGFR* or *ALK* alterations and with low PD-L1 expression (≥1%) as determined by an FDA-approved test (19).

However, advanced NSCLC patients, regardless of PD-L1 expression on TC, benefited from first-line combination therapy with platinum-based chemotherapy and pembrolizumab (KEYNOTE-189 and KEYNOTE-407 studies) (18–21). Regarding 2nd-line treatment, significantly longer survival was observed in locally advanced or advanced NSCLC patients receiving nivolumab (CheckMate-017 and CheckMate-057 studies) or atezolizumab (OAK study) compared with docetaxel regardless of PD-L1 expression although it should be noted that greater benefits were observed in patients with higher percentages of TC with PD-L1 expression (22–24). Pembrolizumab in the 2nd-line treatment could be used in patients with ≥1% of TC with PD-L1 expression (KEYNOTE-010 study) (25, 26). It should be mentioned that significant clinical efficacy of maintenance durvalumab therapy was observed in locally advanced NSCLC patients who received concurrent chemoradiotherapy (PACIFIC trial) (27, 28). In this study, patients were enrolled regardless of PD-L1 expression, but *post hoc* subgroup analysis of progression-free survival (PFS) and OS showed significant clinical benefits from durvalumab therapy when PD-L1 expression was present on ≥1% of TC (27). Recently, the FDA approved three new therapeutic strategies for first-line therapy in metastatic NSCLC patients: atezolizumab in patients with ≥50% of TC with PD-L1 expression (Impower-110 study); combination therapy with nivolumab and ipilimumab in patients with ≥1% of PD-L1 positive TC (CheckMate-227 study); and combination therapy with nivolumab, ipilimumab, and

chemotherapy in patients regardless of PD-L1 expression on TC (CheckMate-9LA study) (29–33).

Additionally, an important issue regarding the benefit from immunotherapy in PD-L1 negative patients was raised in the CheckMate 227 trial (30, 34). In the subset of PD-L1 negative tumors, a significantly stronger survival benefit was observed in patients treated with nivolumab plus ipilimumab compared with chemotherapy. However, direct comparison of OS in PD-L1 negative tumors between combined immunotherapy and chemo-immunotherapy was not performed; a higher response rate for chemo-immunotherapy rather than for the nivolumab plus ipilimumab combination (38% *vs.* 27%) was observed. Based on that, we could speculate that immunotherapy combination and chemo-immunotherapy regimens demonstrated similar efficacy in PD-L1 negative patients (30, 34).

The abovementioned important clinical trials that resulted in immunotherapy registration clearly indicate that PD-L1 expression was an important factor for stratifying patients to receive ICIs and to obtain clinical efficacy (14–16, 35). However, it is surprising that different therapies used in different lines are registered in patients with different PD-L1 status on TC. It should be considered whether PD-L1 expression is an ideal biomarker or whether is it associated with ambiguity and controversy. Among the many unsolved questions concerning PD-L1 expression, the following aspect should be mentioned: 1) use of different cutoff levels for the percentage of PD-L1 positive TC for different ICIs; 2) differences in testing platforms; 3) the heterogeneous expression of this molecule through the tumor; its dependence on the histological type of TC; and the history of treatment (chemotherapy and radiotherapy could change PD-L1 expression on TC).

The scoring system for PD-L1 expression is varied for each immunotherapeutic. There were two categories in clinical trials with pembrolizumab: ≥50% of TC with PD-L1 expression is considered sufficient although <50% was insufficient for qualification for first-line therapy (18, 21). Pembrolizumab could be administered as second-line therapy if PD-L1 expression was observed on ≥1% of TC and in the first line with low PD-L1 expression (≥1%) but only in the United States (18, 19, 21, 35). Unlike this, nivolumab and atezolizumab could be ordered irrespective of PD-L1 expression on TC in second-line therapy (22–24, 31). However, in clinical studies with nivolumab in the second line, patients were stratified for PD-L1 expression into 4 groups: <1%, ≥1%, ≥5%, and ≥10% of PD-L1 positive TC. An even more complex scale was adopted in the OAK study. The efficacy of atezolizumab was assessed in 4 groups based on the PD-L1 expression on TC and tumor-infiltrating IC. Percentages of PD-L1-expressing TC were as follows: TC3 ≥50%, TC2 ≥5% and <50%, TC1 ≥1% and <5%, and TC0 <1% and percentage of tumor area infiltration by IC were as follows: IC3 ≥10%, IC2 ≥5% and <10%, IC1 ≥1% and <5%, and IC0 <1% (24). In the PACIFIC trial, ≥25% and ≥1% of TC with PD-L1 expression was used for assessment of durvalumab therapy efficacy; however, clinical benefits were observed irrespective of PD-L1 expression (27, 28). One should also remember that effectiveness of different treatment lines is also associated with various methods of qualification for treatment based on PD-L1 expression assessment.

As was mentioned, testing for PD-L1 expression by the IHC technique is considered to be a standard in predictive factor diagnosis. Unfortunately, each clinical trial with different immunotherapeutics used different anti-PD-L1 antibody clones and a different commercially available platform for testing. Trials with nivolumab used a 28-8 antibody clone, studies with pembrolizumab used a 22C3 clone, studies with atezolizumab used a 142 clone, and trials with durvalumab used an SP263 clone. The epitopes for anti-PD-L1 binding are in the extracellular domain for 28-8 and 22C3 antibody clones, and those for SP142 and SP263 are in the cytoplasmic domain (12, 21–24, 33, 35, 36).

In previous years, two large studies concerning the specificity and sensitivity of all the anti-PD-L1 antibody clones were conducted (Blueprint-1 and Blueprint-2) (35, 37, 38). In both studies, three companion assays—with 22C3 (used for pembrolizumab), 28-2 (used for nivolumab), and SP263 (used for durvalumab) antibody clones—achieved comparable specificity and sensitivity. Clone SP142, used for atezolizumab, was found to be less sensitive (37–39).

A quite complicated situation was resolved in 2015. The FDA approved the IHC 22C3 pharmDx assay as a companion diagnostic for the identification of NSCLC patients for pembrolizumab therapy (11, 12, 18). Moreover, in 2017, an IHC assay using the SP263 antibody clone, previously used for PD-L1 testing in clinical trials with durvalumab, received the CE mark for its use in PD-L1 testing during qualification for pembrolizumab immunotherapy. Therefore, PD-L1 expression is no longer required to be tested with the 22C3 antibody although this test is still approved for diagnosis (14–16).

The last problem associated with PD-L1 diagnosis is the heterogeneity of its expression within the tumor and its variability observed between primary and metastatic sites (14, 15, 40). Mansfield and colleagues examined paired primary lung tumor tissue and metastatic brain tissue and demonstrated that many of the brain metastases significantly lacked PD-L1 expression even when it was present in the primary lung cancer specimens (40).

During qualification of NSCLC patients for ICI treatment, all the limitations related to PD-L1 expression assessment described above as well as the spatial and temporal heterogeneity of the tumor microenvironment should be kept in mind.

Tumor Mutational Burden

Cancer cells can be recognized by the immune system if there are tumor-specific antigens on their surface, and these arise as a result of somatic mutations in TC (14–16, 41, 42). During TC transformation, their genetic materials are very unstable, and gene reparation does not always occur properly. The total number of mutations within a tumor genome counted per coding area of a tumor genome is defined as the TMB (39). A higher number of somatic mutations causes an increased number of neoantigens, which translates into increased immunogenicity of such tissues (39). Cancer tissues with high TMB are thought to

be more sensitive to immunological checkpoint inhibitors. Therefore, TMB could be a potential important biomarker in qualification to ICI therapy. Indeed, it has been used in numerous clinical studies (39, 41, 42).

The first studies that indicated its predictive value related to second-line therapy were clinical trials with atezolizumab (24, 31, 33). The OAK and POPLAR studies assessed TMB in patients' blood and used a different cutoff level for TMB: 10, 16, and 20 mutations (mut) per megabase (Mb). Both studies reported a positive correlation between the number of mutations and OS as well as PFS of patients treated with atezolizumab. Moreover, both studies used the same platform for TMB assessment (Foundation One) (24, 31, 33, 35, 36).

The CheckMate-227 study is extremely important for the use of TMB as a predictive marker. In this study, combination therapy with ipilimumab and nivolumab was administered in first-line settings for chemotherapy-naive stage IV or recurrent NSCLC patients (32, 43, 44). The cutoff level for TMB was estimated at 10 mut/Mb. A significantly higher OS (18.3 months) was observed for high TMB patients (≥10 mut/Mb) when compared with patients (12.7 months) with low TMB (<10 mut/Mb). Furthermore, the significant efficacy of immunotherapy observed in patients with high TMB was irrespective of PD-L1 expression on tumor cells. The benefit of combination immunotherapy was observed even in patients with high TMB and <1% of TC with PD-L1 expression (32, 43, 44).

This biomarker could serve as a more sensitive predictor of immunotherapy benefit than PD-L1 expression on TC. However, it does not happen because, as a biomarker, TMB has some strong limitations (44–46). First, different clinical studies used various cutoff levels for defining TMB, ranging from 10 to 15 mut/Mb in tissue and from 6 to even 20 mut/Mb in plasma-derived, cell-free DNA (44, 47). In some studies, the cutoff scale was two points and in others 3 points and some of the studies defined TMB as high, medium, or low. In this regard, there is no standard definition of TMB that could be used to determine the level of mutations in further studies. However, the CheckMate 568 study used combination therapy (nivolumab and ipilimumab in untreated advanced NSCLC patients) and demonstrated that there was no evidence of increased immunotherapy efficacy in patients with very high TMB (≥15 mut/Mb) compared to patients with high TMB (≥10 mut/Mb) (43).

Second, different platforms were used for TMB estimation and various genetic techniques, including whole genome sequencing (WGS), whole exome sequencing (WES), or comprehensive genomic sequencing (CGS). Some panels require parallel sequencing of a paired normal specimen to exclude germline variants from analysis; others remove germline variants from tumor sequencing results (39, 45, 46). A harmonization study of TMB determination in NSCLC samples using three different commercially available sequencing methods was conducted by Garido-Martin and colleagues. They suggested that wider sequencing for more accurate TMB assessment is needed to reduced misclassification (48).

Ultimately, different materials were tested from NSCLC patients: tissue biopsies or peripheral blood. It should be noted that, in both peripheral blood and tumor tissue, the percentage of results that could not be analyzed was relatively high (32, 47). In the CheckMate 227 study, which used tissue materials and the Foundation One CDx assay, the rejection rate was estimated at 47% of analyzed specimens (32). This suggests that, for reliable TMB testing, particularly good quality material is required. These facts translated into FDA registration of nivolumab in combination with ipilimumab in advanced NSCLC patients based on the results of the CheckMate 227 study. This combination therapy can be administered as first-line therapy in patients with ≥1% of TC expressing PD-L1. TMB in these patients does not need to be evaluated (11, 32, 39, 43).

Taken together, TMB has a notably great potential as a predictive biomarker. Indeed, further standardization of methods used in TMB assessment and systematic evaluation of TMB across different sequencing platforms should be undertaken before it is fully incorporated into clinics.

Immunoprofile and Gene Expression Signature of Tumor Tissue

Studies carried out in many different cancers have proven that the presence of IC, especially tumor infiltrating T lymphocytes (TILs) in the tumor tissue, is associated with higher benefits from immunotherapy (14–16, 49, 50). These observations are widely described in patients with breast cancer or melanoma (49, 50). Moreover, using data regarding ICI effectiveness in various malignancies, it has been indicated that tumors have three immunoprofiles based on their immune system activation: 1) "hot" tumors, which are strongly infiltrated by T lymphocytes and with many inflammatory signals; 2) "cold" tumors, which have scant IC infiltration or inflammatory signs; and 3) tumors with immune exclusion, in which immune cells are at the periphery or within the stromal tissue (49–51).

Are similar divisions of cancerous tissue described in NSCLC, and even more interestingly, has analysis of the immune system in cancerous tissue ever been used as a predictor for ICI efficacy?

In the case of lung cancer, it seems that a clear division into three types of tumor tissue has never been used prospectively as an ICI predictor in clinical trials. Rather, the immune gene signature profile, particularly those associated with IC activation (e.g., INF-γ signaling), instead of immunological examination, is correlated with immunotherapy outcome (14, 15, 31). In the POPLAR trial, in which the efficacy of atezolizumab was compared with docetaxel in a second-line setting, retrospective analysis showed that significant improvement in patient survival was associated with a high expression of the interferon-γ gene and genes associated with the T-effector activation (defined by *CD8A*, *GZMA*, *GZMB*, *IFN-γ*, *EOMES*, *CXCL9*, *CXCL10*, and *TBX21* gene expression) (31). All these genes had high co-expression in tested tumor specimens, which have been previously associated with activated T cells, immune cytolytic activity, and interferon-γ expression (31). It is obvious that tissue with high expression of

these mentioned genes meets the criteria for a "hot, inflamed tumor," but it was based on genetic, not immunological, examination. The "hot" tumors are associated with denser PD-1-positive T lymphocytes infiltration with a preexisting primed immune response, and they are more likely to respond to an anti-PD-1 or anti-PD-L1 blockade used as monotherapy (31, 49).

Are these relationships also observed when we use a combination of immunotherapy, which seems to have a great importance for the future? In the IMpower 150 study, among patients with no PD-L1 expression on TC, with low expression of the genes responsible for T-effector activation, and with liver metastases, a significantly longer median PFS was observed in the group of patients receiving combination therapy (atezolizumab, bevacizumab, chemotherapy) than in patients receiving only bevacizumab with platinum doublets (33). However, when tissue samples were qualified as "inflamed," more benefits from combination therapy were observed in patients carrying "hot" tumors.

The greater efficacy of immunotherapy in patients with "inflamed" than in patients with "non-inflamed" tumors seems to be well documented in the literature (50, 51). What about the tumor, where the preexisting immune response is located at the invasive tumor margin? Lung cancer trials are rather scarce on this observation. However, it seems, based on melanoma trials, that tumor regression after therapeutic PD-1 blockade requires tumor infiltration by CD8-positive cells (50). Pretreatment samples obtained from melanoma patients who later responded to pembrolizumab therapy showed a higher number of CD8-positive, PD-1-positive, and PD-L1-expressing cells at the invasive tumor margin and inside tumors (52).

Currently, none of anti-PD-1 or anti-PD-L1 antibodies could be administered based on the immunological status of the tumor tissue (11, 12). However, immunological analysis or estimation of the gene expression profile in cancer tissue could be considered to be a reliable biomarker in the prospective qualification for immunotherapy. What should be chosen for the future direction? Immunological analysis of the existing immune response in cancer tissue could be added into the basic pathomorphological diagnosis. This is a relatively quick and inexpensive technique, but it requires several serially cut tissue specimens. Molecular analysis of cancerous tissue evaluated by gene expression could be carried out simultaneously in one tissue fragment, but it requires a specific molecular platform as a microarray. However, for both these methods, a bright future is ahead, and expanding the benefits from immunotherapy based on profiling of immune and genetic characteristic of tumors is possible, but prospective validation is still needed.

Genetics Biomarkers

Very recent studies indicate that some genetic abnormalities could be considered as predictive biomarkers for immunotherapy. *STK11/LKB1*-inactivating mutations have been significantly linked to a primary resistance to PD-1 inhibitors (53). Patients harboring the *STK11* mutation had a significantly lower expression of PD-L1 molecules on TC and higher TMB score and no clinical benefits were observed when they received immune checkpoint inhibitors with a median survival of only 6 months. Moreover, the presence of this mutation strongly correlated with the low immune cell infiltration within the tumor tissue (53). That indicates that an *STK11*-positive tumor could be defined as the cold type, which directly translates into poor immunogenicity. Quite often the *STK11* mutation significantly coexists with the *KRAS* and *KEAP1* mutations in cancer patients. The kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) intracellular pathway is defined as a factor regulating genes related to the cellular protective response as well as to resisting the action of chemotherapy drugs (54, 55). Malfunctioning of *Nrf2* and *Keap1* genes has been observed in lung cancer, and it is possible that they are associated with tumor progression, cytoprotection, and poor prognosis. However, clinical implementation of Nrf2 inhibitors in patients with advanced NSCLC may be a useful therapeutic approach for patients harboring *KEAP1-NRF2* mutations, increasing the chance for clinical response (54, 55).

In the context of genetic markers, their determinations seem to be of great importance in predicting resistance to immunotherapy. T cell–mediated cytotoxicity could be deregulated by mutations in genes involved in chromatin remodeling pathways. The mutations in the *SWI/SNF* (SWItch/sucrose nonfermentable) complex as well as in the *PBAF* complex (PBRM1, ARID2, and BRD7) regulate the chromatin opening for the IFN pathway in TC, resulting in an increased resistance to lymphocyte cytotoxicity. This resistance can be reversed by *PBRM1* as well as *ARID1A* gene inactivation (56).

BIOMARKERS RELATED TO PERIPHERAL BLOOD

One could imagine an ideal situation in which biomarker determinations for ICI qualification is performed in a material as easily accessible as peripheral blood. This is already established for patients progressing on EGFR tyrosine kinase inhibitors when the presence of the Thr790Met mutation in the *EGFR* gene is examined in peripheral blood for osimertinib qualification (57, 58). Is there any chance that biomarkers tested in peripheral blood would indicate a group of patients benefiting more from immunotherapy? To date, most published analysis of peripheral blood biomarkers has been tested retrospectively (12–14). However, it seems that they have a lot of information about the activity of the immune system in cancer patients.

PERIPHERAL BLOOD SOLUBLE BIOMARKERS

The most investigated serum soluble biomarker is blood tumor mutational burden (bTMB), estimated by commercial platforms (e.g., the FoundationOne CDx assay) in cell-free DNA (not in peripheral blood circulating cancer cells) (59, 60). The most recognizable studies that determined bTMB were POPLAR, OAK, and CheckMate 227 (24, 31, 32). Gandara et al. described a novel, technically robust, blood-based assay to measure bTMB based on hybridization-capture methodology,

which is distinct from tissue-based approaches (47). First, they showed positive correlation between blood and tissue TMB in advanced NSCLC patients treated with second- or third-line immunotherapy included in the POPLAR trial. The cutoff points for bTMB that significantly correlated with outcomes of patients treated with atezolizumab were confirmed in the OAK study (24, 31, 47). They found that significantly longer PFS in atezolizumab-treated patients was associated with higher bTMB, and the definition of high TMB was estimated as ≥16 mut/Mb (24, 31, 47). Both studies have undoubtedly shown that bTMB could be a predictive biomarker for ICI qualification. Notwithstanding patients with bTMB ≥16 mut/Mb showed benefits from combination immunotherapy (tremelimumab plus durvalumab) in the MYSTIC clinical trial. In addition, patients with TMB ≥10 mut/Mb in tissue usually had TMB ≥16 mut/Mb in their blood serum (61, 62). The MYSTIC trial was a negative study in which the clinical benefit of combination therapy over chemotherapy was not demonstrated, but this study allowed a prospective determination of the TMB ≥10 mut/Mb cutoff threshold for the CheckMate 227 study.

PERIPHERAL BLOOD–SOLUBLE PD-1 AND PD-L1

The soluble form of PD-L1 (sPD-L1) is usually undetectable in the plasma of healthy people (63). However, detection of sPD-L1 is associated with a poor prognosis in various cancers. Moreover, a high level of sPD-L1 is associated with systemic inflammation and with activation of a nonspecific immune response (63). Taken together, this factor could be considered a predictive marker for immunotherapy qualification. So far, only one study has looked at the use of sPD-L1 as a prognostic factor in NSCLC patients. Interestingly, in the group of EGFR-mutated NSCLC patients, the increased level of sPD-L1 during erlotinib therapy was associated with a better prognosis. It is remarkably interesting because it is obviously known that patients with driving mutations do not receive benefits from immunotherapy (63). Meyo MT et al. showed the potential predictive role of soluble sPD-1 and sPD-L1 expression examination in metastatic NSCLC patients receiving nivolumab therapy (63). After two cycles of nivolumab therapy, increased sPD-1 levels, compared with the baseline value, independently correlated with longer PFS (adjusted HR=3.32; p=0.013) and OS (adjusted HR=0.33; p=0.006), and this relation was not seen when analyzing other soluble biomarkers (e.g., sCD40L, sCD44, or VEGFA). The authors proposed that composite biomarker analysis using sPD-1 and sPD-L1 could predict nivolumab efficacy (63). Zhang J et al. determined the expression of circulating PD-L1 in samples taken from 109 advanced NSCLC and 65 healthy patients (64). The results were analyzed with the association between clinicopathologic features and prognosis. First, Zhang J et al. showed higher PD-L1 expression in advanced NSCLC patients compared with the healthy controls ($p<0.001$). Moreover, high PD-L1 expression was positively correlated with shorter OS compared with low expression (18.7 vs. 26.8 months, $p < 0.001$). The presented results may give hope for the future use of sPD-1 or sPD-L1 determinations as prognostic factors (64). Moreover, the high levels of these molecules were related to intense inflammation and unspecific immune response activation, which is also considered to be a positive factor from immunotherapy benefits.

PLASMA-CIRCULATING TUMOR DNA AND PD-L1-CARRIED EXOSOMES

When we discuss the determination of predictive factors in a patient's blood serum, do not forget about the possibility of examining plasma-circulating tumor DNA (ctDNA). Ricciuti B et al. showed that assessment of plasma ctDNA would enable early detection of response to immunotherapy in NSCLC patients even before radiological examinations (65). Advanced NSCLC patients treated with first-line pembrolizumab +/- platinum doublet chemotherapy were enrolled in this study, and plasma samples were collected prior to starting therapy and serially during treatment. ctDNA was analyzed by NGS using targeted amplification of hot spots (65). Ricciuti B et al. showed that median PFS (mPFS) and median OS (mOS) were significantly longer among patients with low ctDNA levels tested at baseline compared with those with an increase in ctDNA (mPFS: 13.7 vs. 3.4 months, HR:0.20, $P < 0.01$; mOS: 32.8 vs. 14.7 months, HR:0.06, $P < 0.01$) (65). Similarly, Jia N et al., in a study of metastatic colorectal cancer patients treated with a first-line chemotherapy regimen, showed that changes in ctDNA determined by various techniques may have a strong predictive value for the assessment of patients' responses (66). Taken together, these results suggest that rapid changes in ctDNA could be applied as an early pharmacodynamic biomarker of response or resistance to immunotherapies (65, 66). Unfortunately, today it seems that the potential related to ctDNA examination is not fully utilized in the clinic.

The presence of exosomes that carry PD-L1 molecules on their surface may be another predictive factor of response to anti-PD-1 or anti-PD-L1 antibody therapy (67, 68). Exosomes, which are extracellular vehicles, are produced by cancer cells and released into the tumor microenvironment. In malignant melanoma patients, the high level of PD-L1-carried exosomes positively correlated with IFN-γ and indicated a high-level stimulation of an adaptive response in the early course of the disease (67, 68). Studies reported by Chen G et al. indicated that the level of PD-L1-carried exosomes could be a predictive factor that stratifies patients qualified for immunotherapy into two groups: responders with a high level of PD-L1-carried exosomes and nonresponders with a low level of these molecules (69). However, there are two important issues that should be kept in mind. First, there are methodological difficulties in examining the level of exosomes. At present, this test is not performed as a

routine predictive factor but as research used for scientific purposes. Second, we should consider whether patients with high levels of PD-L1-carried exosomes should be treated with anti-PD-L1 or anti-PD-1 monoclonal antibodies or with antibodies specifically blocking PD-L1 exosomes or with combination therapy that can lock both of these points. These questions need to be answered in the future.

PERIPHERAL BLOOD CELLULAR BIOMARKERS

We should consider the use of conventional signs of inflammation tested in peripheral blood, such as LDH, C-reactive protein, or IL-6 concentration. Current data indicate only retrospective analysis of inflammatory-associated factors in NSCLC patients who received immunotherapy (12–15).

The simple analysis of selected peripheral blood parameters provides basic but particularly important information about the patients' immune system status. However, it is problematic to talk about predictive factors based on simple blood testing. This testing could be a great source of information about rapid progression during ICI therapy (70). For instance, the neutrophil-to-lymphocyte ratio (NLR), which could be simply calculated from a complete blood testing report, attracted a lot of interest regarding detection of rapid progression during ICI treatment (71, 72). The studies conducted in the tumor microenvironment of different solid cancers demonstrated that increased neutrophil infiltration should be considered as a factor promoting tumor progression (70–72). The NLR has also been studied in NSCLC patients. Takeda and colleagues reported that NLR could distinguish between nonresponders and responders to nivolumab therapy at an early stage of treatment, which is crucial for rapid progression diagnosis (73). The results show that low NLR (<5) after 4 weeks of nivolumab administration was significantly associated with higher median PFS compared to patients with NLR≥5. Takeda et al. indicate that the expression of these markers fluctuates dramatically during treatment; therefore, repeated evaluation is essential (73). Liu J and colleagues explored the systemic immune-inflammation index (SII), which combines NLR and platelet-to-lymphocyte ratio (PLR) (74). SII is a novel inflammatory marker, and it is considered to be an independent risk factor for the development of solid cancer. Low SII, NLR, and PLR were significantly associated with higher median PFS for metastatic NSCLC patients treated with nivolumab as second- or third-line treatment (74).

A literature review shows that more effort is made to retrospectively assess baseline peripheral blood biomarkers and associate them with clinical outcomes in NSCLC patients treated with immunotherapy (71, 72). Tanizaki and colleagues evaluated the relationship between survival and peripheral blood parameters measured before nivolumab initiation, including absolute neutrophil count (ANC), absolute lymphocyte count (ALC), absolute monocyte count, absolute eosinophil count (AEC), serum C-reactive protein, and lactate dehydrogenase concentrations (70). Low ANC, high ALC, and high AEC were significantly and independently associated with both higher mPFS and mOS in multivariable analysis. Additionally, the patients with only one positive predictive factor had a significantly worse outcome than those with two or three factors. All patients with ≥50% PD-L1 expression on TC had at least two favorable factors (70). This may suggest that high PD-L1 expression on TC influences systemic inflammation parameters, and combined analysis of those parameters could better predict response to ICI therapy. Unfortunately, the studies have been conducted on a small group of patients, and future validation is still necessary.

Some studies report that clinical benefits could be predicted based on regulatory T cell examination in patients with melanoma (75). Moreover, a high percentage of myeloid-derived stem cells (MDSCs) in peripheral blood was negatively correlated with the clinical benefit for ipilimumab-treated patients (75). Regrettably, those examinations are of very marginal importance.

CONCLUSION

Unquestionably, the effectiveness of immunotherapy has been proven in many clinical studies and documented by numerous registration approaches. Nevertheless, the issue that still raises some concerns is the use of appropriate biomarkers for qualification of cancer patients to immunotherapy. The presented work summarizes the most important information about biomarkers that could be used in the clinic. All this information is summarized in **Table 1**, but be aware of the following points. First, NSCLC patients should always be qualified for immunotherapy in regard to the registration summary of each immunotherapeutic based on the predictive factors that are dedicated to them. Currently, expression of the PD-L1 molecule on TC for immunotherapy of advanced NSCLC patients is the only predictive factor validated in prospective clinical trials. However, recently, new immunotherapeutic registrations are based on TMB as a predictive factor although this has not been validated as deeply as PD-L1 expression.

Based on the clinical trials conducted so far, we could conclude that one perfect predictive biomarker does not exist, and during qualifying cancer patients for immunotherapy, at least two biomarkers should be taken into account. One should remember that the immune system is a complex network of intercellular interactions, and it is difficult to talk about a single factor that determines its activity. Unfortunately, it seems that we will never achieve the situation that occurs for molecularly targeted therapy, in which one driving mutation affects treatment effectiveness. In addition, the situation with biomarkers could be more complicated when new immunotherapies targeting the remaining negative immune control points, anti-TIGIT or anti-TIM-3, are introduced in the clinic.

TABLE 1 | Summary of the most important advantages or disadvantages of the described biomarkers used in qualification of NSCLC patients to immunotherapy.

Biomarkers related to tumor tissue	
PD-L1 expression on tumor cells or tumor-infiltrating-immune cells	• only validated biomarker in many prospective trials • positivity for PD-L1 expression was defined using different values of PD-L positive tumor cells percentage • evaluation of percentage of tumor area infiltrated with immune cells expressing PD-L1 is extremely difficult and useless • expression was tested with different platforms • tumor tissue could demonstrate heterogenicity for PD-L1 expression • PD-L1 expression could depend on the histological type of tumor cells and patients' history of treatment
Tumor mutational burden	• proven to be a valuable factor in combination therapy regardless of PD-L1 expression • various cutoff levels for defining TMB level • different platforms were used for TMB estimation • different samples were tested for TMB with high rejection rate related to tumor samples
Immunoprofile of tumor tissue	• has never been used in prospective trials with ICI therapy • immunological analysis could be added into the basic pathomorphological diagnosis • relatively quick and inexpensive technique • requires several serially cut tissue specimens
Gene expression signature	• interferon-γ gene signature retrospectively demonstrated predictive value for ICIs therapy • molecular analysis could be carried out simultaneously in one tissue specimen • required specific molecular platform
Mutations in immunotherapy-resistance genes: *STK11*, *KEAP1*	• estimated by NGS technique or single-gene testing • significantly associated with poorer OS • lacks prospective validation in clinical trials
Biomarkers related to peripheral blood	
Peripheral blood-soluble biomarkers	
Blood TMB	• positive correlation between blood and tissue TMB was shown • determined in the most easily accessible blood samples • has never been used prospectively as an ICI therapy predictor
Soluble PD-1, PD-L1	• increase level at baseline correlated with ICI benefit • used as additional research • used only as retrospective factors
ctDNA and PD-L1-carried exosomes	• rapid changes in ctDNA as an early pharmacodynamic biomarker of response or resistance to ICIs • used as additional research • used only as retrospective factors
Inflammation parameters tested in blood analysis: LDH, C-reactive protein, IL-6 plasma, or serum concentration	• no impact on ICI effectiveness • used as additional research • usually in scientific research
Peripheral blood cellular biomarkers	
Neutrophil-to-lymphocyte ratio (NLR)	• simple analysis performed during completed blood testing • could be a great source of information about the rapid progression during ICI therapy • it fluctuates dramatically during treatment; repeated evaluation is essential
Systemic inflammation parameters: absolute neutrophil count (ANC), absolute lymphocyte count (ALC), absolute monocyte count, absolute eosinophil count (AEC)	• has never been used prospectively as a ICI predictor • significantly and independently associated with PFS and OS • systemic inflammation parameters combined with PD-L1 expression could better predict response for ICIs therapy

Second, we do not dismiss the possibility of using knowledge about additional biomarkers. We should also remember that many biomarkers that have not been registered so far can greatly facilitate monitoring of immunotherapy effectiveness. They are imperfect indeed but still could be important to prevent rapid tumor progression or for identification of the site effects of immunotherapy.

AUTHOR CONTRIBUTIONS

KW-K—literature review, preparing the manuscript, editing the manuscript TK—literature review, preparing the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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The Role of Intratumor Heterogeneity in the Response of Metastatic Non-Small Cell Lung Cancer to Immune Checkpoint Inhibitors

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Immune checkpoint inhibitors (ICIs) represent one of the most promising therapeutic approaches in metastatic non-small cell lung cancer (M-NSCLC). Unfortunately, approximately 50–75% of patients do not respond to this treatment modality. Intratumor heterogeneity (ITH) at the genetic and phenotypic level is considered as a major cause of anticancer therapy failure, including resistance to ICIs. Recent observations suggest that spatial heterogeneity in the composition and spatial organization of the tumor microenvironment plays a major role in the response of M-NSCLC patients to ICIs. In this mini review, we first present a brief overview of the use of ICIs in M-NSCLC. We then discuss the role of genetic and non-genetic ITH on the efficacy of ICIs in patients with M-NSCLC.

Keywords: metastatic non-small cell lung cancer (NSCLC), tumor heterogeneity, immunotherapy, tumor mutation burden, tumor microenvironment, neoantigens, programmed-death 1 (PD-1), programmed-death ligand 1 (PD-L1)

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IMMUNE CHECKPOINT INHIBITORS IN METASTATIC NON-SMALL CELL LUNG CANCER: AN OVERVIEW

Understanding the interactions between the immune system and cancer cells has greatly advanced our knowledge of the mechanisms of tumor growth and progression (1). By now, it is clear that the immune system plays a pivotal role not only in eradicating the disease in cancer patients, but also in promoting a long-lasting immunity (2). This key observation has paved the way to the development of immunomodulating agents and opened the era of cancer immunotherapy (3), which culminated with the assignment of the Nobel Price to James P. Allison and Tasuku Honjo in 2018 (4). Modulation of interactions between T-cells, antigen-presenting cells, and tumor cells has helped unleash suppressed immune responses and increase the effective elimination of cancer cells (1, 2).

The availability of immune checkpoint inhibitors (ICIs) has radically changed the management of patients affected by M-NSCLC (3). Commonly used ICIs in these patients include the monoclonal antibodies: nivolumab (5–8), pembrolizumab (9–12), durvalumab (13), atezolizumab (14–16), and avelumab (17), which act by targeting immune checkpoints expressed by tumor infiltrating lymphocytes (TILs)— programmed-death 1 (PD-1)—or expressed by cancer and tumor infiltrating immune cells— programmed-death ligand 1 (PD-L1) (18, 19). The selection of which ICI to use depends on the expression of PD-L1, which can be evaluated using various assays, whose

clinical validity has been assessed in numerous clinical trials (5, 6, 8–10, 12–16). ICIs have proven to be better tolerated than standard chemotherapy (2, 6, 10). However, the response to single-agent ICI therapy is not durable, and only a minority of patients have a prolonged benefit (2, 9, 14). Moreover, there is now evidence that dual blockade of CTLA-4 and PD-1 receptors is sufficient to induce unique cellular responses compared with agents blocking these receptors given alone to M-NSCLC patients (20).

Most studies conducted so far have shown that the response to ICIs in M-NSCLC patients is independent of the histological subtype (squamous or non-squamous histology) (1, 3). However, many factors contribute to the extent of the response as well as the risk of developing resistance to ICIs in these patients (1, 3). In this review, we discuss how both genetic and non-genetic intratumor heterogeneity (ITH) influences the immunogenicity of M-NSCLC, and highlight the importance of integrated genomic, pathologic and immunologic analyses to refine the selection of M-NSCLC patients who may be candidates to treatment with ICIs.

DETERMINANTS OF RESPONSE TO IMMUNE CHECKPOINT INHIBITORS

The Tumor Microenvironment

A key determinant of the response of M-NSCLC patients to ICI therapy is the tumor microenvironment (TME) (21–23). The TME is the ensemble of tumor cells, non-tumor cells including carcinoma-associated fibroblasts and immune cells, extracellular matrix as well as blood and lymphatic vessels composing a neoplastic lesion (24–27). Among malignant cells, the TME contains tumor cell subclones expressing phenotypic traits that protect them from the hosts immune system and support their ability to invade the extracellular matrix and extravasate (22, 28). On the other hand, the TME also contains a rich repertoire of tumor-infiltrating immune cells including T- and B-cells, neutrophils, dendritic cells, myeloid-derived suppressor cells, or tumor-associated macrophages, that normally constitute a natural barrier to carcinogenesis (22, 24). Inside the TME, the activity of these immune cells is strongly suppressed by cytokines, growth factors and matrix re-modelling enzymes secreted from cancer cells (22, 24, 29). These immunosuppressive effects are further intensified by the intensive aerobic glycolysis metabolism, which is observed in many tumors (22, 24–26). The TME is able to control the accumulation of T-cells inside the tumor by multiple regulatory mechanisms (30, 31). Notably, the type, density and location of immune cells within the TME play an important role in the progression of the disease and have both predictive and prognostic values in patients with M-NSCLC (31, 32). Based on the density and location of CD4 and CD8-positive TILs in the tumor center and infiltration margins of TME, tumors have been classified as "hot" when they have a high number/density of TILs or as "cold" when they contain a low number of TILs (30, 33, 34). M-NSCLCs generally fall into the "hot" category and, accordingly, patients with M-NSCLC respond relatively well to ICIs (21). However, the response is generally limited to a small subset of patients (21, 35), which is likely due to differences in the cellular composition and spatial organization of the TME, as we discuss further.

Programmed-Death 1 and Programmed-Death Ligand 1

A key feature of M-NSCLCs that respond to ICIs is the expression of PD-1 and its ligand PD-L1 in the TILs and tumor cells of the TME (22, 29, 33). PD-L1 is expressed on the surface of tumor cells and binds to its cognate PD-1 receptor on the surface of B- and T-cells, regulatory T-cells, and NK cells (18, 19). In NSCLCs, the expression of PD-L1 protein was shown to be predictive of the response to ICIs (5, 6, 8–15). However, the expression of this protein can vary substantially between primary and metastatic lesions, as well as depending on the TME composition (2, 18). In general, M-NSCLCs are thought to be immunogenic and anti-PD-1 or anti-PD-L1 antibodies are most effective when the TME is characterized by high levels of PD-L1 expression and a high density of TILs (22, 23, 36). In the absence of TILs and positive PD-L1 expression on tumor cells, treatment with anti-PD-1 or anti-PD-L1 antibodies is expected to be less effective (23, 24, 36). One possibility is also that TILs are present in the TME, but do not express PD-1, leading to an alternative immunosuppressive mechanism (24, 36). In addition to the PD-1/PD-L1 axis, other immune regulators such as myeloid-derived suppressor cells, tumor-associated macrophages, NK cells, dendritic cells, B-cells, and various chemokine/cytokine networks operate in the TME, which likely play important roles in defining the sensitivity of M-NSCLCs to ICIs (2, 22, 24, 26).

Some challenges of the prediction of the response to ICI in M-NSCLC come from methodological variabilities, as well as, various clinically approved cut-off scores for PD-L1 expression assessment (37, 38). First of all, there is no uniformity in PD-L1 assessment among numerous clinical trials that evaluated immune checkpoint inhibitors in M-NSCLC (18, 39). Moreover, these trials used different cut-offs for considering a sample as PD-L1 positive, as summarized in **Table 1**. Notably, some studies were based on a single biopsy assessment, making the results more susceptible to intratumor heterogeneity, whereas others relied on archival tissue in which the expression might change over the time (18, 40–42). Furthermore, data on PD-L1 testing in cytological specimens, which are the predominant sample type at some institutions, are limited (43). Moreover, IHC antibodies typically bind PD-L1 at only two small hydrophilic regions that make them structurally unique and might be differentially accessible in fresh frozen *versus* Formalin-fixed paraffin-embedded (FFPE) samples (18, 39, 41). Likewise, also glycosylation of PD-L1 could cause its polypeptide antigens inaccessible to PD-L1 antibodies, which could lead to inaccurate IHC staining (44). Therefore, removal of the glycan moieties from PD-L1 to expose its polypeptide antigens has the potential to improve its detectability and to increase its utilization as a diagnostic biomarker to predict response to ICIs therapy (45).

TABLE 1 | Characterization of IHC assays used for PD-L1 assessment in different clinical trials.

PD-L1 clone (species)	Company (platform)	Tested ICI (target)	Trial(no. of patients)	Cell type for PD-L1 scoring	Percentage of PD-L1 positive cells(cut-offs)	Indication
22C3 (Mouse)	Dako (Autostainer Link 48)	Pembrolizumab (PD-1)	KEYNOTE-001 (12) (495) KEYNOTE-010 (9) (1,034) KEYNOTE-024 (10) (305) KEYNOTE-021 (11) (123)	Tumor cells	TC < 1% TC ⩾ 1%, TC ⩾ 50% (min. of 100 TC)	Second-line (⩾1% of TC) First-line (⩾ 50% of TC)
28-8 (Rabbit)	Dako (Autostainer Link 48)	Nivolumab (PD-1)	Checkmate-017 (5) (272) Checkmate-057 (8) (582) Checkmate-026 (6) (541)	Tumor cells	TC < 1% TC ⩾ 1% TC ⩾ 5% TC ⩾ 10% (min. of 100 TC)	Second-line regardless of PD-L1 expression
SP142 (Rabbit)	Ventana (BenchMark ULTRA)	Atezolizumab (PD-L1)	OAK (14) (850) POPLAR (15) (287)	Tumor cells, Immune cells	TC < 1% and IC < 1% TC ⩾ 1% or IC ⩾ 1% TC ⩾ 5% or IC ⩾ 5% TC ⩾ 50% or IC ⩾ 10% (min. of 50 TC with associated stroma)	Second-line regardless of PD-L1 expression
SP263 (Rabbit)	Ventana (BenchMark ULTRA)	Durvalumab (PD-L1)	PACIFIC (13) (149)	Tumor cells	TC < 1% TC ⩾ 1% TC ⩾ 25% (min. of 100 TC)	Maintenance therapy after chemoradiotherapy (⩾1% of TC)
73-10 (Rabbit)	Dako (Autostainer Link 48)	Avelumab (PD-L1)	JAVELIN (17) (184)	Tumor cells, Immune cells	TC < 1% TC ⩾ 1% (min. no of cells not defined)	Not approved

ICI, immunological checkpoint inhibitor; TC, tumor cells; IC, immune cells.

Tumor Mutation Burden

In addition to the TME and PD1/PD-L1, the amount of mutations expressed by a tumor—known as tumor mutation burden (TMB)—is another major determinant of the response of M-NSCLC patients to ICIs (37, 38, 46). A summary of trials that have evaluated the association between the TMB and ICI efficacy is presented in **Table 2**. Several studies reported that TMB ≥10 mutations per megabase (mut/Mb) is predictive of longer progression-free survival (PFS) and overall survival (OS) during ICI (37, 47). In addition, the B-F1RST trial reported that TMB ≥16 mut/Mb in cell-free DNA is associated with significantly longer PFS (16). A higher TMB (>10 mut/Mb) was found in M-NSCLCs harboring driver mutations in *KRAS* or *BRAF* genes, but not in tumors with *EGFR*, *ALK*, *ROS1*, or *MET* gene mutations (3.1–6.2 mut/Mb) (48). Furthermore, adenocarcinomas were found to carry a lower TMB compared to squamous cell carcinomas (9.1 *vs.* 11.3 mut/Mb on average, respectively) (49). This observation might be explained by the fact that the etiology of adenocarcinomas is independent of tobacco exposure, making these tumors endowed with a lower neoantigen burden and therefore less immunogenic (25, 50).

The TMB is typically estimated based on either whole exome sequencing (WES) or targeted sequencing (TS) of the DNA extracted from a tumor (46, 51). However, these methodologies have different sequencing coverage and depth, and therefore provide a different sensitivity and specificity in estimating the TMB (38, 46, 52). TS, which covers pre-specified small exonic or genomic regions, makes the assessment of the TMB easier, cheaper, and more practical in a clinical setting (37, 38, 46, 52). However, TS panels cover a substantially smaller fraction of the genome compare to WES probes, carrying the risk of actual TMB underestimation (53, 54). It was suggested that TS panels covering less than 300 genes or 1 Mb cause unreliable TMB results and should be avoided (54). Importantly, a crucial step in correctly estimating the TMB is the bioinformatic selection of tumor-specific single-nucleotide variants (SNVs) by filtering out germline or synonymous SNVs, which represent false positives and are unlikely involved in neoantigen generation, respectively (55, 56). Likewise to tumor-specific SNVs, also frameshift indels (small insertions and deletions) are considered a highly immunogenic mutational class that trigger an increased quantity of

TABLE 2 | Summary of clinical trials that have evaluated different TMB cut-offs for predicting the response to immunotherapy.

Trial	Treatment arms	Cut-off (mutation per megabase)	No. of patients	OS		PFS	
				Median	HR	Median	HR
CheckMate 026 (6)	NIVO vs CTH	High TMB	107	18.3	1.1	9.7	0.62
		Low or medium	195	12.7	0.99	4.1	1.82
CheckMate 227 (7)	NIVO + IPI vs CTH	TMB ≥ 10	199			7.2	0.58
		TMB < 10	380			3.2	1.07
OAK (14)	ATEZO vs. CTH	TMB ≥ 10	251		0.69		0.73
		TMB ≥ 16	158		0.64		0.65
		TMB ≥ 20	105		0.65		0.61
POPLAR (13)	ATEZO vs. CTH	TMB ≥ 10	96		0.59		0.67
		TMB ≥ 16	63		0.56		0.57
		TMB ≥ 20	42		0.51		0.58
B-F1RST (16)	ATEZO	bTMB ≥ 12	22			3	0.95
		bTMB < 12	36			3.2	
		bTMB ≥ 14	14			3.4	0.73
		bTMB < 14	44			3.2	
		bTMB ≥ 16	14			9.5	0.49
		bTMB < 16	47			2.8	
		bTMB ≥ 20	8			9.5	0.23
		bTMB < 20	50			2.7	

ATEZO, atezolizumab; CTH, chemotherapy; HR, hazard ratio; IPI, ipilimumab; NIVO, nivolumab; OS, overall survival; PFS, progression-free survival; TMB, tumor mutational burden; bTMB, blood based TMB.

neoantigen, moreover, it was reported that both the SNPs and frameshift burdens are significantly associated with ICIs response (57). In addition, pre-analytical and analytical factors such as the use of FFPE samples as a source of genomic DNA, a low tumor purity or a dense TME reduce the sensitivity of TMB determination, both for TS and WES (38, 46, 47, 51).

Neoantigens

Neoantigens are proteins with modified epitopes because of somatic mutations in their coding genes. These epitopes are loaded onto HLA molecules and displayed on the surface of tumor cells (25, 58). Neoantigens can be recognized as foreign by the host's immune system, ultimately triggering a T-cell mediated antitumor response (50, 58). A higher TMB is expected to increase the likelihood of recognition of the tumor by neoantigen-reactive T-cells (59). In M-NSCLC patients, the co-existence of a high TMB and neoantigen expression has a positive predictive value of the response to anti-PD1, anti-PD-L1 and anti-CTLA-4 therapy (13, 60). Some studies have suggested that neoantigen heterogeneity may influence immune surveillance, however, clonal and subclonal neoantigens do not drive equally immunogenicity (13, 50, 59). Mutations induced by cytotoxic therapy enhance the subclonal neoantigens burden and might not elicit an effective antitumor response (50, 61). On the other hand, the extensive clonal mutational repertoire present in smoking-associated M-NSCLC (5) could render this disease sensitive to T-cell therapies targeting multiple clonal neoantigens, in combination with appropriate modulation of immune checkpoints (50). Likewise, the observation that the expression of neoantigens is subjected to the genetic control may have important implications for predicting the response and resistance to ICIs, and might be harnessed to develop vaccines or adoptive cell therapies (25, 58, 62). Activity of T-cells by the amount of neoantigens expressed within the tumor is regulated by the inflammatory microenvironment that controls the availability of immune-regulatory checkpoints for T-cell (22, 28, 63, 64). Tumor subclones expressing neoantigens may be preferentially eliminated by the immune system resulting in neoantigen loss (25). However, it is unclear which neoantigens are depleted as the result of the response to the therapy or tumor dissemination, and whether such phenomena only lead to tumor escape or may be harnessed to improve the response (25, 50, 58, 62).

THE ROLE OF INTRATUMOR HETEROGENEITY

In M-NSCLC, both spatial and temporal heterogeneity are considered as a main indicators of tumor diversity (65–67). The spatial type of ITH is related to discrepancies between different regions within the same tumor and may be detected at genetic and immunological level leading to a heterogeneous immune response in distinct populations of cancer cells (65, 66, 68–70). The expression of PD-1 or PD-L1 might vary considerably from region to region within the same tumor, as a result of somatically acquired genetic differences. Up to 40% of M-NSCLC patients have substantially different anti-PD-1 resistance scores in different regions of the same tumor, which often leads to discordant predictions of the extent of response to anti-PD-1 or anti-PD-L1 inhibitors (41, 70). Depending on the study, 2–46% of small biopsy samples were found to give false-negative PD-L1 expression results in comparison to surgically resected specimens (40–42, 71, 72). Moreover, in M-NSCLC, the heterogeneity of PD-L1 expression is also observed not only within the primary tumor, but also within and between coexisting metastases

(40, 70–72). The metastatic sites may affect the value of PD-L1 as a predictive biomarker for ICIs treatment in NSCLC. Namely, specimens from lymph node metastases have low PD-L1 expression and are not preferred to guide ICIs treatment in clinical practice or in clinical trials (65). Among distant metastases of NSCLC, liver and adrenal sites have high PD-L1 expression, whereas it is low in brain and bones metastases (65, 73). Low PD-L1 expression in brain metastases may be related to the immune sanctuary features of this site (65, 67), whereas, bone tissues have a small pool of effective cytotoxic immune cells and a relatively large accumulation of suppressor immune cells (65, 74). This immune imbalance may favor the development of bone metastases with less selective pressure from the immune system, making PD-L1 expression in bone metastases less important for immune escape (65, 69, 74). On the other hand, liver and adrenal glands are immunologically equipped for effective tumor surveillance with potent cytotoxic T-cells and, therefore, they require inhibitory mechanisms, like up-regulation of PD-L1 expression, for cancer cells to survive (65, 69, 73).

In contrast to the spatial ITH, the temporal heterogeneity is created in between different time points during the disease course (67). The anticancer therapies may increase genetic ITH by shaping a new subclones with different somatic mutations, moreover, tumors with a highly heterogeneous subclonal structure might not produce enough neoantigens for T-cells to mount an effective anti-tumor response upon treatment with ICIs (46, 47, 50, 75). It was reported that the first line of M-NSCLC treatment may potentially affect immune response during cancer evolution leading to the response to ICIs in various ways (75, 76). In overall, chemotherapy, radiotherapy, and EGFR or ALK tyrosine kinase inhibitors increase PD-L1 expression, suggesting that up-regulation of PD-L1 is one approach that cancer cells may use to evade immune-mediated cell destruction (65, 77–79). It is worth to add that increase of PD-L1 expression after administration of the cytotoxic agents is insignificant, whereas ICIs significantly decrease the PD-L1 expression within M-NSCLC (65, 79). Today, the decision whether to administer ICIs to M-NSCLC patients is based on PD-L1 staining in primary lesions (80). However, considering the above mentioned facts that the PD-L1 status might change during treatment, all M-NSCLC patients should be re-biopsied and tested for PD-L1 expression upon therapy failure or at the time of disease progression (81).

In addition to genetic ITH, also non-genetic heterogeneity might influence the response of M-NSCLC patients to ICIs. As mentioned above, the heterogeneity of the TME can affect pathological stage, treatment efficacy and prognosis (29), and is an important predictor of antitumor response (22, 24). For example, the amount of desmoplastic stroma and the balance between promoting and inhibiting angiogenic factors (*e.g.* the vascular endothelial growth factor, VEGF) may influence the penetration of ICIs in the TME (82, 83). Additional spatial heterogeneities in the TME might cause an uneven penetration of these agents and contribute to the emergence of resistant cell populations or to the development of hypoxic niches that might support cancer stem cell phenotypes and immune evasion (21, 29, 84). Furthermore, in M-NSCLC, the tumor immune evasion capacity may be modulated at different stages of the disease either by factors stimulating the tumor immune escape or through the loss of neoantigens expression (25, 50, 62). Also spatial heterogeneity of intratumoral T-cells may be driven by the intratumoral neoantigen load and sculpted by a mutational background (25, 85).

CONCLUSIONS AND FUTURE PERSPECTIVES

Immunotherapy based on ICIs has drastically changed the natural history of many patients with M-NSCLC. However, the path towards ensuring long-term survival to most M-NSCLC patients remains steep. Inter-patient differences in the composition and spatial structure of the TME and in the TMB influence the type and duration of response to ICIs and ultimately explain why certain patients, unlike others, have only a limited benefit from these agents. Genetic and phenotypic ITH is an important barrier limiting the effects of single-agent immune therapies. On the other hand, ITH might represent a vulnerable "Achilles' heel" that might be targeted by combinatorial therapies and/or adaptative strategies (29, 86). More studies on ITH are needed to understand the complex interplay between tumor and immune cells and the role of spatio-temporal tumor heterogeneity in the response of M-NSCLC patients to immunotherapies. Single-cell approaches based on single-cell-sequencing or spatial transcriptomic may bring us an important step closer to understanding the role of ITH on response to ICI (87–89). Ultimately, this should lead to the development of novel therapeutic agents and/or treatment modalities, improving the prognosis of this still largely prevalent and deadly cancer.

AUTHOR CONTRIBUTIONS

All authors contributed to conception of the minireview. MN wrote the first draft of the manuscript and sections of the manuscript. PK, NC, and JM contributed to the draft of the manuscript revision. All authors contributed to the article and approved the submitted version.

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Efficacy and Safety of PD-1/PD-L1 Inhibitors Plus Chemotherapy Versus PD-1/PD-L1 Inhibitors in Advanced Non-Small Cell Lung Cancer: A Network Analysis of Randomized Controlled Trials

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Immune checkpoint inhibitors (ICIs) are recommended as first-line treatment for late-stage non-small cell lung cancer (NSCLC), either as monotherapy or in combination with chemotherapy. However, efficacy and safety comparisons between ICIs as monotherapy and ICIs with chemotherapy are lacking. We searched PubMed, Embase, and Cochrane Library for randomized controlled trials published before February 29th, 2020, with the search terms "immunotherapy" and "chemotherapy". 10 eligible trials were identified with a total of 5,956 patients. Of these patients, 3,204 received immune therapy and 2,752 received chemotherapy. PD-1 inhibitors with chemotherapy improved OS (HR 0.84, 0.77–0.92), PFS (HR 0.80, 0.75–0.85), and objective response rate (ORR) (odds ratio (OR) 2.55, 1.20–5.28) compared to PD-1 inhibitors as monotherapy. In contrast, PD-L1 inhibitors plus chemotherapy showed no significant differences in OS, PFS, or ORR compared with PD-L1 inhibitors as monotherapy. When patients were stratified according to PD-L1 expression level, patients with high PD-L1 expression (≥ 50%) receiving PD-1 inhibitors plus chemotherapy had improved PFS, but not other outcomes, compared to PD-1 inhibitors as monotherapy. In these patients, PD-L1 inhibitors plus chemotherapy showed no significant difference in survival compared with PD-L1 inhibitors. In the low PD-L1 expression group (1%–49%), PD-1 inhibitors plus chemotherapy improved OS and PFS, but no advantage was observed in PD-L1 inhibitors plus chemotherapy in OS, PFS, or ORR compared with PD-L1 inhibitor monotherapy. When comparing PD-1/PD-L1 inhibitors plus chemotherapy with PD-1/PD-L1 inhibitors monotherapy, no significant differences were observed in the rate of immune-related adverse events (AEs). In summary, for treating patients with late-stage NSCLC, PD-1 inhibitors plus chemotherapy have improved efficacy compared with PD-1 inhibitor monotherapy, but PD-L1 inhibitors plus chemotherapy have

similar efficacy as PD-L1 monotherapy. Survival benefits of PD-1/PD-L1 inhibitors combined with chemotherapy were particularly significant in patients with low PD-L1 expression levels.

Systematic Review Registration: PROSPERO, identifier CRD42020166678 (https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=166678).

Keywords: PD-1/PD-L1 inhibitors, late-stage non-small cell lung cancer, PD-L1 expression level, survival efficacy, safety, first line treatment

INTRODUCTION

Worldwide in 2018, 2,093,876 new lung cancer patients were diagnosed and lung cancer caused 1,761,007 deaths (1). Non-small cell lung cancer (NSCLC) accounts for 80%–85% of these cases (2). Therapeutic regimes for patients with NSCLC in stage III or beyond include radical radiotherapy, chemo-radiotherapy, gene targeted therapy, and immune checkpoint inhibitors (ICIs).

ICI therapies, targeting T-cell regulatory pathways to provide significant clinical benefits against cancers (3, 4), have been heralded as a promising treatment for lung cancer. The receptor PD-1 and its ligands PD-L1 and PD-L2 play a vital role in the maintenance of immunologic self-tolerance (5). Cancers can exploit this pathway to escape T-cell-mediated attack by the immune system. Clinical practice has attempted to enhance anti-tumor immune responses by augmenting costimulatory signals, but coinhibitory signals that block anti-tumor T-cell responses have been shown to be more effective than costimulatory signals (6).

MDX1105 (nivolumab) was the first PD-1 inhibitor used in cancer therapy. In 2010, it was reported that one patient with colorectal cancer achieved a complete response and two patients with melanoma and renal cancer achieved partial response (7). More recent data have indicated that 18% of NSCLC patients respond to treatment with MDX11105 (8).

NCCN guidelines recommend the PD-1 inhibitor pembrolizumab as first-line treatment for NSCLC with PD-L1 expression level ≥50%. KEYNOTE-024 (9) demonstrated that pembrolizumab improved median OS compared to chemotherapy, with OS of 30 months with pembrolizumab and 14.2 months with chemotherapy. Similarly, KEYNOTE-042 (10) showed that median OS was 22.3 months in patients treated with pembrolizumab and 10.5 months in patients treated with chemotherapy. KEYNOTE-407 (11) evaluated pembrolizumab plus chemotherapy in patients with stage IV squamous NSCLC. In this study, median OS was 15.9 months in patients receiving combination therapy and 11.3 months in patients receiving chemotherapy. A similar trend was observed in non-squamous NSCLC patients in IMpower 130, in which the median OS was 18.6 months in patients receiving the PD-L1 inhibitor atezolizumab plus chemotherapy and 13.9 months in patients receiving chemotherapy. Comparisons between PD-1/PD-L1 inhibitor monotherapy and chemotherapy confirmed the survival benefits of immunotherapy (9, 10, 12, 13). The NCCN NSCLC panel recommended single-agent pembrolizumab as first-line therapy for NSCLC patients with PD-L1 expression level >1%, and ICIs plus chemotherapy were recommended for patients who could tolerate adverse events (AEs) (14, 15).

Although immunotherapy has gradually become the mainstay in NSCLC therapy, optimization of the treatment plan for advanced NSCLC patients is still facing challenge. This network analysis aims to compare the efficacy and safety of PD-1/PD-L1 inhibitors plus chemotherapy with PD-1/PD-L1 monotherapy.

METHODS

Data Sources and Searches

This network analysis was conducted using Preferred Reporting Item for Systemic Reviews and Meta-Analyses guidelines (16). The review is registered on the PROSPERO website as No. CRD42020166678. PubMed, Embase, and Cochrane Library were systematically searched up to February 29th, 2020, using the search terms "non-small cell lung cancer," "immune checkpoint inhibitors," "immune checkpoint blockade," "pembrolizumab," "nivolumab," "atezolizumab," "durvalumab," and "chemotherapy." See **Supplementary Material** for more information.

Study Selection

Two reviewers (XL and SY) independently screened the study titles and abstracts based on predefined inclusion and exclusion criteria. The reviewers discussed any discrepancies or, if necessary, by seeking a decision from a third reviewer (NW).

Inclusion criteria for studies were as follows: (i) Patients had histologically confirmed previously untreated unresectable advanced (stage IIIB/IIIC/IV) NSCLC without *EGFR* or *ALK* mutations. (ii) Interventions were PD-1/PD-L1 inhibitor plus platinum-based chemotherapy or PD-1/PD-L1 inhibitor alone. (iii) Comparators were platinum-based chemotherapy. (iv) Outcomes were efficacy outcomes, including OS, PFS, ORR; and safety outcomes, including AEs. OS was defined as the time from when patients enrolled into trials to death with any cause. PFS was defined as the time from randomization until progression for any cause. ORR was defined as the proportion of patients achieving partial or complete remission. AEs were defined and graded according to the common terminology criteria from the National Cancer Institute (17). (v) Only randomized controlled trials (RCTs) were included.

Studies were excluded according to the following exclusion criteria: (i) Patients had cytologically or histologically confirmed small cell lung cancer or other kinds of lung cancer. Patients with driver gene-mutations were excluded. Patients who had received previous systemic treatment were excluded. (ii) Patients who

received spontaneous anti-CTLA-4 were excluded. Patients who received concurrent or sequential radio-chemotherapy were excluded. (iii) Studies other than RCTs were excluded.

Data Extraction and Risk of Bias Assessment

Data were extracted using ADDIS 16.7 software. Two investigators independently reviewed the full text of included studies and extracted information, including first author, year of publication, patient characteristics, inclusion and exclusion criteria, treatment protocol, outcomes, HR for OS, HR for PFS, OR for ORR, and OR for AEs. We concentrated on treatment-related severe AEs, defined as grades 3–5. The included trials were performed at multiple sites worldwide over long periods, so we extracted data from the most recent published articles or conference reports possible. Risk of bias of trials was assessed independently by two investigators using the Cochrane risk of bias tool (18). Differences in data extraction were mediated by Prof. N.W.

Data Synthesis and Statistical Analysis

Direct Comparison

Pooled HRs with 95% confidence intervals (95% CI) were calculated for OS and PFS and pooled ORs with 95% CI were calculated for ORR and the rate of AEs using the random-effects model in REVMAN 5.3 (Cochrane). The quantity I^2 was used to describe heterogeneity between studies. We included only low risk of bias in the sensitivity analysis.

Indirect and Mixed Comparisons

A random-effects network meta-analysis (NMA) within a Bayesian framework was then performed using OpenBUGS version 3.2.3. Pooled HRs and ORs with 95% CI were also summarized. Each treatment was ranked using the surface under the cumulative ranking curve (SUCRA) and a treatment hierarchy was generated. A treatment ranked as 100% is certain to be the best and a treatment ranked as 0% is certain to be the worst in terms of efficacy and safety outcomes. We also used the contribution plot to measure the percent contribution of each direct comparison to the mixed estimates, the indirect estimates, and the entire network, as shown in the **Supplementary Material**.

Examination of Assumptions in Network Meta-Analysis

To check the consistency of the NMA, we used the node-splitting model to assess inconsistencies between direct and indirect treatment effects. A predictive interval plot was used to estimate heterogeneity for all comparisons.

Additional Analyses

We used the comparison-adjusted funnel plot to assess small-study effects. The synthesized endpoints included OS, PFS, ORR, and treatment-related grade 3–5 AEs. HRs of OS and PFS were preferentially reported and were adjusted for confounders in individual studies (19). HR could also be estimated according to the method described by Tierney and colleagues (20). Consistency was assessed by comparing synthesized HR of NMA with pairwise head-to-head meta-analyses. The ORs of ORR and AEs were calculated using Bayesian statistics.

Bayesian NMA was done using a Markov Chain Monte Carlo simulation technique in OpenBUGS version 3.2.3. We used non-informative uniform and normal prior distributions (21) and three different sets of initial values to fit the model. For OS and PFS, 30,000 sample iterations were generated with 5,000 burn-ins and a thinning interval of 10. For ORR and AE, 50,000 sample iterations were generated with 20,000 burn-ins and a thinning interval of 10.

Transitivity was estimated by assessing studies that compared two treatments and evaluating direct and indirect comparisons (22). All included studies were RCTs that compared experimental treatments with platinum-based chemotherapy, providing convincing and stable transitivity.

Patients were stratified according to PD-L1 expression (≥ 50% or 1%–49%). PD-1 inhibitors and PD-L1 inhibitors were evaluated to determine their effect on the NMA.

RESULTS

Systematic Review and Characteristics

We screened 1,426 records (**Figure 1**) and identified 24 studies for full-text reads. Ten eligible RCTs were included in this NMA (**Table 1**), with a total of 5,956 patients receiving one of six treatment strategies as first-line therapy for advanced unresectable lung cancer. Of these patients, 3,204 patients received immune therapy and 2,752 patients received chemotherapy. Most of the included trials were published with low bias (see **Supplementary Material**).

The included trials (9–13, 23–27) reported HRs for OS and PFS. Four of the trials (9, 10, 12, 13) evaluated a PD-1 or PD-L1 inhibitor as monotherapy while six of the trials (11, 23–27) evaluated a PD-1 or PD-L1 inhibitor in combination with chemotherapy. Six trials (9, 12, 13, 23, 24, 26) used a platinum-based chemotherapy plus pemetrexed or gemcitabine, three trials (11, 25, 27) used a platinum-based chemotherapy plus paclitaxel, and one trial (10) used a platinum-based chemotherapy plus pemetrexed/paclitaxel (**Table 1**). Some studies reported efficacy outcomes stratified according to PD-L1 expression; eight trials (9–13, 23, 25, 27) reported survival data in patients with high PD-L1 expression (≥ 50%) and five trials (10, 11, 23, 25, 27) reported survival data in patients with low PD-L1 expression (1-49%) (**Figure 2**).

Results of Pairwise Meta-Analysis

Head-to-head comparisons revealed that compared with chemotherapy, OS was improved in patients treated with PD-1 inhibitors (HR 0.85, 0.76–0.95), PD-1 inhibitors plus chemotherapy (HR 0.57, 0.48–0.69), and PD-L1 inhibitors plus chemotherapy (HR 0.83, 0.74–0.94). PFS was also improved in patients treated with PD-L1 inhibitors (HR 0.77, 0.63-0.94), PD-1 inhibitors plus chemotherapy (HR 0.54, 0.47–0.62), and PD-L1 inhibitors plus chemotherapy (HR 0.65, 0.59–0.72). No significant difference in PFS was observed when comparing

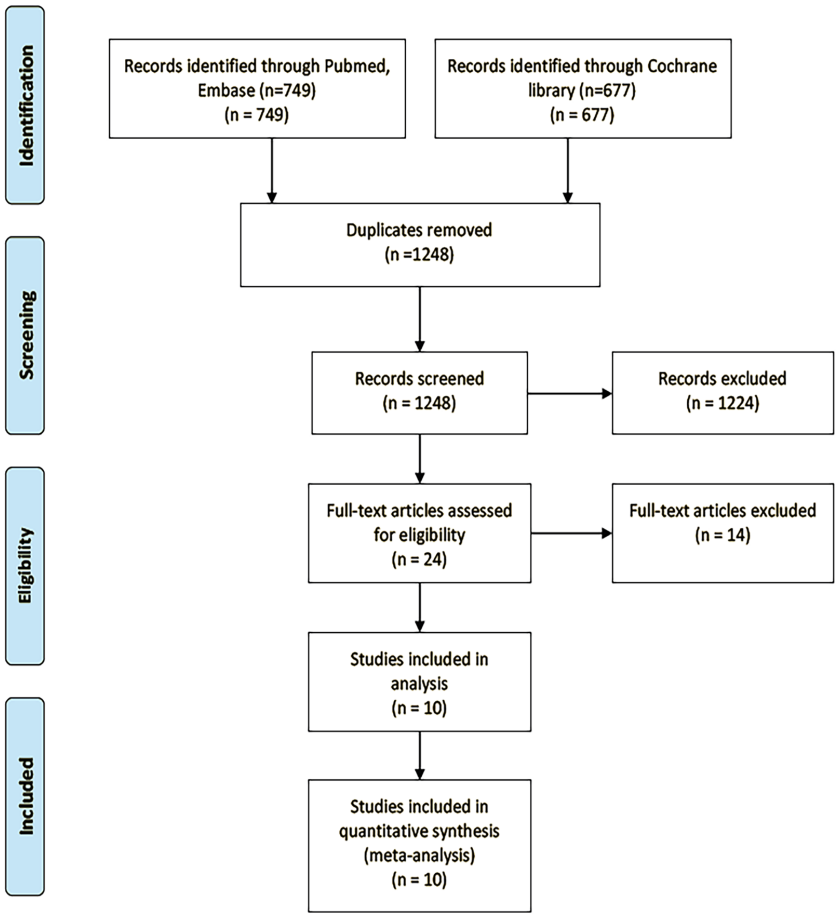

FIGURE 1 | The flow diagram of studies selection.

PD-1 inhibitors as monotherapy with chemotherapy (HR 1.0, 0.91–1.1).

Efficacy Outcomes

Compared with PD-1/PD-L1 inhibitors as monotherapy, PD-1 and PD-L1 inhibitors combined with chemotherapy did not significantly improve OS (HR 0.94, 0.90–1.01), but the combination therapy did significantly improve PFS (HR 0.82, 0.78–0.87).

Compared with PD-1 inhibitor monotherapy, PD-1 inhibitors plus chemotherapy significantly improved OS (HR 0.84, 0.77–0.92), PFS (HR 0.80, 0.75–0.85), and ORR (OR 2.55, 1.20–5.28).

Compared with PD-L1 inhibitor monotherapy, PD-L1 inhibitors plus chemotherapy showed no significant difference in OS (HR 1.01, 0.89–1.13), PFS (0.91, 0.82–1.01), and ORR (OR 2.02, 0.68–5.80) (**Figure 3**).

Compared with PD-L1 inhibitor plus chemotherapy, PD-1 inhibitor plus chemotherapy significantly improved OS (HR 0.85, 0.76–0.93) but not PFS (HR 0.99, 0.92–1.06) (**Figure 4**).

High PD-L1 Expression Level

In patients with high PD-L1 expression levels (≥ 50%), PD-1/PD-L1 inhibitors plus chemotherapy did not significantly improve OS compared to PD-1/PD-L1 inhibitors as monotherapy (HR 0.94, 0.82–1.07), but did significantly improve PFS (HR 0.78, 0.70–0.86). PD-1 inhibitors plus chemotherapy did not significantly improve OS compared with PD-1 inhibitors (HR 0.86, 0.73–1.03), but did significantly improve PFS (HR 0.72, 0.63–0.83). PD-L1 inhibitors plus chemotherapy did not significantly improve OS (HR 1.08, 0.85–1.37) or PFS (HR 0.89, 0.73–1.08) compared with PD-L1 inhibitors as monotherapy. PD-1 inhibitors plus chemotherapy significantly improved OS (HR 0.85, 0.76–0.93), but not PFS (HR 0.99, 0.92–1.06) compared with PD-L1 inhibitors plus chemotherapy.

Low PD-L1 Expression Level

In patients with low PD-L1 expression levels (1%–49%), PD-1/PD-L1 inhibitors plus chemotherapy significantly improved OS (HR 0.84, 0.73–0.96) and PFS (HR 0.79, 0.73–0.85) compared

TABLE 1 | Characteristics and results of included trials.

Patient Numbers		HR (95% CI)		ORR (%)	AEs	
		OS	PFS		All	Severe
IMpower-110	572					
Atezolizumab 1200 mg Q3W	286	0.83	0.77	29.2	258	91
Cisplatin 75 mg/m^2 or carboplatin AUC 6, pemetrexed 500 mg/m^2 IV Q3W; cisplatin 75 mg/m^2 + gemcitabine 1,250 mg/m^2 or carboplatin AUC 5 + gemcitabine 1,000 mg/m^2 IV Q3W	286	(0.65–1.07)	(0.63–0.94)	31.8	249	144
IMpower-130	705					
Atezolizumab 1,200 mg Q3W, then carboplatin 6 mg/ml Q3W plus nab-paclitaxel 100 mg/m2 QW	473	0.79	0.64	49.2	455	354
Carboplatin 6 mg/mL Q3W + nab-paclitaxel 100 mg/m2 QW	232	(0.64–0.98)	(0.54–0.77)	31.9	215	141
IMpower-131	683					
Atezolizumab 1,200 mg IV Q3W, then carboplatin AUC 6 IV Q3W, nab-paclitaxel 100 mg/m^2 IV QW	343	0.96	0.61	49	332	274
Carboplatin AUC 6 IV Q3W, nab-paclitaxel 100 mg/m^2 IV QW	340	(0.78–1.18)	(0.48–0.77)	41	324	234
IMpower-132	578					
Atezolizumab 1,200 mg IV Q3W, then carboplatin AUC 6 mg/ml/min IV Q3W or cisplatin 75 mg/m^2 IV Q3W, pemetrexed 500 mg/m^2 IV Q3W	292	0.81	0.60	47	286	202
Carboplatin AUC 6 mg/ml/min IV Q3W or cisplatin 75 mg/m^2 IV Q3W, pemetrexed 500 mg/m^2 IV Q3W	286	(0.64–1.03)	(0.49–0.72)	32	266	161
CheckMate-026	541					
Nivolumab 3 mg/kg Q2W	271	1.02	1.15	26	190	47
Cisplatin 75 mg/m^2 + gemcitabine 1,250 mg/m^2 or carboplatin AUC 5 + gemcitabine 1,000 mg/m^2 or carboplatin AUC 6 + paclitaxel 2,000 mg/m^2; cisplatin 75 mg/m^2 or carboplatin AUC 6 + pemetrexed 500 mg/m^2	270	(0.8–1.3)	(0.91–1.45)	33	243	133
KEYNOTE-021	123					
Pembrolizumab 200 mg + carboplatin AUC 5 + pemetrexed 500 mg/m^2 IV Q3W	60	0.90	1.17	42.7	55	23
Carboplatin AUC 5 + pemetrexed 500 mg/m^2 IV Q3W	63	(0.42–1.91)	(0.95–1.43)	18.4	56	16
KEYNOTE-024	305					
Pembrolizumab 200 mg Q3W, 35 cycles	154	0.60	0.50	44.8	113	41
Carboplatin AUC 5/6 Q3W or cisplatin 75 mg/m^2 + pemetrexed 500 mg/m^2 Q3W; cisplatin 75 mg/m^2 or carboplatin AUC 5/6 + gemcitabine 1,250 mg/m^2 Q3W or paclitaxel 200 mg/m^2 Q3W	151	(0.41–0.89)	(0.37–0.68)	41.98	135	80
KEYNOTE-042	1274					
Pembrolizumab 200 mg Q3W, 35 cycles	637	0.79	0.64	27	399	113
Carboplatin AUC 5/6 Q3W + paclitaxel 200 mg/m^2 Q3W or carboplatin AUC 5/6 Q3W + pemetrexed 500 mg/m^2 Q3W, 6 cycles	637	(0.64–0.98)	(0.54–0.77)	27	553	252
KEYNOTE-189	616					
Pembrolizumab 200 mg + pemetrexed 500 mg/m^2 + carboplatin AUC 5 or cisplatin 75 mg/m^2 Q3W, 4 cycles	410	0.79	0.64	47.6	404	272
Placebo (saline) + pemetrexed 500 mg/m^2 + carboplatin AUC 5 or cisplatin 75 mg/m^2 Q3W, 4 cycles	206	(0.64–0.98)	(0.54–0.77)	18.9	200	133
KEYNOTE-407	559					
Pembrolizumab 200 mg Q3W + carboplatin AUC 6 + paclitaxel 200 mg/m^2 or nab-paclitaxel 100 mg/m^2 Q3W, then pembrolizumab 200 mg Q3W	278	0.64	0.56	57.4 58.7	273	194
Carboplatin AUC 6 + paclitaxel 200 mg/m^2 or nab-paclitaxel 100 mg/m^2 Q3W, then pembrolizumab 200 mg Q3W	281	(0.49–0.85)	(0.45–0.70)	37.7 39.5	274	191

OS = overall survival; PFS = progression-free survival; ORR = objective response rate; AEs = adverse events; all = all AEs; severe = grade3-5 AEs.

with PD-1/PD-L1 inhibitors as monotherapy. PD-1 inhibitors combined with chemotherapy significantly improved OS (HR 0.81, 0.68–0.95) and PFS (HR 0.75, 0.67–0.85) compared with PD-1 inhibitors as monotherapy. No significant differences were observed in OS (HR 0.91, 0.71–1.15) or PFS (HR 0.93, 0.80–1.07) when comparing PD-1 inhibitors plus chemotherapy with PD-L1 inhibitors plus chemotherapy (**Figure 5**).

Safety Outcomes

In this network analysis, PD-1/PD-L1 inhibitors combined with chemotherapy were more likely to cause AEs, and especially severe AEs (grade 3–5) than PD-1/PD-L1 inhibitors as monotherapy (**Figure 6**). PD-1 inhibitors plus chemotherapy caused more AEs than PD-1 inhibitors as monotherapy when considering any AEs (OR 7.73, 2.99–19.88) and also when considering only severe AEs (OR 4.55, 2.94–7.69). Compared with PD-L1 inhibitor monotherapy, PD-L1 inhibitor plus chemotherapy caused more AEs overall (OR 4.96, 1.39–20.34) and more severe AEs (OR 4.76, 2.27–10.0). Compared with PD-1 inhibitor plus chemotherapy, PD-L1 inhibitor plus chemotherapy caused more AEs overall (OR 1.60, 0.51–5.20) and more severe AEs (OR 1.52, 0.88–2.56), but no significant differences were observed. Compared with PD-L1 inhibitors as monotherapy, PD-1 inhibitors as monotherapy did not cause significantly more overall AEs (OR 0.69, 0.14–1.35) or severe AEs (grade 3–5) (OR 0.41, 0.14–1.33) (**Figure 7**).

When comparing PD-1 inhibitors plus chemotherapy with PD-1 inhibitors as monotherapy, no significant differences were observed in the rate of immune-related AEs, including hypothyroidism (OR 0.41, 0.12–1.46), hyperthyroidism (OR

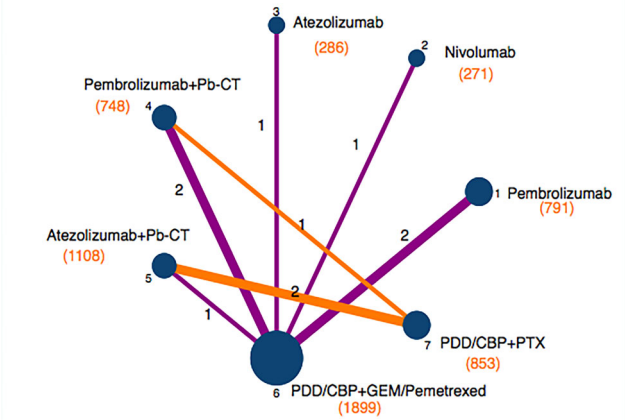

FIGURE 2 | Net plot of included trials.

0.38, 0.04–3.75), pneumonia (OR 0.18, 0.03–1.41), and skin reactions (OR 0.10, 0.00–1.45). When comparing PD-L1 plus chemotherapy with PD-1 plus chemotherapy, no significant differences were observed in the rate of immune-related AEs, including hypothyroidism (OR 3.57, 0.68–24.47), hyperthyroidism (OR 4.82, 0.19–291.1), and pneumonia (OR 1.17, 0.17–13.44).

Rank Probabilities

Ranking probabilities of the six treatments were summarized for OS, PFS, ORR, and AEs (**Supplementary Material**). PD-1 inhibitor plus chemotherapy provided the most favorable balance between efficacy and safety. For ORR, PD-1 inhibitor plus chemotherapy ranked first (89%) and PD-L1 inhibitor plus chemotherapy ranked second (77%). Similarly, PD-1 inhibitor plus chemotherapy had the highest SUCRA ranking. Nivolumab had the highest SUCRA ranking for AEs, causing the fewest severe AEs (98.9%) and overall AEs (90.5%). Pembrolizumab plus chemotherapy ranked first in OS (98.9%) and PFS (98.4%), pembrolizumab ranked second in OS (52.5%), and atezolizumab plus chemotherapy ranked second (91.2%) in PFS.

Assessment of Heterogeneity

Heterogeneity estimates were calculated in four sub-group pairwise analyses (see **Supplementary Figures 8**, **9**, **40**). During analysis, no heterogeneity (I^2 = 0%) or low heterogeneity (I^2<50%) was used to assess comparisons. Notably, the I^2 values of "PD-1 inhibitors versus platinum-based chemotherapy" were 75% for OS, 91% for PFS, and 33% for severe AEs. Meanwhile, the I^2 value of "PD-1 inhibitors plus platinum-based chemotherapy versus platinum-based chemotherapy" showed moderated heterogeneity (41%). Other comparisons showed minimal heterogeneity (I^2 = 0%).

Node-split plots for ORR and AEs were listed (see **Supplementary Material**) and consistency was confirmed for p-values > 0.05. Forest plots of direct and indirect comparisons were generated for OS (**Figure 8**), PFS (**Figure 9**), ORR (**Supplementary Material**), and AEs (**Supplementary Material**). Funnel plots indicated little report bias among trials (**Supplementary Material**).

DISCUSSION

Immunotherapy has been proved to be an effective treatment in NSCLC and its use has gradually increased in clinical practice. The appropriate choice of ICI and treatment regime requires solid evidence. ICIs monotherapy and ICIs with chemotherapy are both recommended in NCCN guidelines. ICIs target T-cell

PD-1	0.80 (0.28 to 2.31)	2.55 (1.20 to 5.28)	1.62 (0.75 to 3.51)	0.90 (0.51 to 1.55)
1.25 (0.43 to 3.56)	**PD-L1**	3.18 (1.08 to 8.80)	2.02 (0.68 to 5.80)	1.12 (0.43 to 2.80)
0.39 (0.19 to 0.84)	0.31 (0.11 to 0.93)	**PD-1+Pb-CT**	0.63 (0.31 to 1.36)	0.35 (0.22 to 0.59)
0.62 (0.29 to 1.34)	0.50 (0.17 to 1.48)	1.58 (0.73 to 3.21)	**PD-L1+Pb-CT**	0.56 (0.33 to 0.96)
1.11 (0.65 to 1.95)	0.89 (0.36 to 2.31)	2.82 (1.71 to 4.58)	1.79 (1.05 to 3.07)	**Pb-CT**

FIGURE 3 | Network meta-analysis of objective response rate in all patients.

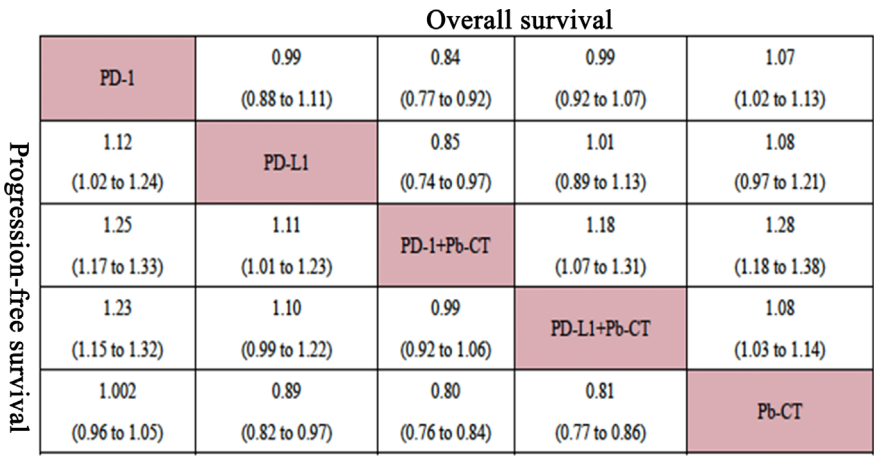

FIGURE 4 | Network meta-analysis of all patients.

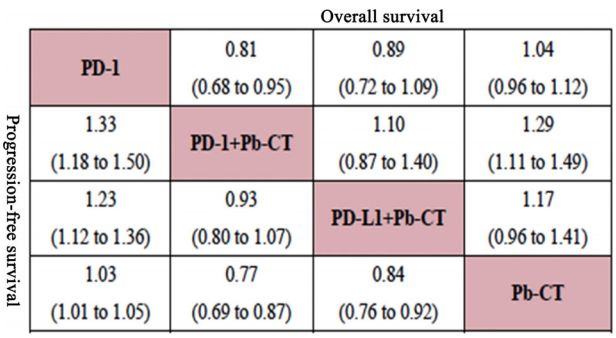

FIGURE 5 | Network meta-analysis of patients with PD-L1 expression level 1% to 49%.

regulatory pathways to enhance the anti-tumor immune response, providing significant benefit in cancer therapy (3, 4). Further, ICIs as monotherapy have performed better than chemotherapy alone in several large multicenter trials (9, 10, 13). The NCCN NSCLC panel has recommended single-agent pembrolizumab as first-line treatment for eligible patients with metastatic advanced NSCLC regardless of histology for patients with PD-L1 expression levels greater than 50% and without EGFR, ALK, ROS1, and BRAF V600E mutations. Pembrolizumab is also recommended as monotherapy in patients with low PD-L1 expression (1%–49%) (15), and recent research has suggested that patients with PD-L1 expression levels just below and just above 50% are likely to have a similar response (28). Combining ICIs and chemotherapy sometimes performed better than ICIs as monotherapy, but there is not consistent clinical evidence to support this treatment approach. This study was designed to

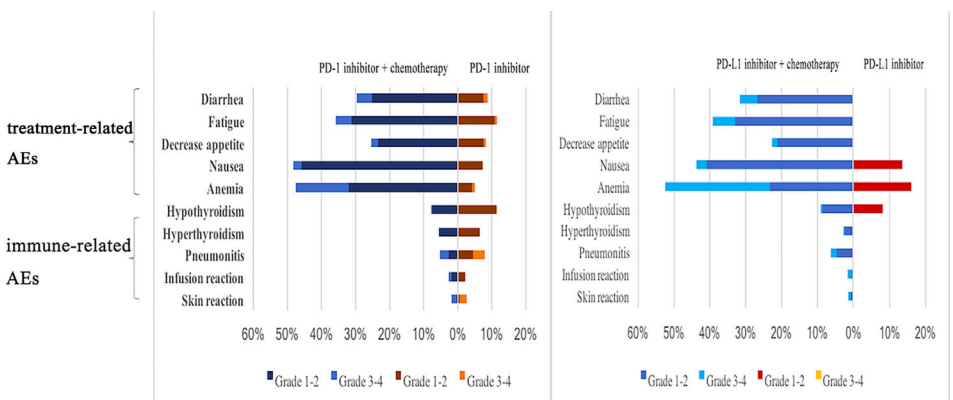

FIGURE 6 | Proportion of adverse events in all patients.

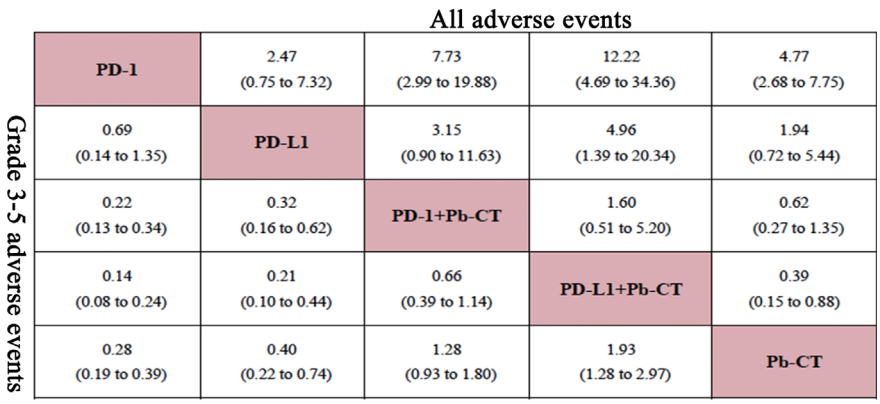

FIGURE 7 | Network meta-analysis of adverse events in all patients.

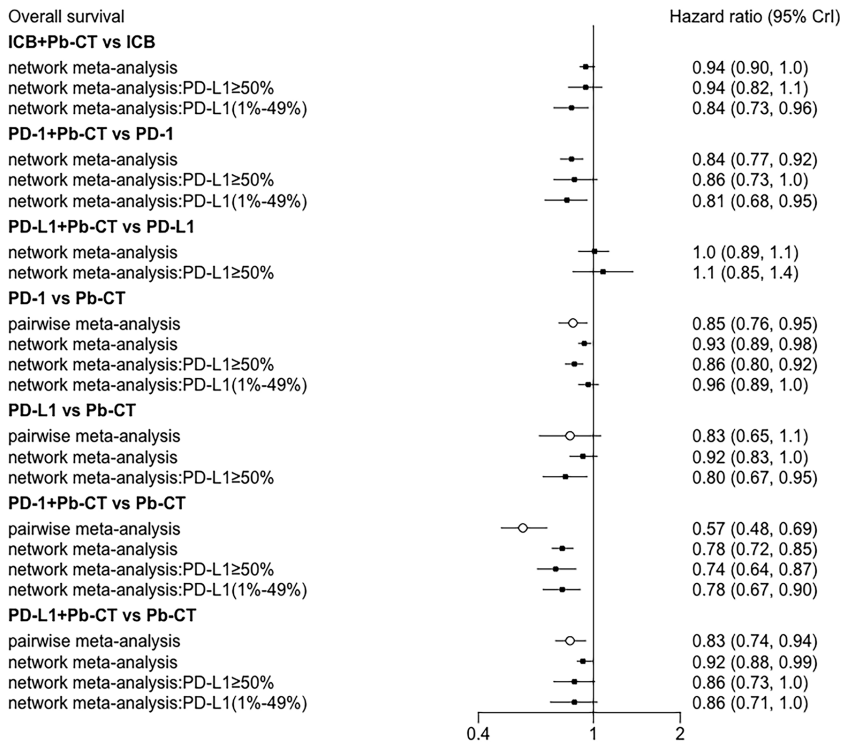

FIGURE 8 | Consistency analysis for overall survival.

provide additional guidance on choosing the optimal treatment plan for NSCLC patients with different PD-L1 expression levels.

10 trials evaluating PD-1/PD-L1 inhibitors were included in this analysis. We considered efficacy outcomes (OS, PFS, and ORR) and safety outcomes (AEs) to compare treatment with PD-1/PD-L1 inhibitor plus chemotherapy with PD-1/PD-L1 inhibitors as monotherapy. The trial results were also examined for effect modifiers. Consistency analysis indicated well-behaved data with robust stability.

Our results demonstrated: (i) PD-1/PD-L1 inhibitors plus chemotherapy performed better than PD-1/PD-L1 inhibitors as monotherapy, particularly in patients with low PD-L1 expression levels (1-49%). (ii) PD-1 inhibitors plus chemotherapy improved OS compared with PD-L1 inhibitors plus chemotherapy. (iii) PD-1/PD-L1 inhibitors plus chemotherapy caused more AEs than PD-1/PD-L1 inhibitors as monotherapy.

PD-1/PD-L1 inhibitors plus chemotherapy improved PFS compared with PD-1/PD-L1 inhibitors as monotherapy, but no

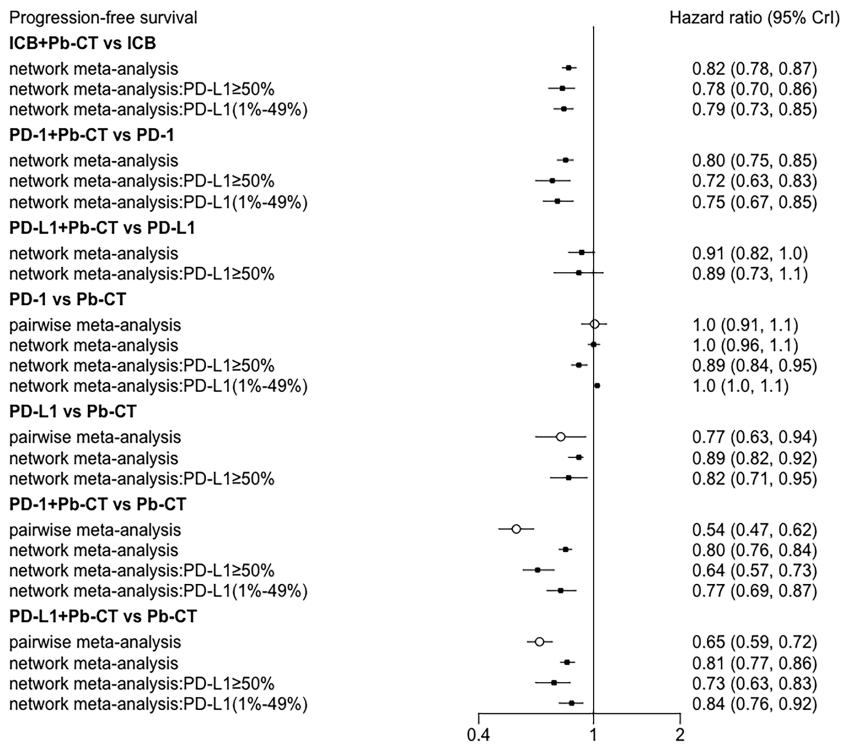

FIGURE 9 | Consistency analysis for progression-free survival.

significant differences were observed for OS. Nonsynonymous mutations and neo-antigens in tumors are associated with improved efficacy, durable clinical benefits, and PFS (5). Stratifying patients based on PD-L1 expression demonstrated that patients with lower PD-L1 expression level (1%–49%) may obtain better OS and PFS benefits from immunotherapy plus chemotherapy compared to immunotherapy alone. Likewise, patients receiving PD-1 inhibitors plus chemotherapy had improved OS and PFS compared with patients receiving PD-1 inhibitors as monotherapy, regardless of PD-L1 expression level. Multiple factors may impact the clinical efficacy of immunotherapy (29). In such cases, ICIs plus chemotherapy may be a better option for patients (30) than platinum-based chemotherapy (11, 23–25, 27).

Basic research has revealed that synergistic anti-tumor effects of immunotherapy and chemotherapy could be mediated through several pathways. First, cytotoxic T lymphocyte (CTL) could generate an immune response to kill tumor cells by releasing perforin or cytokines or through FasL-mediated apoptosis. Chemotherapy increased the expression level of mannose-6-phosphate receptors, with more Granzyme B expressed on CTLs allowing them to enter tumor cells (31). Second, chemotherapy can enhance tumor cell immunogenicity by increasing HSP 70/90 expression on the surface of tumor cells or increasing DNA cross-linking. Tumors with high autophagy would release more adenosine triphosphate than those lacking autophagy, as the latter cannot adequately stimulate T lymphocytes or recruit CD4 and CD8, while the former could raise more dendritic cells (DC) and T lymphocytes (32). Third, chemotherapy causes immunogenic cell death in tumors, causing calreticulin/HSP exposure and adenosine triphosphate/HMGB1 (high-mobility group box 1) release (33). Fourth, chemotherapy could help clear immune-suppressing cells. Along with decreased lymphocytes, immunosuppressive CD4+, CD25+, Foxp3+ regulatory T cells, and myeloid derived suppressor cells could also be cleared (34). Finally, chemotherapy could change the tumor micro-environment to promote antigen presentation and anti-tumor immune response, causing tumor cells to release HMGB1. This could recruit and activate DC as well as induce DC maturation. The combination of HMGB1 and toll-like receptor 4 on DC may also prevent degradation of tumor antigens (35).

PD-L1 expression level may serve as a biomarker to predict ICI efficacy. In this study, patients with lower PD-L1 expression levels (1%–49%) obtained improved survival benefits from PD-1/PD-L1 inhibitors plus chemotherapy compared with PD-1/PD-L1 inhibitors as monotherapy. Meanwhile, PD-1/PD-L1 inhibitors plus chemotherapy showed no survival advantage for patients with high PD-L1 expression level (≥ 50%). Recent research has revealed that T cells secrete cytokines, inducing multiple positive feedback loops. Chemokines promote T cell infiltration, and the altered antigen presentation could help T cells recognize tumor cells. Accordingly, the activation of the PD-1 or PD-L1 pathway is more important than PD-L1 expression level (36).

Interestingly, when PD-1 inhibitors plus chemotherapy were compared with PD-L1 inhibitors plus chemotherapy, the former improved OS, but no significant difference was observed for PFS.

Patient stratification according to PD-L1 expression level removed any advantage for OS, PFS, and ORR. Pembrolizumab plus chemotherapy did have a significant advantage compared with atezolizumab plus chemotherapy in terms of OS and PFS.

AEs caused by ICIs may be enhanced by chemotherapy as these treatments cause different kinds of toxicities. Other studies have shown that severe AEs after immune therapy, and especially immune-related AEs, hinder the efficacy of immune treatments. Previous studies have revealed several mechanisms causing AEs (37). Immune therapy increases T-cell activity against antigens present on both tumors and healthy tissue, and immune therapy causes increased levels of preexisting autoantibodies. In addition, immune therapy increases the level of inflammatory cytokines and enhances complement-mediated inflammation due to direct binding of an anti-CTLA-4 antibody to CTLA-4 expressed by normal tissue (38).

Compared with PD-L1 inhibitor monotherapy, PD-1 inhibitor monotherapy improved treatment outcomes in multiple tumor types (39). In treating NSCLC, no significant differences were observed between monotherapy with these two ICIs. PD-1 inhibitors block the interaction between PD-1 and B7.1/PD-L1, while PD-L1 inhibitors block the interaction between PD-L1 and PD-1/RGMB. Once the PD-1-PD-L1 pathway is suppressed, T cells can kill tumor cells. This may explain the similar efficacy of PD-1 inhibitor monotherapy and PD-L1 inhibitor monotherapy in terms of OS, PFS, ORR, and AEs.

IMPLICATION

This network analysis provides new evidence which helps clinician to choose optimal treatments for previously untreated advanced NSCLC patients. PD-1/PD-L1 inhibitors plus chemotherapy did not significantly improve survival compared to PD-1/PD-L1 monotherapy. However, PD-1 inhibitors plus chemotherapy did significantly improve OS, PFS, and ORR, compared to PD-1 inhibitor monotherapy, but also caused increased AEs. PD-L1 inhibitors plus chemotherapy showed no significant improvement in OS, PFS, or ORR compared with PD-L1 inhibitor monotherapy. In addition, patients were stratified according to their PD-L1 expression level. Our results suggest that patients with high PD-L1 expression level (≥ 50%) might be optimally treated with PD-1/PD-L1 inhibitor monotherapy, while patients with low PD-L1 expression level (1-49%) may obtain more benefits from PD-L1 inhibitors plus chemotherapy compared with PD-1 inhibitors as monotherapy, as long as patients could tolerate increased immune-related AEs. Consistent with the comparison between PD-1 inhibitors and PD-L1 inhibitors given as monotherapy, no significant differences in PFS, ORR, or AEs were observed between PD-1 inhibitors plus chemotherapy and PD-L1 inhibitors plus chemotherapy.

LIMITATIONS

This study has several limitations. Due to the essence of network analysis, this research provides a starting point for clinical practice. Several of the multicenter RCTs included in this study are ongoing and this research will need to be updated as more data are available. In addition, patients were not stratified according to histology (adenocarcinoma or squamous cell cancer) for analysis. Another limitation was the high heterogeneity observed when comparing PD-1 to platinum-based chemotherapy, both in OS (I^2 = 75%) and PFS (I^2 = 91%). In subgroup analysis, we did not show efficiency in patients with negative PD-L1 expression (<1%), because data within these patients were not available in enrolled published studies, which needs further exploration. Furthermore, this study only included trials evaluating the PD-L1 inhibitor atezolizumab as trials with durvalumab did not meet inclusion criteria. Finally, the KEYNOTE studies measured PD-L1 expression level with 22C3 pharmDx assays and IMpower trials used SP142 assays. These difference in detection methods could potentially cause patients to be misclassified (38).

CONCLUSIONS

PD-1 inhibitors combined with chemotherapy improved outcomes for patients with late-stage NSCLC compared with PD-1 inhibitor monotherapy, while PD-L1 inhibitors combined with chemotherapy had similar outcomes as PD-L1 monotherapy. The survival benefits of PD-1/PD-L1 inhibitors combined with chemotherapy were particularly striking in patients with low PD-L1 expression levels.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

NW, MZ, and XL conceptualized and designed the study, collected and organized the data, and drafted the initial manuscript. XL, SY, YW, and SL collected and organized the data, review the included articles, and conducted the analyses. XL, JY, CL, and JZ collected and organized the data and reviewed the included articles. NW and MZ conceptualized and designed the study, coordinated and supervised data collected, and critically reviewed and revised the manuscript. YY critically reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.574752/full#supplementary-material

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Tissue MicroRNA Expression as a Predictor of Response to Immunotherapy in NSCLC Patients

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Introduction: Expression of PD-L1 protein on tumor cells, which is so far the only validated predictive factor for immunotherapy, is regulated by epigenetic and genetic factors. Among the most important ones that regulate gene expression are microRNAs.

Materials and Methods: The study included 60 patients with NSCLC who underwent first or second line immunotherapy with pembrolizumab or nivolumab. FFPE materials were collected before the start of immunotherapy. We examined relative expression of microRNAs (miR-141, miR-200a, miR-200b, miR-200c, miR-429, miR-508-3p, miR-1184, miR-1255a) and *PD-L1* mRNA expression. Copy number variation (CNV) of *PD-L1* gene by qPCR and FISH methods were assessed. Two single nucleotide polymorphisms (SNPs) in promoter region of *PD-L1* gene (rs822335 and rs822336) were examined. Expression of PD-L1 protein on tumor cells was assessed by immunohistochemistry (IHC). The response rate to immunotherapy and progression free survival (PFS) measured in weeks and overall survival (OS) measured in months from the start of immunotherapy were evaluated.

Results: Response to immunotherapy was observed in nine patients (15%, including one complete response), disease stabilization in 22 patients (36.7%), and progression in 29 patients (48.3%). Significantly higher (p=0.015) expression of miR-200b and significantly lower (p=0.043) expression of miR-429 were observed in responders compared to patients who did not respond to immunotherapy. The median PFS in the whole group of patients was 16 weeks, and the median OS was 10.5 month. In univariate analysis, the median PFS was significantly higher in patients with high miR-200b

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expression (HR=0.4253, 95%CI: 0.1737–1.0417, p=0.05) and high miR-508 expression (HR=0.4401, 95%CI: 0.1903–1.0178, p=0.05) and with low expression of miR-429 (HR=0.1288, 95%CI: 0.01727–0.9606, p=0.0456) compared to patients with low and high expression of these molecules, respectively. The median OS was higher in patients with low expression of miR-429 (HR=0,6288, 95%CI: 0,3053–1,2949, p=0.06) compared with patients with high expression of this microRNA. In multivariate analysis, we found that patients with PD-L1 expression on ≥1% of tumor cells compared to patients without PD-L1 expression on cancer cells had a significantly lower risk of progression (HR=0.3857, 95%CI: 0.1612–0.9226, p=0.0323) and death (HR=0.377, 95%CI: 0.1636–0.8688, p=0.022).

Conclusion: The miR-200b and miR-429 molecules in tumor cells seem to have greatest impact on the effectiveness of immunotherapy in NSCLC patients.

Keywords: PD-L1, immunotherapy, microRNA, non-small cell lung cancer, SNP, copy number variation

INTRODUCTION

Immunotherapy with anti-PD-1 or anti-PD-L1 antibodies has become one of the leading treatment method in patients with advanced non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (1–4).

Predictive factors enabling precise qualification of patients for immunotherapy have not been sufficiently defined, and expression of PD-L1 protein on tumor cells is the only validated factor used in clinical practice in the qualification of NSCLC patients to first line therapy with pembrolizumab (5–8). Unfortunately, only less than 50% of patients with PD-L1 expression on ≥50% of tumor cells respond to first line immunotherapy (9, 10). Reasons for this situation can be sought in the molecular basis of PD-L1 expression.

The variability in the number of copies of the *PD-L1* gene, its polymorphisms, regulatory epigenetic mechanisms, especially microRNA expression in cancer cells, can have a big impact on the expression of PD-L1 protein, and thus on the effectiveness of immunotherapy in patients with various types of cancers (11–15).

In our study, we attempted to correlate genetic and epigenetic factors associated with PD-L1 expression with effectiveness of anti-PD-1 monoclonal antibodies.

MATERIAL AND METHODS

Patients

We enrolled (from July 2018 to September 2019) 60 NSCLC patients (41 men and 19 women) with a mean age of 67 years qualified for first or second line immunotherapy with pembrolizumab (n=12, 20%) or nivolumab (n=48, 80%). PD-L1 expression status was assessed in all patients included in the study. Patients received second line immunotherapy regardless of PD-L1 expression on tumor cells if they received chemotherapy in their first-line treatment. First line therapy with pembrolizumab was used only in patients with PD-L1 expression on ≥50% of tumor cells. All patients were in good (n=42, 70%) or very good (n=18, 30%) condition. Fourteen patients (23%) were in stage IIIB and 46 patients (77%) were in stage IV. Adenocarcinoma (AC) was diagnosed in 24 (40%) patients, squamous cell carcinoma (SCC) - in 30 patients (50%), and NSCLC NOS (not-otherwise specified) - in six (10%) patients. **Table 1** shows the demographic and clinical data of our patients.

The inclusion criteria for treatment were as follows: age over 18 years, very good or good performance status (PS=0 or 1 according to ECOGS scale), diagnosis of NSCLC (regardless of the pathomorphological type), no mutations in the *EGFR* (*epidermal growth factor receptor*) gene and no rearrangement of the *ALK* (*anaplastic lymphoma kinase*) gene in patients with non-SCC, PD-L1 expression on ≥50% of tumor cells in qualification to first line treatment with pembrolizumab, stage IIIB or IV, presence of measurable neoplastic lesions in computed tomography according to RECIST 1.1 (response evaluation *criteria* in solid tumors), no other contraindications to the use of immunotherapy in accordance with the summary of product characteristic for individual drugs (e.g. autoimmune diseases). Imaging to assess PFS and ORR were performed every 3 months during immunotherapy, and then depending on the clinical situation. In the absence of disease progression after immunotherapy, the computed tomography were continued every 3 months until progression. These criteria were in compliance with the reimbursement regulations in Poland. All patients qualified for immunotherapy who had signed a written consent to participate in the study were included in the study. One hundred twenty-seven patients qualified for immunotherapy were provided with information on the methodology and purpose of the study. The small number of patients results from delays in the reimbursement of immunotherapy in Poland compared to other European Union countries.

We performed a routine examination of PD-L1 expression in formalin-fixed paraffin-embedded (FFPE) material immediately after bronchoscopy and after obtaining the result of a pathomorphological examination. At the same time, material

TABLE 1 | Demographic and clinical features of the studied group of patients.

Characteristic	Percentage of tumor cells with PD-L1 expression							
	<50% (n=42, 70%)	≥50% (n=18, 30%)	*p-value*	X^2	<1% (n=19, 32%)	≥1% (n=41, 68%)	*p-value*	X^2
Age								
<65 (n=28)	21 (75)	7 (25)	0.43	0.625	11 (39)	17 (61)	0.23	1.408
≥65 (n=32)	21 (66)	11 (34)			8 (25)	24 (75)		
Gender								
Male (n=41)	28 (68)	13 (32)	0.18	0.67	14 (34)	27 (66)	0.54	0.368
Female (n=19)	14 (78)	5 (26)			5 (36)	14 (74)		
Histological type								
SqSc (n=30)	**25 (83)**	**5 (17)**	**0.02**	**5.079**	11 (37)	19 (63)	0.40	0.693
AC+NOS (n=24+6 respectively =30)	**17 (57)**	**13 (43)**			8 (27)	22 (73)		
Stage								
IIIB (n=14)	**14 (100)**	**0 (0)**	**0.005**	**7.826**	7 (50)	7 (50)	0.09	2.836
IV (n=46)	**28 (61)**	**18 (39)**			12 (26)	34 (74)		
Smoking status								
Yes (n=49)	33 (67)	16 (33)	0.34	0.896	17 (35)	32 (65)	0.29	1.132
No (n=11)	9 (82)	2 (18)			2 (18)	9 (82)		
Response to treatment								
CR+PR+SD (n=1+8+22)	22 (71)	9 (29)	0.86	0.029	10 (32)	21 (68)	0.92	0.010
PD (29)	20 (69)	9 (31)			9 (31)	20 (69)		

for genetic testing was secured and DNA as well as total RNA was isolated (there were no archival materials). The following factors have been genetically tested:

-relative expression of selected microRNA examined by qPCR (quantitative PCR) method,

-relative mRNA expression of *PD-L1* gene examined by qPCR method,

-copy number of *PD-L1* gene assessed by FISH (fluorescence *in situ* hybridisation) and qPCR methods,

-polymorphisms of the *PD-L1* gene promoter examined by qPCR method,

-protein expression on tumor cells assessed by IHC method (immunochistochemistry).

RNA Isolation

Total RNA including microRNA was extracted from FFPE tissues using the miRNeasy FFPE Kit (Qiagen Inc., Germany) according to the manufacturers' instructions. RNA samples were stored at −80°C until synthesis of complementary DNA (cDNA) was performed.

microRNA Expression

We examined relative expression of microRNAs (miR-141, miR-200a, miR-200b, miR-200c, miR-429, miR-508-3p, miR-1184, miR-1255a) complementary to the 3'UTR region (3'untranslated region) of *PD-L1* mRNA (according to the TargetScan 7.2 and miRBase Sanger). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and U6 RNA were used as internal control. cDNA was prepared using TaqMan Advanced miRNA cDNA Synthesis Kit (Life Technologies, USA) according to manufacturers' instructions. cDNA was amplified in real-time PCR performed on Illumina Eco Real-Time PCR System (Illumina Inc, San Diego, USA). The 20 µl of PCR mixture contained: 10 µl of TaqMan Fast Advanced Master Mix, 1 µl of TaqMan Fast Advanced miRNA Assay, 4 µl of RNase free water and 5 µl of cDNA. Reaction conditions were as follows: 95°C for 20 s (enzyme activation) and 40 cycles for 95°C for 5 s and 60°C for 30 s. Ct values were obtained for each examined microRNAs and for internal controls. Analysis was performed using $2^{-\Delta Ct}$ method.

PD-L1 Messenger RNA (mRNA) Expression

RT-PCR (reverse transcription PCR) for *PD-L1* mRNA was conducted using High-Capacity RNA-to-cDNA Kit (Life Technologies, USA) according to the manufactures' instructions.

mRNA expression was assessed by using *GAPDH* mRNA as an internal control. Real-time PCR was performed on Illumina Eco Real-Time PCR System (Illumina Inc, San Diego, USA). The qPCR mixture contained: 10 µl of TaqMan Fast Advanced Master Mix (Life Technologies, USA), 1 µl of TaqMan Gene Expression Assay (for PD-L1 or GAPDH separate reactions, Life Technologies, USA), 5 µl of RNaze-free water and 4 µl of cDNA. Reaction was conducted in subsequent conditions: 95°C for 20 s (enzyme activation) and 40 cycles: 95°C for 3 s, 62°C for 30 s. Ct values were obtained for *PD-L1* mRNA and for *GAPDH* mRNA. Analysis was performed using $2^{-\Delta Ct}$ method.

DNA Extraction

DNA was isolated from FFPE tissues using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) according to the manufactures' instruction. The quantity and quality of extracted DNA were analyzed using a BioPhotometer UV/Vis Spectrophotometer (Eppendorf, Germany).

PD-L1 Promoter Polymorphism (Single Nucleotide Variation - SNV)

Using quantitative real-time PCR, we examined two SNPs of *PD-L1* promoter region: rs822335 (C>T) and rs822336 (C>G). qPCR

reaction was performed using 5.5 µl of Genotyping MasterMix (Life Technologies, USA), 4 µl of DNA (5 ng/µl), 0.5 µl of TaqMan SNP Genotyping Assay (for rs822335 and rs822336 separately, Life Technologies, USA). Real-time PCR was performed on Illumina Eco Real-Time PCR System (Illumina Inc, San Diego, USA) in following conditions: initial denaturation and enzyme activation: 95°C for 10 min, and 40 cycles: 95°C for 15 s, 62°C for 90 s.

CNV of *PD-L1* Gene Assessed by qPCR Method

Copy number variation of *PD-L1* gene were studied using quantitative real-time PCR method based on RNazeP (TaqMan™ Copy Number Reference Assay) as a housekeeping gene. DNA from lymphocytes of sixteen healthy persons were taken as a control. PCR reaction was performed using 5.5 µl of Genotyping MasterMix (Life Technologies, USA), 4 µl of DNA (5ng/µl), 0.5 µl of TaqMan CNV Assay (Life Technologies, USA) on Illumina Eco Real-Time PCR System (Illumina Inc, San Diego, USA) in the following conditions: denaturation and enzyme activation: 95°C for 10 min, and 40 cycles: 95°C for 15 s, 62°C for 90 s. CNV was scored by $2^{-\Delta\Delta Ct}$ method.

CNV of *PD-L1* Gene Assessed by FISH Method

The ZytoLight SPEC CD274, PDCD1LG2/CEN9 Dual Color Probe (CE-IVD marked, Zytovision, Germany) was used to detect *PD-L1* gene copy number by fluorescence *in situ* hybridization technique. ZytoLight FISH-Tissue Implementation Kit (Zytovision, Germany) was used for pre-staining procedure. For this procedure 3–5 µm FFPE sections were placed on positively-charged glass slides. First, the specimen was kept for 10 min. at 70°C on the hot plate. Slides with samples were then washed twice in xylen for 10 min and dehydrated two times in subsequent solutions of alcohol: in 100% ethanol for 5 min, and in 90% and 70% ethanol for 5 min each. In sequence, the slides were washed twice in deionized water for 2 min and then were immersed for 15 min in pre-warmed Heat Pretreatment Solution Citric at 98°C. Then, the slides were put twice to deionized water for 2 min. After drying, the appropriate amount of pepsin solution was applied on the samples and they were incubated for 12 min at 37°C in a humidity chamber. The slides were put into Wash Buffer for 5 min and were dehydrated in 70%, 90%, and 100% ethanol for 1 min each. After drying, 10 µl of probe mixture was applied to a slide (in the dark) and immediately coverslipped and sealed with rubber cement. The slides were placed for 10 min on hotplate at 75°C and then at 37°C for overnight hybridization. Next day rubber cement was removed, and slides were placed in Wash Buffer at room temperature to allow the coverslips to float off the slides. Afterwards, the slides were washed twice for 5 min in Wash Buffer previously warmed to 37°C. Then they were dehydrated in 70%, 90% and 100% ethanol for 1 min each and allowed to dry in dark room. 10 µl of DAPI counterstaining was applied to the target area, then coverslipped, and the specimens were scored in fluorescence microscope (Nicon Eclipse 55i, Japan).

The SPEC *CD274*, *PDCD1LG2*/CEN 9 Dual Color probe is a mixture of a green fluorochrome direct-labeled probe specific for *CD274* (*PD-L1*) and *PDCD1LG2* (*CD273* or *PD-L2*) genes in chromosome 9 at 9p24.1 and orange fluorochrome direct-labeled probe specific for the classical satellite III region of chromosome 9 centromere. In "healthy" nucleus, two orange and two green signals are expected. In a cell with polysomy or amplification of *PD-L1* and *PD-L2* genes, multiple copies of the green signal or large green signal clusters are observed. The ratio (R) of the number of green signals from the probe complementary to the *PD-L1* gene to the number of red signals from the probe complementary to the centromere was calculated.

At least 60 non-overlapping nuclei was analyzed in each sample in three different regions of interest.

PD-L1 Protein Expression

Immunohistochemical analyses (IHC) of PD-L1 protein expression were performed on FFPE tissue cut into 3 µm sections and fixed on Thermo Scientific Superfrost Plus™ glass slides. Glass slides with tissue sections were preheated in 59°C on hotplate prior to IHC staining for at least 3 h. PD-L1 protein IHC staining was conducted using VENTANA SP263 antibody on Ventana Benchmark GX equipment according to the manufacturers' instruction. After staining all glass slides were washed and dehydrated twice in a series of two 96% ethanol and two xylene washing steps, and then coverslipped.

The cut of points for the assessment of cancer cell percentages with PD-L1 expression (<50% and ≥50% of tumor cells with PD-L1 expression or <1% and ≥1% of tumor cells with PD-L1 expression) were adopted from the Updated Analysis of KEYNOTE-024 and KEYNOTE-010 clinical trials, which compared the efficacy of pembrolizumab and first or second line chemotherapy based on platinum compounds or docetaxel (16, 17).

Statistical Analysis

The response rate to immunotherapy and progression free survival (PFS) measured in weeks as well as overall survival (OS) measured in months from the start of immunotherapy were evaluated. The statistical analysis was made using chi square, U Mann-Whitney, Spearman, Pearson, and Kaplan-Meier tests. Multivariate analysis using Cox proportional hazards regression method with stepwise selection procedures by minimum AIC was used to establish factors affecting patients' survival. Receiver operating curves (ROC) with area under the curves (AUC) were used to determine the diagnostic value of microRNAs to predict the PFS or OS. The Youden Index has been determined. Analysis were conducted using MedCalc and Statistica softwares.

The study was approved by the Ethics Committee of the Medical University of Lublin, Poland (No. KE-0254/95/2018). In order to collect blood from the patient, we obtained informed consents. The language of informed consents is Polish.

RESULTS

Response to Immunotherapy and Molecular Factors

Response to immunotherapy was observed in 9 patients (15%, including one complete response), disease stabilization - in 22 patients (36.7%), and progression - in 29 patients (48.3%). Median PFS in the whole group of patients reached 16 weeks and median OS was 10.5 month.

Significantly higher and lower expression of miR-200b and miR-429 respectively, was observed in patients with disease control (p=0.015 and p=0.043 respectively, compared to patients with disease progression (**Figures 1A, B** respectively). There was no differences in percentage of tumor cells with PD-L1 expression in responders and non-responders' group (p=0.85, **Figure 1C**). The other examined genetic predictive factors, and clinical factors including gender, age, performance status (PS=0 vs. PS=1), stage of disease, pathomorphological diagnosis, line of immunotherapy did not affect treatment response.

In univariate analysis, we observed that the median PFS was significantly higher in patients with high miR-200b expression (HR=0.4253, 95% CI: 0.1737–1.0417, p=0.05, **Figure 2A**) and in patients with high miR-508 expression (HR=0.4401, 95% CI: 0.1903-1.0178, p=0.05, **Figure 2B**) and in patients with low expression of miR-429 (HR=0.1288, 95% CI: 0.01727–0.9606, p=0.04, **Figure 2C**) compared to patients with low and high expression of these molecules, respectively. Moreover, in patients with high mRNA expression of the *PD-L1* gene, the median PFS was not significantly higher than in patients with low mRNA expression for the *PD-L1* gene (HR=0.4965, 95% CI: 0.2013–1.2249, p=0.12, **Figure 2D**). Patients with CC genotype in rs822336 polymorphic site of *PD-L1* gene had insignificantly lower median PFS (HR=0.5330; 95% CI: 0.2473–1.1484; p=0.1) than patients with CG or GG genotypes of this polymorphism. The other examined genetic predictive factors, PD-L1 protein expression on tumor cells and clinical factors did not affect progression free survival of immunotherapy treated patients.

In multivariate analysis with Cox proportional hazards regression method, we found that patients with PD-L1 expression on ≥1% of tumor cells compared to patients without PD-L1 expression on cancer cells had a significantly lower risk of progression (HR=0.3857, 95% CI: 0.1612–0.9226, p=0.0323). Moreover, patients with CC or CG genotypes in rs822336 of *PD-L1* gene as well as with high miR200b expression compared to patients with CC genotype of this polymorphism and with low miR200b expression had an insignificantly lower risk of progression (**Table 2**).

Diagnostic value of genetic factors for PFS prediction was calculated in ROC analysis. We found that AUC for miR-200b was 0.848 with specificity of 87% and sensitivity of 67% (95% CI: 0.689–1, p<0.0000, Youden index=0.54), for miR-429 - 0.711 with specificity of 66% and sensitivity of 77% (95% CI:0.413–1, p=0.16, Youden index=0.42), for miR-508-3p –0.674 with specificity of 88% and sensitivity of 73% (95% CI: 0.43–0.918, p=0.16, Youden index=0.42) and for *PD-L1* mRNA - 0 with specificity of 92% and sensitivity of 65% (95% CI: 0.473–1, p=0.07, Youden index=0.59).

In univariate analysis, the median OS was non significantly higher in patients with low expression of miR-429 (HR=0.6288, 95%CI: 0.3053–1.2949, p=0.06) compared with patients with high expression of this microRNA. The median OS in patients treated with pembrolizumab in first-line therapy was not reached, and the differences in death risk reduction between first and second line immunotherapy was not statistically significant (HR=0.7429, 95% CI: 0.3261–1.6923, p=0.4792). An imbalance in the number of patients treated with first and second line of immunotherapy could explain the absence of differences in outcome according to PD-L1 expression. Moreover, most of the patients had metastatic lung cancer (n=46, 77%). This could cause the inability to demonstrate a statistically significant correlation between the stage of the disease and disease outcome. Other examined genetic, immunological, and clinical factors did not influence the median OS according to a univariate analysis.

In multivariate analysis with Cox proportional hazards regression method, we found that patients with PD-L1 expression on ≥1% of tumor cells compared to patients without PD-L1 expression on cancer cells had a significantly lower risk of death (HR=0.377, 95% CI: 0.1636–0.8688, p=0.022, **Table 2**).

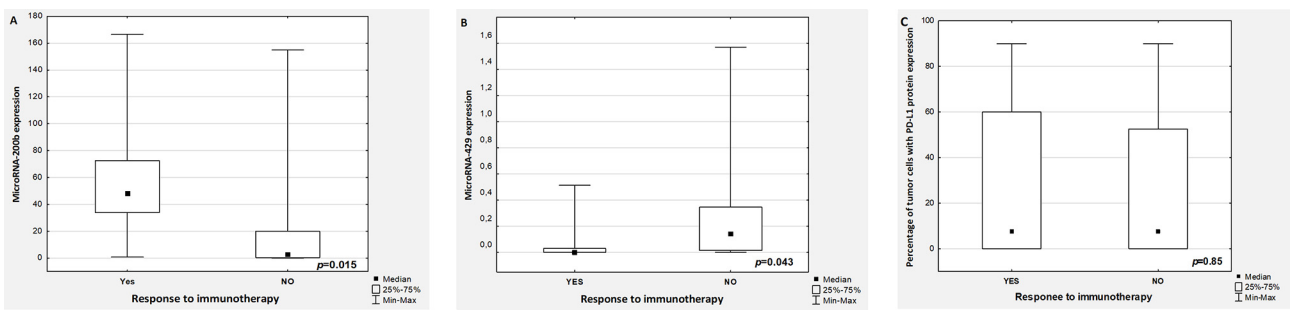

FIGURE 1 | The expression of miR-200b **(A)**, miR-429 **(B),** and percentage of tumor cells with PD-L1 expression **(C)** in patients with and without disease control (YES: stable disease or partial response or complete response, NO: progression disease).

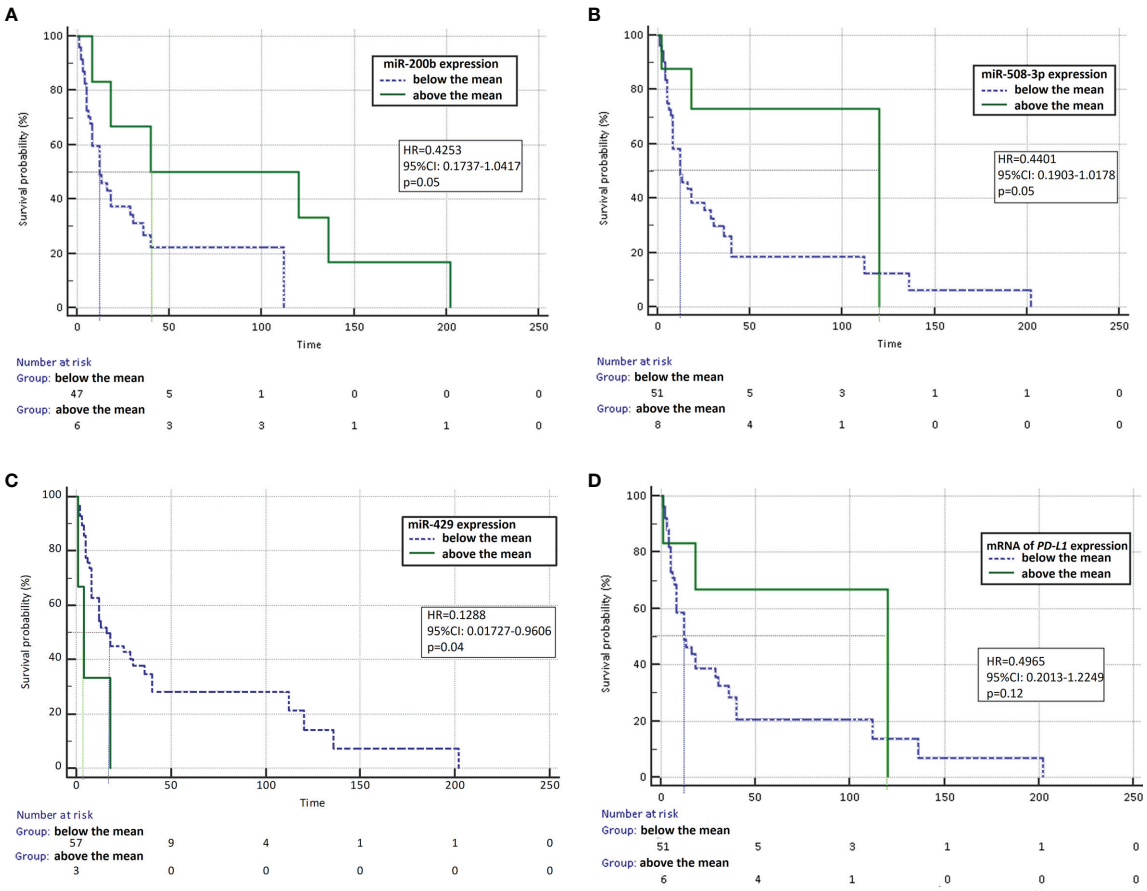

FIGURE 2 | Progression-free survival analysis using Kaplan-Meier test. Survival curves were calculated for patients expressing individual microRNAs and mRNA above and below the median: miR-200b **(A)**, miR-508-3p **(B)**, miR-429 **(C)**, and mRNA of the *PD-L1* gene **(D)**.

TABLE 2 | The factors significantly affected progression free survival and overall survival in patients treated with immunotherapy in multiparameter analysis using Cox proportional hazards regression method.

Factor	p	Hazard ratio	95% CI
PFS			
PD-L1 expression on ≥1% of tumor cells	**0.0323**	0.3857	0,1612–0.9226
CG or GG genotype in rs822336 of PD-L1 gene	0.0917	0.3358	0,0945–1.1937
High miRNA-200b expression	0.0771	0.528	0,0190–1.2266
Overall model fit: χ^{2} = 4.88, p=0.0272			
OS			
PD-L1 expression on ≥1% of tumor cells	**0.0220**	0.3770	0.1636–0.8688
Overall model fit: χ^{2} = 13.467, p=0.0037			

Influence of Molecular Factors on PD-L1 Protein Expression

Percentage of PD-L1-positive cancer cells was significantly correlated with the number of *PD-L1* gene copies in the tumor cells' nuclei found with the FISH method (Spearman's R=0.3320, p=0.04, Pearson's R=0.333,2, p=0.033, **Figure 3A**). There was no correlation between PD-L1 protein and *PD-L1* mRNA expression (p=0.6). Moreover, there was a significant positive correlation between the number of copies of the *PD-L1* gene found in FISH method and PD-L1 gene copies number assessed in qPCR method (Spearman's R=0.4284, p=0.009, Pearson's R=0.3388, p=0.014, **Figure 3B**). **Figure 4** shows sample images from the FISH and IHC analysis used to assess *PD-L1* gene copy number and to assess the percentage of tumor cells with PD-L1 protein expression.

The expression of miR-200b and miR-200c significantly negatively correlated with the percentage of tumor cells with expression of PD-L1 protein (R=-0326, p=0.027, Pearson's

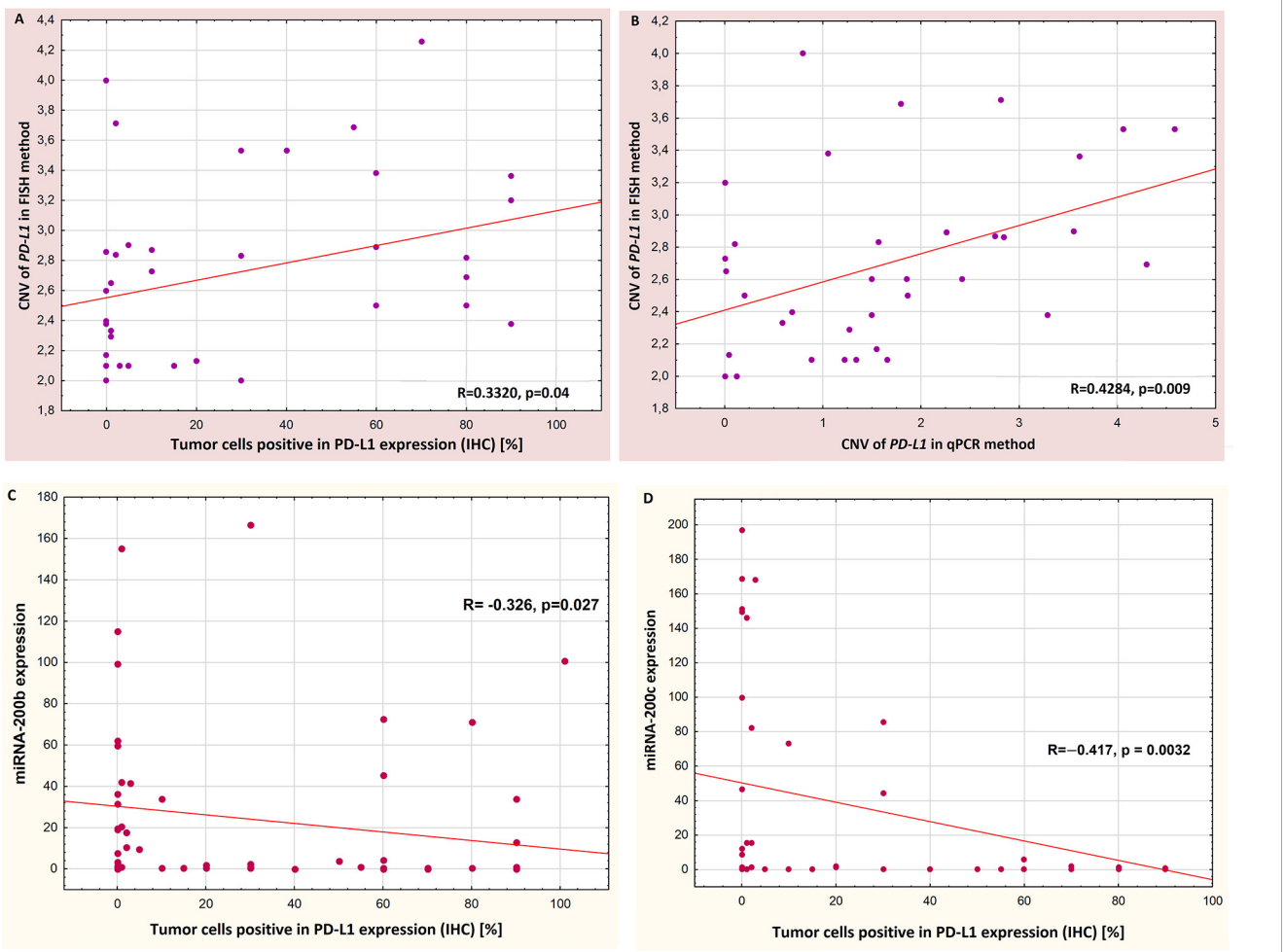

FIGURE 3 | Correlations between: percentage of PD-L1-positive tumor cells and copy number of *PD-L1* gene detected by FISH method **(A)**, copy number of *PD-L1* gene detected by FISH and qPCR methods **(B)**, percentage of PD-L1-positive tumor cells and expression of miR-200b **(C)** and percentage of PD-L1-positive tumor cells and expression of miR-200c **(D)**.

R=−0.221, p=0.08 and R=−0.417, p=0.0032, Pearson's R=0.29, p=0.037, respectively, **Figures 3C, D**).

Patients with CC genotype of the *PD-L1* gene in rs822336 polymorphic site showed significantly lower percentage of tumor cells with PD-L1 protein expression than patients with CG and GG genotype of this polymorphism (p= 0.025).

DISCUSSION

Based on the KEYTNOTE-024 clinical trial results, the advanced NSCLC patients with ≥50% of PD-L1-positive tumor cells could be treated with pembrolizumab in the first line of therapy. While, locally advanced and advanced patients with PD-L1 expression on ≥1% of tumor cells may be eligible for second line therapy with pembrolizumab (KEYNOTE 010 study). NSCLC patients in stage IIIB and IV, regardless of their PD-L1 expression status, can also be treated in the second line with nivolumab (CheckMate 017 and 057) or atezolizumab (OAK study). Currently, many combination therapies involving immunotherapy and new possibilities for immunotherapy have emerged. Atezolizumab was approved for first line treatment in patients with advanced NSCLC with PD-L1 on ≥50% of tumor cells, and pembrolizumab - in patients with PD-L1 expression on ≥1% of neoplastic cells. Chemotherapy in combination with pembrolizumab in the first line of treatment in patients with advanced NSCLC, regardless of PD-L1 expression on tumor cells, has become common. The combination of nivolumab and ipilimumab is used in the first line of treatment in advanced patients with PD-L1 expression on ≥1% of neoplastic cells (2–4, 16–18).

Tumor PD-L1 expression is still widely used in qualification for immunotherapy. Rapid progression is also observed in patients with PD-L1-positive tumors. On the other hand, treatment

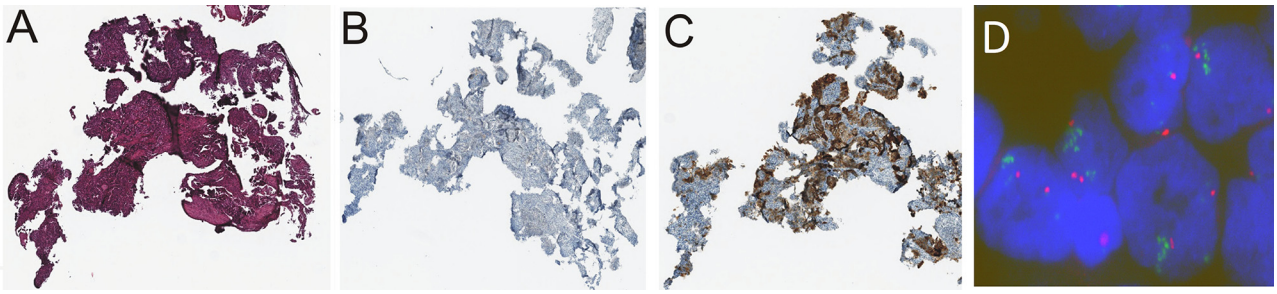

FIGURE 4 | Sample immunohistochemistry (IHC) and FISH staining images taken on the same patient: hematoxylin and eosin staining performed to localize tumor cells **(A)**, negative control of IHC staining **(B)**, IHC staining with the SP263 antibody that detected PD-L1 expression on 70% of tumor cells **(C)**, cell nuclei with visible amplification of the *PD-L1* gene (green); red signals come from the probe complemented to the centromere of chromosome 9; R=4.25 **(D)**.

response may occur in patients without PD-L1 expression on tumor cells. However, expression of PD-L1 on tumor cells is not an ideal predictive factor for immunotherapy (19).

In our study, we found that there were no differences in the percentage of tumor cells with PD-L1 expression (analyzed as a continuous variable) in patients with disease control and progression occurred during immunotherapy. However, in multivariate analysis, we showed that patients with PD-L1 expression on ≥1% of tumor cells compared to patients without PD-L1 expression on cancer cells had a lower risk of progression and death. In our study the high percentage of patients with squamous cell carcinoma should be explained by the use of bronchoscopic methods used in diagnosis of advanced lung cancer patients. Squamous cell carcinoma is usually a central tumor and the tumor material is easy to collect by bronchoscopy. Moreover, due to the high percentage of smokers in Poland, we still observe a high incidence of squamous cell carcinoma. In a group of 1,923 lung cancer patients diagnosed with bronchoscopy in our clinical center, we found 32.07% patients with squamous cell lung cancer (data not shown in this article).

Previously, clinical studies have been proven that immunotherapy is ineffective in NSCLC patients with *EGFR* gene mutations and *ALK* gene rearrangements. Moreover, the researchers found that high tumor mutation burden (TMB) may be a favorable predictor of immunotherapy in NSCLC patients. Currently, there are many studies to link efficacy of ICIs with presence of abnormalities in different genes, including mutations in *STK11* (*serine-treonine kinase 11*) and *KEAP1* genes. Expression of genes encoding immunomodulatory factors (e.g. cytokines or chemokines) is also considered as a predictive factor for immunotherapy. Many studies devoted to biomarkers that would distinguish hyperprogression an pseudoprogression in patients treated with immunotherapy (20–22). However, only in single studies microRNAs expression is considered as predictor factor for immunotherapy. Investigation on numerous genetic factors that may affect PD-L1 expression are also important.

Therefore, our attention has been focused on microRNAs molecules as potential predictors of response to immunotherapy. In addition, CNV measured by two different methods (qPCR and FISH), assessment of SNPs in the promoter region of the *PD-L1* gene or *PD-L1* mRNA expression were considered as tests of potential utility in qualification to immunotherapy. Our observations show that among mentioned factors profile of microRNAs could identify the patients most likely to benefit from immunotherapy. We tested 8 microRNAs molecules that regulate *PD-L1* mRNA expression according to the TargetScan base.

We found that miR-200b and miR-429 expression could distinguish between NSCLC patients who benefit from immunotherapy and those with disease progression. Their expression and expression of miR-508-3p also influenced the progression free survival in NSCLC patients treated with immunotherapy. On the other hand, there were no differences in the percentage of PD-L1-positive cancer cells in groups of patients with disease control and disease progression. However, the only significant predictive factor which increased the risk of progression or death in a multivariate analysis was PD-L1 expression on ≥1% of tumor cells. Therefore, based on our empirical data, we are joining the opinion that PD-L1 protein expression on tumor cells is not a perfect predictive biomarker for qualification to immunotherapy.

The "microRNAs market" is very wide and a single microRNA molecule has regulatory capacity for dozens or even hundreds of genes. This creates complicated regulatory networks. Therefore, scientific research on these molecules is not easy (23). For example, Tao and colleagues looked for biomarkers of immunotherapy response in patients with prostate cancer (24). They detected that high expression of miR-195 and miR-16 were positively correlated with the biochemical recurrence-free survival of prostate cancer patients. Moreover, the expression of these two molecules were negatively correlated with PD-L1, PD-1, CD80 and CTLA-4 proteins expression (24).

In our study we investigated microRNAs expression in cancer tissue. However, researchers tend to lean toward liquid biopsy in their scientific reports on biomarkers related to the effectiveness of immunotherapy. Boeri and colleagues established plasma immune-related microRNAs-signature classifier (MSC) to identify the risk for an adverse course of the disease in patients with early stages of NSCLC (25). MSC stratified individuals into

high, intermediate, and low risk of unfavorable course of the disease. Afterwards, they tested the efficacy of the MSC as prognostic marker in patients with advanced NSCLC treated with nivolumab, pembrolizumab, avelumab, atezolizumab, durvalumab or durvalumab and tremelimumab combination (25). They study included a panel of 24 microRNAs in Custom Taq Array MicroRNA. The study showed that MSC was significantly associated with progression free survival and overall survival. Patients with intermediate and low risk of unfavorable course of the disease estimated based on MSC had higher median of PFS and OS than patients with high risk of disease progression (25). Researchers indicated also that the plasma MSC test could supplement PD-L1 tumor expression test to identify a subgroup of patients with advanced lung cancer who could benefit from immunotherapy. This specific approach using circulating microRNAs profile could be a promising diagnostic tool to assess patients' chances of responding to immunotherapy.

In our study we also analyzed *PD-L1* mRNA expression. There was no correlation between *PD-L1* mRNA and protein expression. In our opinion, this indicates (which was also pointed out by Wei et al), that PD-L1 expression is subjected to post-transcriptional regulatory mechanisms of microRNAs, protein modification and their transport (26). In this context, we also noted that *PD-L1* mRNA expression, SNP of *PD-L1* gene promoter or CNV of *PD-L1* gene were not a predictor of progression-free survival or overall survival in patients treated with immunotherapy. Unfortunately, the group of patients included in our study was not large and it is limitation of our study. This limitation can be seen especially in the case of subgroups analyzed. The number of patients qualified for our study was due to two problematic aspects. Firstly reimbursement of immune checkpoint inhibitors in NSCLC patients began later in Poland than in other European Union countries. Therefore, we did not manage to collect more patients. Secondly, the number of genetic and immunological tests needed to be performed was large. Therefore, we could only include patients with sufficient tumor materials (in terms of the number and percentage of cancer cells). Due to this limitation of our study, further experiments should be carried out in an enlarged group of patients treated with immunotherapy.

Other researchers also looked at the number of the *PD-L1* gene copies as a predictive marker for immunotherapy. Ikeda examined samples of 94 patients who underwent surgical resection of lung cancer. The authors considered the three copies of the gene as *PD-L1* amplification and they found amplification of *PD-L1* gene in 5% of patients. Also, they noticed the co-amplification of the *PD-L1* gene and the *JAK2* gene in some cases. These genes are located quite close on chromosome 9 (27). Additionally, they tested PD-L1 protein expression on tumor cells by IHC method. No increased expression of PD-L1 protein was found in patients with amplification of the *PD-L1* gene.

Goodman and colleagues examined the number of *PD-L1* gene copies by FISH method in 221 of NSCLC patients. They showed an increase in the number of *PD-L1* gene copies in 11 patients, representing 5% of the study population. In contrast to results obtained by Ikeda et al. (12), all samples with increased *PD-L1* gene copy number had increased expression of PD-L1 protein (≥1% of tumor cells with PD-L1 expression) (12). The results of Goodman's study are consistent with our results, in which we found a positive correlation between the PD-L1 gene copy number in FISH examination and the percentage of tumor cells with PD-L1 expression in IHC test (12).

Lamberti at al. compared the percentage of PD-L1-positive tumor cells with the results of targeted NGS (next generation sequencing) in large group of 909 non-squamous NSCLC patients (28). They noticed that *PD-L1* gene copy loss is associated with lower response rate and shorter PFS in NSCLC patients treated with immune checkpoint inhibitors. The expression of PD-L1 protein were lower in patients with mutations in the following genes: *STK11, EGFR*, *CTNNB1* (*catenin beta 1*), *APC* (*adenomatous polyposis coli*), and *SMARCA4* (*SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4*).

The results of these studies showed that it is legitimate to pay attention to the number of PD-L1 gene copies in NSCLC patients as a predictive factor for immunotherapy. It is also important to examine how *PD-L1* gene CNV and other genetic factors (e.g. genes mutations) affect the expression of PD-L1 protein on tumor cells.

CONCLUSION

It seems that evaluation of microRNAs expression in plasma or in tissue of NSCLC patients is a good direction in the search for new predictive factors useful in the qualification of NSCLC patients for immunotherapy. The miR-200b and miR-429 molecules in tumor cells seem to have greatest impact on the effectiveness of immunotherapy in NSCLC patients. However, it should be noted that this is a study involving a small group of patients and further studies on circulating/tissue microRNAs, on a larger group of patients, should be carried out.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical University of Lublin, Poland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception and design: AG, PK, IC, TKuc. Acquisition of data: AG, IC, TKub, SK, DŚ, MB-B, MF, RK, MSz, AK, SM, DK, MSa, ES, RR, JS, MK, JM. Analysis and interpretation of data: AG,

KW-K, TKuc, BJ, IP, KR, PK, KK. Drafting the article: AG, PK, TKuc, KW-K. Critically revising: JM, JS, ES, TKub, SM. Final approval of the version to be submitted: AG, PK, JB, IC, TKuc, SK, KW-K, TKub, BJ, IP, DŚ, MB-B, MF, RK, MSz, KR, KK, AK, SM, DK, MSa, ES, RR, JS, MK, JM. All authors contributed to the article and approved the submitted version.

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nab-Paclitaxel Plus Durvalumab in Patients With Previously Treated Advanced Stage Non-small Cell Lung Cancer (ABOUND.2L+)

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Background: The standard therapy for advanced stage non-small cell lung cancer (NSCLC) with no actionable gene alterations is a platinum-based chemotherapy doublet and immune checkpoint blocker (ICB), either concurrently or sequentially, followed by docetaxel at the time of tumor progression. However, more effective treatments are needed. We evaluated the *nab*-paclitaxel and durvalumab combination in patients with previously treated advanced stage NSCLC.

Methods: Patients with advanced stage NSCLC previously treated with one line of platinum-based doublet with or without an ICB and no activating *EGFR* mutations or *ALK* translocations received *nab*-paclitaxel 100 mg/m^2 (days 1 and 8) plus durvalumab 1,125 mg (day 15) every 21 days. The primary endpoint was progression-free survival (PFS). Key secondary endpoints included overall survival (OS) and safety.

Results: Between February 2016 and December 2016, 79 patients were enrolled. The median age was 63 years. Most patients were males (68.4%), had non-squamous histology (69.6%), and had no prior ICB treatment (88.6%). The median PFS was 4.5 months; median OS was 10.1 months. A *post hoc* analysis of survival by prior ICB treatment revealed a median PFS and OS of 4.4 and 9.9 months, respectively, in ICB-naive patients and 6.9 months and not estimable, respectively, in patients previously treated with ICB. The most common treatment-emergent adverse events were asthenia (46.2%) and diarrhea (34.6%); four treatment-related deaths (5.1%) occurred.

Conclusions: The *nab*-paclitaxel and durvalumab combination is feasible and demonstrated antitumor activity without new safety signals. Additional studies using taxanes and ICB in patients with previously treated NSCLC are warranted.

Clinical Trial Registration: ClinicalTrials.gov registration (NCT02250326).

EudraCT number: 2014-001105-41

Keywords: advanced stage non-small cell lung cancer, durvalumab, immune checkpoint blocker plus chemotherapy, *nab*-paclitaxel, second-line therapy

INTRODUCTION

The standard initial therapy for patients with advanced stage non-small cell lung cancer (NSCLC) and no actionable gene alterations includes platinum-based chemotherapy doublet and immune checkpoint blockers (ICB) either sequentially or concurrently (1, 2). For patients previously treated with chemotherapy alone, monoclonal antibodies against programmed death-1 (PD-1) or its ligand (PD-L1) are associated with improved overall survival (OS) when compared to docetaxel in the second-line setting, although prolonged benefit is observed only in a small percentage of patients (3–6). For those already treated with both chemotherapy and ICB, docetaxel with or without ramucirumab remains the standard option (7). Response rates and survival, however, remain poor for the majority of patients treated with second-line ICB monotherapy or docetaxel, indicating the need for new treatment options.

Nanoparticle albumin-bound paclitaxel (*nab*-paclitaxel), a cremophor-free formulation that can be administered without dexamethasone premedication (8), is one of the recommended drugs for locally advanced or metastatic NSCLC in combination with carboplatin, with or without pembrolizumab, in the first-line setting for patients who are not candidates for curative surgery or radiation therapy (2, 9). Single-agent *nab*-paclitaxel has been associated with promising results in previously treated patients with metastatic NSCLC (10, 11) and better tolerability compared with docetaxel in a randomized clinical trial for patients with metastatic breast cancer (12).

Durvalumab is a human IgG1 monoclonal antibody against PD-L1, approved as consolidation therapy after chemoradiation in patients with stage III NSCLC (13). In patients with advanced stage NSCLC, single-agent durvalumab is associated with similar activity and safety profiles when compared with other ICBs (14).

Based on both preclinical (15) and clinical (16–18) studies demonstrating a benefit from concurrent chemotherapy and ICB in NSCLC, we postulated that the same principles may apply to patients treated with *nab*-paclitaxel after progression on platinum-based chemotherapy with or without ICB.

ABOUND.2L+ was a randomized clinical trial comparing *nab*-paclitaxel alone or in combination with CC-486, an oral formulation of azacitidine (19). The study showed no benefit from the addition of azacitidine to *nab*-paclitaxel in the randomized cohorts of the study, although single-agent *nab*-paclitaxel was associated with a tolerable safety profile and promising outcomes, including response rates, median progression-free survival (PFS), and median OS of 16.3%, 4.2, and 17.0 months, respectively. Here we present the results of the third arm of the study evaluating the combination of *nab*-paclitaxel and durvalumab, which was non-randomized and added as an amendment.

MATERIALS AND METHODS

Patients

Eligible patients were 18 years of age or older and had histologically or cytologically confirmed advanced stage NSCLC, radiologically documented measurable disease by Response Evaluation Criteria In Solid Tumors (RECIST) 1.1, an Eastern Cooperative Oncology Group performance status 0 or 1, adequate hematologic, renal, and hepatic function, and no other current active malignancy requiring anticancer therapy. One prior line of platinum-based chemotherapy regimen for metastatic or recurrent disease was allowed, with the exception of taxanes, which were allowed only if used in the adjuvant setting more than 12 months prior to enrollment into the trial. Prior use of ICBs, either as a component of the first-line therapy or in the second line, was allowed. Key exclusion criteria included known activating *EGFR* mutations or *ALK* translocations, peripheral neuropathy grade 2 or higher by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.0, active or prior documented autoimmune or inflammatory disorder, use of systemic immunosuppressive therapy within 14 days from starting durvalumab except for corticosteroids, at doses up to 10 mg per day of prednisone or its equivalent, and brain metastases unless asymptomatic and clinically stable for at least 8 weeks following completion of therapy.

The study was approved by the institutional review board or independent ethics committee at participating sites and conducted in accordance with the principles of good clinical practice and the Declaration of Helsinki. All patients provided written informed consent prior to treatment initiation.

Study Design

This was an open-label phase II study. Initially, patients were randomized 1:1 to receive *nab*-paclitaxel 100 mg/m^2 on days 8 and 15 plus CC-486 200 mg on days 1 to 14 or *nab*-paclitaxel 100 mg/m^2 alone on days 1 and 8 of each 21-day cycle (19). After enrollment for the *nab*-paclitaxel alone and *nab*-paclitaxel plus CC-486 arms was completed, the protocol was amended to include a third arm, which enrolled patients with advanced stage non-squamous or squamous NSCLC and one prior platinum-based chemotherapy. Patients were assigned to this arm and received *nab*-paclitaxel 100 mg/m^2 infused over 30 min on days 1 and 8 plus durvalumab 1,125 mg infused over 1 h on day 15, with the cycles repeating every 21 days. Hence, randomization did not occur between the *nab*-paclitaxel

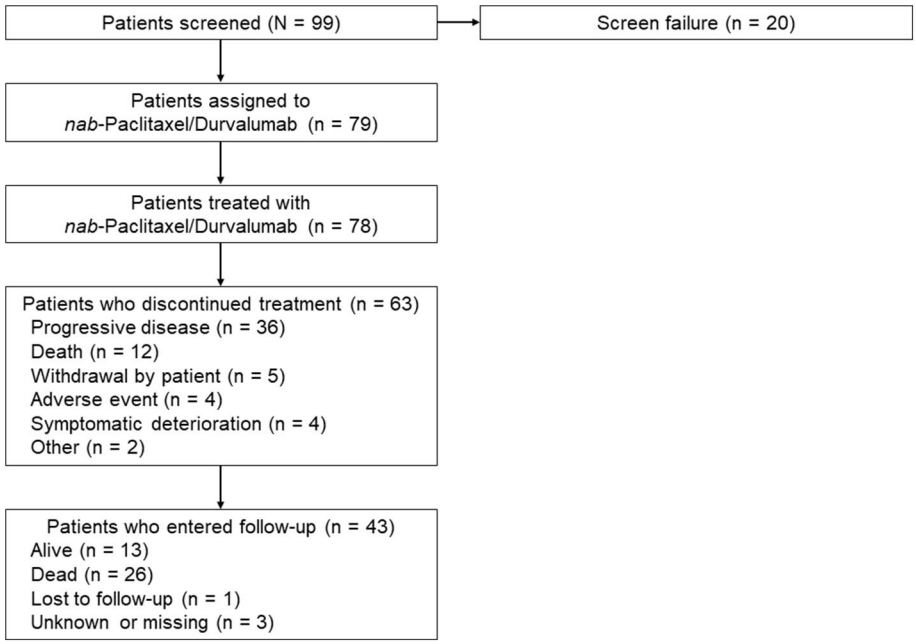

FIGURE 1 | CONSORT diagram.

plus durvalumab and *nab*-paclitaxel alone arms. Treatment was continued until documented tumor progression, unacceptable toxicity, withdrawal of consent, lost to follow-up, or death.

Endpoints and Assessments

The primary endpoint was the duration of PFS in the intent-to-treat (ITT) population, defined as the time from date of treatment initiation to the date of disease progression, based on investigator assessment using RECIST version 1.1, or death from any cause. Secondary endpoints included OS, defined as the time between the first treatment and death from any cause, overall response rate (ORR), disease control rate (DCR), and safety. Imaging studies with computer tomography scans were performed at baseline and every 42 days until treatment discontinuation. All patients who received at least one treatment dose underwent safety analysis, with documentation of treatment-emergent adverse events (TEAEs) graded based on NCI-CTCAE version 4.0.

Statistical Analyses

The median PFS and median OS were estimated using the Kaplan-Meier estimates with corresponding two-sided 95% confidence intervals (CI). The sample size estimation was based on the expected median PFS of 4.25 months for *nab*-paclitaxel plus durvalumab and 2.5 months for *nab*-paclitaxel alone based on historical data with docetaxel alone (7, 20, 21).

In the randomized part of the trial, it was estimated that a total of 160 patients would be needed to observe 120 PFS events, which would have provided 80% power to detect a hazard ratio of 0.60 using a one-sided test at the 2.5% level of significance. After enrollment in the *nab*-paclitaxel plus CC-486 and *nab*-paclitaxel monotherapy arms was completed (each arm had reached a total of approximately 80 patients), all patients were assigned to the *nab*-paclitaxel plus durvalumab arm until approximately 80 patients were enrolled in that arm. The statistical assumptions used were identical to the *nab*-paclitaxel plus CC-486 arm. An interim analysis for PFS comparing the *nab*-paclitaxel plus durvalumab and *nab*-paclitaxel monotherapy arms was conducted when approximately 30 PFS events were observed in the *nab*-paclitaxel plus durvalumab combination arm.

RESULTS

Patients

Between September 2016 and December 2016, 99 patients were screened and 79 were enrolled into the study (**Figure 1**). The median age was 63 years (range 29–84 years); most patient were males (68.4%) and had non-squamous histology (69.6%) and no prior use of ICB (88.6%) (**Table 1**). Prior chemotherapies included a platinum (97.5%), pemetrexed (50.6%), vinorelbine (25.3%), and gemcitabine (26.6%). The median duration of prior platinum plus pemetrexed (39 patients) was 10.4 weeks (range 1.6–43.4 weeks). In total, nine patients (11.4%) received prior ICB, which was their most immediate prior line of therapy. Among these nine patients, six received nivolumab (66.7%), two received pembrolizumab (22.2%), and one received avelumab (11.1%), with the latter in the first-line setting. Of these nine patients, eight (88.9%) received prior ICB monotherapy. The remaining patient (11.1%) received prior combination therapy with ICB and carboplatin. One patient did not receive the study treatment. In total, 63 patients (80.8%) discontinued treatment due to progressive disease (36 [46.2%]), death (12 [15.4%]), patient withdrawal (5 [6.4%]), clinical progression

TABLE 1 | Demographic and baseline clinical characteristics.

Characteristic	*nab*-Paclitaxel + Durvalumab (*N* = 79)
Age, years	
Mean (SD)	62.7 (10.74)
Median (range)	63.0 (29–84)
Male sex, number (%)	54 (68.4%)
ECOG performance status, number (%)	
0	18 (22.8%)
1	61 (77.2%)
Stage IV disease, number (%)	75 (94.9%)
Histology	
Squamous	23 (29.1%)
Non-squamous	55 (69.6%)
Not specified	1 (1.3%)
Prior therapy	
No prior ICB	70 (88.6%)
Prior ICB	9 (11.4%)[a]

[a]*One patient with prior ICB treatment received first-line avelumab without platinum-based chemotherapy.*
ECOG, Eastern Cooperative Oncology Group; ICB, immune checkpoint blocker.

(4 [5.1%]), adverse events (4 [5.1%]), and other reasons (2 [2.6%]). The adverse events (AEs) leading to *nab*-paclitaxel and durvalumab discontinuation were pneumonitis, urinary tract infection, and *Pneumocystis jirovecii* pneumonia (one patient each) and increased white blood cell count, abnormal liver function, localized edema, and peripheral edema (one patient). The median follow-up for survival was 12.9 months.

Efficacy

For the primary analysis of investigator-assessed PFS in the ITT population, 56 patients (70.9%) had progressive disease (PD) or died. The median PFS was 4.5 months (95% CI, 3.5–5.9 months), with an estimated PFS rate at 12 months of 25.7% (95% CI, 16.3–36.2%; **Figure 2A**).

For the OS analysis in the ITT population, 44 patients (55.7%) had died. The median OS was 10.1 months (95% CI, 7.8 months-not estimable [NE]), with estimated survival at 12 months of 43.8% (95% CI, 32.3–54.7%; **Figure 2B**).

The ORR was 27.8% (95% CI, 18.3–39.1%), with complete response (CR) in one patient (1.3%) and partial response (PR) in 21 patients (26.6%). The DCR was 70.9% (95% CI, 59.6–80.6%), with 34 patients (43.0%) achieving stable disease (SD).

Due to the heterogeneity of the patient population, a *post hoc* analysis was performed to evaluate outcomes according to prior ICB treatment and histology in the 78 patients with known histology. The median PFS was 4.4 months (95% CI, 2.96–5.68 months) in ICB-naive patients and 6.9 months (95% CI, 1.38 months-NE) in patients previously treated with ICB (**Figure 3A**). Among ICB-naive patients, the median PFS was 5.6 months (95% CI, 1.3–7.8 months) in those with squamous histology and 4.1 months (2.7–5.7 months) in those with non-squamous histology, with corresponding 12-month PFS of 27.1% (95% CI, 9.0–49.2%) and 20.9% (95% CI, 10.6–33.6%), respectively (**Figure 3B**). The median OS was 9.9 months (95% CI, 7.52–12.94 months) in ICB-naive patients and NE in those previously treated with ICB (**Figure 3C**). Among ICB-naive patients, the median OS for squamous and non-squamous histologies was 8.9 months (95% CI, 2.99 months-NE) and 10.3 months (95% CI, 6.57 months-NE), respectively (**Figure 3D**).

The median percentage change from baseline in sum of diameters of target lesions was −17.3% (range −100.0 to +65.4%) for ICB-naive patients and −21.4% (range −76.2 to +28.1%) for those previously treated with ICB (**Figure 4**). Among ICB-naive patients, one achieved CR (1.4%), 17 achieved PR (24.6%), and 30 had SD (43.5%) for a DCR of 69.6%. Of the remaining patients, 10 had PD (14.5%) and 11 (15.9%) had no post-treatment response assessment. Among patients previously treated with ICB, four achieved PR (44.4%), four achieved SD (44.4%), and one had PD (11.1%).

Treatment Exposure

The median number of cycles and treatment duration were 7 (range 1–21) and 24.4 weeks (range 1.4–66.1 weeks), respectively. The median cumulative doses of *nab*-paclitaxel and durvalumab were 1,250 mg/m^2 and 6,750 mg, respectively. Dose reductions for *nab*-paclitaxel due to toxicity occurred in 11 patients (14.1%); per protocol, durvalumab dose reductions were not allowed. Dose delays of *nab*-paclitaxel and durvalumab occurred in 39 patients (50%) and 24 patients (30.8%), respectively.

Safety

All patients developed at least one TEAE, with grade 3 or 4 TEAEs occurring in 43 patients (55.1%) (**Table 2**). The most common TEAEs of any grade were asthenia (46.2%), diarrhea (34.6%), and decreased appetite (33.3%), while the most common grade 3 or 4 TEAEs were asthenia (12.8%), dyspnea (7.7%), and pneumonia (7.7%). Peripheral neuropathy was seen in 29 patients (37.2%), of which 3 (3.8%) were grade 3 or 4.

Immune-related TEAEs of grade 3 or 4 were observed in seven patients (9.0%). The grade 3 or 4 immune-related TEAEs were diarrhea (1 [1.3%]), rash (2 [2.6%]), pneumonitis (1 [1.3%]), and adrenal insufficiency (3 [3.8%]). Among the nine patients who received prior ICB, immune-related TEAEs of grade 3 or 4 were observed in two patients (22.2%). The grade 3 or 4 immune-related TEAEs were adrenal insufficiency and rash (1 patient [11.1%] each). Other AEs of interest included grade 1 or 2 dermatitis (10.3%) and thyroid dysfunction (hypothyroidism, 6.4%; hyperthyroidism, 2.6%; thyroiditis, 1.3%).

Overall, four patients (5.1%) experienced a grade 5 TEAE suspected to be treatment related. The specific grade 5 treatment-related TEAEs were pneumonitis, pulmonary hemorrhage, *Pneumocystis jirovecii* pneumonia, and clinical deterioration.

DISCUSSION

The median PFS of 4.5 months exceeded the pre-specified threshold, and the response rate of 27.8% is higher than previously described in patients treated with either docetaxel or ICB monotherapy (3–6).

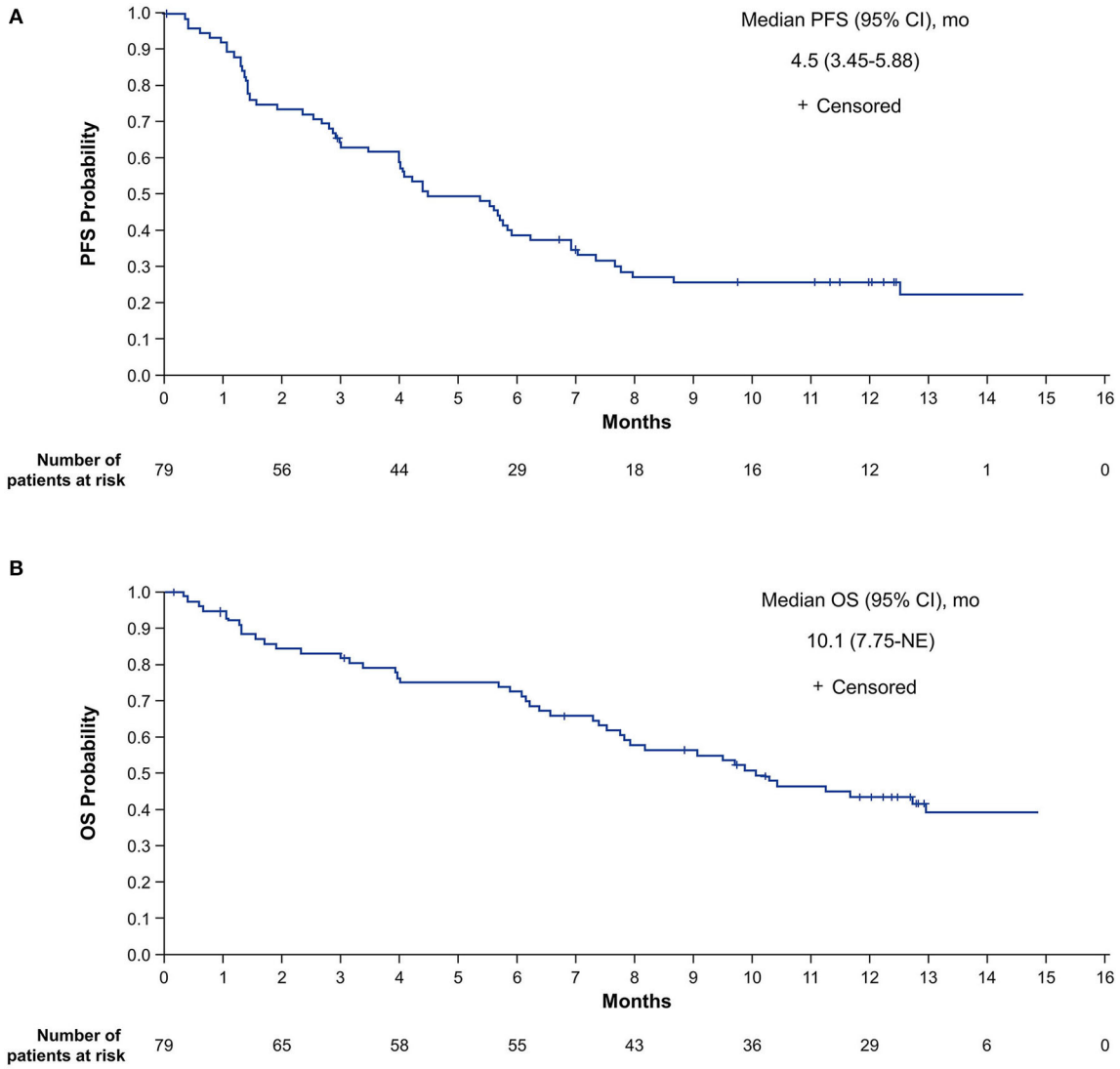

FIGURE 2 | Investigator-assessed PFS **(A)** and OS **(B)** in the intent-to-treat population. NE, not estimable; OS, overall survival; PFS, progression-free survival.

There are increasing data suggesting that the efficacy of conventional chemotherapy drugs relies not only on their cytotoxic effects, but also on the ability to stimulate the immune system. In the case of paclitaxel, there are many postulated mechanisms for its immunostimulatory effects in addition to tumor debulking in case of effective cytotoxic activity, with reduction of the systemic immunosuppression caused by malignant cells. Paclitaxel induces immunogenic cell death through increased chromosomal content, which causes endoplasmic stress response and calreticulin exposure, stimulates toll-like receptor 4 increasing T cell priming by dendritic cells, and depletes myeloid derived suppressor cells (22, 23). Paclitaxel may also increase the antigenicity of cancer cells by stimulating their production of interferon-β, leading to increasing MHC class I expression (24, 25). Another mechanism is the sensitization to cytotoxic T lymphocytes by upregulating mannose-6-phosphate receptors on tumor cells, which increases the permeability of the membrane to granzyme B, leading to cancer cell death independent from perforin (26). Since *nab*-paclitaxel does not require the use of premedication with corticosteroids, it may be a better partner for combination with ICB when compared with other taxanes since, at least in patients treated with single-agent ICB, use of corticosteroids at doses of 10 mg or higher has been associated with worse outcomes compared with no use within 30 days (27).

There are limited data on the combination of taxanes and ICB without platinum in patients with previously treated NSCLC. In a small phase Ib study evaluating the combination of chemotherapy and nivolumab 10 mg/kg every 3 weeks, there were six patients previously treated with platinum-doublets who were enrolled into the docetaxel arm (28). One patient

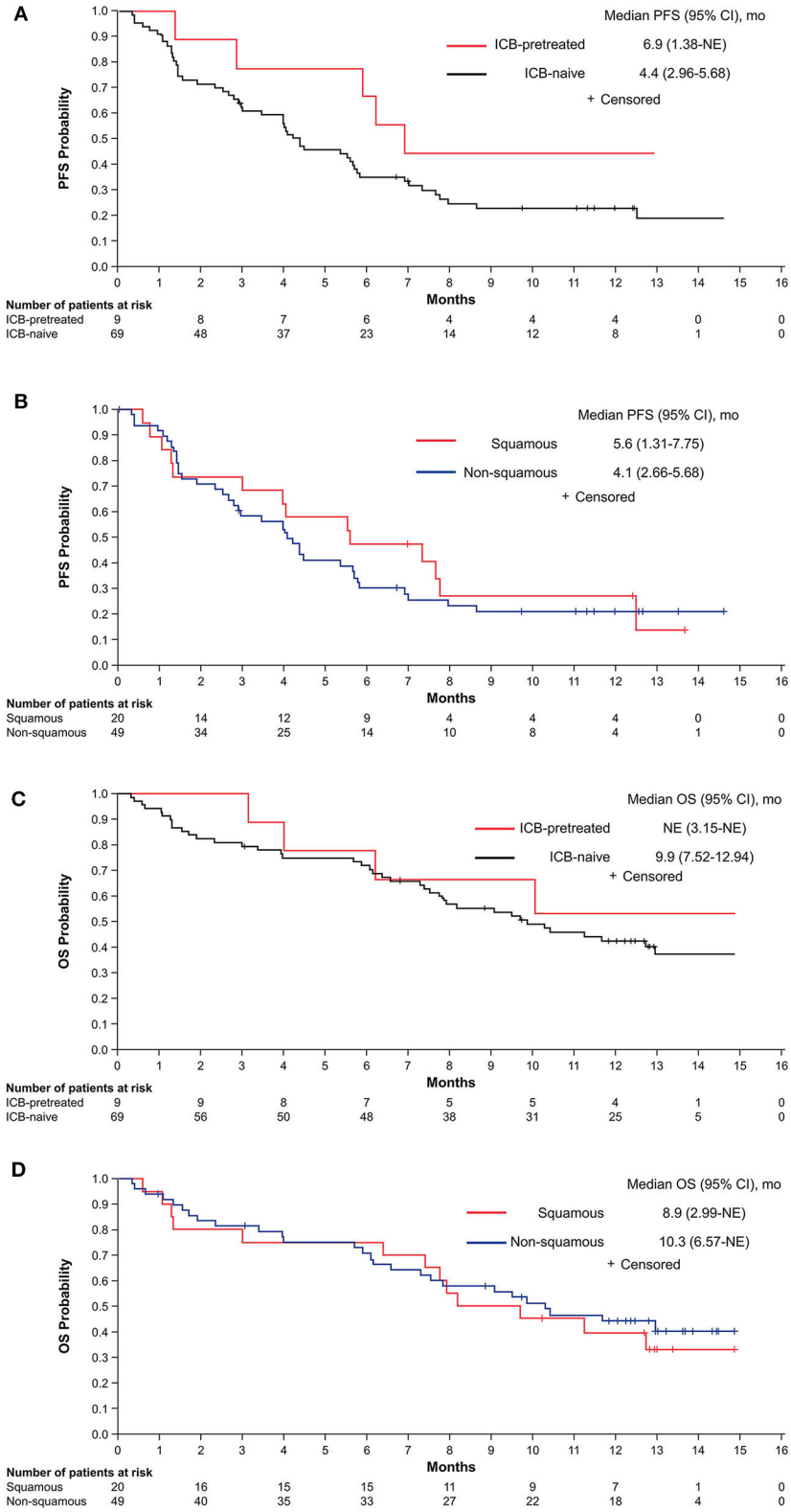

FIGURE 3 | PFS by ICB treatment status **(A)** and in ICB-naive patients according to histology **(B)** and OS by ICB treatment status **(C)** and in ICB-naive patients according to histology **(D)**. ICB, immune checkpoint blocker; NE, not estimable; OS, overall survival; PFS, progression-free survival.

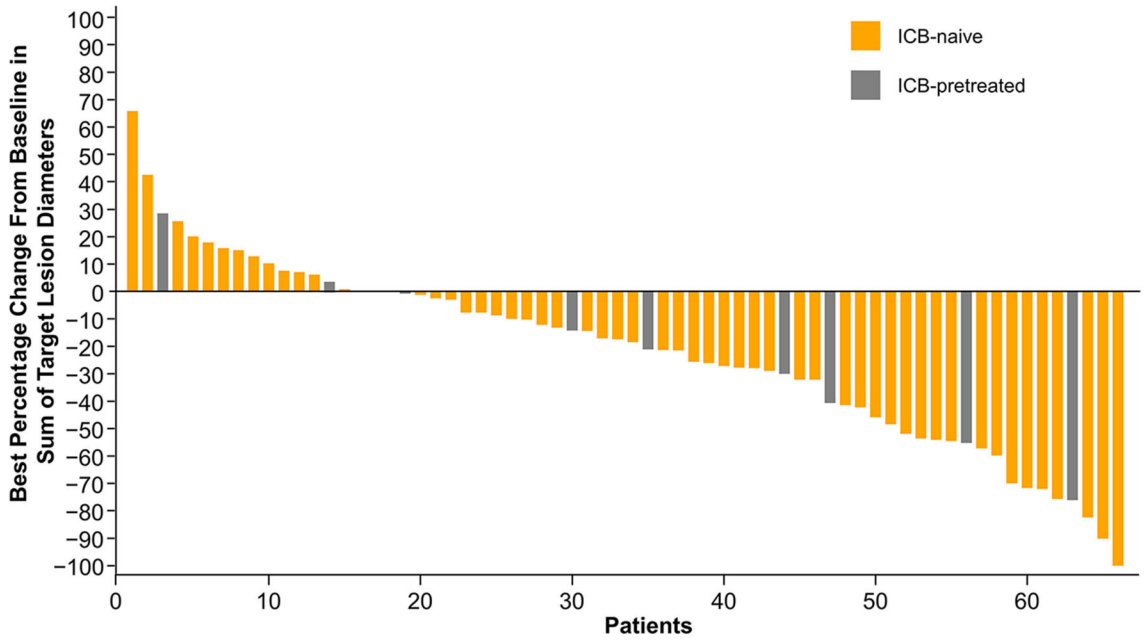

FIGURE 4 | Percentage of tumor change from baseline. ICB, immune checkpoint blocker.

TABLE 2 | Treatment-emergent adverse events occurring in at least 15% of patients.

TEAE, *n* (%)	*nab*-Paclitaxel + Durvalumab (*n* = 78)	
	Any grade	Grade 3 or 4
Any event	78 (100.0%)	43 (55.1%)
Asthenia	36 (46.2%)	10 (12.8%)
Diarrhea	27 (34.6%)	1 (1.3%)
Decreased appetite	26 (33.3%)	1 (1.3%)
Alopecia	25 (32.1%)	0
Anemia	24 (30.8%)	4 (5.1%)
Peripheral neuropathy	29 (37.2%)	3 (3.8%)
Fatigue	22 (28.2%)	2 (2.6%)
Upper respiratory tract infection	22 (28.2%)	2 (2.6%)
Constipation	20 (25.6%)	0
Dyspnea	20 (25.6%)	6 (7.7%)
Nausea	19 (24.4%)	0
Cough	19 (24.4%)	0
Pyrexia	15 (19.2%)	0
Neutropenia	14 (17.9%)	5 (6.4%)
Lower respiratory tract infection	12 (15.4%)	1 (1.3%)

TEAE, treatment-emergent adverse event.

(16.5%) responded to the treatment, and the median PFS was 3.1 months. All patients developed grade 3 or 4 AEs, which were mostly hematologic.

In our study, the combination of *nab*-paclitaxel plus durvalumab was generally well-tolerated; however, the 4 grade 5 treatment-related TEAEs were an unexpected finding. Patients in the *nab*-paclitaxel plus durvalumab arm received more treatment cycles and a greater cumulative dose of *nab*-paclitaxel compared with those who received *nab*-paclitaxel with or without CC-486 in the randomized portion of this trial (19). Therefore, it is reasonable to speculate that the grade 5 treatment-related TEAEs were due, at least in part, to a greater treatment exposure with second-line combination therapy. Although there were no grade 5 AEs reported with second-line pembrolizumab plus docetaxel in the phase II PROLUNG trial (29), that study accrued patients considerably younger than those treated with *nab*-paclitaxel plus durvalumab in the current study (mean, 50.1 vs. 62.7 years).

Our study has several limitations. The durvalumab arm started enrollment after the completion of the randomized *nab*-paclitaxel with or without CC-486, precluding a more reliable comparison to single-agent *nab*-paclitaxel, and the increased use of pembrolizumab or atezolizumab in the first-line setting decreased the number of ICB-naive patients eligible for the *nab*-paclitaxel plus durvalumab combination in the clinical setting (16–18). Furthermore, we did not collect data on PD-L1 status of the tumors or genetic biomarkers, which are known predictors for response to ICBs in previously untreated patients (5, 30), although the role is not clear in patients with resistance to ICBs.

Nevertheless, despite these limitations, our study provides the initial data on the use of *nab*-paclitaxel plus durvalumab after progression on ICB, a setting with increased relevance since the trial was designed. Despite the multiple ongoing studies evaluating combinations involving antibodies against PD-1 or PD-L1 with other immunostimulatory antibodies, antiangiogenic agents and targeted drugs (31–33), none has an established role in NSCLC patients previously treated with ICBs. Although the

number of patients previously treated with ICB in our study was small, the preliminary results are promising, with all but one patient achieving tumor control and a prolonged benefit observed in four of the nine patients.

Since there are limited data on the efficacy of docetaxel after tumor progression on ICB and we cannot clearly separate the effects of *nab*-paclitaxel and durvalumab, this question could only be addressed in a randomized clinical trial comparing a taxane, either docetaxel or *nab*-paclitaxel, alone or in combination with an ICB.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available. Data requests may be submitted to Celgene, A Bristol Myers Squibb Company, at: https://vivli.org/ourmember/celgene/ and must include a description of the research proposal.

ETHICS STATEMENT

Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors satisfied the following criteria: contributed to the conception or design of the research or the acquisition, analysis, or interpretation of data for the research, drafted the manuscript or critically revised it for important intellectual content, gave final approval of the version to be published, agreed to be accountable for all aspects of the research in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of Interest: DM has been an advisory board member for AbbVie, Bristol Myers Squibb, PharmaMar, Takeda, Gilead, and Boehringer Ingelheim. PEP has been an advisory board member for AstraZeneca, Boehringer Ingelheim, Bristol Myers Squibb, Celgene (a Bristol Myers Squibb Company), Clovis, Janssen, MSD, and Roche. JB has been an advisory board member for Amgen, AstraZeneca, Bristol Myers Squibb, Novartis, and Roche. OJ-V has been an advisory board member for AstraZeneca, AbbVie, Boehringer Ingelheim, Bristol Myers Squibb, Merck Sharp & Dohme, and Roche/Genentech. DS has served as an advisory for Roche Canada; he has received grant support from AstraZeneca, Boehringer Ingelheim, Bristol Myers Squibb, Celgene (a Bristol Myers Squibb Company), and Novartis. AA has received honoraria from Boehringer Ingelheim, Bristol Myers Squibb, Eli Lilly, and MSD. RB and TJO are employees of and hold stock in Bristol Myers Squibb Company. MW is contracted for medical review by Celgene (a Bristol Myers Squibb Company) and was employed by Pharmaceutical Research Associates Inc. (PRA) Health Sciences. MR served as a consultant or advisory for AstraZeneca, Boehringer Ingelheim, Bristol Myers Squibb, Celgene (a Bristol Myers Squibb Company), Lilly, Merck Sharp & Dohme, Pfizer, and Roche. DT has been an advisory board member and received honorarium from Celgene (a Bristol Myers Squibb Company). RG serves in an advisory role for AbbVie, AstraZeneca, Baxalta, Boehringer Ingelheim, Celgene (a Bristol Myers Squibb Company), Merck, MSK, Pfizer, and Roche; he serves as a consultant for AbbVie, ARAID, Astellas, Bristol Myers Squibb, Genentech, and INC Research; he has received honoraria from AbbVie. JF was employed by the company Lungenklinik Löwenstein gGmbH.

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The Impact of Programmed Death-Ligand 1 Expression on the Prognosis of Early Stage Resected Non-Small Cell Lung Cancer: A Meta-Analysis of Literatures

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Introduction: Previous studies have demonstrated that programmed cell death-ligand 1 (PD-L1) serves as biomarker for poor prognosis and survival in advanced-stage non-small cell lung cancer (NSCLC) patients. However, the merit of PD-L1 expression to predict the prognosis of early stage NSCLC patients who underwent complete resection remains controversial. In the present study, we performed a meta-analysis to investigate the relationship between PD-L1 expression and prognosis in patients with early stage resected NSCLC.

Methods: Electronic databases, including PubMed, EMBASE, and the Cochrane Library, were searched until July 23 2020 for studies evaluating the expression of PD-L1 and the prognosis of resected NSCLCs. Hazard ratios (HRs) with 95% confidence intervals (CIs) of overall survival (OS) and disease-free survival (DFS) were pooled and analyzed. Heterogeneity and publication bias analyses were also assessed.

Results: A total of 15 studies involving 3,790 patients were considered in the present meta-analysis. The pooled HR indicated that PD-L1 expression related to a much shorter DFS (HR = 1.56, 95% CI: 1.18–2.05, $p < 0.01$), as well a significantly worse OS (HR = 1.68, 95% CI: 1.29–2.18, $p < 0.01$). Furthermore, our analysis indicated that PD-L1 expression was significantly associated with gender (male *vs.* female: OR = 1.27, 95% CI:1.01–1.59, p = 0.038), histology (ADC *vs*. SCC: OR = 0.54, 95% CI:0.38–0.77, p = 0.001), TNM stage (I *vs*. II–III: OR = 0.45, 95% CI:0.34–0.60, p = 0.000), smoking status (Yes *vs* No: OR = 1.43, 95% CI:1.14–1.80, p = 0.002) and lymph node metastasis (N+ *vs* N–: OR = 1.97, 95% CI:1.26–3.08, p = 0.003).

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Conclusions: The results of this meta-analysis suggest that PD-L1 expression predicts an unfavorable prognosis in early stage resected NSCLCs. The role of personalized anti-PD-L1/PD-1 immunotherapy in the adjuvant settings of resected NSCLC warrants further investigation.

Keywords: PD-L1, NSCLC, meta-analysis, prognosis, resection

INTRODUCTION

Lung cancer is the most commonly diagnosed cancer and a leading cause of cancer-related deaths (1). Surgery is the standard treatment for early stage non-small cell lung cancer (NSCLC); however, postoperative prognosis remains unsatisfactory, with a 5-year survival rate ranging between 71 and 83% (2).Therefore, it is essential to identify new biomarkers for efficient clinical decision making and improve patient outcomes. Currently, blockade of the programmed cell death 1 (PD-1)/PD-1 ligand 1 (PD-L1) signaling pathway is one of the most promising immunotherapeutic strategies in boosting the immune system in the fight against cancer (3, 4). Programmed death 1 (PD-1), an important immune checkpoint molecule, is an immune-inhibitory receptor expressed on the surface of activated T cells in response to persistent inflammatory stimuli (5, 6). PD-L1 expressed on the tumor cells binds to the PD-1 receptors on activated T cells, resulting in the inhibition of the cytotoxic T cells. Blockade of the PD-1/PD-L1 pathway with monoclonal antibodies is a promising therapeutic strategy, with prominent clinical benefits of this checkpoint-blockade observed in recent clinical trials (7, 8).

Previously, a study has demonstrated that PD-L1 is a marker of poor prognosis and survival in advanced-stage NSCLC patients (9). A meta-analysis, performed on six studies with 1,157 patients, demonstrated that NSCLC patients with positive PD-L1 expression exhibited a much poorer OS (10). Another meta-analysis by Li et al., which included the largest number of patients (fifty studies with 11,383 patients), also indicated that PD-L1 IHC expression was related to poor overall survival (11). However, the searching deadline for Li's study was January 2018, and more recent studies focusing on PD-L1 and prognosis in NSCLC were missing. More importantly, all of previous studies, including Li's study, were performed on the NSCLC patients of both early and advanced stages, and they only took OS into account. Therefore, the impact of PD-L1 expression in the prognosis of patients with early stage NSCLCs following complete resection remains controversial. In the present study, we performed a meta-analysis of all available evidence not only to analyze OS but also assess the correlation between PD-L1 expression and DFS in early stage surgically resected NSCLC patients, which is more accurate and valuable to reflect the influence of PD-L1 expression on the survival of NSCLC.

METHODS

This study was reported on the basis of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement guidelines (**Supplementary Data 1**).

Relevant studies were retrieved by searching PubMed, Embase, and the Central Registry of Controlled Trials of the Cochrane Library, using the following terms: "Carcinoma, Non-Small Cell Lung" AND "PD-L1 protein, human" AND "Prognosis". (**Supplementary Data 2**) The final search period was July 23 2020. Two authors (SX and SZ) performed the search independently. We restricted our research to studies published in English.

Eligible studies were in agreement with the following criteria: (1) the histology type of cancer was NSCLC; (2) valid TNM stage and cancer differentiation, as well as sufficient survival data, such as hazard ratio (HR) with 95% confidence intervals (CI), OS, and DFS were available (3) were published in English; (4) evaluated the association between PD-L1 expression and prognosis or pathological features; (5) involved early stage resectable NSCLC patients; (6) had similar research experimental design and methods; (7) PD-L1 expression was divided into high (positive) and low (negative) categories; and (8) relevant information could be extracted from the full-text study. Exclusion criteria included: (1) duplicate reports, ongoing studies, letters, conference papers, and reviews; (2) studies regarding lung cancer cell lines, animal models, and other types of cancer; (3) studies with insufficient survival data for which HR and CI could not be determined; (4) papers not published in English; (5) methods and experimental design differed from those of the selected studies.

The Newcastle–Ottawa quality assessment scale (NOS) and National Institute for Clinical Excellence (NICE) quality assessment scale was performed to assess methodological quality and risk of bias for cohort studies and case series studies, respectively. The primary outcomes of our study were disease-free survival (DFS) and overall survival (OS). The characteristic details of the publications, including the first author's name, publication year, tumor type, PD-L1 level, stage, the evaluation method of the PD-L1expression, were extracted by two independent investigators. Any disagreement was discussed between investigators to reach a consensus. Multivariate analysis results were extracted as some included studies performed univariate analysis. We used the data directly from the included studies, providing precise HR (95% CI). In the case of studies only providing Kaplan–Meier survival curves, the Engauge Digitizer version 2.11 software was used to extract relevant numerical values from survival curves and calculate the HR (95% CI) (12, 13).

The STATA 15.0 (Stata Corporation, College Station, USA) software and R package 4.0.2 were used for data synthesis and analysis.

The random-effects model was employed in case of potential heterogeneity and to avoid underestimation of standard errors of

pooled estimates in this meta-analysis. HRs for DFS and OS with 95% CIs according to the expression status of PD-L1 were pooled. The pooled odds ratios (ORs) were used to investigate the correlation between PD-L1 expression and clinicopathological features.

The heterogeneity test was performed using the Cochrane's Q test (Chi-squared test; Chi^2) and I^2 metric (14). A chi-squared P value <0.1 or an I^2 statistic >50% was defined as statistically significant heterogeneity (15). Moreover, the potential publication bias was assessed through Begg's funnel plots (16). P <0.05 was considered as statistically significant based on the two-sided test. Subgroup analysis was conducted according to gender, histology, TNM stage, smoking status, and lymph node metastasis. An HR >1 reflected longer OS or DFS for PD-L1 negative patients.

Sensitivity analyses (17) were conducted to investigate the influence of a specific study on the pooled risk estimate by removing one study in each turn (**Figures 2D**, **3D**, **Supplementary Data 3**).

RESULTS

We identified 1,719 potentially relevant records through our search. A total of 1,558 studies were excluded after reviewing the title and abstract as their research contents failed to meet our inclusion criteria. Subsequently, the full texts of 92 articles were carefully screened, and a total of 15 studies (18–32) were found eligible for the final analysis. **Figure 1** summarizes the flow chart depicting the process followed for study selection. No article was excluded by methodological quality and risk of bias and sensitivity analysis for significant heterogeneity (**Figures 2D**, **3D**).

In all articles, resectability was a necessary intervention. All 15 studies were retrospective and published between 2016 and 2020. Fourteen of 15 studies recorded OS data, while only eight out of 15 studies presented only DFS information. Overall, 15 studies comprising of 3,790 patients were included in the pooled analysis and, all selected studies used immunohistochemistry or H-score

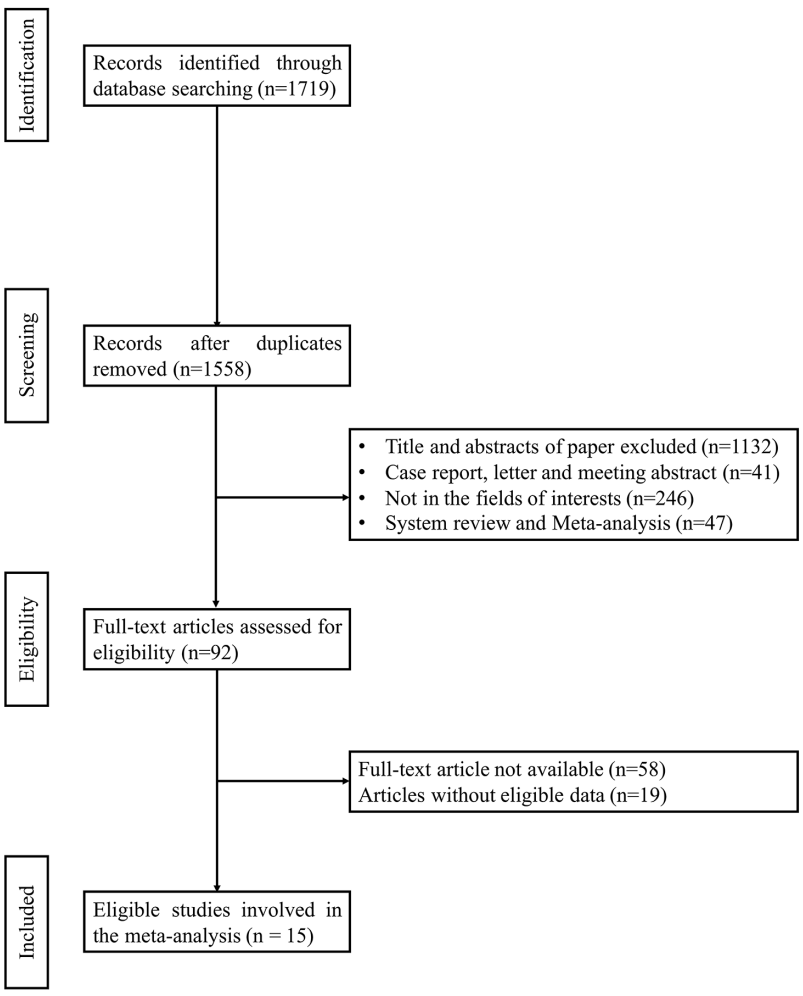

FIGURE 1 | Flow chart of selection process to identify studies eligible for pooling.

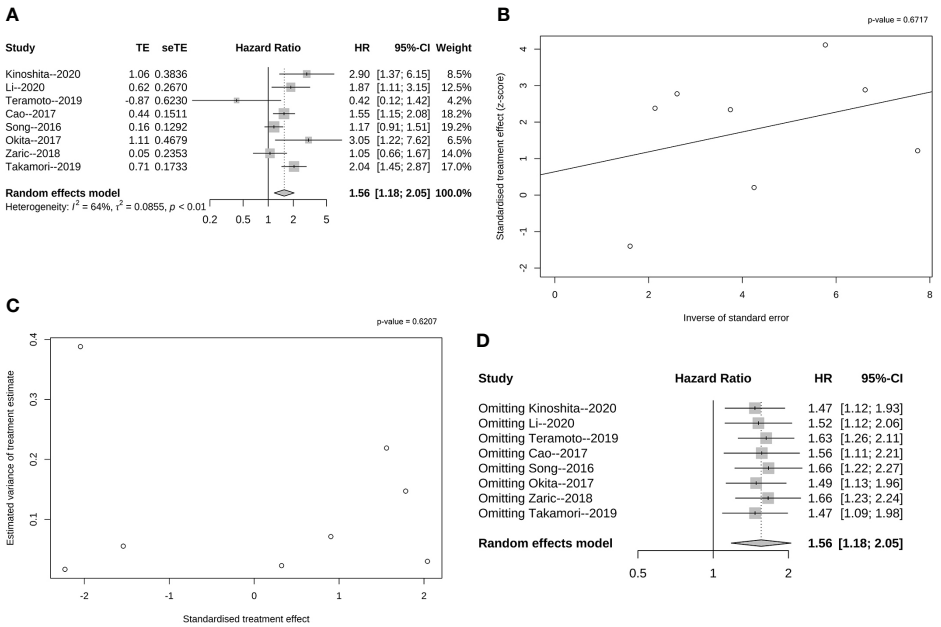

FIGURE 2 | Pooled analysis of DFS according to PD-L1 expression. **(A)** Forest plot of HR for the association between PD-L1 expression and DFS with early stage resected non-small cell lung cancer; **(B)** Funnel graph of potential publication bias of HR for DFS in the eligible studies by Egger's test; **(C)** Funnel graph of potential publication bias of HR for DFS in the eligible studies by Begg's test. **(D)** Sensitivity analysis for DFS *via* elimination of each study in turn.

assays to evaluate PD-L1 expression in tumor cells and/or tumor-infiltrating lymphocytes (TILs). **Table 1** summarized the characteristics of all involved studies, including peri-operative therapy and PD-L1 detection information.

As shown in **Figure 2A**, the overall pooled HR indicated that the high PD-L1 expression was related to a significantly shorter DFS (HR = 1.56, 95% CI: 1.18–2.05, p < 0.01). Further analysis demonstrated heterogeneity among the eight studies (I^2 = 64%, p<< 0.01), and we hence performed Egger's and Begg's tests. According to Egger's test (p = 0.671, **Figure 2B**) and Begg's test (p = 0.620, **Figure 2C**), no publication bias influenced the HRs for DFS. Additionally, this confirmed that a negative correlation existed between PD-L1 expression and DFS in the case of resectable NSCLC patients.

In NSCLC patients, the positive expression of PD-L1 on tumor tissues was associated with significantly poorer OS when compared to those indicating negative PD-L1 expression (HR = 1.68, 95% CI: 1.29–2.18, p < 0.01, **Figure 3A**). Furthermore, heterogeneity was observed among the 15 studies (I^2 = 73%, P < 0.01). However, Egger's (p = 0.595, **Figure 3B**) and Begg's tests (p = 0.411, **Figure 3C**) demonstrated no publication bias influencing the HRs for OS, confirming the negative correlation of PD-L1 expression and OS for resectable NSCLC patients.

The correlation of PD-L1 expression and clinical characteristics was further analyzed, including gender, histology, tumor stage, smoking status, and lymph node metastasis. We observed that PD-L1 expression was significantly associated with gender (male *vs.* female: OR = 1.27, 95% CI:1.01–1.59, p = 0.038), histology (ADC *vs.* SCC: OR = 0.54, 95% CI:0.38–0.77, p = 0.001), TNM stage (I *vs.* II–III: OR = 0.45, 95% CI:0.34–0.60, p = 0.000), smoking status (Yes *vs* No: OR = 1.43,95% CI:1.14–1.80, p = 0.002) and lymph node metastasis (N+ *vs* N−:OR = 1.97, 95%CI:1.26–3.08, p = 0.003) (**Table 2**).

In addition, we also investigated the different cutoff values of PD-L1 expression on the survival. We found that PD-L1 expression at 5% cutoff value was not correlated to DFS (HR = 1.39, 95% CI: 0.89–2.17, p = 0.142) (**Supplementary Data 4**), but indicated a significantly worse OS (HR = 1.93, 95% CI: 1.57–2.36, p = 0.000) (**Supplementary Data 5**). However, PD-L1 expression at 1% cutoff value was correlated with a poor DFS (HR = 1.76, 95% CI: 1.03–3.02, p = 0.040) (**Supplementary Data 6**) but not for OS (HR = 2.19, 95% CI: 0.66–7.22, p = 0.199) (**Supplementary Data 7**). We propose that the discrepancy for these findings may be attributed to limited data and non-uniform PD-L1 detection platforms.

DISCUSSION

Immune checkpoint inhibitors targeting PD-1/PD-L1 have improved survival in patients with advanced NSCLC in second- and first-line settings (33–37). The PACIFIC trial reported a DFS benefit in patients with locally advanced, unresectable stage III NSCLC who received durvalumab consolidation therapy (38). Owing to the success of immune checkpoint inhibitors in advanced NSCLC, these agents are currently under investigation in the neo- or adjuvant setting for resected NSCLC patients (39–42) Therefore, it is important to understand the impact of PD-1/PD-L1 expression on the prognosis of early stage, resected NSCLC patients. Several studies have demonstrated that PD-L1 is a

TABLE 1 | Summary characteristics of included studies.

Author	Years	Region	NOS (star) /NICE	Cancer type	Perioperative adjuvant therapy	Stage	Method	PD-L1 expression			Number	Antibody				Outcome
								Cut-off values	Negative	Positive		Company	Source	Type	Clone	
Kinoshita et al. (18)	2020	Japan	7	AD	NP	I	IHC	1%	155	48	203	Spring Biosclence, USA	Mouse	MAB	SP142	OS PFS
Gachechiladze et al. (19)	2020	Europe	8	NSCLC	12% neoadjuvant CT or CRT; 31% adjuvant CT; 18% adjuvant RT	I-III	IHC	NA	755	109	864	NA	Rabbit	PAB	SP263	OS
Li et al. (20)	2020	China	6	NSCLC	NP	I-III	IHC	5%	56	31	87	NA	Rabbit	DAB	66248-1-Ig	OS PFS
Meng et al. (21)	2019	China	7	NSCLC	29% adjuvant CT; 22% adjuvant CRT	I-III	IHC	10%	NA	NA	197	Abcam, USA	Rabbit	NA	28-8	OS
Kim et al. (22)	2019	Korea	7	AD	NP	I-III	IHC	5%	241	60	301	Spring Bioscience, USA	Rabbit	DAB	SP142	OS
Teramoto et al. (23)	2019	Japan	6	NSCLC	25% adjuvant CT	I-III	IHC	50%	104	21	125	Cell Signaling Technology, USA	Mouse	DAB	E1L3N	PFS
Cao et al. (24)	2017	China	7	NSCLC	61% adjuvant therapy*	I-III	IHC	50%	283	81	364	Cell Signaling Technology, USA	Mouse	DAB	EIL3N	OS PFS
Song et al. (25)	2016	China	7	AD	70% adjuvant therapy*	I-III	IHC	5%	199	186	385	Proteintech Group Inc.,USA	Rabbit	NA	66248-1-Ig	OS PFS
Okita et al. (26)	2017	Japan	6	NSCLC	NP	I-III	H-socre	100	78	13	91	Spring Biosclence, USA	Mouse	MAB	SP142	OS PFS
Sepesi et al. (27)	2017	America	7	NSCLC	21% adjuvant therapy*	I	IHC	4.69%	87	26	113	Cell Signaling Technology,USA	NA	NA	E1L3N	OS
Imanishi et al. (28)	2018	Japan	6	NSCLC	35% adjuvant therapy*	I-III	IHC	15%	16	10	26	Cell Signaling Technology, Japan	Rabbit	NA	E1L3N	OS
Zaric et al. (29)	2018	Austria	7	AD	46% adjuvant CT;7% adjuvant RT	I-III	IHC	1%	102	59	161	Cell Signaling	Rabbit	NA	E1L3N	OS PFS
Takamori et al. (30)	2019	Japan	8	AD	NP	I-III	IHC	1%	287	146	433	Spring Bioscience,USA	NA	NA	SP142	OS PFS
Matsubara et al. (31)	2019	Japan	7	SCC	NP	I-III	IHC	5%	134	77	211	Spring Bioscience, USA	Rabbit	DAB	SP142	OS
Igawa et al. (32)	2017	Japan	7	NSCLC	15% adjuvant therapy*	I-III	H-socre	100	109	120	229	Ventana Medical Systems, USA	Rabbit	PAB	SP263	OS

NSCLC, non-small cell lung cancer; AD, adenocarcinoma; SCC, squamous cell carcinoma; IHC, immunohistochemistry; OS, overall survival; DFS, disease-free survival; NA, not available; NP, not provided; CT, chemotherapy; CRT, chemo/radiotherapy; RT, radiotherapy.

**The exact means of therapy (CT, RT or CRT) is not clearly mentioned.*

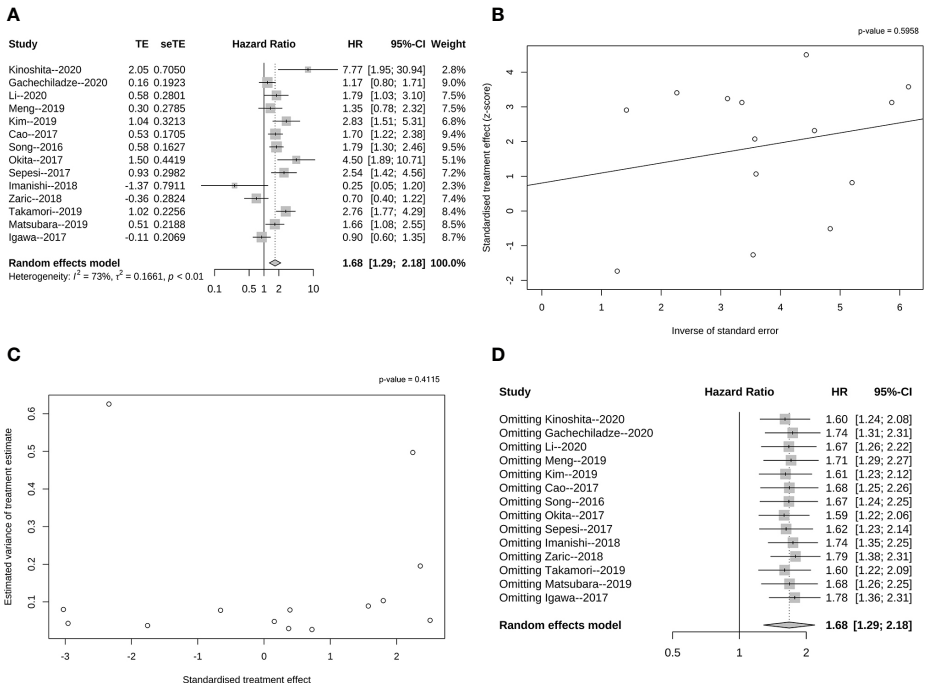

FIGURE 3 | Pooled analysis of OS according to PD-L1 expression. **(A)** Forrest plot of HR for the association between PD-L1 expression and OS with early stage resected non-small cell lung cancer; **(B)** Funnel graph of potential publication bias of HR for OS in the eligible studies by Egger's test; **(C)** Funnel graph of potential publication bias of HR for OS in the eligible studies by Begg's test. **(D)** Sensitivity analysis for OS *via* elimination of each study in turn.

TABLE 2 | The relationship between PD-L1 expression and clinicopathologic features.

Clinicopathological characteristics	No. of studies	Heterogeneity		OR	95%CI	P-value
		P-value	I² (%)			
Gender (male vs. female)	8	0.023	16.2	1.27	1.01-1.59	0.038
Histology (ADC vs. SCC)	5	0.044	8.12	0.54	0.38-0.77	0.001
TNM stage (I vs. II-III)	5	0.000	40.2	0.45	0.34-0.60	0.000
Smoking status (yes vs. no)	8	0.008	18.9	1.43	1.14-1.80	0.002
Lymph node metastasis (N+ vs. N0)	3	0.425	1.71	1.97	1.26-3.08	0.003

AD, adenocarcinoma; SCC, squamous cell carcinoma; OR, odds ratio; 95%CI, 95%confidence intervals.

biomarker indicating poor prognosis and survival in advanced-stage NSCLC patients. However, as reported by previous studies, the expression of PD-L1 on the prognosis of early stage resected NSCLC remains controversial. A comprehensive analysis is required to integrate all available data and provide further insight on this issue.

By summarizing the data available from included studies, our results confirmed that the PD-L1 expression indicates an unfavorable prognosis in early stage resected NSCLC as well. Our conclusion that PD-L1 positive or high expression indicated a significantly inferior OS in early stage resected NSCLC, is consistent with the previous analysis for all stages or advanced stage NSCLC patients (9–11). Additionally, we demonstrate that DFS, which was not explored by the previous studies, is also negatively correlated with PD-L1 expression in early stage resected NSCLC. Lastly, based on subgroup analysis, we observed that the PD-L1 expression was associated with gender, histology, tumor stage, smoking status, and lymph node metastasis.

Our analysis provided evidence to support that the PD-L1 expression may have prognostic value in predicting survival of patients with resected NSCLC. However, there were several limitations to our study. Firstly, this was based on a retrospective analysis. A prospective analysis is required to further clarify these issues. Secondly, 12 out of 15 included studies were performed in Asian population. Although it was not our intention to ignore the data from non-Asian population, the lack of data from non-Asian population is still a limitation for our meta-analysis. Therefore, future investigation should focus more on the PD-L1 expression in early stage non-Asian NSCLC patients. Thirdly, the different adjuvant treatment strategies post-surgery and follow-up also influence the survival of patients with NSCLC undergoing resection, which could have influenced the analysis. Moreover, the cutoff value of the defined PD-L1 expression was rather

different among the included studies. We have to categorize high (positive) and low (negative) PD-L1 expression and study the correlation between PD-L1 expression and post-operative survival. Finally, the platform and antibody of PD-L1 detection was not uniform either in the included studies. We have mentioned this issue in **Table 1**.

CONCLUSIONS

In conclusion, our meta-analysis suggests that PD-L1 expression indicates an unfavorable prognosis in early stage resected NSCLC patients. In the adjuvant settings resected NSCLC, the role of individualized anti-PD-L1/PD-1 immunotherapy merits further investigated.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

TS, SZ, and HG retrieved and analyzed all of the data in the study. XFL, YW, XL, and SKZ revised the manuscript for important intellectual contents. LP and DH reviewed and edited the manuscript. SX, ZL, and LP designed, checked, and supervised all study processes. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.567978/full#supplementary-material

Supplementary Data Sheet 1 | The PRISMA checklist of the manuscript.

Supplementary Data Sheet 2 | The search strategy of the manuscript.

Supplementary Data Sheet 3 | The data of sensitivity analyses.

Supplementary Data Sheet 4 | Forest plot of HR for the association between PD-L1 expression and DFS with early stage resected non-small cell lung cancer. (PD-L1 expression at 5% cutoff value).

Supplementary Data Sheet 5 | Forest plot of HR for the association between PD-L1 expression and OS with early stage resected non-small cell lung cancer. (PD-L1 expression at 5% cutoff value).

Supplementary Data Sheet 6 | Forest plot of HR for the association between PD-L1 expression and DFS with early stage resected non-small cell lung cancer. (PD-L1 expression at 1% cutoff value).

Supplementary Data Sheet 7 | Forest plot of HR for the association between PD-L1 expression and OS with early stage resected non-small cell lung cancer. (PD-L1 expression at 1% cutoff value).

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Concordance of PD-L1 Status Between Image-Guided Percutaneous Biopsies and Matched Surgical Specimen in Non-Small Cell Lung Cancer

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Objective: Programmed death-ligand 1 (PD-L1) expression status is a crucial index for identifying patients who will benefit from anti-programmed cell death protein 1 (PD-1)/PD-L1 therapy for non-small cell lung cancer (NSCLC). However, the concordance of Tumor Proportion Score (TPS) between biopsies and matched surgical specimens remains controversial. This study aims to evaluate the concordance of PD-L1 expression between image-guided percutaneous biopsies and matched surgical specimens.

Method: We evaluated 157 patients diagnosed with operable NSCLC on both surgical tissue sections and matched lung biopsies retrospectively. The patients underwent either regular computed tomography (CT)-guided biopsy (n = 82) or positron emission tomography (PET)/CT-guided biopsy (n = 75). The concordance between surgical specimens and lung biopsies for PD-L1 TPS was evaluated using Cohen's kappa (κ) coefficient.

Results: Immunohistochemical expression of PD-L1 was evaluated in both surgical resected specimens and matched biopsies in the eligible 138 patients. The concordance rate of PD-L1 expression between surgical tissue sections and matched biopsies was fairly high at 84.1% (116/138), and the κ value was 0.73 (95% CI: 0.63–0.83, $P < 0.001$). The concordance rate was higher for tissue sections from PET/CT-guided biopsy than for tissue sections from CT-guided biopsy [88.6% (62/70, κ value: 0.81) vs 79.4% (54/68, κ value: 0.66)].

Conclusion: PD-L1 TPS was strongly concordant between surgical specimens and matched lung biopsies. Thus, the routine evaluation of PD-L1 expression in diagnostic percutaneous biopsies could be reliable for identifying patients who will benefit from anti-PD-1/PD-L1 immunotherapy.

Keywords: programmed death-ligand 1 (PD-L1), biopsy, surgical resected specimen, PET/CT, non-small cell lung cancer

INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide, and it is projected to account for over 22% of deaths in 2020 in the United States (1). Non-small cell lung cancer (NSCLC) accounts for about 80% of lung cancers. NSCLC has poor prognosis, but immunotherapy has markedly improved survival in patients with advanced NSCLC without driver alterations. In the KEYNOTE-024 and KEYNOTE-042 phase III clinical trials, programmed cell death protein 1 (PD-1)-targeted immunotherapy significantly improved the overall survival (OS) rate compared with standard therapy in advanced NSCLC (2, 3). Based on these studies, the FDA approved pembrolizumab as the first-line treatment for advanced NSCLC patients with a Tumor Proportion Score (TPS) of ≥50% (based on KEYNOTE-024) and 1% (based on KEYNOTE-042), respectively.

Other immunotherapies targeted against PD-1 or programmed death-ligand 1 (PD-L1), such as nivolumab, atezolizumab, and durvalumab (**Supplemental Table 1**), also showed OS benefit with similar PD-L1 cut-off values (4, 5). Thus, determining PD-L1 expression status may help select patients who will optimally benefit from immunotherapy. Immunohistochemistry (IHC) has been widely used to evaluate PD-L1 status in both clinical trials and routine clinical practice. Currently, PD-L1 testing is mainly performed on biopsy samples, which may not be representative of the whole tumor. Thus, the concordance of TPS between lung biopsies and matched surgical specimens remains controversial (6), which may result in decreased confidence on the reliability of biopsies for PD-L1 testing.

Image-guided percutaneous biopsy is currently widely used as it is minimally invasive and is associated with fewer complications. CT-guided biopsy is the most common approach for sampling of lung lesions, but it has varying diagnostic performance depending upon the target organ and type of needle used (7, 8). Meanwhile, ^{18}F-FDG PET/CT provides both anatomic structures and metabolic features and has therefore been suggested to improve the diagnostic accuracy of image-guided biopsy (9–12). We have previously reported the importance of PET/CT-guided biopsy for patient evaluation at various stages of cancers (9, 13, 14). Applying PET/CT information to image-guided biopsy may facilitate accurate histopathological diagnosis and help with staging. However, only few studies to date have compared the reliability and reproducibility of PET/CT-guided biopsy with corresponding resected surgical specimens. Moreover, the use of PD-L1 as a biomarker for determining sensitivity to PD-1/PD-L1 checkpoint blockades has raised concerns on the reliability of biopsy samples compared with surgical specimens. Thus, the present study aimed to evaluate the concordance of immunohistochemical expression of PD-L1 between image-guided biopsies and matched surgical specimens.

MATERIALS AND METHODS

Patient Population

The study protocol was discussed and approved by the institutional review board of the First Affiliated Hospital of Xiamen University (ID, KY2017-001), and written informed consent was obtained from all patients.

We retrospectively evaluated 157 patients with operable NSCLC who underwent both diagnostic percutaneous lung biopsy and surgical resection at the First Affiliated Hospital of Xiamen University, between December 2016 and October 2019. The exclusion criteria were as follows: (i) history of chemotherapy or radiotherapy before biopsy and surgery and (ii) incomplete preoperative clinical record, including data on body mass index, smoking history, blood routine examination, and blood biochemistry examination. Of the 157 patients, 82 underwent CT-guided biopsy, while the other 75 patients underwent ^{18}F-FDG PET/CT-guided biopsy. Eventually, 138 matched biopsy and surgical resection specimens were obtained for further IHC analysis of PD-L1 expression. There were 72 men and 66 women in the derivation cohort, and the median patient age was 63 years (range: 25–81 years). In total, 53 (38.4%) patients were current or former smokers. All patients were staged or restaged according to the 8th International Association for the Study of Lung Cancer staging system based on postoperative pathological result. Overall, 61 (44.2%) patients had stages II–III disease, and majority (n = 116, 84.1%) had non-squamous histology. The clinicodemographic characteristics of the 138 patients are summarized in **Table 1**.

Image-Guided Percutaneous Biopsy

Diagnostic percutaneous lung biopsy was performed under either CT guidance or ^{18}F-FDG PET/CT guidance. ^{18}F-FDG PET/CT protocol and imaging analysis were performed as previously described (15). The imaging modality was determined based on discussions with the referring oncologists. The biopsy target was decided by the referring oncologists and interventional radiologists based on the results of ^{18}F-FDG PET/CT or CT scan.

Image-guided biopsy was performed with an 18G or 20G semiautomatic core needle (Coaxial Achieve, Bard, IL, USA) with coaxial guide needle. The percutaneous biopsy was performed by a board-certified interventional radiologist following a step-by-step technique as previously described (8, 13). Briefly, the patients were positioned in a supine or prone position in accordance with factors such as the location of FDG-avid lesion, shortest skin-to-target distance, and optimal needle path. Interventions were conducted under aseptic conditions after administration of local anesthesia using 2.0% lidocaine. The needle was introduced in a stepwise manner under fused PET/CT and CT imaging guidance. Three or four specimens were obtained from each patient, after which histopathological examination and immunohistochemical staining were performed on each specimen. After the biopsy, the patients were observed by referring oncologists for at least 24 h, and the patients were asked to report any abnormality.

Immunohistochemistry

The samples were prepared and stained as previously described (15). In brief, formalin-fixed, paraffin-embedded tumor tissues were sliced into 4 μm-thick sections. For IHC detection of PD-L1, we used the BenchMark GX automated slide stainer (SP263,

TABLE 1 | Clinicodemographic characteristics of the study patients.

Characteristic	Number	%
Age (years)		
<65	79	57.2%
≥65	59	42.8%
Median (range)	63 (25–81)	
Sex		
Male	72	52.2%
Female	66	47.8%
Smoking history		
Nonsmoker	85	61.6%
Smoker	53	38.4%
Histology		
Squamous cell carcinoma	22	15.9%
Adenocarcinoma	116	84.1%
Clinical stage (International Association for the Study of Lung Cancer 8th)		
I	77	55.8%
II/III	61	44.2%
Tumor size (cm)		
≤3	94	68.1%
>3	44	31.9%
Derived neutrophil–lymphocyte ratio		
<3	133	96.4%
≥3	5	3.6%
Lactate dehydrogenase (U/L)		
<240	129	93.5%
≥240	9	6.5%
Body mass index		
<25	102	73.9%
≥25	36	26.1%

Ventana, Oro Valley, AZ, USA) to stain the sections with the PD-L1 antibody according to the manufacturer's recommended protocol. Positive control (placenta) and negative control samples were run simultaneously.

The immunostained tissue sections were scored according to the PD-L1 scoring algorithm (16, 17) by three independent experienced pathologists who were blinded to the clinical data. Discrepancies in the PD-L1 score were resolved by reviewing the slides again. The cut-off values for PD-L1 expression were set to 1 and 50%.

Statistical Analysis

All statistical analyses were conducted using the SPSS 22.0 statistical analysis software (IBM, Armonk, NY, USA). For continuous data, we used the t-test or the Wilcoxon test for analyses, as appropriate. The concordance of PD-L1 TPS between surgical specimens and lung biopsies was evaluated using Cohen's kappa (κ) coefficient (18, 19). The relative strength of agreement was interpreted as follows: κ <0, poor; 0.01–0.20, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial; and 0.81–1.00, almost perfect (18, 19). Between group comparisons were performed using the chi-squared test, Yates' correction of chi-squared test, or Fisher's exact test. The correlation between different variables was analyzed using the non-parametric Spearman's rank test. All tests were two-sided, and a *P* value lower than 0.05 was considered statistically significant.

RESULTS

The diagnostic success rates of CT- and PET/CT-guided biopsy were 82.9% (68/82) and 93.3% (70/75), respectively. There were 11 central and 146 peripheral NSCLCs. Fourteen (8.9%) patients underwent pneumothorax, while 10 (6.4%) patients happened hemoptysis. PD-L1 TPS was <1, 1–49, and ≥50% in 72, 51, and 15 percutaneous biopsy specimens, respectively, and in 58, 63, and 17 surgical resection specimens, respectively (**Figure 1A**). Compared with the whole tumor section, 19 biopsy specimens underestimated the PD-L1 TPS, while only three biopsy section overestimated the TPS. Regarding the concern that biopsy specimens may underestimate the PD-L1 expression, four cases (one with PET/CT-guided biopsy, and three with CT-guided biopsy) in our study showed lower PD-L1 TPS in diagnostic biopsies (1–49%) as compared to the surgical samples (> 50%).

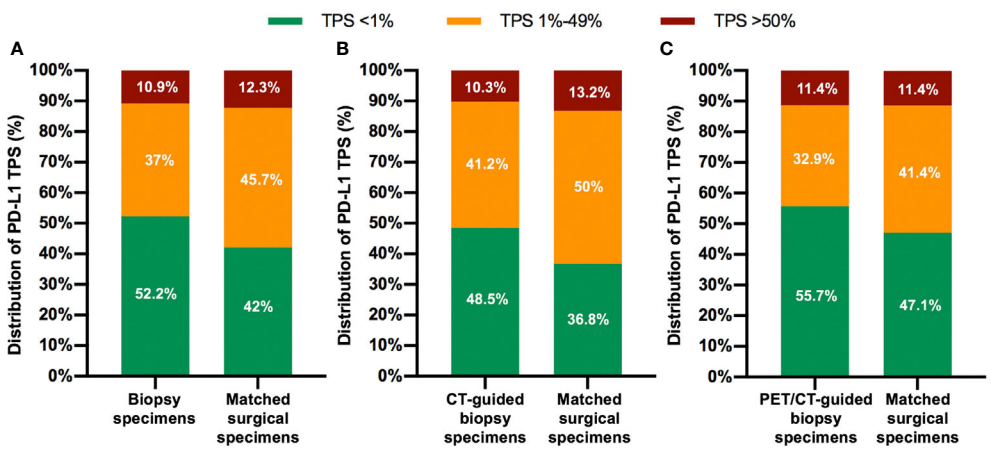

FIGURE 1 | Distribution of PD-L1 Tumor Proportion Score (TPS) for both biopsy specimens and matched surgical specimens in the overall patient population **(A)**, in the CT-guided biopsy group **(B)**, and in the PET/CT-guided biopsy group **(C)**.

The tissue sections from the biopsy and surgical resection in the four cases were further cautiously analyzed by our experienced pathologists, and a highly heterogenous expression of PD-L1 in the surgical specimen was observed in two out of four cases. There was no significant difference in PD-L1 TPS between biopsy and surgical tissue sections (P = 0.24).

The overall concordance rate for PD-L1 TPS between percutaneous biopsy specimens and matched surgical specimens was 84.1% (116/138). The Cohen's κ value was equal to 0.73 (95% CI: 0.63–0.83, P < 0.001), indicating substantial agreement. The concordance rate at a cut-off value of 1% PD-L1 TPS was 88.4%, and the κ value was equal to 0.77. The concordance rate at a cut-off value of PD-L1 TPS 50% also indicated substantial agreement (κ = 0.79). Representative images of concordant and discordant PD-L1 between biopsy and matched surgical specimens are shown in **Figures 2** and **3**, respectively. The Cohen's κ value according to histological subtypes was 0.79 (95% CI: 0.57–1, P < 0.001) for squamous cell carcinoma and 0.71 (95% CI: 0.59–0.83, P < 0.001) for lung adenocarcinoma.

There was no statistical difference in the distribution of PD-L1 TPS between biopsy and surgical specimens (**Figures 1B, C**). The concordance rate for PD-L1 TPS between CT-guided biopsy and matched surgical tissue was 79.4% (54/68), and the Cohen's κ value was 0.66 (95% CI: 0.50–0.82, P < 0.001), which indicated substantial agreement. Meanwhile, the concordance rate between PET/CT-guided biopsy specimen and matched surgical resection specimen was higher at 88.6% (62/70), and the Cohen's κ value was 0.81 (95% CI: 0.68–0.94, P < 0.001), which indicated almost perfect agreement (**Table 2**). PD-L1 TPS was significantly associated with SUVmax on Spearman correction analysis (P = 0.048, **Supplemental Table 2**). Representative ^{18}F-FDG PET/CT images for lung biopsy are shown in **Figure 4**.

As the CT and PET/CT groups had different concordance between percutaneous biopsy and matched surgical specimens, between-group comparisons were performed. The results showed no signifcant difference in the measured PD-L1 TPS between CT-guided biopsies and PET/CT-guided biopsies (P = 0.47). There was no significant difference in the primary clinicodemographic characteristics between the two cohorts (**Supplemental Table 3**).

DISCUSSION

Anti-PD-1/PD-L1 immunotherapy has improved the prognosis of NSCLC. In particular, nivolumab and pembrolizumab have

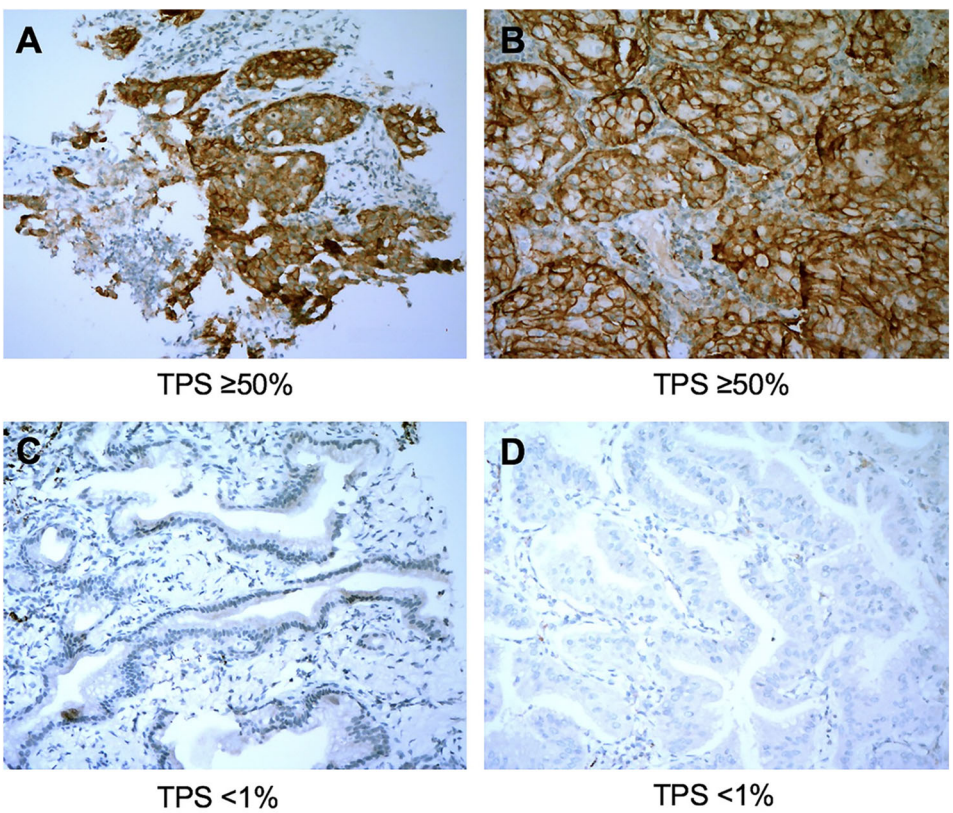

FIGURE 2 | Representative images of concordant cases between biopsy specimens (left panel) and matched surgical resection specimens (right panel). The PD-L1 Tumor Proportion Score (TPS) in the biopsy tumor specimen **(A)** and the corresponding resected tumor **(B)** were both ≥50%. PD-L1 TPS in the biopsy tumor **(C)** and the matched resected specimen **(D)** were both <1%. All images are at ×40 magnification.

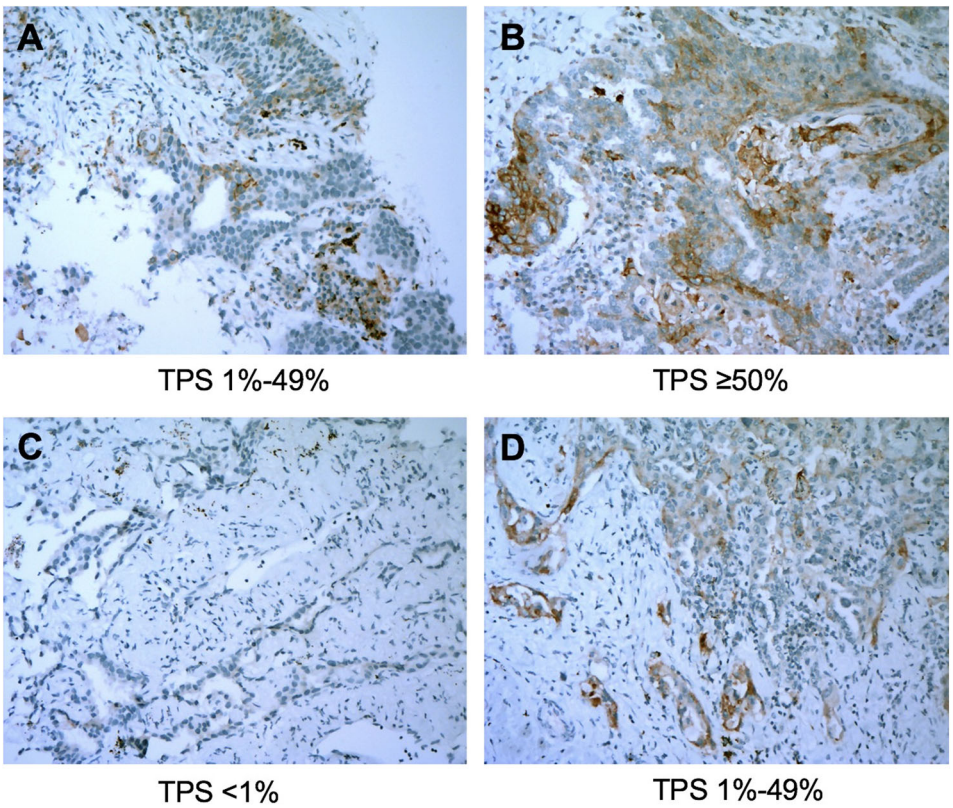

FIGURE 3 | Representative images of discordant cases between biopsy specimens (left panel) and matched resected specimens (right panel). The PD-L1 Tumor Proportion Score (TPS) was 10% in the biopsy tumor specimen **(A)** and was ≥50% in the corresponding resected tumor **(B)**. PD-L1 TPS in the biopsy specimen was <1% **(C)** and was 20% in the matched surgical specimen **(D)**. All images are at ×40 magnification.

TABLE 2 | Cohen's κ for concordance between percutaneous biopsy and matched surgical specimens based on TPS.

PD-L1 cutoff	Cohen's kappa (κ)		
	Overall population (N = 138)	CT group (N = 68)	PET/CT group (N = 70)
Tumor Proportion Score (TPS) ≥1%	0.77	0.7	0.83
TPS ≥50%	0.79	0.72	0.86
Overall	0.73	0.66	0.81

significantly improved the long-term survival of patients with NSCLC (20, 21). Compared to the usual OS rate of 5% with standard chemotherapy, the 5-year OS rate of pembrolizumab treatment was 29.6% in NSCLC patients with a PD-L1 TPS of over 50% in the KEYNOTE-001 study (21). Therefore, determining the PD-L1 TPS may help identify patients who will benefit from PD-1/PD-L1 blockade therapy, allowing for a more individualized treatment approach and avoiding unnecessary treatment. In this study, the percentage of NSCLCs with PD-L1 TPS <1, 1–49, and ≥50% were 42.0, 45.7, and 12.3%, respectively, which was in agreement with the previous publication (22). Accordingly, our study evaluated the concordance of tumor PD-L1 expression between image-guided percutaneous biopsies and matched surgical specimens in patients with NSCLC. Our results indicated substantial agreement of the PD-L1 TPS between surgical specimens and matched lung biopsies. Notably, samples from PET/CT-guided biopsy demonstrated higher success rate and concordance with surgical tissue sections than those from CT-guided biopsy.

PD-L1 testing using surgical specimens is rarely feasible in patients with NSCLC because of diagnosis at the advanced stage. Our results support the reliability of PD-L1 TPS determined using image-guided percutaneous biopsy specimens. These results can help establish PD-L1 expression assessed using image-guided biopsy specimen as a reliable biomarker for predicting benefit from anti-PD-1/PD-L1 immunotherapy.

Previous investigations have shown that intratumoral heterogeneity of PD-L1 expression exists within the entire surgical specimen (23), which may result in low concordance with the results obtained on diagnostic biopsies. As such, the concordance of PD-L1 status between biopsy samples and matched resected specimens varied across previous studies. For example, Tsunoda et al. and Kitazono et al. showed good concordance (24, 25), whereas Ilie et al. and Erik et al. demonstrated poor concordance (6, 26). It is worth noting that

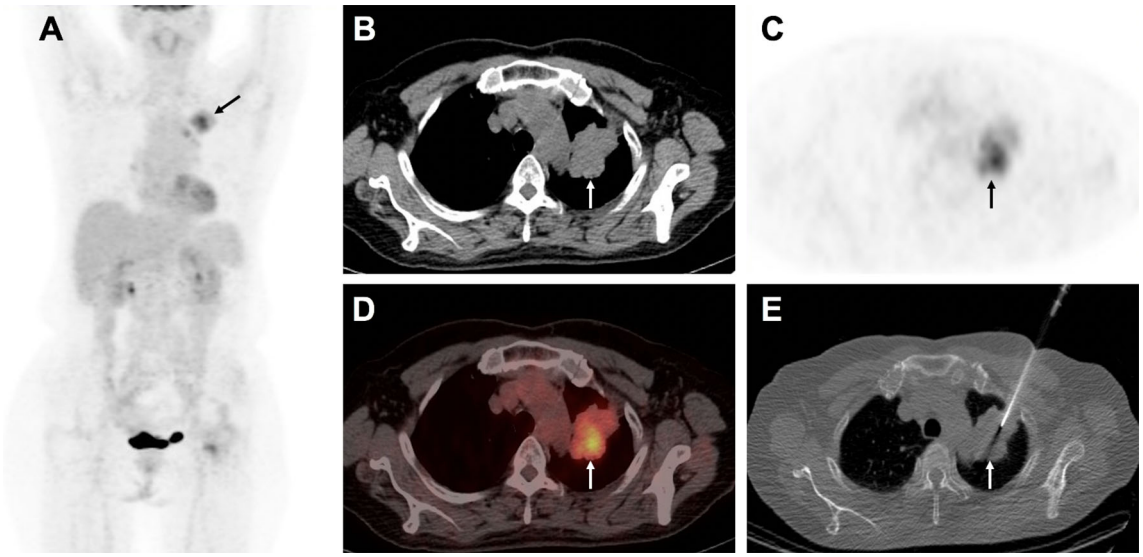

FIGURE 4 | A 58-year-old man with suspected lung carcinoma underwent ^{18}F-FDG PET/CT for tumor staging before treatment. The maximum intensity projection image **(A)** shows intense activity in the left upper lobe of the lung (arrow). Axial view of PET/CT **(B)** CT scan, **(C)** PET image, **(D)**, combined PET/CT image, shows the metabolic active mass (arrow) with atelectasis in its caudal part. Axial view of CT image **(E)** shows biopsy needle positioned into the metabolic active area (arrow). Histological examination confirmed the lung lesion as primary adenocarcinoma.

the biopsy samples in all of these studies were mainly obtained from bronchoscopic biopsy (includes EBUS-TBNA, transbronchial or endobronchial biopsy), and part of the specimen were metastatic lymph nodes instead of primary lung tumors (6, 24–26). Despite being minimally invasive, a tissue core usually is not obtained with bronchoscopic biopsy, limiting a detailed morphologic examination and therefore (27), may affect the observation on PD-L1 expression of tumor cell membrane. In our study, all biopsy specimens were obtained percutaneously using image guidance, which allowed sufficient tissues (generally 3–4 biopsy fragments were obtained for each patient, 8–12 mm * 0.5 mm for each fragment) than bronchoscopic biopsy and tissue microarrays. Although previous study reported a significant difference regarding the number of biopsy tissues between concordant and discordant case (6), we still cannot conclude that more sufficient tissues obtained *via* percutaneous biopsy is the reason for the high PD-L1 concordance observed in our study relative to studies wherein most tissue was obtained with bronchoscopy. Further investigation that comparing percutaneous to bronchoscopic biopsy with respect to PD-L1 testing, is in of itself an important subject matter, but not one addressed in this study.

A successful biopsy procedure must provide a diagnostic sample of tissue, which means the sample of tissue must adequately show the presence of malignancy and specific histopathologic features. Although, PET/CT-guided percutaneously obtained biopsy is not the universal standard of care for image guided biopsies, PET/CT guidance has been widely used to improve the biopsy success rate (9–12). In our study, PET/CT-guided biopsy samples demonstrated a higher success rate (93.3%) than did CT-guided biopsy samples (82.9%), and no significant difference regarding patients' characteristics was observed between those who underwent PET/CT-guided biopsy and CT-guided biopsy. According to our experience, PET/CT-guided biopsy demonstrates the following advantages: First, reducing the frequency of sampling failure can improve biopsy results and patient experiences. Second, resulting in a higher percentage of malignant lesions than did CT-guided biopsy, which could be explained by better identification of the lesion site based on the integration of anatomic structure with metabolic features. For example, we found that ^{18}F-FDG PET/CT-guided biopsy has better accuracy in NSCLC with pulmonary atelectasis in this study (**Figure 4**). Moreover, PET/CT allows identification of FDG-avid lesions that are most accessible to biopsy from among multiple lesions with similar uptake (9–11). Biopsy of the most accessible lesion could simultaneously reduce the risk of complications and minimize the sampling error.

Another important finding of this study is that samples from PET/CT-guided biopsy showed stronger concordance with surgical tissue sections than those from CT-guided biopsy, despite that, there was no significant between-group difference in patient characteristics. Considering the possibility of intra-tumor heterogeneity of PD-L1 expression in NSCLC, sampling the specimen that accurately reflects the PD-L1 status is important (28). Increasing evidence show ^{18}F-FDG uptake in NSCLC samples was positively correlated with PD-L1 expression (29–32), which has been further confirmed in our study. This might be one of the reasons that the FDG uptake of the primary lesion was able to predict the immunotherapy response (33, 34). In some cases, ^{18}F-FDG PET/CT can show the heterogeneity of

metabolism in lesions, which are of equal density in CT. Puncture sampling of areas with ^{18}F-FDG-avid focus may help obtain representative specimens for measuring PD-L1 expression.

Compared with previous investigations, our study has the following advantages. First, we compared PD-L1 expression between surgical tissue sections and image-guided percutaneous biopsies in the same group of patients. In the ATLANTIC study that included over 1500 NSCLC patients, PD-L1 expression status was not significantly different between biopsy and surgical samples (35). However, the samples (1,365 samples were obtained by biopsy and 180 by surgical resection) were not matched from the same patients. Second, we used relatively new samples for PD-L1 staining; the specimen in our cohort was less than 3 years old. A previous study showed that samples older than 3 years may show lower PD-L1 TPS on IHC (35). Third, we used SP263 as the antibody for PD-L1 measurement, and the cut-off value was clinically relevant (2–5). SP263 antibody in PD-L1 assay has been reported to have high reliability and reproducibility for NSCLC tumor samples (16, 36, 37). Blueprint PD-L1 IHC Assay Comparability Project Phase 2 consolidates the analytical evidence for interchangeability of the 22C3, SP263, and 28-8 assays because of the similar analytical performance (38).

Despite its advantages, our study also has some notable limitations that need to be addressed. First, our cohort did not include patients with stage IV NSCLC, and all patients had resectable disease. Consequently, the concordant between biopsy samples and matched surgical specimens was mostly applied to early stage NSCLCs, rather than advanced disease. Second, anti-PD-1/PD-L1 immunotherapy to date was mainly used in unresectable NSCLC patients, but the patients in our cohort were treated with surgery rather than immunotherapy. However, the apparent survival benefit of immunotherapy has transformed it from being an alternative modality to being the recommended first-line treatment in the real world. Anti-PD-1/PD-L1 immunotherapy in the neoadjuvant setting also showed encouraging results in patients with resectable lung cancer (39, 40). Third, we did not examine multiple areas of the surgical specimens to evaluate the PD-L1 expression. Since the PD-L1 protein levels in NSCLC reveals heterogeneity within tumors, the PD-L1 expression of the whole tumor in this study may not be fully evaluated. Additionally, this was a retrospective, single-center study with a moderate number of patients. Prospective, multicenter studies with a larger patient population are needed.

In conclusion, PD-L1 expression is concordant between diagnostic percutaneous biopsy samples and matched surgical specimens. Thus, PD-L1 expression in image-guided percutaneous biopsies could be a reliable biomarker for screening patients who will benefit from anti-PD-1/PD-L1 immunotherapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of the First Affiliated Hospital of Xiamen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LZ, HC, and QL: study conception and design. KF and YD: literature research. KF, PC, YW, and YZ: data acquisition. PC, JL, YD, and LS: data analysis and interpretation. LZ and HC: manuscript drafting. HC and LZ: manuscript editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.551367/full#supplementary-material

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A CT-Based Radiomics Approach to Predict Nivolumab Response in Advanced Non-Small-Cell Lung Cancer

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Purpose: This study aims to develop a CT-based radiomics model to predict clinical outcomes of advanced non-small-cell lung cancer (NSCLC) patients treated with nivolumab.

Methods: Forty-six stage IIIB/IV NSCLC patients without EGFR mutation or ALK rearrangement who received nivolumab were enrolled. After segmenting primary tumors depicting on the pre-anti-PD1 treatment CT images, 1,106 radiomics features were computed and extracted to decode the imaging phenotypes of these tumors. A L1-based feature selection method was applied to remove the redundant features and build an optimal feature pool. To predict the risk of progression-free survival (PFS) and overall survival (OS), the selected image features were used to train and test three machine-learning classifiers namely, support vector machine classifier, logistic regression classifier, and Gaussian Naïve Bayes classifier. Finally, the overall patients were stratified into high and low risk subgroups by using prediction scores obtained from three classifiers, and Kaplan–Meier survival analysis was conduct to evaluate the prognostic values of these patients.

Results: To predict the risk of PFS and OS, the average area under a receiver operating characteristic curve (AUC) value of three classifiers were 0.73 ± 0.07 and 0.61 ± 0.08, respectively; the corresponding average Harrell's concordance indexes for three classifiers were 0.92 and 0.79. The average hazard ratios (HR) of three models for predicting PFS and OS were 6.22 and 3.54, which suggested the significant difference of the two subgroup's PFS and OS ($p<0.05$).

Conclusion: The pre-treatment CT-based radiomics model provided a promising way to predict clinical outcomes for advanced NSCLC patients treated with nivolumab.

Keywords: CT-based radiomics approach, nivolumab, NSCLC, machine-learning, immunotherapy

INTRODUCTION

Over the past decade, immune checkpoint inhibitors targeting programmed cell death protein-1 (PD-1) or programmed cell death protein ligand-1 (PD-L1) have opened a new epoch of treatment for advanced non-small-cell lung cancer (NSCLC), with improved survival and durable responses compared with chemotherapy in patients both in first- and second-line treatment (1–5). PD-1 or PD-L1 inhibitors pembrolizumab, nivolumab, and atezolizumab, prolonged overall survival (OS) compared with chemotherapy in patients with previously treated advanced NSCLC based on the results of Keynote-010 (1), CheckMate 017/057 (2, 3) and OAK studies (4). The phase III study CheckMate 078 has demonstrated consistent results of superior OS by nivolumab compared with docetaxel in a predominantly Chinese population with previously treated advanced NSCLC (6).

Despite remarkable success of immunotherapy, up to 60% of patients with advanced NSCLC could not benefit from PD-1 or PD-L1 inhibitors (7). Different biomarkers have been investigated to predict the efficacy and prognosis, such as PD-L1 expression and copy number gains (1–4, 8–11), tumor mutation burden (TMB) (12–14), microsatellite instability (MSI) (15), tumor infiltrating lymphocytes (16–18) and inflammatory cytokines (19). Even though the PD-L1 expression of tumor cell has been identified as a predictive biomarker for response of immunotherapy in both newly diagnosed or previously treated NSCLC (3, 4, 11), the relationship between the PD-L1 expression and the therapeutic effects of nivolumab is still unclear. Tumor heterogeneity, instability of tissue specimens, non-standardized detection techniques and the dynamic nature of the immune microenvironment are also limitations of PD-L1 expression as a predictive biomarker (20, 21). The urgent need to discover and validate non-invasive, stable predictive biomarkers to select patients who will benefit from immunotherapy remains an ongoing challenge.

Since non-invasive diagnostic images can depict the phenotypes of lung tumor, recently studies have illustrated that utilization of imaging biomarker to predict the survival stratification of advanced NSCLC patients with different therapies is feasible. Among these non-invasive imaging based prediction or classification models, CT image based radiomics approach has been developed and applied to build the prognostic prediction model for evaluating the effectiveness and necessity of developing different therapies, e.g., targeted therapeutics, chemotherapy, radiation therapy, and for early prediction of clinical outcome. The non-invasive quantitative imaging technique may provide a new approach to assess the clinical outcome at an early stage of updated PD-1 therapeutic process.

In this study, we proposed a novel CT-based radiomics model to predict the progression probability to the recommended nivolumab therapy for individually patient. To decode the imaging phenotypes of lung tumor, we computed and extracted thousands of pretherapy CT features to deeply interpret the patients treated with immunotherapy to select critical PD-1/PD-L1 associated phenotypic features. Then, we used three machine-learning classifiers to develop the CT-based radiomics models to stratify the risk of progression-free survival (PFS) and overall survival (OS) in advanced stage NSCLC patients. Finally, we analyzed and compared the Kaplan–Meier survival estimators of the stratified subgroups with high and low risk for progression and death (**Figure 1**).

MATERIALS AND METHODS

Patients

Forty-six patients with previously treated NSCLC were prescribed with nivolumab from CheckMate 078 study, CheckMate 870 study or clinical practice between Apr 2016 and Jan 2019 at Fudan University Shanghai Cancer Center. All patients were histologically or cytologically-diagnosed with locally advanced or metastatic NSCLC. Patients were included regardless of tumor PD-L1 expression. Patients with epidermal growth factor receptor (EGFR)-mutation or anaplastic lymphoma kinase (ALK) translocation-positive tumors were excluded. We retrospectively collected clinical data and treatment outcomes from the patients' medical records. The clinical stage was assigned according to the 8th edition of the TNM staging system.

The institutional review board of Fudan University Shanghai Cancer Center approved this study.

Treatment

Patients received intravenous nivolumab at dose of 3 mg/kg or fixed dose of 240mg every two weeks until disease progression or discontinuation owing to intolerance of toxicity. All patients received a diagnostic contrast-enhanced chest CT prior to immunotherapy. All the CT scans were reconstructed by using the standard convolution kernel. The pixel spacing of CT image ranges from 0.672 mm to 0.822 mm, and the slice thickness is 1 mm or 1.5 mm. Each axial slice image was reconstructed with a matrix 512×512 pixels. The pre-treatment CT scan was collected and used as baseline imaging data.

Efficacy

Efficacy was assessed by determining PFS, OS, overall response rate (ORR) and the disease control rate (DCR). PFS was defined as the time from initiation of nivolumab therapy to disease progression or death. Patients alive without progression at the time of analysis were censored at their last follow-up. OS was defined as the time from initiation of nivolumab therapy to death. DCR was defined as the percentage of patients with a complete response (CR), partial response (PR), and stable disease (SD), while ORR was defined as the percentage with CRs and PRs. The tumor response was initially assessed after 8 weeks of nivolumab therapy and subsequently thereafter every 8 weeks using the Response Evaluation Criteria In Solid Tumors (RECIST, version 1.1). Responses were defined as the best response from the start of treatment until disease progression.

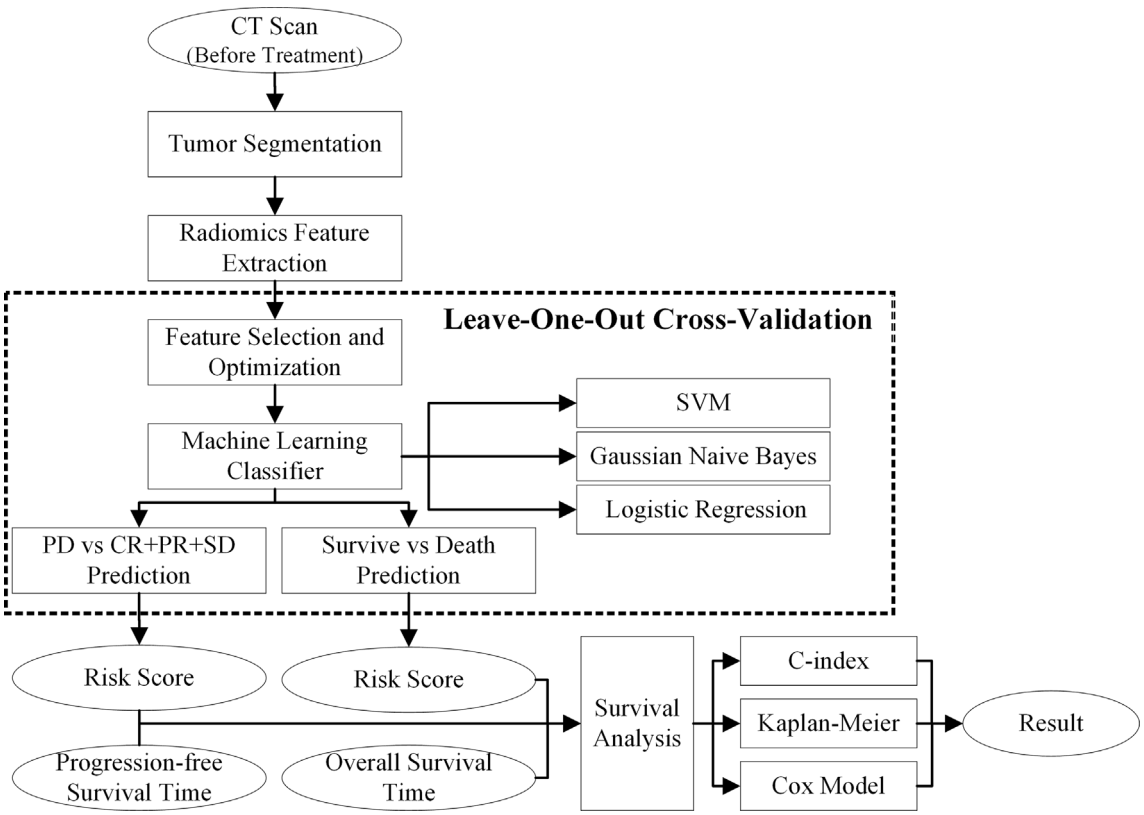

FIGURE 1 | Flowchart of our proposed model.

Statistical Analysis

CT images were first interpreted qualitatively and quantitatively by two radiologists (Dr. Shengping Wang with 15 years experience and Dr. Quan Liu with 28 years experience). **Figure 2** shows the radiomics feature extraction process. To evaluate the therapeutic effect, radiologists provided a standardized report to record lymph node status and common sites of distant metastasis (i.e., bones, liver, and brain) for each patient during the treatment cycles.

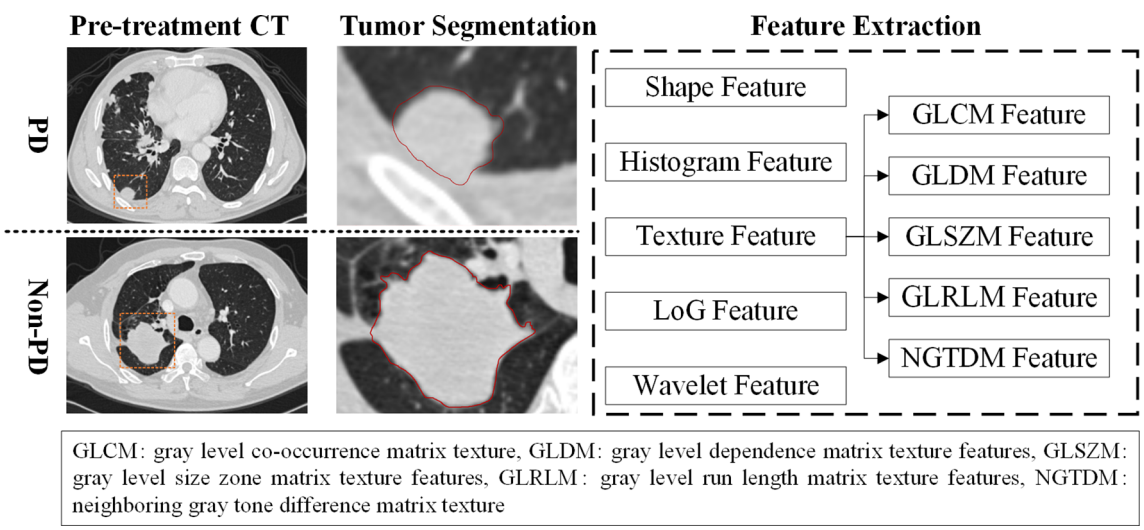

FIGURE 2 | The radiomics feature extraction process.

Then, the radiologists delineated the 3D boundary of each primary tumor and segmented the tumor volume by using the CT scan examined before immune treatment. The largest tumor was defined as target lesion for subject with multiple lesions in this study. All the primary tumors were segmented manually in slice by slice fashion on CT images. Due to the variant of CT parameters, a B-spline curve interpolation algorithm was used to resample the 3D CT images to a spacing of (1, 1, and 1mm). In order to character the imaging phenotypes of each tumor, 1,106 CT based radiomics features were initially computed and extracted to quantitative the tumor. Among these features, 274 LoG features, 728 wavelet features, 14 shape features, 18 histogram features, and 68 texture features were involved. The LoG features were calculated based on image filtered with Laplacian of Gaussian (LOG) filter, and wavelet features were extracted by using image filtered with wavelet filter. Since each phenotypic feature has different value range, a feature normalization technique was used to transform these radiomics features to [0, 1].

Due to a large number of redundant features in the initial feature pool, a L1-based feature selection method was applied to the redundant features and reduce the dimensionality of radiomics features remove. During this process, a linear support vector classifier was used to build the meta-transformer to select the robust imaging features. After feature selection, classification models were built by training three different machine-learning classifiers namely, support vector machine (SVM) classifier, logistic regression classifier (LRC), and Gaussian Naïve Bayes (GNB) classifier, respectively. To evaluate the performance of our proposed models, a leave-one-out cross-validation (LOOCV) was used to train and test the classifier. In order to avoid biases in portioning dataset, the feature selection process and machine-learning classifier were embedded into the LOOCV training/testing cycles.

Finally, several statistical data analysis methods were applied to measure the association between the model's predicted low or high risk scores and patients' PFS and OS, which include 1) a Harrell's concordance index (C-index) analysis, 2) Kaplan–Meier plots, and 3) Cox proportional hazards regression models. To assess the model's performance, the cases were divided into two groups of low and high risk in cancer progression by applying an operation threshold of 0.5 to the prediction scores generated by three classifiers namely, SVM, NBC, and LRC.

In this study, the prediction models were built by using Python programming software (version 3.6, https://www.python.org), and the statistical data analysis process was implemented on R software (version 3.5.2, https://www.r-project.org). To evaluate the performance of our proposed model, a maximum likelihood based receiver-operating characteristic (ROC) fitting program (ROCKIT, http://www-radiology.uchicago.edu/krl, University of Chicago) was used to compute the area under a ROC curve (AUC) value and the corresponding 95% confidence interval (CI). PFS and OS were estimated by the Kaplan-Meier method, along with hazard ratios (HRs). All outcome measures were calculated with 95% CIs, which were estimated by use of the Cox proportional hazard model. The significance level of statistical tests was set at $p < 0.05$. All expressed p values and CIs were two-tailed. All the medical image processes and performance evaluation processes were performed on a computer with Intel Core i7-8700 CPU 3.2GHz × 2, 16 GB RAM.

RESULTS

Patient Characteristics

A total of 46 patients with previously treated advanced NSCLC were administrated with nivolumab at Fudan University Shanghai Cancer Center between Apr 2016 and Jan 2019. Their baseline characteristics at the initiation of nivolumab therapy are shown in **Table 1**. The patients' median age was 62.0 years (range, 46 to 77 years). There was a higher proportion of males (34/46, 73.9%) than females, and of current/former smokers (30/46, 65.2%) than never smokers. Thirty-four patients (73.9%) were diagnosed with adenocarcinoma while 12 patients (26.1%) were diagnosed with squamous cell carcinoma; 42 (91.3%) had stage IV disease at baseline. All 46 patients had an Eastern Cooperative Oncology Group performance status (ECOG PS) of 1.

All patients had a routine examination before initiation of nivolumab treatment, 22 patients (47.8%) had one metastatic site, 16 patients (34.8%) had two metastatic sites and 8 patients (17.4%) had more than two metastatic sites. In 42 patients (91.3%), nivolumab was used as second-line treatment and in 4 patients (8.7%) as third-line or later treatment.

Efficacy

Tumor responses are shown in **Table 2**. One patient (2.2%) achieved CR, 6 patients (13.0%) achieved PR and 12 (26.1%) had

TABLE 1 | Baseline patient characteristics (N = 46).

Characteristic	All, No. of patients (%)
Age, median (range), years	62 (46–77)
Sex	
Male	34 (73.9)
Female	12 (26.1)
ECOG PS	
1	46 (100)
Smoking status	
Current/former smoker	30 (65.2)
Never smoker	16 (34.8)
Number of lines of prior systemic cancer therapy	
1	42 (91.3)
≥2	4 (8.7)
Tumor histology	
Adenocarcinoma	34 (73.9)
Squamous cell carcinoma	12 (26.1)
Tumor Stage	
IIIB	4 (8.7)
IV	42 (91.3)
No. of metastatic sites at baseline	
1	22 (47.8)
2	16 (34.8)
≥3	8 (17.4)

ECOG PS, Eastern Cooperative Oncology Group performance status.

TABLE 2 | Tumor responses.

Responses	All patients (*n* = 46) (*n* or %)
CR	1 (2.2)
PR	6 (13.0)
SD	12 (26.1)
PD	27 (58.7)
ORR	15.2% (95 CI, 4.7–25.7%)
DCR	43.5% (95% CI, 29.0–58.0%)

CR, complete response; DCR, disease control rate; ORR, objective response rate; PD, progressive disease; PR, partial response; SD stable disease.

SD, resulting in an ORR of 15.2% (95% CI, 4.7–25.7%) and a DCR of 43.5% (95% CI, 29.0–58.0%).

At the cutoff date Dec 13th 2019, median follow-up time was 11.5 months (range, 1.0–46.0 months). Nineteen patients (41.3%) were still alive, 2 patients (4.3%) were lost to follow-up and 25 patients (54.3%) were dead at the cutoff date. The median PFS (**Figure 3**) was 3.0 months (95% CI, 1.9 to 4.1 months) and the estimated median OS (**Figure 4**) was 17.0 months (95%CI, 7.3–26.7 months).

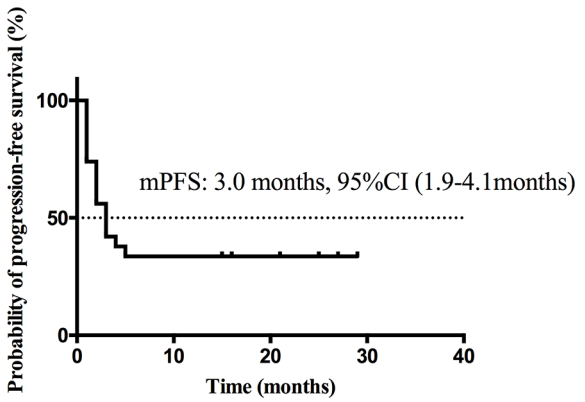

FIGURE 3 | Kaplan-Meier curve of PFS of all patients.

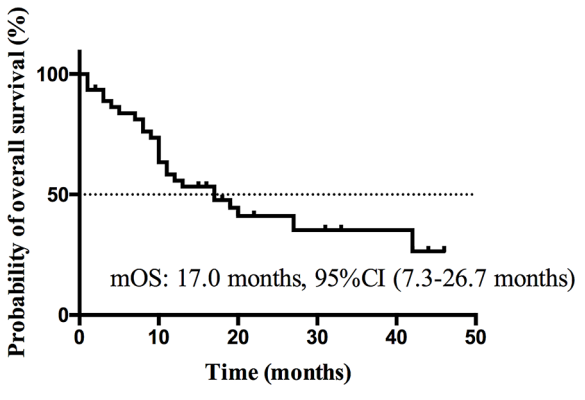

FIGURE 4 | Kaplan-Meier curve of OS of all patients.

Development of Prediction Model and Survival Analysis

Figure 5 shows the boxplots of three imaging features frequently selected in LOOCV process. By using the feature selection method, three imaging features were selected from the initial 1,106 feature pool in PFS prediction process. Two LoG image features and one wavelet feature were involved. The boxplots showed that PD and non-PD category have different distributions in three features. It indicated that the selected features had a potential to classify between PD and non-PD cases. Meanwhile, four imaging features were selected to build OS prediction model, involving three LoG image features and one wavelet feature.

Figure 6 illustrates the ROC curves of PFS and OS classification models built with three classifiers. To predict the risk of PD, SVM, LRC and GNB generated AUC values of 0.73 ± 0.07 [95% CI: (0.57, 0.85)], 0.73 ± 0.07 [95% CI: (0.57, 0.86)], and 0.74 ± 0.07 [95% CI: (0.58, 0.86)], respectively. Meanwhile, SVM, LRC and GNB generated AUC values of 0.60 ± 0.08 [95% CI: (0.43, 0.75)], 0.60 ± 0.08 [95% CI: (0.44, 0.75)], and 0.64 ± 0.08 [95% CI: (0.48, 0.79)]. To evaluate the inter classifier differences; the p-values of the prediction scores generated by three classifiers were computed by using a univariate z-score test. It showed that the AUC values of three classifiers were no significant difference ($p>0.05$).

Figure 7 shows the survival analysis results of three PFS classification models. **Figure 6A** compares the Harrell's C-indexes for PFS generated by three classifiers. For SVM, LRC and GNB classifier, the C-index was 0.93 [95% CI: (0.83, 1.0)], 0.91 [95% CI: (0.79, 1.0)], and 0.92 [95% CI: (0.80, 1.0)], respectively. **Figures 6B–D** illustrates the Kaplan–Meier plots of PFS by using SVM, LRC and GNB classifier, respectively. The Kaplan–Meier survival curves demonstrated that the low risk cohort predicted by three classifiers was significantly different from the high-risk group by using immune therapy ($p<0.05$). **Table 3** lists the summary of data analyses of three cox regression models for PFS. The hazard ratios (HR) of three models reach over 5.6, which suggested the dramatic difference of the two subgroup's PFS in immune treatment ($p<0.05$).

Figure 8 shows the survival analysis results of three OS classification models. **Figure 8A** compares the Harrell's C-indexes for OS generated by three classifiers. For SVM, LRC and GNB classifier, the C-index was 0.76 [95% CI: (0.60, 0.93)], 0.76 [95% CI: (0.60, 0.92)], and 0.86 [95% CI: (0.74, 0.97)], respectively. **Figures 8B–D** illustrates the Kaplan–Meier plots of OS by three prediction models. It shows that the low risk OS cohort was significantly different from the high-risk OS group in Kaplan–Meier curve ($p<0.05$). **Table 3** lists the summary of data analyses of three cox regression models for OS. The hazard ratios (HR) of three models reach over 2.5 ($p<0.05$).

DISCUSSION

Although immunotherapy has been a pivotal development in the management of advanced NSCLC, durable responses and

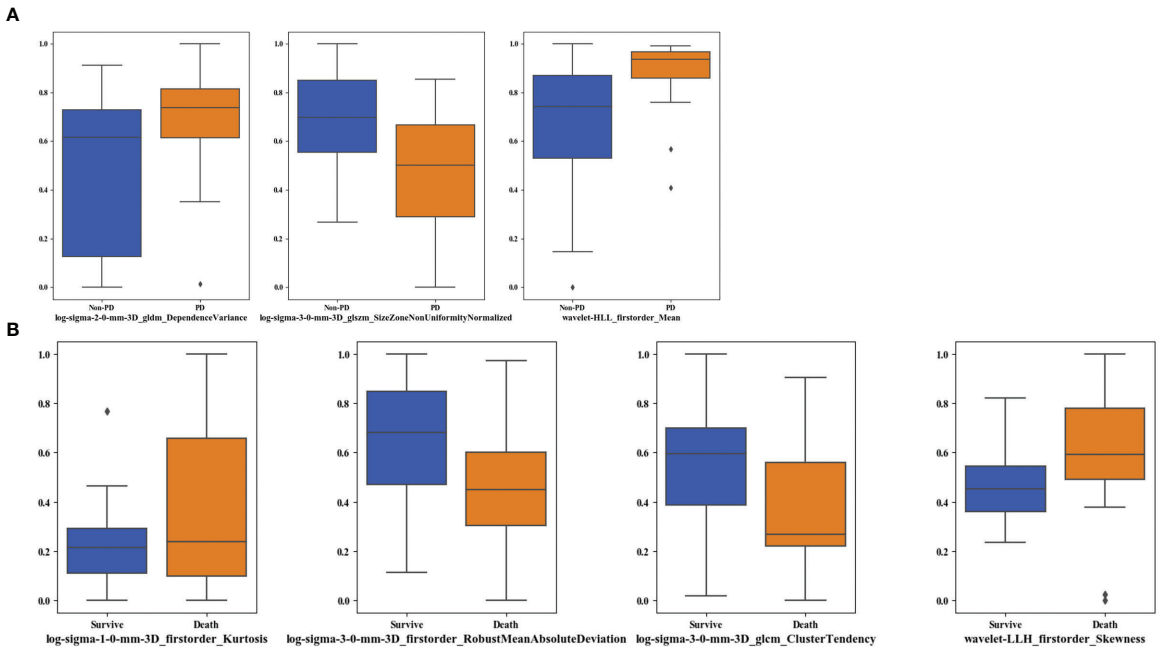

FIGURE 5 | Boxplots of the frequently selected imaging features in the LOOCV process. **(A)** shows the imaging features selected in PFS prediction process, **(B)** shows the imaging features in OS prediction process.

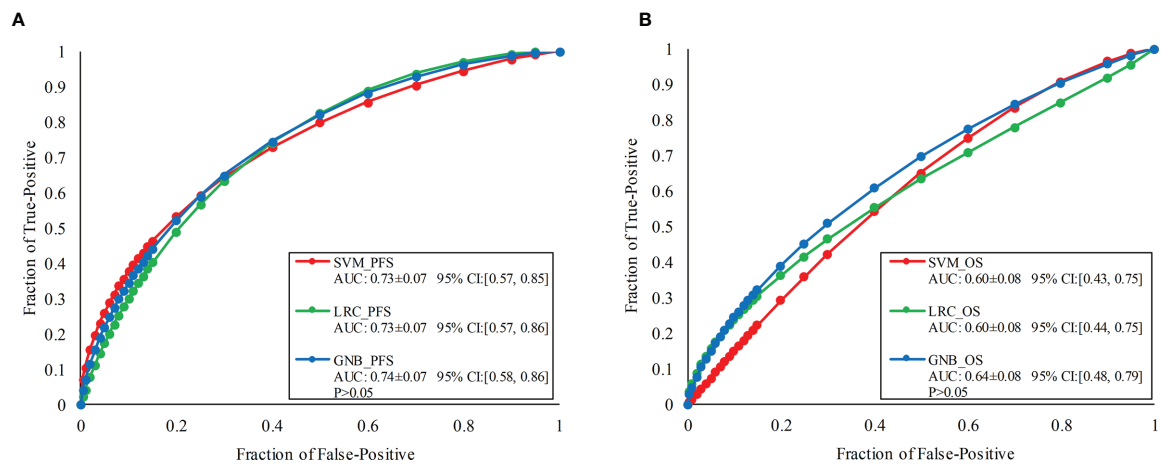

FIGURE 6 | ROC comparisons of PFS and OS classification models built with three classifiers namely, SVM, LRC, and GNB, respectively. **(A)** Illustrates the ROCs of PFS classification models, **(B)** illustrates the ROCs of OS prediction models.

improved survival have been observed only in 20–50% of patients (1–5, 11, 22). Predictive biomarkers of response for immunotherapy and superior survival are urgently needed to improve patient selection and avoid toxicity in potential non-responders.

PD-L1 expression is the only biomarker currently approved by the US Food and Drug Administration (FDA) to select patients who are most likely to benefit from immunotherapy. Compared with docetaxel, nivolumab demonstrated better overall survival, with PD-L1 expression conferring enhanced efficacy in pretreated patients with advanced non-squamous NSCLC in Checkmate 057 study (3). However, among patients with advanced, previously treated squamous-cell NSCLC in Checkmate 017 study (2), OS, ORR, and PFS were significantly better with nivolumab than with docetaxel, regardless of PD-L1 expression level. The survival benefit with nivolumab was also observed regardless of PD-L1 expression level in Chinese patients with previously treated NSCLC in Checkmate 078

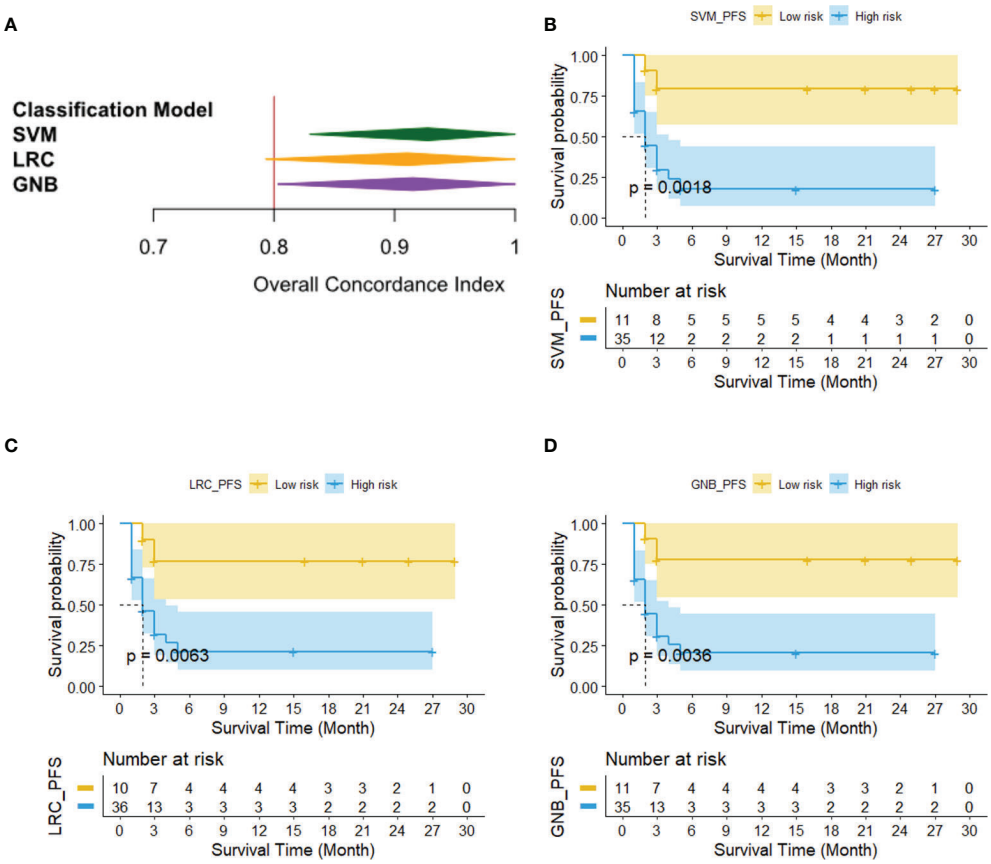

FIGURE 7 | The results of survival analysis of three PFS classification models. **(A)** C-indexes generated by three PFS prediction models, **(B–D)** Kaplan–Meier PFS estimates from all 46 patients by using SVM, LRC and GNB classifier, respectively.

TABLE 3 | Summary of data analyses of three cox regression models for PFS and OS.

	PFS			OS		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
SVM	6.85	(1.61, 29.15)	0.0092	2.95	(1.17, 7.40)	0.021
LRC	5.63	(1.33, 23.85)	0.019	5.17	(2.04, 13.10)	0.00054
GNB	6.18	(1.46, 26.18)	0.013	2.50	(1.03, 6.03)	0.042

study (6). Another study explained that PD-L1 expression alone was insufficient to determine whether patients should receive PD-1 or PD-L1 blockade therapy (23). Furthermore, more and more studies demonstrated that there were many factors associated with the PD-L1 expression, including copy number gains (24), heterogeneity (25), dynamic changes (20) and other participants of immune cell subsets (26–28) in NSCLC. In addition to PD-L1 expression, recent research indicated that TMB of 10 or more mut/Mb was associated with improved response and prolonged PFS in both tumor PD-L1 expression 1% or greater and less than 1% subgroups and was thus identified as a potential biomarker for first-line therapy of nivolumab plus ipilimumab in advanced NSCLC (29). Although research on predictors of response to immunotherapy has sprung up, there are still few well-recognized non-invasive biomarkers of immunotherapy with high-specificity, high-sensitivity and stability.

To assess the immunotherapy response, irRECIST (Immune-related Response Evaluation Criteria in Solid Tumors), iRECIST and imRECIST (Immune-Modified Response Evaluation Criteria In Solid Tumors) were proposed (30–33). In the previously reported studies, PET/CT based response evaluation models have been investigated and developed to evaluate the short-term or long-term response of immunotherapy for lung cancer (34, 35). These studies evaluated the treatment response of immunotherapy effectively, but series PET/CT images during the immunotherapy process were needed to analyze to build

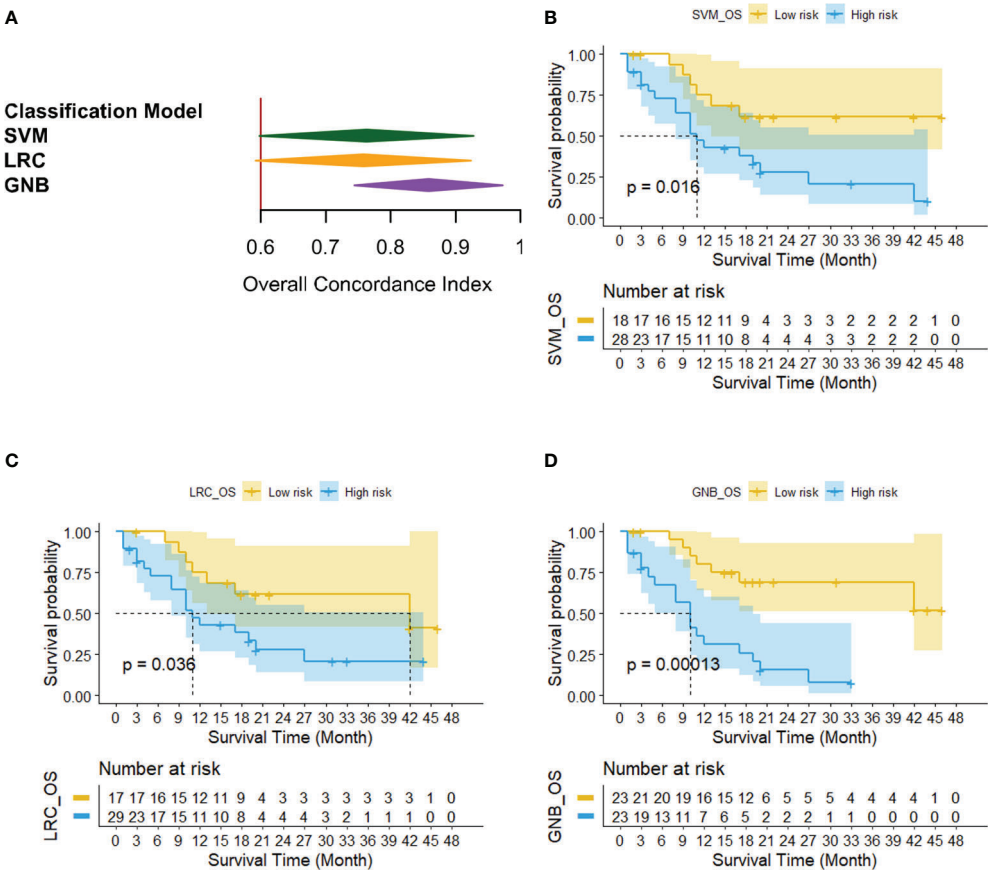

FIGURE 8 | The results of survival analysis of three OS classification models. **(A)** C-indexes generated by three OS prediction models, **(B–D)** Kaplan–Meier OS estimates from all 46 patients by using SVM, LRC and GNB classifier, respectively.

prediction models. In recent years, numerous studies have evaluated the potential clinical utility of radiomics features from CT images of NSCLC, correlated with tumor histology, staging and patient prognosis (36–48). Nardone V et al. (37) used pre- and post-contrast CT sequences to contour the gross tumor volume (GTV) of the target lesions prior to nivolumab treatment. The impact of variations on contouring was analyzed by two delineations, which were performed on each patient, and the CT texture analysis (TA) parameters were tested for reliability using the Intraclass Coefficient Correlation method (ICC). The study indicated that TA parameters could identify patients that will benefit from PD-1 blockage by defining the radiological settings that were potentially suggestive of an active immune response. Xu et al. (41) evaluated deep-learning networks for predicting clinical outcomes through analyzing time-series CT-images of locally advanced NSCLC patients. In our study, only pre-immunotherapy CT images were used to evaluate and predict the response results of immunotherapy. Thus, the treatment response might be predicted before conducting the immunotherapy by using CT images.

In this study, a non-invasive CT-based radiomics model was developed to predict the effectiveness of immunotherapy for advanced NSCLC patients. Thousands of quantitative imaging features were computed and investigated to decode the phenotypes of primary lung tumor. Then, the optimal feature pool selected from initial radiomics features were used to train and test three machine-learning classifiers to build prognosis prediction models. A LOOCV method was applied to test and evaluate the model performance. The results demonstrated that it was an effective way to predict the effectiveness of immunotherapy for advanced NSCLC patients by using machine-learning based models (i.e., results showed in **Figure 6**). If our models were robust by testing on the more diverse and larger dataset in future studies, it would provide a new way to predict patient's short-term treatment response before immunotherapy prescribed in the advanced lung cancer.

To further investigate that how much extra benefit we could obtain for predicting individual patient's PFS and OS by using the risk scores predicted by our machine-learning models, we also analyzed the survival analysis to evaluate and compare the outcomes of patients with different risk factor. Three machine-learning classifiers yielded high concordance with clinical evaluation outcomes determined by independent radiology review (IRR) for predicting PFS (i.e., C-index for SVM: 0.93,

LRC: 0.91, and GNB: 0.92) and OS (i.e., C-index for SVM: 0.60, LRC: 0.60, and GNB: 0.64). Then, the Kaplan–Meier survival analysis illustrated significant difference between the high and low risk patient group for PFS and OS analysis ($p<0.05$).

Despite of promising results, our study had some limitations. Firstly, to develop CT radiomics models, three machine-learning classifiers were trained and tested on a relatively small dataset with only forty-six cases. Although the LOOCV method was applied in the classifier training and testing process to avoid biases, the robustness and effectiveness of our model were still needed to be evaluated by using more diverse and larger data sets. Secondly, only CT-based radiomics features were used to predict the PFS and OS of advanced NSCLC patients. Some of the other potentially useful clinical information and image features (i.e., biomarkers, MRI image, PET image) have not been explored. Thus, different kinds of features needed to be investigated in our future studies. Thirdly, only the selected target lesions were analyzed instead of all the lesions; nevertheless, the degree of enhancement after CT enhanced scanning of target lesions in different tissues will be different. Lastly, this was only a primary technology development study that just developed a CT-based radiomics model to predict clinical outcomes of advanced NSCLC patients treated with nivolumab. Due to the incomplete data of retrospective studies, we did not include clinical data, genomics and other factors for analysis. Before our prediction models were applied into clinical practice, we will conduct more clinical validation studies to improve the performance of prediction model by combining imaging technologies, clinical characteristics, genomics and other factors.

In conclusion, the novel CT-based radiomics model has the ability to predict the progression probability for patients with advanced NSCLC receiving nivolumab therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board of Fudan University Shanghai Cancer Center. Written informed consent to participate in this study was provided by the patient/participants.

AUTHOR CONTRIBUTIONS

JW and SW designed the study, collected data and verified its integrity, and helped writing the manuscript. CL, JG, and HY were responsible for statistical analysis and writing the manuscript. SW and QL were responsible for interpreting CT images and verifying the integrity of the data. All authors contributed to the article and approved the submitted version.

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Immune-Related Adverse Events and Their Association With the Effectiveness of PD-1/PD-L1 Inhibitors in Non-Small Cell Lung Cancer: A Real-World Study From China

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Background: Programmed cell death-1/programmed cell death ligand-1 (PD-1/PD-L1) inhibitors are increasingly used in China, but no real-world data are available about the immune-related adverse events (irAEs). This real-world retrospective study aimed to assess the safety and effectiveness of PD-1/PD-L1 inhibitors in patients with non-small cell lung cancer (NSCLC) and to analyze the association between irAEs and effectiveness.

Methods: This was a retrospective study of the clinical data of patients with NSCLC treated with PD-1/PD-L1 inhibitors from August 2016 to November 2019 at Beijing Cancer Hospital. The patients were divided into the irAE or non-irAE groups. Overall adverse events, the impact of irAE on tumor response, and the association of irAEs with effectiveness were evaluated.

Results: One hundred and ninety-one patients were included, including 70 (36.6%) patients in the irAE group and 121 (63.4%) patients in the non-irAE group. AE, grades 3–5 AEs, and irAE occurred in 107 (56.0%), 24 (12.6%), and 70 (36.6%) of the patients, respectively. The objective response rate (ORR) and disease control rate (DCR) were higher in the irAE group compared with the non-irAE group (42.0% vs. 25.8%, P=0.038; 91.9% vs. 70.8%, P=0.002). Multivariable analyses identified that irAE were associated with progression-free survival (HR=0.62, 95%CI: 0.43–0.91; P=0.015), but not with overall survival (HR=0.76, 95%CI: 0.44–1.28; P=0.299).

Conclusion: In NSCLC treated with PD-1/PD-L1 inhibitors, patients with irAEs showed improved effectiveness over patients without irAEs. Future studies of anti-PD-1/PD-L1 immunotherapy should explore this association and the underlying biological mechanisms of efficacy.

Keywords: non-small cell lung cancer, immunotherapy, adverse events, real-world evidence, objective response, survival

INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide (1–3), with an incidence of 31.5 per 100,000 men and 14.6 per 100,000 women (3). It is mainly categorized as non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (4). NSCLC accounts for about 80% of all lung cancers, of which 75% of the patients are in an advanced stage at diagnosis (4).

In recent years, inhibitors of the programmed cell death 1 (PD-1)/programmed death-ligand 1 (PD-L1) have shown strong anti-tumor activity and become standard anti-tumor treatments for patients with lung cancer (5). To date, two PD-1 inhibitors (pembrolizumab and nivolumab) and one PD-L1 inhibitor (atezolizumab) have been approved for first- or second-line treatment of NSCLC by the US Food and Drug Administration (6, <x>7 </x>,7). Other PD-1/PD-L1 inhibitors (avelumab, camrelizumab, sintilimab, tislelizumab, toripalimab, camrelizumab, sintilimab, tislelizumab, and toripalimab) are at different stages of clinical development (8–11). Those drugs inhibit the immune escape mechanism of the tumor cells, allowing the body's immune system to recognize the cancer cells as non-self and killing them (8–11).

The immune checkpoint inhibitors (ICIs), including the PD-1 axis inhibitors, activate the body's immune system and can cause adverse events (AEs), including damaging the normal tissues and organs in the form of an inflammatory response, which is known as immune-related adverse events (irAEs) (12). Those irAEs can affect different organs and show different clinical symptoms such as skin (rash and pruritus), gastrointestinal (diarrhea and colitis), liver (hepatitis), endocrine (hyperthyroidism, hypothyroidism, and adrenal insufficiency), lung (pneumonitis), and kidney (renal insufficiency) (12). In addition, treatment-related adverse events (trAEs), including fatigue, anorexia, and nausea, are also among the adverse events of ICIs (13–15). In general, these toxicities are mild, but some specific irAEs can affect the treatment course, and can even be life-threatening (16).

At present, irAEs are thought to represent the bystander effect of activated T cells (e.g., due to a more competent/treatment-responsive immune system or cross-reactivity between tumor and host tissue) (17–19), and it is a reasonable assumption that the patients who respond to ICI are more likely to develop autoimmune toxicity. Previous studies have shown that irAE onset may represent one clinical biomarker for ICI response (20, 21). Several retrospective studies showed that irAEs were associated with durable response to ICIs and clinical benefit in patients with melanoma (22, 23), and several studies have shown similar associations with NSCLC treated with nivolumab (24–26). Nevertheless, to our knowledge, no similar studies have been reported for the treatment of PD-1/PD-L1 inhibitors (alone or in combination) in patients with advanced NSCLC in China.

Considering that PD-1/PD-L1 inhibitors are increasingly being used, it is important to fully understand their AEs in the treatment of NSCLC. Most of the current data on them come from clinical trials, which were mainly conducted in Caucasians, and only a small number of Asian participants were included in multi-ethnic trials. Therefore, the data about the AEs, especially irAEs of PD-1/PD-L1 inhibitors in the Chinese population, are not exhaustive. Therefore, the purpose of this study was to assess the incidence of irAEs and analyze the association of irAEs with the effectiveness of PD-1/PD-L1 inhibitors for patients with advanced NSCLC in the real-world Chinese population.

METHODS

Patients

This was a retrospective study of patients with NSCLC treated with PD-1/PD-L1 inhibitors from August 2016 to November 2019 at the Second Department of Thoracic Medicine of Beijing Cancer Hospital. The study was approved by the Ethics Committee of Beijing Cancer Hospital (No. 2020YJZ24). The need for individual consent was waived by the committee because of the retrospective nature of the study.

The inclusion criteria were 1) cytological or histological confirmation of NSCLC (6, 27), 2) patients who received monotherapy with PD-1/PD-L1 inhibitors or PD-1/PD-L1 inhibitors with chemotherapy or PD-L1 plus CTLA-4 with or without chemotherapy, or any other regimen that includes PD-1/PD-L1 inhibitors, 3) completed at least one cycle of immunotherapy, and 4) available data about the AEs. The exclusion criteria were 1) important organ dysfunction before treatment or 2) did not complete an AE follow-up visit of one cycle at the time of data collection.

Data Collection

Patients' data were collected through the information system of Beijing Cancer Hospital (HIS), which is a comprehensive electronic patient chart system that is fully indexed and searchable (28). Clinical data included age, sex, pathological type, Eastern Cooperative Oncology Group (ECOG) score, weight change before treatment, smoking history, driver gene variants [epidermal growth factor receptor (EGFR)/anaplastic lymphoma kinase (ALK)], tumor-node-metastasis (TNM) stage, line of immunotherapy, and other basic clinical characteristics as well as medication regimen, adverse events, clinical effectiveness, and prognosis were recorded. All patients routinely underwent preoperative systematic physical examination, complete blood count, and routine biochemical examination. Weight loss was defined as a weight loss >5% within 6 months.

AEs and Effectiveness Evaluation

AEs were judged according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.03 (29) and were classified as grades I–V. irAEs were defined according to the guidelines on the management of immunotherapy-related toxicities (12, 30). The time to onset of an irAE was defined as the time from the start of immunotherapy to the occurrence of irAE. irAEs were defined as having a potential immunological basis that required more frequent monitoring and potential intervention. Based on this, the patients were divided into two groups (the irAE and non-irAE groups), and the overall response rate (ORR), progression-

free survival (PFS), and overall survival (OS) were evaluated in each group.

According to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (31), the response was divided into the complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). The ORR was (CR+PR)/(CR+PR+SD+PD) ×100%. The disease control rate (DCR) was (CR+PR+SD)/(CR+PR+SD+PD) ×100%. Target and non-target lesions were measured routinely by radiologists and effectiveness were evaluated routinely by physician internists as part of the routine clinical workup. In the present study, the assessment was performed unblinded and retrospectively by two attending physicians with >8 years of experience and two associate physicians with >3 years of experience in medical oncology.

The PFS was defined as the time from the start of treatment to progression or death from any cause. The OS was defined as the time from the start of treatment to death from any cause. The values of the patients who were lost to follow-up, and those who did not progress were treated as censored values. The censored time was the last follow-up that confirmed that the patients had neither progressed nor died.

Outcomes and Follow-Up

The outcome of the study was the effectiveness of PD-1/PD-L1 inhibitors, including ORR, PFS, and OS. The effectiveness of the 3-week regimens was assessed every 6 weeks, and that of the 2-week regimens every 8 weeks. Thyroid function, myocardial enzymes, B-type brain natriuretic peptide (BNP), lipase, and amylase were examined before the first treatment and every 3 months. After completing the treatment, the patients were followed for progression or survival every 3 months by clinical visits and telephone interviews. In this study, there were three researchers responsible for follow-up. They all have participated in nearly eight clinical trials of PD-1/PD-L1 inhibitor treatment as sub-investigators, and they received training to identify AE and irAE. Besides, one person, a staff of the Statistics Department of the Beijing Cancer Hospital with 15-year-experience, was responsible for collecting the survival data of the patients. The follow-up was censored on May 27, 2020.

Statistical Analysis

SPSS 23.0 (IBM, Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) were used for statistical analysis and graph plotting. The Kolmogorov-Smirnov test was applied to assess the normal distribution of continuous data. The continuous data are presented as medians (ranges) and were analyzed using the Mann-Whitney U-test. The categorical data are presented as numbers (percentages) and were analyzed using the chi-square test or Fisher's exact test. The time to the onset of irAE (grades 1–2 vs. grades 3–5) was compared using the Kruskal-Wallis rank-sum test. The Kaplan-Meier method and the log-rank test were used for comparison of survival data between groups. The univariable and multivariable Cox regression model was used for the analysis of PFS and OS. The data filtering method used in the multivariable Cox regression was backward stepwise (likelihood ratio). Factors with P<0.10 in the univariable analyses and factors with unbalanced baseline characteristics were included in the multivariable analysis. P-values <0.05 were considered statistically significant.

RESULTS

Characteristics of the Patients

A total of 222 patients were screened: 191 were included, and 31 were excluded (the treatment (active vs. placebo) could not be confirmed in 19 patients due to inclusion in a randomized, double-blind clinical trial; six patients were enrolled in phase I clinical trials to receive combined anti-PD-1 and its downstream target double-inhibitor; two patients had an abnormal liver function; one patient had abnormal kidney function before treatment; three patients did not complete the evaluation of adverse reactions after the first cycle).

The characteristics of the patients are shown in **Table 1**. The 191 patients were divided into two groups: the irAE group (70, 36.6%) and the non-irAE group (121, 63.4%). The median age was 62 years (range, 30–87). There were no differences between the two groups in terms of sex, NSCLC histology, ECOG, changes in weight before treatment, smoking history, stage, EGFR status, and ALK status. There were differences between patients with or without irAE, including age, the lines of immunotherapy, type of drug, and therapeutic modalities (all P<0.05). Treatment drugs and treatment cycles were summarized in **Supplementary Table S1**. Among the 191 patients, 69 patients participated in clinical trials.

Adverse Events

As shown in **Table 2**, AE, grades 3–5 AEs, and irAE were occurred in 107 (56%), 24 (12.6%), and 70 (36.6%) of the 191 patients, respectively.

Among all 191 patients enrolled in this study, the most common overall AEs were poor appetite (14.7%), fatigue (14.1%), nausea (12.6%), and fever (10.5%). The endocrine AEs were mainly hypothyroidism (4.7%), hyperthyroidism (2.6%), and hypophysitis (0.5%). The gastrointestinal toxicities were diarrhea (4.2%) and immune-related pancreatitis (1.0%). Nervous system and musculoskeletal toxicities included drooping eyelids (0.5%), myalgia (3.1%), and arthralgia (3.1%).

In all patients enrolled in this study, the most common irAEs were rash (11.0%), pruritus (8.4%), immune-related pneumonitis (7.9%), and elevated ALT (7.3%). The most common grades 3–5 irAEs were immune-related pneumonitis (2.1%), increased creatinine (1.6%), increased GGT (1.0%), increased ALT (1.0%), and increased lipase (1.0%). Most irAEs were mild, and the incidence of grades 3–5 irAEs was low. Only one drug-related death (hypophysitis) was observed. The patient who developed grade 5 hypophysitis was a 72-year-old man treated with pembrolizumab (2 mg/kg q 3 weeks). After five cycles, he developed hypophysitis that showed as drowsiness, fatigue, consciousness disorder, and hypotension. Laboratory examination showed mild hyponatremia, secondary adrenal insufficiency, and secondary hypothyroidism. Steroid treatment was ineffective. The patient also had immune-related pneumonitis.

TABLE 1 | Characteristics of the patients.

Characteristics, N (%)		irAE Group (n=70)	Non-irAE Group (n=121)	P
Sex	Male	54 (38.8)	85 (61.2)	0.302
	Female	16 (30.8)	36 (69.2)	
Age (years)	<65	52 (41.9)	72 (58.1)	0.039
	≥65	18 (26.9)	49 (73.1)	
NSCLC histology	Adenocarcinoma	41 (38.0)	67 (62.0)	0.497
	Squamous carcinoma	27 (37.5)	45 (62.5)	
	Others	2 (18.2)	9 (81.8)	
ECOG	0–1	68 (38.2)	110 (61.8)	0.138
	≥2	2 (15.4)	11 (84.6)	
Changes in weight before treatment	No change	55 (37.4)	92 (62.6)	0.688
	Loss of weight	15 (34.1)	29 (65.9)	
Smoking history	Yes	27 (38.0)	44 (62.0)	0.945
	No	42 (36.2)	74 (63.8)	
	Unknown	1 (25.0)	3 (75.0)	
Stage	I	1 (20.0)	4 (80.0)	0.675
	II	4 (25.0)	12 (75.0)	
	III	17 (34.0)	33 (66.0)	
	IV	47 (39.8)	71 (60.2)	
	Unknown	1(50)	1 (50)	
EGFR status	Wild type	46 (38.7)	73 (61.3)	0.746
	Mutant	5 (31.3)	11 (68.8)	
	Unknown	19 (33.9)	37 (66.1)	
ALK status	Wild type	55 (38.7)	87 (61.3)	0.614
	Fusion	0 (0)	1 (100.0)	
	Unknown	15 (31.3)	33 (68.8)	
Lines of immunotherapy	First line	39 (52.0)	36 (48.0)	0.005
	Second line	15 (27.3)	40 (72.7)	
	Third line and above	11 (24.4)	34 (75.6)	
	Others	5 (31.3)	11 (68.7)	
Type of drug	Anti-PD-1	43 (27.9)	111 (72.1)	<0.001
	Anti-PD-L1	27 (73.0)	10 (27.0)	
Therapeutic modalities	Single drug	29 (29.3)	70 (70.7)	0.019
	Combined with chemotherapy	35 (42.7)	47 (57.3)	
	Combined with CTLA-4	5 (83.3)	1 (16.7)	
	Others	1 (25.0)	3 (75.0)	

irAE, immune-related adverse events; NSCLC, non-small cell lung cancer; ECOG, Eastern Cooperative Oncology Group; EGFR, epithelial growth factor receptor; ALK, anaplastic lymphoma kinase; PD-1, programmed cell death protein 1; PD-L1, programmed cell death protein ligand 1; CTLA-4, cytotoxic T-lymphocyte-associated protein 4. P-values <0.05 were considered statistically significant.

TABLE 2 | Overall adverse events.

AE, N (%)	All AEs (n=191)	Grades 3–5 AEs (n=191)
Total	107 (56.0)	24 (12.6)
Poor appetite	28 (14.7)	1 (0.5)
Fatigue	27 (14.1)	0
Nausea	24 (12.6)	1 (0.5)
Fever	20 (10.5)	1 (0.5)
Pneumonia	19 (9.9)	5 (2.6)
Vomiting	12 (6.3)	1 (0.5)
Influenza-like symptoms	6 (3.1)	0
Dizziness	5 (2.6)	0
Reactive capillary hemangiomas	5 (2.6)	0
Headache	4 (2.1)	1 (0.5)
Stomachache	4 (2.1)	0
Mucosal ulcer	4 (2.1)	0
Transfusion reaction	3 (1.6)	0
Dry mouth	2 (1.0)	0
Dysphagia	2 (1.0)	1 (0.5)
Hyperglycemia	2 (1.0)	1 (0.5)
irAE	70 (36.6)	14 (7.3)
Rash	21 (11.0)	0
Pruritus	16 (8.4)	0
Pneumonitis	15 (7.9)	4 (2.1)
ALT increase	14 (7.3)	2 (1.0)
Hypothyroidism	9 (4.7)	1 (0.5)
Creatinine increased	8 (4.2)	3 (1.6)
Diarrhea	8 (4.2)	0
AST increased	8 (4.2)	1 (0.5)
GGT increased	7 (3.7)	2 (1.0)
Myalgia	6 (3.1)	0
Arthralgia	6 (3.1)	1 (0.5)
Hyperthyroidism	5 (2.6)	0
Amylase increase	5 (2.6)	1 (0.5)
Elevated muscle enzymes	3 (1.6)	1 (0.5)
Lipase increase	3 (1.6)	2 (1.0)
Bilirubin increased	2 (1.0)	0
Pancreatitis	2 (1.0)	0
Neurotoxicity[a]	1 (0.5)	0
Hypophysitis[b]	1 (0.5)	1 (0.5)
Hepatitis	1 (0.5)	1 (0.5)

[a]*One patient with neurotoxicity presented with a drooping eyelid.*
[b]*Only one drug-related death was hypophysitis.*
AE, adverse event; irAE, immune-related adverse events; ALT, alanine transaminase; AST, aspartate transaminase; GGT, γ-glutamyltransferase.

The median time to onset of irAEs was shown in **Supplementary Figure S1**. As shown in **Supplementary Figure S2**, grades 1–2 irAEs occurred earlier than grades 3–4 irAEs (P=0.005).

Among the patients with irAE, 19 were treated with steroids (13 with immune-related pneumonitis, two with immune-related pancreatitis, one with elevated amylase and lipase, one with immune-related hypophysitis combined with immune-related pneumonitis, one with immune-related hepatitis, and one with elevated creatinine), and one patient with immune-related pneumonitis was treated with low-dose cyclophosphamide. No Patients were treated with infliximab or tocilizumab. Six patients were treated with thyroid hormone replacement.

This study included four patients with abnormal liver function and one patient with abnormal renal function at baseline. After immunotherapy, there was no aggravation of abnormal liver function or renal function. A grade 2 rash was observed in a patient with psoriasis (28 days after the combination of PD-L1 inhibitor + CTLA-4 inhibitor), a grade 2 immune-related pneumonitis was observed in one patient with rheumatoid arthritis (68 days after starting the combination of chemotherapy and PD-L1 inhibitor), and one patient presented with a tuberculosis relapse (178 days after chemotherapy combined with PD-L1 inhibitor).

Association Between irAEs and Effectiveness

The last follow-up time was in May 2020. The average follow-up time was 9.8 months, and the longest follow-up time was 43.5 months. There were 175 cases of stage IV or recurrent NSCLC among those patients: 64 in the irAE group and 111 in the non-irAE group.

As shown in **Table 3**, there were 191 cases of NSCLC, and the response was evaluated in 151 patients: none had CR, 49 (32.5%)

had PR, 71 (47.0%) had SD, and 31 (20.6%) had PD; ORR was 32.5%, and DCR was 80.1%. The CR, PR, SD, and PD rates of the irAE and non-irAE groups were 0, 42.0%, 50.0%, and 8.0% vs. 0, 25.8%, 44.9%, and 29.2%, respectively (P=0.002). The ORR and DCR rates of the irAE were higher than in the non-irAE group (42.0% vs. 25.8%, P=0.038; 91.9% vs. 70.8%, P=0.002).

As shown in **Figure 1**, the PFS of the irAE group was longer than in the non-irAE group in advanced NSCLC (8.8 vs. 3.9 months, 95% CI: 6.5–11.1 vs. 2.5–5.3, P=0.001). As shown in **Figure 2**, the OS of the irAE group was longer than the non-irAE group in advanced NSCLC (21.0 vs. 14.8 months, 95% CI:12.0–30.0 vs. 8.3–21.3, P=0.033).

As shown in **Table 4**, the univariable analysis suggested that age, ECOG, EGFR status, lines of immunotherapy, type of drug, therapeutic modalities, and irAE might be prognostic factors of PFS (all P<0.10). And the multivariable analysis of advanced NSCLC showed that ECOG≥2 (HR=1.97, 95%CI: 1.03–3.76, P=0.04), second line of immunotherapy (vs. first line, HR=1.75, 95%CI: 1.15–2.65, P=0.008) and irAE (HR=0.62, 95%CI: 0.43–0.91, P=0.015) were independent associated with PFS.

TABLE 3 | Impact of irAE on tumor response.

Effectiveness of treatment	All (N = 151)	irAE Group (n = 62)	non-irAE Group (n = 89)	P
CR	0	0	0	0.002
PR	49 (32.5%)	26 (42.0%)	23 (25.8%)	
SD	71 (47.0%)	31 (50.0%)	40 (44.9%)	
PD	31 (20.6%)	5 (8.0%)	26 (29.2%)	
ORR%	49 (32.5%)	26 (42.0%)	23 (25.8%)	0.038
DCR%	120 (80.1%)	57 (91.9%)	63 (70.8%)	0.002

irAE, immune-related adverse events; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ORR, objective response rate; DCR, disease control rate.

P-values <0.05 were considered statistically significant.

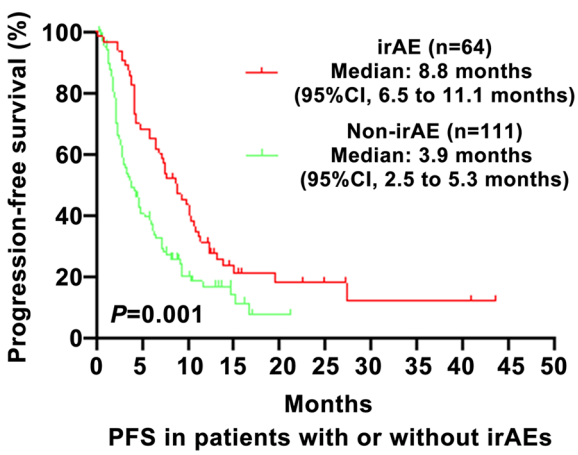

FIGURE 1 | Impact of immune-related adverse events (irAEs) on progression-free survival (PFS). PFS, progression-free survival; CI, confidence interval; irAE, immune-related adverse events; P-values <0.05 were considered statistically significant.

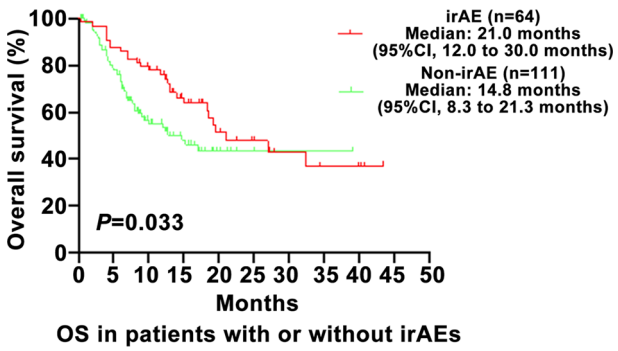

FIGURE 2 | Impact of immune-related adverse events (irAEs) on overall survival (OS). OS, overall survival; CI, confidence interval; irAE, immune-related adverse events; P-values <0.05 were considered statistically significant.

As shown in **Table 5**, the univariable analysis suggested that age, ECOG, smoking history, lines of immunotherapy, type of drug, and irAE might be prognostic factors of OS (all P<0.10). And the multivariable analysis of advanced NSCLC showed that age ≥65 years (HR=1.81, 95%CI: 1.13–2.88, P=0.013) and ECOG≥2 (HR=4.92, 95%CI: 2.40–10.05, P<0.001) were independent associated with OS.

Sixty-five percent of all irAEs in the patients in this study occurred within 3 months. The median time to the first irAE was 1 month. Therefore, to account for a possible immortal time bias, the patients were divided into five groups: ICI >1 month, non-irAE; ICI <1 month, non-irAE; ICI >1 month, irAE <1 month; ICI <1 month, irAE <1 month; and ICI >1 month, irAE >1 month. The PFS was different for all patients (P<0.001) and ICI >1 month (P=0.006), but not for ICI <1 month (P=0.747). The OS was different for all patients (P<0.001), but not for ICI >1 month (P=0.072) and ICI <1 month (P=0.456).

Analysis of Association in Different Status

The differences in PFS and OS between the irAE and non-irAE groups according to specific organs (skin, pneumonitis, hepatotoxicity, and thyroid dysfunction) were not statistically significant (**Supplementary Figure S3**). In this study, 17 patients discontinued PD-1/PD-L1 inhibitors due to irAE among 175 patients with metastasis or recurrence, including eight patients with PR and nine patients with SD. The median PFS from treatment initiation was 13.9 months for PR patients and 12.4 months for SD patients (P=0.301) (**Supplementary Figure S4A**). The median PFS from the discontinuation was 11.1 months for PR patients and 5.1 months for SD patients (P=0.279) (**Supplementary Figure S4B**). The median OS from the initiation was NR for PR or SD patients (P=0.04) (**Supplementary Figure S4C**). The median OS from the discontinuation was NR for PR and 9.9 months for SD patients (P=0.041) (**Supplementary Figure S4D**). There were 64 patients with irAE in this study, 17 of whom were treated with

TABLE 4 | Univariate and multivariable analyses of factors associated with PFS.

Factor	Univariable		Multivariable	
	HR (95% CI)	P	HR (95% CI)	P
Male	0.88 (0.60–1.29)	0.513		
Age ≥65 years	1.54 (1.08–2.20)	**0.018**	1.37 (0.95–1.97)	0.091
NSCLC histology				
Adenocarcinoma	1			
Squamous	0.88 (0.61–1.27)	0.498		
Other	1.13 (0.52–2.45)	0.764		
ECOG ≥2	2.51 (1.34–4.71)	**0.004**	1.97 (1.03–3.76)	**0.04**
No change in weight before treatment	1.15 (0.92–1.43)	0.216		
Smoking history				
No	1			
Yes	0.92 (0.64–1.32)	0.64		
Unknown	1.36 (0.49–3.77)	0.555		
EGFR status				
Wild type	1		1	
Mutant	1.89 (1.03–3.47)	**0.039**	1.76 (0.93–3.31)	0.082
Unknown	0.86 (0.57–1.31)	0.491	0.86 (0.56–1.30)	0.469
ALK status				
Wild type	1			
Fusion	0.00 (0.00–1.25E162)	0.959		
Unknown	0.73 (0.48–1.12)	0.154		
Lines of immunotherapy				
First line	1		1	
Second line	1.99 (1.32–3.00)	**0.001**	1.75 (1.15–2.65)	**0.008**
Third line and above	1.64 (1.06–2.53)	**0.026**	1.43 (0.91–2.25)	0.119
Type of drug, Anti-PD-L1	0.56 (0.36–0.87)	**0.01**	0.73 (0.45–1.19)	0.204
Therapeutic modalities				
Single drug	1		1	
Combined with chemotherapy	0.73 (0.51–1.04)	**0.083**	0.82 (0.48–1.39)	0.459
Combined with CTLA-4	0.79 (0.34–1.84)	0.588	1.82 (0.69–4.82)	0.23
Others	0.32 (0.04–2.27)	0.252	0.34 (0.04–2.65)	0.305
irAE	0.54 (0.37–0.77)	**0.001**	0.62 (0.43–0.91)	**0.015**

PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; irAE, immunotherapy-related adverse events; ECOG, Eastern Cooperative Oncology Group; EGFR, epithelial growth factor receptor; ALK, anaplastic lymphoma kinase; Bold values indicate P-values <0.05, considered statistically significant.

steroid therapy for irAE. PFS was 4.2 (95%CI: 2.3–6.2) months in the patients with steroid use and 9.3 (95%CI: 6.7–11.9) months in those without (P=0.291) (**Supplementary Figure S5A**). OS was 12.4 (95%CI: 4.0–20.8) months in steroid-treated patients and 27.0 (95%CI: 9.4–44.6) months in non-steroid-treated patients (P=0.005) (**Supplementary Figure S5B**). After adjusting for the type of drug, therapeutic modalities, lines of immunotherapy, age, smoking history, EGFR status, and ECOG, the multivariate Cox analysis showed that steroid use was independently associated with OS (HR=2.86, 95%CI: 1.30–6.30, P=0.009) but not with PFS (HR=1.34, 95%CI: 0.69–2.59, P=0.386).

Subgroup Analysis in the Patients With Stage IV

The overall number of therapy lines were 1–8 lines (mean of 2.3 lines) and 1–8 lines (mean of 2.7 lines) in the irAE and non-irAE groups of stage IV patients, respectively. Among the 118 stage IV patients, 47 were in the irAE group, and 71 were in the no-irAE group. The median progression-free survival (PFS) was 8.7 (95% CI: 7.0–10.4) months in the irAE group and 3.9 (95% CI: 2.4–5.4) months in the non-irAE group (P=0.002) (**Supplementary Figure S6A**). The median overall survival (OS) was 27.0 (95% CI: 13.4–40.6) months in the irAE group and 14.8 (95%CI: 10.7–18.9) months in the non-irAE group (P=0.069) (**Supplementary Figure S6B**).

DISCUSSION

PD-1/PD-L1 inhibitors are increasingly used in China, but few real-world data are available about the irAEs to our knowledge. This real-world retrospective study aimed to assess the safety and effectiveness of PD-1/PD-L1 inhibitors in patients with NSCLC and to analyze the association between irAEs and effectiveness. The results suggest that the TRAEs of PD-1/PD-L1 inhibitors in NSCLC were generally of low grade. irAEs presented mainly as skin toxicity, immune-related pneumonitis, and hepatotoxicity. irAEs were associated with higher ORR and DCR and longer PFS. To the best of our knowledge, this study on the irAEs and their association with the effectiveness of PD-1/PD-L1 inhibitors in NSCLC is by far the one with the largest number of patients in the real-world NSCLC Chinese population.

Approximately 56.0% of the patients developed AEs, and 36.6% developed irAEs among all 191 patients in this study. The most common AEs were poor appetite, fatigue, nausea, and fever.

TABLE 5 | Univariable and multivariable analyses of factors associated with OS.

Factor	Univariable		Multivariable	
	HR (95% CI)	P	HR (95% CI)	P
Male	1.27 (0.76–2.11)	0.364		
Age ≥65 years	2.08 (1.32–3.26)	**0.001**	1.81 (1.13–2.88)	**0.013**
NSCLC histology				
Adenocarcinoma	1			
Squamous	1.02 (0.63–1.63)	0.945		
Others	0.85 (0.30–2.35)	0.747		
ECOG ≥2	6.15 (3.06–12.37)	**<0.001**	4.92 (2.40–10.05)	**<0.001**
No change in weight before treatment	0.91 (0.70–1.18)	0.469		
Smoking history				
No	1		1	
Yes	1.26 (0.78–2.02)	0.345	1.31 (0.79–2.18)	0.302
Unknown	2.60 (0.91–7.45)	**0.075**	1.13 (0.37–3.48)	0.829
EGFR status				
Wild type	1			
Mutant	0.68 (0.27–1.70)	0.408		
Unknown	0.87 (0.51–1.48)	0.599		
ALK status				
Wild type	1			
Fusion	0.00 (0.00–1.46E225)	0.967		
Unknown	0.76 (0.43–1.33)	0.334		
Lines of immunotherapy				
First line	1		1	
Second line	1.62 (0.96–2.71)	**0.069**	1.31 (0.74–2.32)	0.360
Third line and above	1.19 (0.68–2.10)	0.548	0.95 (0.46–1.97)	0.883
Type of drug, Anti-PD-L1	0.51 (0.28–0.94)	**0.03**	0.61 (0.32–1.13)	0.117
Therapeutic modalities				
Single drug	1		1	
Combined with chemotherapy	0.68 (0.42–1.09)	0.111	0.72 (0.43–1.19)	0.197
Combined with CTLA-4	0.97 (0.35–2.70)	0.95	1.70 (0.54–5.40)	0.367
Others	0.00 (0.00–3.45E267)	0.97	0.00 (0.00–2.10E222)	0.964
irAE	0.60 (0.37–0.97)	**0.036**	0.76 (0.44–1.28)	0.299

OS, overall survival; HR, hazard ratio; CI, confidence interval; irAE, immunotherapy-related adverse events; ECOG, Eastern Cooperative Oncology Group; EGFR, epithelial growth factor receptor; ALK, anaplastic lymphoma kinase; Bold values indicate P-values <0.05, considered statistically significant.

The most common grades 3–5 treatment-related AE was pneumonia (2.6%). The most common irAEs were rash and pruritus, immune-related pneumonitis, and increased ALT. The most common grades 3–5 irAEs were immune-related pneumonitis (2.1%), increased creatinine (1.6%), increased GGT (1.0%), increased ALT (1.0%), and increased lipase (1.0%). The intensity of irAE was generally mild to moderate, with only 7.3% of patients with grades 3–5. The type of irAEs observed in this study was similar to those observed in the previous studies about PD-1/PD-L1 inhibitors, but the incidence of immune-related pneumonitis was higher than that reported in previous studies (13, 32, 33). This might be due to the selection criteria used in the various trials. Nevertheless, no new safety signal was observed.

Among the 191 patients in this study, the most common irAEs were rash and pruritus, immune-associated pneumonitis, and increased ALT. The most common grades 3–5 irAEs were immune-associated pneumonitis, increased creatinine, increased GGT, and increased ALT. Immune-associated pneumonitis is a potentially lethal irAE, and it is a focus of attention among irAEs of lung cancer. In this study, the incidence of immune-associated pneumonitis was 7.9%, and the incidence of grades 3–5 was 2.1%. This was more frequent than in a previous study (33). The possible reason might be that patients with basic lung diseases (such as chronic interstitial bronchitis) were excluded from the clinical trials. In addition, many previous studies of AEs included a variety of tumors (melanoma, kidney cancer, and non-small cell lung cancer). Because the microenvironment of lung cancer is different from that of other tumors, the incidence of lung cancer immune-associated pneumonitis could be slightly higher. Third, this study included patients treated with PD-1/PD-L1 combined with other drugs, which might increase the likelihood of developing immune-related pneumonitis. There were no deaths due to immune-related pneumonitis in this study, and it could usually be relieved by timely detection and standard treatment. Skin toxicity was the most common irAEs in all patients enrolled in this study, mainly rash (11.0%) and pruritus (8.4%) of grades 1–2. This is consistent with previous studies (13, 32). Hepatotoxicity was another of the most common irAE, with an overall incidence of 7.3% (increased ALT), and the incidence of grades 3–5 was 1.0% (increased ALT or GGT). The higher incidence compared with previous studies might be related to the combined medication regimens included in this study. Endocrine toxicity mainly manifested as hypothyroidism (4.7%) and hyperthyroidism (2.6%), with only one case of grades 3–5 hypothyroidism, similar to previous researches (13, 34). Uncommon irAEs also occurred in our study: two cases of pancreatitis, one of neurotoxicity, and one

of hypophysitis. The proportion of pneumonia was high (9.9% of any-grade and 2.6% of grades 3–5), which is significantly higher than the incidence rate during chemotherapy. During PD-1/PD-L1 combined with chemotherapy, the decrease of leukocytes and neutrophils may increase the risk of pneumonia during immunotherapy, and it needs to be clinically distinguished from immune-related pneumonitis.

Although the precise mechanisms of irAEs have not been fully revealed, they are thought to be the bystander effect of activating T cells, which is consistent with the mechanism of ICIs (12). irAEs might be triggered by antigens that are common to both tumor and inflamed organs. Second, the link between T-cells and irAEs focuses on the gut microbiome, and significant differences in microbial diversity might be observed in responding versus non-responding patients. Third, pre-existing organ-specific antigen expression may be another cause of irAEs without representing a shared effect from anti-tumor activity, which could be mechanisms of autoimmune toxicity that are independent of the anti-tumor response (20). irAE onset may be a clinical biomarker for the response of immune checkpoint blocking drugs. This phenomenon was first seen in melanoma patients, although not all evidence supports this hypothesis. Several recent retrospective analyses showed that among patients receiving nivolumab, patients with irAE had a better response (ORR or DCR or PFS or OS) than patients without irAE (22–24, 35, 36).

The patients with irAE had higher ORR and DCR and a longer median PFS, but not OS. It is suggested that irAE may be a clinical biomarker for the benefit of immunotherapy, including PD-1/PD-L1 inhibitors. It reminds the medical team to monitor and detect irAE in time in order to reduce or avoid the occurrence of serious irAE, thereby reducing the proportion of termination and suspension of treatment. Some previous studies suggested that skin toxicity and thyroid function damage were related to the effectiveness of immunotherapy (37, 38). Previous studies suggested that specific types of irAEs were related to prognosis (39–43), but this was not observed here. In addition, cancers with a high tumor mutational burden (TMB) are associated with a higher risk of irAEs, and the possible cause is the different neoantigenic load across cancer types (44), but TMB could not be examined in this study. In this study, 17 patients discontinued treatments due to irAEs; among them, those with PR at discontinuation had a longer OS than those with SD, as supported by a previous study (45). Steroid use also had an impact on survival, as suggested by a previous study (46). The relationship between different irAEs and immunotherapy needs to be confirmed by a larger number of studies. In the future, prospective research is needed to verify the exact impact of specific irAEs on prognosis.

The median age (65 years old) was used as the cut-off, and there was a significant difference between <65 and ≥65 years in the irAE rates. That might be caused by slightly more patients under 65 years of age received a four-drug combination therapy (chemotherapy + immunotherapy + anti-angiogenic therapy) than patients over 65 years of age, and some patients over 65 years of age were given immunotherapy combined with anti-vascular therapy without chemotherapy in our clinical practice.

PD-1/PD-L1 inhibitors are increasingly used in China, but no real-world data are available. This study revealed that the irAEs of PD-1/PD-L1 inhibitors (either as monotherapy or combination therapy) for lung cancer were mainly low grade and suggested that patients with irAEs showed improved effectiveness over patients without irAEs. These data come from real-world results from the Chinese population, so it could better reflect the impact of the irAEs in the actual clinical practice. In addition, this study explored the correlation between the time of occurrence of irAEs or irAEs in different organs with the prognosis and provided data and clues for an in-depth study of the relationship between irAE and prognosis. This study provides a theoretical basis for the clinical use of PD-1/PD-L1 inhibitors in the Chinese population and provides clues for exploring the mechanism of the association of irAEs with effectiveness.

In this study, the patients with elevated creatinine as irAE had no combined medications and related disease history that could explain the renal damage. They included the patients treated with ICI monotherapy and patients treated with combined chemotherapy who did not display elevated creatinine during combined chemotherapy. Patients with elevated creatinine in the maintenance phase were considered to be more likely to be caused by immunotherapy. Nevertheless, because these patients did not undergo a kidney biopsy, the side effects of chemotherapy cannot be excluded, which is a limitation of this study. Besides, patients with pneumonia had clear evidence of infection, including elevated WBC, elevated NE%, high PCT, high CRP, or conditional pathogens from sputum culture or blood culture, and patients who improved after antibiotic treatment. The patients with pneumonitis had no clear clinical evidence of infection (hematological examination and pathogenic bacteria), and interstitial pneumonitis was considered in the imaging or patients who are ineffective in antibiotic therapy and get better after hormone therapy.

This study has limitations. First, this study is a retrospective study with data offsets. Second, at present, there is no clear diagnostic standard for irAE, and some of them are clinical symptoms that might be subjective. The present study determined irAE based on previous research and guidelines (12). There might be deviations in irAE determination. Because of the retrospective nature of the study, the exact timing of irAE occurrence (early vs. late) could not be determined with any accuracy and could not be examined against survival, but this relationship has been described (24).

In conclusion, we found that irAEs of PD-1/PD-L1 inhibitors for lung cancer were mainly low grade and that the occurrence of irAE was positively correlated with ORR, DCR, and PFS, suggesting that patients with irAEs are more likely to benefit from immunotherapy. These data come from real-world results from the Chinese population, including patients with some previous autoimmune diseases, tuberculosis, chronic bronchitis, and ECOG grade 2/3, so it could better reflect the irAE performance in the actual clinical practice environment. Future

prospective studies are needed to confirm those results and explore the mechanism of irAEs' association with effectiveness.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Beijing Cancer Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

XC and JF contributed to the conception, design, analysis of data, and drafted the manuscript. JN, LD, and WH participated in the design of the study and acquisition of data. JZ, JH, XM, GT, SH, DW, YW, JL, and ZZ contributed to the acquisition of data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.607531/full#supplementary-material

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Absolute Lymphocyte Count Predicts Immune-Related Adverse Events in Patients With Non-Small-Cell Lung Cancer Treated With Nivolumab Monotherapy: A Multicenter Retrospective Study

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Background: Among patients with advanced non-small-cell lung cancer who were treated with nivolumab monotherapy, the association of peripheral blood count data (at baseline and 2 weeks after treatment initiation) with the early onset of immune-related adverse events (irAEs) and treatment efficacy has not been clearly established. This study aimed to identify peripheral blood count data that may be predictive of the development of nivolumab-induced irAEs in a real-world clinical setting.

Materials and Methods: This multicenter observational study retrospectively evaluated consecutive patients with advanced non-small-cell lung cancer undergoing nivolumab monotherapy in the second- or later-line setting between December 2015 and November 2018 at the National Cancer Center Hospital and Keio University Hospital in Japan. The primary endpoint was the association between peripheral blood count data and irAEs during the 6-week study period. Receiver operating characteristic curve and multivariable logistic regression analyses were performed.

Results: Of the 171 patients evaluated, 73 (42.7%) had ≥1 irAE during the first 6 weeks following treatment initiation. The median time to irAEs from the initiation of nivolumab was 15 (interquartile range: 13–28) days. Receiver operating characteristic curve analyses revealed that the optimal cut-off values of the absolute lymphocyte count, neutrophil-to-lymphocyte ratio, and lymphocyte-to-monocyte ratio 2 weeks after treatment initiation for early irAE onset were 820, 4.3, and 2.2, respectively. In multivariable logistic regression

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analyses, absolute lymphocyte count >820 at 2 weeks after treatment initiation was significantly associated with an increased risk of early onset of any irAE. In contrast, no significant association was observed for the neutrophil-to-lymphocyte ratio (>4.3) or the lymphocyte-to-monocyte ratio (>2.2) at 2 weeks following treatment initiation.

Conclusions: The absolute lymphocyte count >820 at 2 weeks following nivolumab initiation predicts early onset of irAEs during a 6-week study period. Routinely available absolute lymphocyte count, which is measured after the initiation of nivolumab, may be useful for identifying patients at risk of early onset of irAEs.

Keywords: absolute lymphocyte count, biomarker, lymphocyte-to-monocyte ratio, neutrophil-to-lymphocyte ratio, nivolumab, non-small-cell lung cancer

INTRODUCTION

Lung cancer is a major cause of morbidity and mortality worldwide (1). The development of immune checkpoint inhibitors (ICIs) has markedly modified the treatment paradigm in cancer, leading to durable responses in patients with malignant tumors. Pivotal phase III trials (2–4) have found that ICI monotherapy is markedly superior to standard second-line docetaxel chemotherapy in prolonging survival of previously treated patients with advanced non-small-cell lung cancer (NSCLC). However, ICIs are also associated with the development of immune-related adverse events (irAEs), which remain an unresolved issue in clinical practice (5, 6). Further, Shah et al. (7) reported an association between age and irAEs and identified the risk factors for irAEs in patients treated with ICIs. In contrast, programmed death-ligand 1 (PD-L1) expression was only a predictive biomarker for the efficacy of pembrolizumab. More importantly, routinely available peripheral blood biomarkers predictive of irAEs were inconsistent with those reported in previous studies and thus, remain controversial (8, 9).

In recent years, irAEs induced by the anti-PD-1 antibodies, nivolumab and pembrolizumab, have been shown to be associated with a survival benefit in patients with advanced NSCLC and malignant melanoma (10–19). Early detection of nivolumab-induced irAEs is crucial because of their negative impact on the patient's quality of life and associated burden on healthcare resources and costs. Routinely available peripheral blood count data, such as the absolute lymphocyte count (ALC), neutrophil-to-lymphocyte ratio (NLR), and lymphocyte-to-monocyte ratio (LMR), have the potential to predict treatment efficacy in patients with advanced NSCLC (20–23). Hence, we hypothesized that peripheral blood count data can also predict nivolumab-induced irAEs in patients with advanced NSCLC. ICIs interrupt immune suppression and activate CD8-positive T lymphocytes in the tumor microenvironment. These activated T lymphocytes not only attack the tumors but also cause irAEs, suggesting that these activated CD8-positive T lymphocytes exert systemic actions (22). Taken together, we explored the hypothesis that an increase in ALC and a related change in blood count ratios may help predict irAEs. To the best of our knowledge, no large-scale multicenter study has investigated the association of peripheral blood count data (at baseline and 2 weeks after treatment initiation) with the early onset of irAEs and treatment efficacy in patients with advanced NSCLC treated with nivolumab monotherapy.

Therefore, this study aimed to identify peripheral blood count data that may be predictive of the development of nivolumab-induced irAEs in a real-world clinical setting.

Abbreviations: ALC, absolute lymphocyte count; AMC, absolute monocyte count; ANC, absolute neutrophil count; CI, confidence interval; ICI, immune checkpoint inhibitor; IQR, interquartile range; irAE, immune-related adverse event; LMR, lymphocyte-monocyte-ratio; NLR, neutrophil-to-lymphocyte ratio; NSCLC, non-small-cell lung cancer; OR, odds ratio; PD-L1, programmed death-ligand 1.

MATERIALS AND METHODS

Study Design and Patients

This multicenter, retrospective observational study was conducted at the National Cancer Center Hospital, Keio University Hospital, and Keio University Faculty of Pharmacy, Tokyo, Japan. Research members from the Keio University Faculty of Pharmacy acquired data from electronic medical records at the National Cancer Center Hospital and Keio University Hospital. Data integration was performed at Keio University Faculty of Pharmacy, and subsequent statistical analyses were performed at Kyoto University Graduate School of Medicine, Kyoto, Japan. The methodology of this study has been previously reported by our co-author (24).

The subjects were consecutive patients, who were aged ≥20 years, diagnosed with advanced NSCLC, and underwent nivolumab monotherapy in the second- or later-line setting between December 2015 and November 2018 at the National Cancer Center Hospital and Keio University Hospital. Nivolumab was administered at a dose of 3 mg/kg every 2 weeks until August 2018, and thereafter, at a dose of 240 mg/body every 2 weeks according to the prescribing information contained in the package insert. The treatment schedule and follow-up were modified at the clinicians' discretion according to toxicity profiles. Clinic visits and imaging evaluations were conducted every 6 to 8 weeks starting at treatment initiation according to the Response Evaluation Criteria in Solid Tumors

(version 1.1). Patient records were de-identified and analyzed anonymously. The exclusion criteria were as follows (1): history of prior administration of any ICIs and/or investigational drugs as part of a clinical trial or at a previous hospital before the investigation period (2), discontinuation of treatment owing to death or hospital transfer during the first 6 weeks, (3) discontinuation of treatment after the first cycle because of disease progression or adverse events, (4) lack of laboratory data 2 weeks after the first cycle (acceptable range from day 12 to 16), and (5) study participation shorter than 6 weeks (*i.e.*, patients who started treatment between October and November 2018).

Study Protocol

We used a landmark analysis considering the lead-time bias owing to the time-dependent nature of irAEs (10, 11). Haratani et al. (10) reported that this approach reduced overestimation. Adverse events after the first cycle could be easily detected because the patients were hospitalized. Thus, we focused on the early onset of irAEs and included only patients with controlled disease and those who were alive 6 weeks after treatment initiation. The collected data included patients' baseline characteristics [age, sex, Eastern Cooperative Oncology Group performance status (scores ranging from 0 to 5, with higher numbers reflecting greater disability)], treatment lines, peripheral blood count data [ALC, absolute neutrophil count (ANC), and absolute monocyte count (AMC) at baseline (defined as the most recent blood count within 1 week before treatment initiation) and 2 weeks after treatment initiation], and the incidence and types of irAEs. In the present study, we adopted irAEs as routinely assessed by physicians. Any irAEs that occurred after 6 weeks of nivolumab administration were not counted. In addition, infusion reactions, which can be observed with the use of any monoclonal antibody, were not included as irAEs, according to previous studies (12, 25, 26).

The studies involving human participants were reviewed and approved by the ethics committees of the National Cancer Center Hospital, Keio University Hospital, and Keio University Faculty of Pharmacy, Tokyo, Japan (approval numbers: 2019-199, 20180313, and 200918-2, respectively). The study was conducted in accordance with the Declaration of Helsinki and the Ministry of Education, Culture, Sports, Science, and Technology and Ministry of Health, Labour and Welfare Ethical Guidelines for Medical and Health Research Involving Human Subjects. Japanese law does not require individual informed consent from participants in non-invasive observational trials, such as the present study. Therefore, we used the National Cancer Center Hospital and Keio University Hospital official website to provide an opt-out option rather than acquiring written or verbal informed consent from each participant.

Endpoint

The primary endpoint was the association between peripheral blood count data (at baseline and 2 weeks after treatment initiation) and the early onset of irAEs. Consistently, with the use of potential peripheral blood biomarkers reported in previous studies (20–23), peripheral blood count data were used to calculate the ALC, NLR, and LMR. Changes in the ALC, NLR, and LMR were evaluated by comparing the 2-week values with their respective baseline values. Additionally, decrements or increments in ALC, ANC, and AMC 2 weeks after treatment initiation relative to baseline levels were evaluated. The secondary endpoint was the association of peripheral blood count data (at baseline and 2 weeks after treatment initiation) with skin reactions and diarrhea, the most frequently observed irAEs.

Statistical Analyses

Receiver operating characteristic curve analyses and Youden's index (27) were used to determine the optimal cut-off values of the abovementioned potential peripheral blood biomarkers to predict the early onset of irAEs. The maximum Youden's index was calculated as sensitivity – (1 – specificity). Subsequently, positive predictive values and negative predictive values were also calculated. Multivariable logistic regression analyses were performed to assess the association between peripheral blood biomarkers and the early onset of irAEs. Potential explanatory variables concerning the patient's background such as age, Eastern Cooperative Oncology Group performance status (2 *vs.* 0–1), and treatment line (later- *vs.* second-line treatment) were included as independent variables in the multivariable models. Shah et al. (7) reported an association between age and irAEs. Other explanatory variables were determined through clinical judgment. All statistical analyses were performed using SAS (version 9.4) and JMP (version 15.0.0; SAS Institute Inc., Cary, NC, USA). A two-sided P-value <0.05 was considered statistically significant.

RESULTS

Patient Characteristics

Of the 348 patients initially identified, 177 were excluded. Thus, 171 patients were included in the analysis. The patient inclusion flowchart is shown in **Figure 1**. Baseline patient characteristics are listed in **Table 1**. The median age of the patients was 64 [interquartile range (IQR): 56–69] years. In total, 102 (59.6%), 31 (18.1%), and 38 (22.2%) patients underwent nivolumab monotherapy as second-line, third-, and ≥fourth-line treatment, respectively. PD-L1 expression was not measured because it was not mandatory in the second- or later-line setting.

Endpoints

As shown in **Table 2**, 73 (42.7%) patients had early onset irAEs. Skin reactions and diarrhea were observed in 44 (25.7%) and 20 (11.7%) patients, respectively. The median time to irAEs from the initiation of nivolumab was 15 (IQR: 13–28) days. The median values for ALC, ANC, and AMC at baseline were 1,192 cells/mm^3 (IQR: 860–1,612 cells/mm^3), 4,688 cells/mm^3 (IQR: 3,463–5,841 cells/mm^3), and 449 cells/mm^3 (IQR: 350–616 cells/mm^3), respectively. The median values for ALC, ANC, and AMC at 2 weeks were 1,303 cells/mm^3 (IQR: 917–1,700 cells/mm^3), 4,590 cells/mm^3 (IQR: 3,468–6,219 cells/mm^3), and 459 cells/mm^3 (IQR: 374–599 cells/mm^3), respectively.

Results of the receiver operating characteristic curve analyses of continuous variables are shown in **Figure 2**. Overall, the ALC,

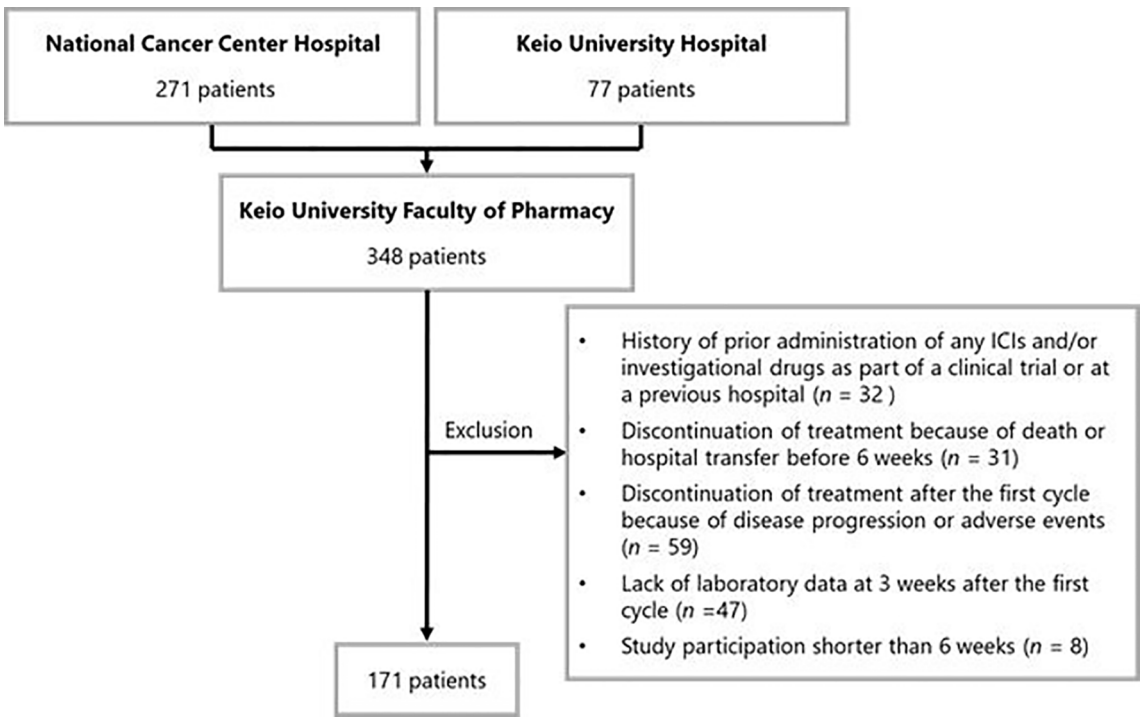

FIGURE 1 | Patient enrollment flowchart. ICI, immune checkpoint inhibitor.

TABLE 1 | Baseline clinicodemographic characteristics.

Characteristic	Patients (*N* = 171)
Age (years), median (IQR)	64 (56–69)
Sex, *N* (%)	
Male	113 (66.1)
Female	58 (33.9)
ECOG PS, *N* (%)	
0	53 (31.0)
1	101 (59.1)
2	17 (9.9)
Treatment line, *N* (%)	
Second	102 (59.6)
Third	31 (18.1)
≥Fourth	38 (22.2)

ECOG PS, Eastern Cooperative Oncology Group performance status; IQR, interquartile range.

TABLE 2 | Immune-related adverse events within 6 weeks of initiating nivolumab treatment.

Event	Patients (*N* = 171)
Any irAE, *N* (%)	73 (42.7)
Skin reaction	44 (25.7)
Diarrhea	20 (11.7)
Thyroiditis/hypothyroidism	15 (8.8)
Liver dysfunction	3 (1.8)
Pneumonitis	2 (1.2)
Encephalitis	1 (0.6)
Myasthenia gravis	1 (0.6)
Venous blood thromboembolism	1 (0.6)

irAE, immune-related adverse event.

NLR, and LMR 2 weeks after treatment initiation showed relatively larger areas under the curve in the receiver operating characteristic analyses of early onset irAEs than the ALC, NLR, and LMR at baseline and changes in ALC, NLR, and LMR from baseline to 2 weeks after treatment initiation. The areas under the curve for the ALC, NLR, and LMR 2 weeks after treatment initiation were 0.572, 0.560, and 0.535, respectively. The optimal cut-off values for the ALC, NLR, and LMR were 820, 4.3, and 2.2, respectively, whereas the Youden's index values were 0.180, 0.151, and 0.141, respectively. The sensitivity, specificity, positive predictive value, and negative predictive value for those cut-offs of ALC were 90.4, 27.6, 66.0, and 27.0%, respectively. The sensitivity, specificity, positive predictive value, and negative predictive value for those cut-offs of NLR were 67.1, 48.0, 49.0, and 47.0%, respectively. The sensitivity, specificity, positive predictive value, and negative predictive value for those cut-offs of LMR were 75.3, 38.8, 55.0, and 38.0%, respectively.

As shown in **Table 3**, multivariable logistic regression analyses revealed that ALC >820 at 2 weeks after treatment initiation was significantly associated with an increased risk of early onset of any irAE [adjusted odds ratio (OR): 3.58, 95% confidence interval (CI): 1.42–9.05; $P = 0.007$]. In contrast, no significant association was observed for NLR (>4.3; adjusted OR: 0.57, 95% CI: 0.30–1.08; $P = 0.083$) or LMR (>2.2; adjusted OR: 1.79, 95% CI: 0.90–3.56; $P = 0.095$) 2 weeks after treatment initiation. In addition, multivariable logistic regression analyses

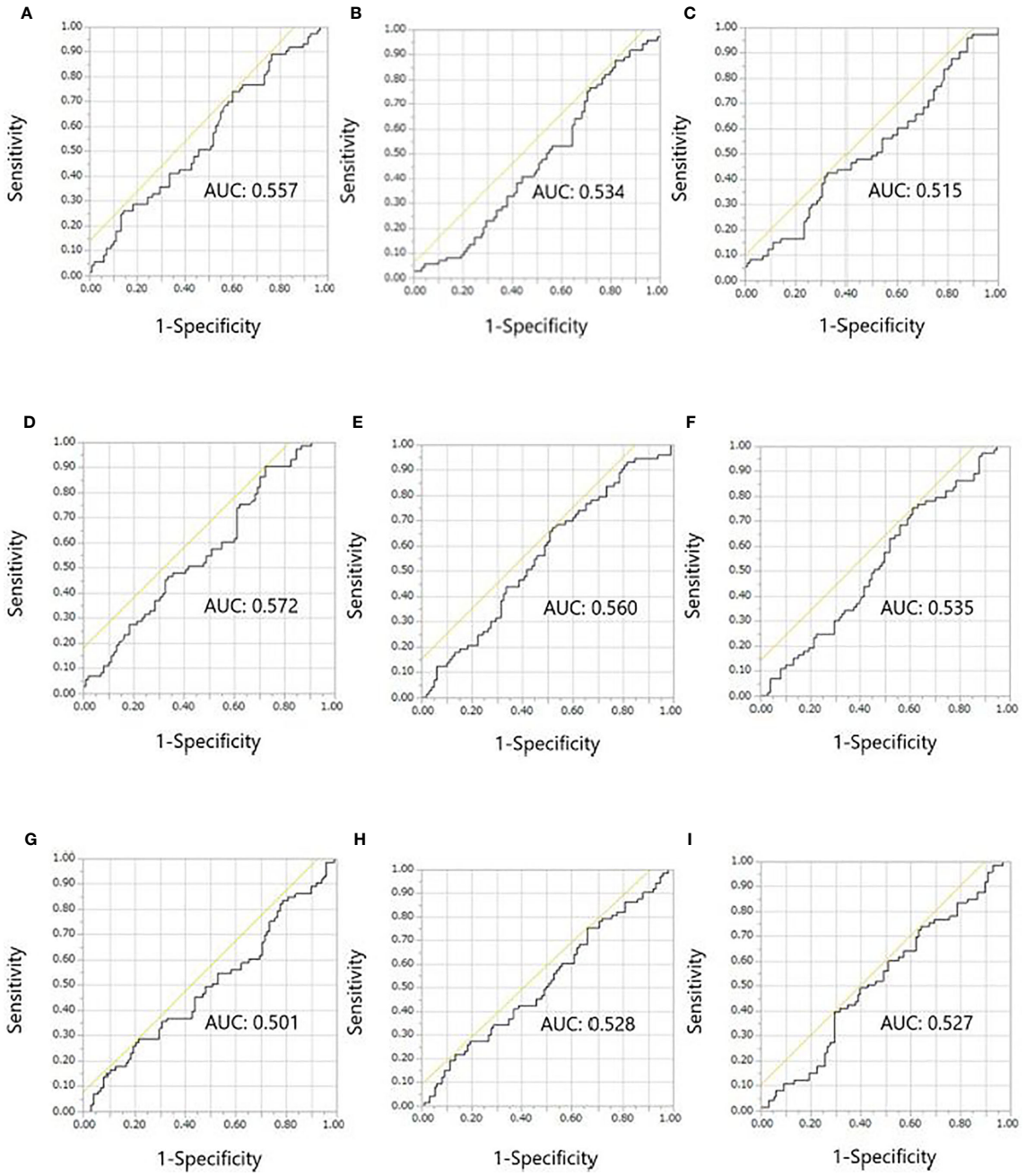

FIGURE 2 | Receiver operating characteristic curves for the early onset of any immune-related adverse event. **(A)** ALC, **(B)** NLR, and **(C)** LMR at baseline. **(D)** ALC, **(E)** NLR, and **(F)** LMR at 2 weeks after treatment initiation. **(G)** ALC, **(H)** NLR, and **(I)** LMR at 2 weeks/baseline. ALC, absolute lymphocyte count; AUC, areas under the curve; LMR, lymphocyte-to-monocyte ratio; NLR, neutrophil-to-lymphocyte ratio.

revealed that there was no significant association between decrements in ALC, ANC, and AMC from 2 weeks after treatment initiation to baseline and early onset of any irAE (adjusted OR: 1.26, 95% CI: 0.68–2.35; $P = 0.460$, adjusted OR: 1.17, 95% CI: 0.63–2.16; $P = 0.612$, and adjusted OR: 1.42, 95% CI: 0.77–2.62; $P = 0.267$, respectively).

In relation to the secondary endpoint, there was no significant association of ALC >820, NLR >4.3, and LMR >2.2 at 2 weeks after treatment initiation with skin reactions and diarrhea (adjusted OR: 2.15, 95% CI: 0.85–5.45; $P = 0.108$, adjusted OR: 0.79, 95% CI: 0.41–1.54; $P = 0.492$, and adjusted OR: 1.47, 95% CI: 0.72–3.03; $P = 0.293$, respectively).

TABLE 3 | Multivariable logistic regression analyses of the early onset of immune-related adverse events.

(A) ALC at 2 weeks after treatment initiation

Variables	Adjusted OR	95% CI	*P*-value
ALC (>820) at 2 weeks	3.58	1.42–9.05	0.007
Age (years)	1.00	0.97–1.03	0.899
ECOG PS (2)	1.07	0.35–3.29	0.908
Treatment line (later-line)	0.74	0.39–1.41	0.359

(B) NLR at 2 weeks after treatment initiation

Variables	Adjusted OR	95% CI	*P*-value
NLR (>4.3) at 2 weeks	0.57	0.30–1.08	0.083
Age (years)	1.00	0.97–1.03	0.956
ECOG PS (2)	0.81	0.28–2.36	0.702
Treatment line (later-line)	0.74	0.39–1.40	0.357

(C) LMR at 2 weeks after treatment initiation

Variables	Adjusted OR	95% CI	*P*-value
LMR (>2.2) at 2 weeks	1.79	0.90–3.56	0.095
Age (years)	1.00	0.97–1.03	0.989
ECOG PS (2)	0.84	0.29–2.45	0.746
Treatment line (later-line)	0.70	0.37–1.31	0.262

The multivariable logistic regression models were adjusted for age, ECOG PS (2 vs. 0–1), and treatment line (later- vs. second-line treatment).
ALC, absolute lymphocyte count; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; LMR, lymphocyte-to-monocyte ratio; NLR, neutrophil-to-lymphocyte ratio; OR, odds ratio.

DISCUSSION

The peripheral blood biomarkers predictive of irAEs have been inconsistent in previous studies (8, 9) and thus, remain controversial. The present study showed that ALC >820 at 2 weeks after treatment initiation was significantly associated with an increased risk of early onset of any irAE in patients with advanced NSCLC who received nivolumab monotherapy in the second- or later-line setting. This confirms our hypothesis that peripheral blood count data can predict nivolumab-induced irAEs in patients with advanced NSCLC. To the best of our knowledge, this is the first multicenter study to investigate whether routinely available ALC can predict nivolumab-induced irAEs using a landmark analysis.

Previous studies (20–23) have focused on the association between peripheral blood count data and ICI treatment efficacy. Additionally, there have been multiple studies already published that examine the role of peripheral blood count data and the association with the development of irAEs (8, 9, 22, 28, 29). However, most of them have combined several ICI treatments such as niolumab, pembrolizumab, and atezolizumab. This could have bolstered their total numbers of patients. Nivolumab was a first in class as a second-line treatment in advanced NSCLC. Moreover, there is a difference between the treatment intervals of nivolumab (every 2 weeks) and pembrolizumab (every 3 weeks). The present study evaluated two points, at baseline and after treatment. Thus, we focused on nivolumab alone. Diehl et al. (8) reported that in patients with solid tumors who were treated with nivolumab (N = 125) or pembrolizumab (N = 42) with or without ipilimumab, ALC >2,000 at baseline or 4 weeks after treatment initiation was significantly associated with the development of grade ≥2 irAEs. Pavan et al. (9) also reported that in patients with advanced NSCLC who were treated with nivolumab (N = 145), pembrolizumab (N = 32), or atezolizumab (N = 7), a baseline NLR <3 and platelet-to-lymphocyte ratio <180 were significantly associated with the development of irAEs. Furthermore, our co-author previously investigated the association of pre- or post-treatment LMR and NLR values (2 and 4 weeks after nivolumab treatment) with the treatment efficacy of nivolumab and the early onset of irAEs (defined as the presentation of irAEs within 4 weeks of treatment initiation) (22). The results showed that these peripheral blood biomarkers at baseline and 2 weeks after treatment initiation were not associated with the early onset of irAEs. However, those studies did not use landmark analysis. To account for the presence of lead-time bias associated with the time-dependent development of irAEs, we conducted a 6-week analysis in accordance with previous studies (10, 11). Other reasons for choosing a 6-week period included the occurrence rate of irAEs and the timing of computed tomography. The occurrence rate of irAEs is approximately 50% within 6 weeks of initiating ICI therapy (10–14, 21). In terms of imaging, the follow-up period for patients with advanced NSCLC in Japan is generally between 6 and 8 weeks following treatment initiation (10).

The findings of the current study reveal that the ALC 2 weeks after treatment initiation may predict the early onset of irAEs in patients with advanced NSCLC and is consistent with the results of a previous study (8). In contrast, there was a discrepancy between our findings and the predictive capacity of NLR and LMR reported in some previous studies (9, 22). The reason for this discrepancy remains unclear. However, in our view, ALC is a key parameter. The interaction between anti-PD-1 and PD-L1 prevents the activation and proliferation of T cells. Inhibition of PD-L1 binding with anti-PD-1 induces T cell activation in the priming phase and increases the number of cytotoxic T cells (30). The increase in activity or number of activated T cells may result in an increased frequency of irAEs. The results of this study were consistent with our hypothesis. These findings suggest that peripheral blood biomarkers, which are evaluated after the initiation of nivolumab treatment may be useful for identifying patients at risk of early irAE onset. Importantly, the early detection of irAEs can help in reducing the negative effect on the patient's quality of life. Our results of no association between ALC >820 and the development of skin reactions and diarrhea were underpowered (P = 0.108) to detect statistical significance. The number of events of skin reactions and diarrhea was 64.

The processes underlying the presentation of irAEs have not been completely clarified. Early studies (31–35) suggest several potential mechanisms, ranging from shared antigens between the tumor and the affected tissue to preexisting autoantibodies and microbiome. In this study, we primarily focused on the association of an increase in ALC and associated changes in blood count ratios with any irAE according to the potential mechanisms. These biomarkers, once validated, are easily

available and do not require additional costs or setup for use in the clinical setting.

In the present study, irAEs and skin reactions were observed in 42.7 and 25.7% of patients, respectively. These incidence rates are comparable with those previously reported in the Japanese population (10–14, 19, 20, 22, 23). Overall, the incidence rate of irAEs in this study was relatively higher than that reported in other countries, and the majority of irAEs were skin reactions.

This study has some limitations. First, it was a retrospective, observational study, rather than a prospective study, and its retrospective nature does not allow formulating valid conclusions but only aids in generating a hypothesis that would require prospective validation. Patient follow-up was at the clinicians' discretion. Thus, the possibility of information bias cannot be excluded. However, we performed multivariable analyses to reduce the effect of potential confounding factors that may be associated with observational studies and clinical differences in patient characteristics. Nevertheless, unmeasured confounders cannot be controlled during multivariate analyses because controlling these could affect the results. Moreover, we adopted irAEs, as routinely assessed by physicians. Research members retrieved the data from the electronic medical records at two hospitals. Therefore, we could not fully assess the grade of irAEs using the Common Terminology Criteria for Adverse Events (version 4.0). However, timely detection of irAEs could contribute to the proper clinical management by optimizing the treatment benefit for patients undergoing nivolumab monotherapy. Second, the sample size was relatively small despite the multicenter design. We did not conduct an additional analysis of results including only second-line treatment because the present study focused on the early onset of irAEs and not the treatment efficacy of nivolumab. Third, other types of irAEs, except those affecting the skin, rarely occurred, thereby limiting our evaluation of the types of irAEs most strongly associated with pre- or post-treatment peripheral blood biomarkers. Fourth, the complexity of the pathophysiology of irAEs is not fully understood and difficult to assess retrospectively. In a preclinical setting, specific subpopulations of lymphocytes such as CD8- and CD4-positive T lymphocytes may be associated with irAE onset (36, 37). Thus, flow cytometry analysis should be considered. Prospective efforts based on stronger scientific rationale are needed to advance in this critical field.

In conclusion, this multicenter study demonstrates that among the peripheral blood biomarkers, ALC >820 at 2 weeks after treatment initiation is significantly associated with nivolumab-induced irAEs in patients with advanced NSCLC. Clinicians should consider using ALC 2 weeks after treatment initiation for the risk stratification of patients within a 6-week study period. These findings suggest that considering the peripheral blood count data after the initiation of nivolumab monotherapy may be useful for predicting the early onset of irAEs in clinical practice. Early detection and cautious management of irAEs can optimize the treatment benefit for patients who are undergoing nivolumab monotherapy. Our data provide preliminary evidence of an association between peripheral blood biomarkers and the early onset of irAEs in Japanese patients with advanced NSCLC. These findings are likely generalizable to other Asian populations, highlighting the need for additional research in this field.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation. The datasets presented in this article are not readily available because of privacy and ethical restrictions.

ETHICS STATEMENT

The studies were reviewed and approved by the ethics committees of the National Cancer Center Hospital, Keio University Hospital, and Keio University Faculty of Pharmacy, Tokyo, Japan (approval numbers: 2019-199, 20180313, and 200403-2). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SE and HK: conception and design. SE, HK, HH, and HN: acquisition of data (acquired the data and managed patients). SE, HK, and RU: analysis and interpretation of the data. SE and HK: writing, review, and/or revision of the manuscript. TN: designed and supervised the study. All authors contributed to manuscript revision and read and approved the submitted version.

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Nivolumab in Non-Small Cell Lung Cancer: Real World Long-Term Survival Results and Blood-Based Efficacy Biomarkers

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Objectives: We aimed to examine clinical data and baseline blood test results as potential predictive biomarkers for benefit from nivolumab, in advanced non-small cell lung cancer patients (NSCLC).

Materials and Methods: A chart review was performed of 108 advanced NSCLC patients who commenced treatment with nivolumab between 2015-6 at three Israeli cancer centers, and for whom laboratory tests results were available. Data collected included sex, age, ECOG-PS, histology and number of previous lines of treatment. Baseline blood test results collected: absolute lymphocyte and neutrophil count (ANC), white blood cells (WBC), hemoglobin, platelets, albumin and lactate dehydrogenase (LDH). Neutrophil to Lymphocyte Ratio and 'derived NLR' (dNLR = (ANC/[WBC-ANC])) were calculated. Disease control at six months (DC6) was defined as any tumor shrinkage or stable disease during the first six months of nivolumab treatment. The association between clinical/laboratory variables and survival was tested with a Cox proportional hazard model. Data cut-off occurred in November 2019.

Results: 35 patients (32.4%) achieved DC6. Median overall survival (OS) of entire study population was 5.4 months. Four year survival rate was 16%. Achievement of DC6 strongly correlated with longer OS (HR 0.12, 95% C.I. 0.07-0.21, p<0.001). In univariate and multivariate analysis, dNLR, albumin and LDH correlated significantly with OS. No variables correlated significantly with DC6 in multivariate analysis. Based on albumin and LDH, we produced a score called CLAS (combined LDH and albumin score), including four prognostic groups of patients. Patients having low albumin and high LDH had the worst prognosis.

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Conclusion: In real-life setting, long-term efficacy of nivolumab in advanced line treatment of NSCLC is consistent with clinical trials. Response or stability of disease during first six months of treatment is associated with prolonged survival. We propose a novel score (CLAS) that may be useful for predicting outcome in nivolumab-treated NSCLC patients, but further validation is required.

Keywords: nivolumab, NSCLC, long term survival, real life data, biomarker

INTRODUCTION

Lung cancer is the leading cause of cancer -related death worldwide (1). Immune checkpoint inhibitors (ICI) for treatment of NSCLCdemonstrated superior survival of patients treated with the anti-PD-1 antibody nivolumab compared with docetaxel after failure of platinum-based chemotherapy (2–4). Pembrolizumab, another PD-1 inhibitor, and atezolizumab, an anti-PD-L1 inhibitor, have both also demonstrated significantly better overall survival (OS) in similarly designed trials, comparing each of them with 2nd-line docetaxel (5, 6).

Based on these trials, in 2015 nivolumab has received US Food and Drug Administration agency (FDA) and European Medicines Agency (EMA) approval for second-line treatment of NSCLC. Atezolizumab is similarly approved by FDA and EMA, while pembrolizumab is approved only for the treatment of patients with PD-L1 positive tumors (as included in the KEYNOTE 010 trial) (5). Response rate (RR) for each of these drugs in the 2nd line setting is roughly 15-20%. Combined updated OS results from checkmate 017 and checkmate 057 show 13.4% OS rate at five years follow-up (3). In a landmark analysis of OS by response category at six months in checkmate 017 and checkmate 057, the OS rate at four years in nivolumab treated patients with CR/PR was 58%, and 19% for patients with SD at six months (3).

Efforts are being undertaken to identify biomarkers predicting response to immunotherapeutic agents (7). PD-L1 expression level and tumor mutational burden (TMB) are predictive for benefit from immunotherapy but are not always available and their predictive accuracy is limited (7–11). Additional molecular biomarkers are being investigated, such as STK11/LKB1 and KRAS (12–15). STK11/LKB1 genomic alterations were associated with shorter PFS and shorter OS in first line ICI treated NSCLC patients (12). On the other hand, several studies showed similar efficacy of ICI in KRAS mutant compared with KRAS wild type NSCLC patients (14, 15).

Numerous studies are onging, aiming to identify immune-related gene signatures correlating with clinical benefit from ICI. One of these assessed the utility of the 18-gene expression tumor inflammation signature in predicting ICI treatment outcome, and found it to predict clinical benefit of ICI in several tumors, including NSCLC (16). Another study reported predictability of selected gene signatures and genes for discriminating patients with durable clinical benefit from ICI from those with non durable benefit. These signatures and genes included the M1 signature, peripheral T cell signature, CD137 and PSMB9 mRNA expression (17). An additional study identified two signatures predicting outcome from ICI in chemotherapy-refractory advanced NSCLC patients, one reflecting the degree of immune infiltration and upregulation of interferon-gamma-induced genes, and a second reflected the epithelial-to-mesenchymal transition status (18).

Potential predictive biomarkers from the tumor microenvironment are being investigated in the PIONeeR study. The analysis for its first 100 patients, presented recently, suggested a predictive value for PDL1 positive cell density and density of cytotoxic T cells and immunosuppressive cells (regulatory T cells and myeloid-derived suppressor cells) in the tumor (19).

Levels of peripheral blood components, blood cells and various circulating molecules are an accessible potential non-invasive source of predictive biomarkers in NSCLC patients, and possibly relevant also for ICI-treated patients. Some of these biomarkers are validated prognostic markers for cancer in various scenarios. For example, high levels of serum lactate dehydrogenase (LDH) has been associated with poorer cancer-specific survival in several malignancies, including lung, colorectal and prostate cancer (20). Another example is albumin serum levels, known to be a robust predictor of survival in cancer patients in general and NSCLC patients in particular (21, 22). Interestingly, retrospective studies have demonstrated a correlation between high serum LDH levels at baseline and lower response rate (RR), and shorter (PFS) and OS in several types of cancer treated with anti-PD-1 and anti-PD-L1 antibodies (23–25). Hemoglobin levels can also serve as prognostic or potentially a predictive biomarker; several studies have demonstrated that anemia is linked to poorer prognosis in NSCLC patients (26, 27).

A number of studies investigated the peripheral blood cellular components as biomarkers of ICI efficacy. For ipilimumab-treated melanoma patients, improved OS and PFS were associated with low absolute neutrophil count, low neutrophil-to-lymphocyte ratio and high lymphocyte levels (28, 29). In a retrospective study including 607 pembrolizumab-treated melanoma patients, baseline elevated eosinophil count and elevated lymphocyte count were both associated with improved OS (30). Retrospective studies demonstrated elevated pre-treatment neutrophil-to-lymphocyte ratio (NLR) to be associated with shorter OS and PFS and with lower response rates in ICI-treated metastatic NSCLC patients (31–33). High NLR has been shown to be associated with decreased OS in a retrospective study analyzing the records of metastatic NSCLC patients enrolled on a number of phase I immunotherapy trials at the MD Anderson Cancer Center (32). However, NLR may not

be relevant for all patients; a study demonstrated that in NSCLC, low NLR was correlated with favorable OS and PFS for patients with TMB > 10, while in patients with TMB ≤ 10, the differences between high and low NLR were not significant (33).

Conceivably, combining such biomarkers may derive a more accurate predictive or prognostic score. A group from Gustave Roussy analyzed data from a retrospective study of ICI *versus* chemotherapy for NSCLC patients and compiled a Lung Immune Prognostic Index (LIPI) (34). In this study, LIPI was correlated with worse outcomes for immunotherapy but not for chemotherapy. Other studies examining the applicability of LIPI in NSCLC and in solid tumors other than NSCLC reported results supporting its correlation with outcomes in ICI-treated patients (35, 36). In contrast, an exploratory pooled analysis of clinical studies of immunotherapy and targeted therapies for advanced NSCLC patients has shown LIPI to be associated with OS and PFS irrespective of treatment, emphasizing its prognostic rather than predictive role (37).

We report here real-world long-term survival results of NSCLC patients treated with nivolumab as a second or later treatment line in three Oncology centers, with a median follow-up of four years. We have comprehensively assessed clinical data and baseline blood levels of LDH, albumin and complete blood count results, as potential predictive biomarkers for benefit from nivolumab.

MATERIALS AND METHODS

Study Conduct

This is a retrospective pharmacoepidemiological study, conducted, analyzed and reported according to REporting of studies Conducted using Observational Routinely collected health Data (RECORD) guidelines, the Strengthening the Reporting of OBservational studies in Epidemiology (STROBE), and ROCORD-pharmacoepidemiological research (RECORD-PE) guidelines (38–40). A chart review was performed of patients with advanced NSCLC that fit the study inclusion criteria.

Patients

Inclusion criteria were age of 18 years and above, progression on first-line platinum-based chemotherapy, treatment at one of three participating Israeli cancer centers (Sheba Medical center (MC), Rabin MC and Shamir MC), administration of at least one cycle of nivolumab, with treatment commencing during 2015-2016. Exclusion criteria was lack of blood test results from the relevant time window (**Figure S1, Supplementary Data**).

Data Collection

Data collected included sex, age, Eastern Cooperative Oncology Group performance status (ECOG-PS), histology and number of previous lines of treatment. Baseline blood test results collected included: absolute lymphocyte count (ALC), absolute neutrophil count (ANC), white blood cells (WBC), hemoglobin (HGB), platelets (PLT), albumin (ALB) and LDH. Baseline blood tests were defined as those performed prior to and within two weeks of the first nivolumab treatment. These parameters were categorized as high or low relative to the median of the study cohort, except for LDH, categorized as normal or above the upper limit of normal for the relevant clinical laboratory and albumin, categorized as normal or below the lower limit of normal for the relevant clinical laboratory. LIPI calculation is based on LDH greater than upper limit of normal (ULN) and 'derived NLR' (dNLR, calculated by: (ANC/[WBC-ANC])) > 3. Three risk groups were characterized: good: none of these factors, intermediate: one factor, poor: two factors. NLR, dNLR and LIPI score were calculated. In addition, CLAS ("Combined LDH and Albumin Score") was defined as a suggested novel score and calculated as described in the results section.

Tumor assessments were performed by the treating physicians based on computerized tomography (CT) scans performed as part of the standard of care. CT scans were usually performed at two-three months intervals, at the treating physicians' discretion. Two major outcomes have been assessed in our study: Disease control at six months (DC6) and OS. DC6 was defined as either present (any tumor shrinkage at any time during the first six months after initiating nivolumab treatment, or no change in tumors' size for at least six months after starting nivolumab) or absent (any tumor growth or death at any time within the first six months).

OS was defined as time from initiation of nivolumab treatment until death, or censored at the last date patient was known to be alive.

Statistics

Variables analyzed as categorial included sex and histology. In addition, LDH was redefined as a binary variable based upon whether the values being above or below the upper-limit of normal, and ALB was redefined as a binary variable based upon whether the values were above or below the lower-limit of normal. Variables analyzed as continuous included age, HGB, WBC, ANC, ALC and PLT. Variables analyzed as ordinal were ECOG-PS and number of previous lines.

The impact of covariates on survival was assessed using a Cox proportional hazard model. Multivariate analysis incorporated putative covariates found at univariate analysis to be significant at $p < 0.1$. Statistical comparisons were performed using Chi-squared test for categorical data and t-test for continuous variables. All p-values were two-sided, and $p < 0.05$ was considered statistically significant. The goodness-of-fit of different models were compared by examining pseudo-R^2 values. Calculations were performed using Stata (version I/C 16.0, StataCorp). Data cut-off was at November 2019.

Ethics

The study was approved by the institutional ethics committees of each of the participating centers. (Shamir Medical Center (MC): IRB #8993-11; Rabin Medical Center (MC): IRB #0391-14; Shamir MC: IRB #0062-17).

Investigators had complete access to the database of population included in the study. No patient identifying information was included in the study database.

RESULTS

Patient Characteristics

A total of 108 patients treated with nivolumab were included in this study, mostly men (65%). Out of the study cohort, 35 patients (32.4%) had achieved DC6. More patients in the non-DC6 group had ECOG-PS of two or higher compared with DC group (p=0.030). The two groups were balanced in terms of other parameters (**Table 1**). Median follow-up was 48.7 months (IQR 47.4m – 53.0m).

Survival and DC6 Analysis

The median OS of the entire study population was 5.4 months (95% CI 3.9-7.4, **Figure 1**).

Survival rates after one, two, three and four years were as follows: 34%, 27%, 19% and 16%, respectively.

Of patients that have achieved DC6, 46% were alive at data cut-off, compared to 1% of patients that did not achieve DC6. In addition, Patients with DC6 demonstrated longer OS compared to patients without DC6 (HR 0.12, 95% confidence interval (CI) 0.07-0.21, p<0.001; **Figure 2**).

Biomarkers

We next tested the effects of the variables collected (demographic, hematological and biochemical) on OS. On univariate analysis, ECOG-PS and baseline values of WBC, ANC, NLR, dNLR, LDH and albumin were significantly correlated with OS. Due to overlap with dNLR, the variables WBC, ANC and NLR were not included in the multivariate analyses. On multivariate analysis, dNLR, albumin and LDH significantly correlated with OS (**Table 2** and **Figures 3A, B**), with high dNLR, low albumin and high LDH being adverse factors correlating with shorter survival. The statistical value of albumin and LDH was more significant than that of dNLR.

Regarding the binomial outcome DC6; age, ECOG-PS and baseline values of WBC, ANC, NLR, dNLR and albumin were all correlated with DC6 on univariate analysis. However, none of the investigated factors were found to be significant on multivariate analysis (**Table S1, Supplementary Data**).

We aimed to validate the prognostic role of LIPI in our cohort. Indeed, in our cohort LIPI was independently associated with OS (HR 1.8, 95% CI, 1.35-2.49, p < 0.001), leading to median OS for poor, intermediate and good LIPI of 2.1 months, 6.4 months, and 9.8 months, respectively (**Figure 4**). Therefore, our data provide further validation of the prognostic value of LIPI for nivolumab-treated advanced NSCLC patients.

Of the parameters examined in our study, baseline LDH and albumin levels were found to be most significantly correlated with survival. We attempted to produce a predictive score by combining these two variables, naming it 'CLAS'. CLAS includes four prognostic groups of patients: high LDH + low albumin (CLAS 0), low LDH + low albumin (CLAS 1), high LDH + high albumin (CLAS 2), low LDH + high albumin (CLAS 3). Indeed, patients classified as CLAS 0 had the worst survival (p<0.001), while patients with CLAS 2 had better survival than patients with CLAS 1, suggesting that low albumin is worse than high LDH (**Figure 3C**).

DISCUSSION

Based on long-term (four years) follow up of our cohort, we report now real-world data of survival of a set of NSCLC patients treated with 2nd-line or higher ICI. In our study, the median OS of nivolumab-treated patients compares unfavorably with the

TABLE 1 | Baseline patient and disease characteristics.

Baseline patient/disease characteristics	All study cohort (108)	DC6 (35)	Non-DC6 (73)	P Value
Age – median (range), years	68 (40-96)	66 (40-85)	69 (43-96)	0.090
Sex – men – n (%)	70 (65%)	23 (66%)	47 (64%)	0.890
ECOG-PS – n (%)				0.030
0	4 (4%)	3 (9%)	1 (1%)	
1	46 (42%)	17 (48%)	29 (40%)	
2	40 (37%)	11 (31%)	29 (40%)	
3	17 (16%)	3 (9%)	14 (19%)	
Unknown	1 (1%)	1 (3%)	0 (0%)	
Histology – n (%)				0.220
Adenocarcinoma	64 (59%)	22 (63%)	42 (57%)	
Squamous cell carcinoma	29 (27%)	10 (28%)	19 (26%)	
NSCLC other*	15 (14%)	3 (9%)	12 (17%)	
No. of previous anticancer treatment lines$ - n (%)				0.440
0	12 (11%)	0 (0%)	12 (17%)	
1	79 (73%)	31 (88%)	48 (66%)	
2	10 (9%)	1 (3%)	9 (12%)	
≥3	7 (7%)	3 (9%)	4 (5%)	

*DC6, Disease Control 6; ECOG-PS, Eastern Cooperative Oncology Group performance status; NSCLC, Non Small Cell Lung Carcinoma. *Other histologies included: large cell neuroendocrine (6), large cell undifferentiated (3), adenosquamous (1), NSCLC non-otherwise-specified (NOS; 5). $ Treatment lines for advanced disease prior to nivolumab treatment.*

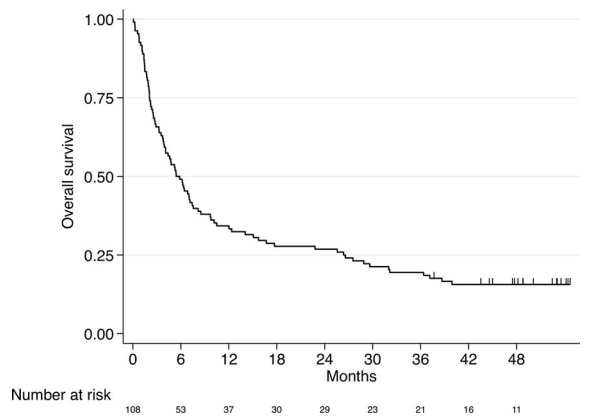

FIGURE 1 | Kaplan–Meier curve for overall survival (entire study cohort, n=108). Median OS 5.4 months (95% CI 3.9-7.4).

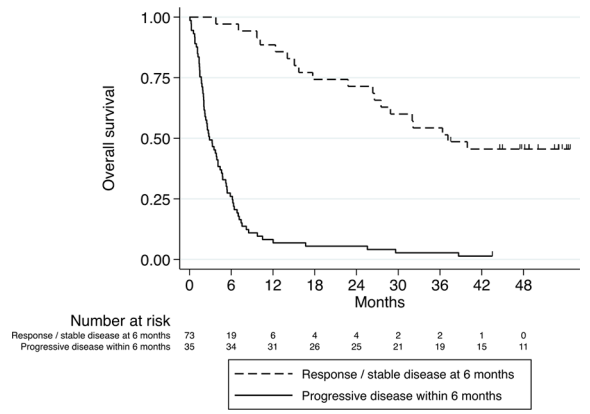

FIGURE 2 | Kaplan–Meier curve for overall survival stratified according to DC6 (n=35) *vs.* non-DC6 (n=73) patients. HR 0.12, 95% confidence interval (CI) 0.07-0.21, p<0.001.

TABLE 2 | Univariate and multivariate analysis for OS of the parameters investigated.

	Univariate Analysis		Multivariate Analysis	
	HR (95% C.I.)	**P value**	**HR (95% C.I.)**	**P value**
Age	1.02 (0.99 - 1.04)	0.148		
Sex	1.18 (0.76 - 1.83)	0.455		
ECOG-PS	**1.46 (1.12 - 1.89)**	**0.005**	1.31 (0.91 - 1.89)	0.141
Histology				
Adenocarcinoma	Comparator			
Squamous	1.02 (0.63 - 1.65)	0.933		
Other	1.47 (0.81 - 2.65)	0.207		
HGB	0.94 (0.83 - 1.05)	0.270		
WBC	**1.12 (1.06 - 1.18)**	**<0.001**		
ANC	**1.15 (1.09 - 1.22)**	**<0.001**		
ALC	0.97 (0.73 - 1.30)	0.860		
PLT	1.00 (1.00 - 1.00)	0.119		
LDH	**1.15 (1.06 - 1.25)**	**0.001**	**1.12 (1.03 - 1.22)**	**0.006**
ALB	**0.33 (0.20 - 0.52)**	**<0.001**	**0.36 (0.20 - 0.63)**	**<0.001**
NLR	**1.05 (1.02 - 1.07)**	**<0.001**		
dNLR	**1.18 (1.10 - 1.27)**	**<0.001**	**1.12 (1.01 - 1.24)**	**0.032**
Number of Previous Lines	0.85 (0.63 - 1.13)	0.260		

Statistically significant values are highlighted as bold. Due to overlap with dNLR, the variables WBC, ANC and NLR were not included in the multivariate analysis. HR, Hazard Ratio; C.I., Confidence Interval; ECOG-PS, Performance Status; HGB, Hemoglobin; WBC, White Blood Cells; ANC, Absolute Neutrophil Count; ALC, Absolute Lymphocyte Count; PLT, Platelets; LDH, Lactate Dehydrogenase; ALB, Albumin; NLR, Neutrophil to Lymphocyte Ratio; dNLR, derived Neutrophil to Lymphocyte Ratio. HR for LDH reflects LDH as calculated for each 100 international units per liter (IU/L). Number of patients with missing data: ECOG-PS: 1, HGB: 2, WBC: 3, ANC: 3, ALC: 4, PLT: 3, LDH: 28 and ALB: 26.

median OS of 9.2 months and 12.2 months for patients with squamous and non-squamous NSCLC in CheckMate 017 and CheckMate 057 trials, respectively (2–4), as may be expected when comparing real-world data to clinical trial data. DC6 was found to be a strong predictor of OS in our cohort, consistent with published data analysis from these two checkmate trials (3). We also report baseline dNLR, ALB and LDH to be significantly and strongly associated with OS for these patients, with none of the examined variables found to be associated with DC6 on multivariate analysis. LIPI score was significantly correlated with survival as well, with poor LIPI group of patients having the worst outcomes, consistent with previous publications. Based on our data, we have produced a score we named CLAS combining ALB and LDH, which demonstrated strong correlation with survival, with low baseline albumin and high baseline LDH correlating with worst outcome. CLAS requires further validation in larger cohorts to clarify its contribution to the management of advanced NSCLC patients.

The relatively shorter median OS in our study may be attributed to the inclusion of patients with ECOG-PS 2/3 that constituted 52% (57 patients) of our cohort, while the randomized studies included only ECOG-PS 0/1 patients. Furthermore, 16% of the patients in our study were heavily pretreated, receiving nivolumab as a later than 2nd treatment line. It should be noted that despite these poor prognostic characteristics, the long-term survival curve looks similar, with 16% long term survivors at data cut-off, consistent with data from the previously mentioned checkmate trials (2–4).

The two parameters outstanding from our data, which were included in the suggested 'CLAS' reflect tumor burden (LDH) and the patients' nutritional status (ALB). The three variables that make up CLAS and LIPI score (LDH, albumin,

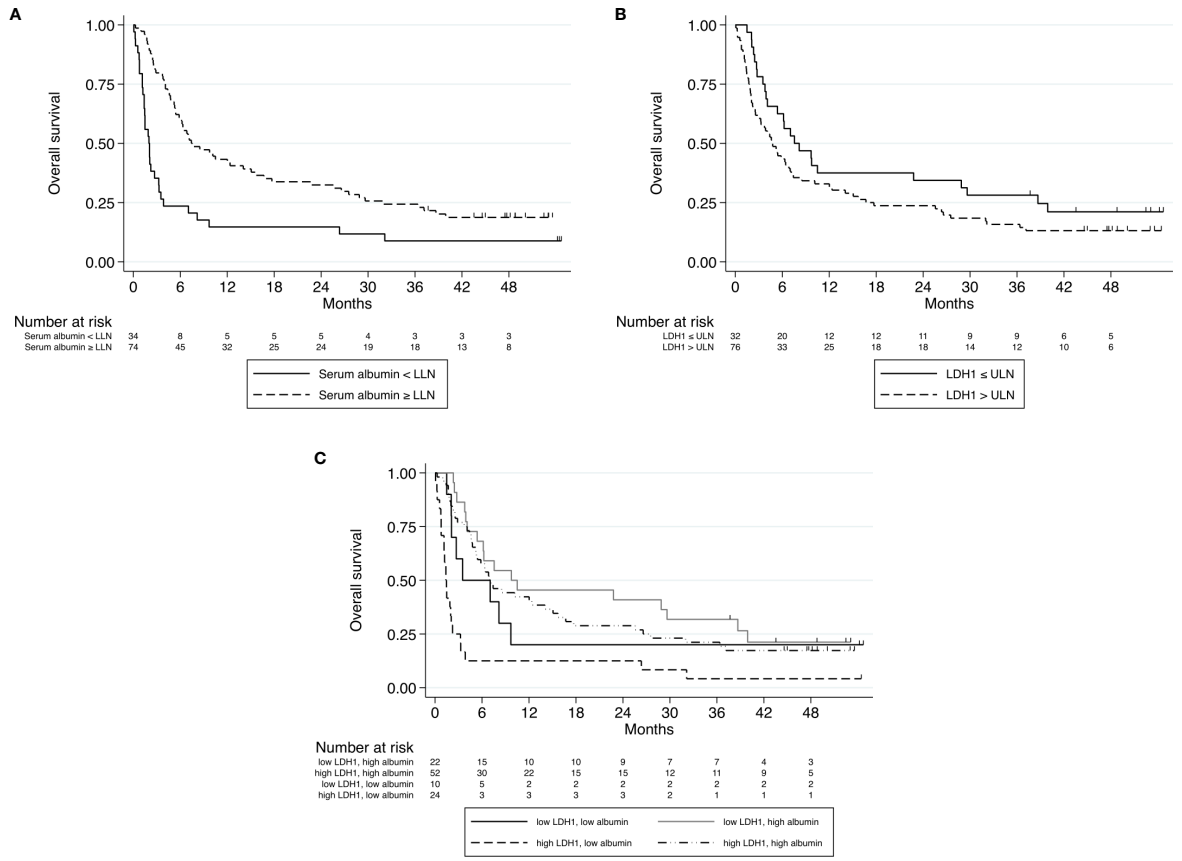

FIGURE 3 | **(A)** Kaplan–Meier curve for overall survival stratified according to Albumin level at baseline (normal or below the lower limit of normal, n=108). **(B)** Kaplan–Meier curve for overall survival stratified according to LDH level at baseline (above upper limit of normal\below upper limit of normal, n=108). **(C)** Kaplan–Meier curves for overall survival stratified according to combined Albumin and LDH levels at baseline. **(A)** HR 0.33, 95% CI (0.20 - 0.52), p<0.001 **(B)** HR 1.15, 95% CI (1.06 - 1.25), p=0.001 **(C)** P<0.001.

dLNR) surprisingly performed better than ECOG-PS as predictors of OS. Despite the recognized validity of ECOG-PS as a strong prognostic factor, the inter-observer variability of this parameter may limit its usefulness in some cases (41). An important question relates to the predictive value of these parameters regarding chemotherapy treatment. Clinical and laboratory factors found to be prognostic in IO-treated NSCLC patients should be examined in parallel in large cohorts of chemotherapy-treated NSCLC patients, all treated with a similar type of chemotherapy. Such analyses would allow the assessment of the role of these factors as potentially predictive for outcome with IO treatments *versus* being general prognostic biomarkers.

A limitation of the study is its retrospective nature, with recognized drawbacks in terms of data accuracy and non-regular follow-up intervals. Furthermore, radiological response to treatment was based on investigators' assessment and not on RECIST. In addition, the relevance of this cohort can be questioned, as most patients are now treated front-line with ICI (either as monotherapy or combined with chemotherapy). We raise here the possibility CLAS score could be applicable in the first-line setting as well, and examining our score in this setting is justified.

Another limitation is the relatively small size of the study, and the lack of a validation cohort to assess the accuracy of the suggested CLAS classifier. Such a validation cohort with equivalent prolonged follow up will be available in the future. However, our data of correlates of long-term survival allows insight into factors correlating with significant ICI efficacy, potentially with the postulated chance of cure. A larger data set is required for comparing the utility of LIPI and CLAS.

In conclusion, unlike median OS, the real life long-term efficacy of nivolumab in the advanced line setting in NSCLC is similar to data from published randomized trials. In addition, we propose a novel score we named 'CLAS' based on baseline albumin and LDH results as a potentially useful score for predicting outcome in nivolumab-treated NSCLC patients. The simple and unbiased measurement of these values adds to their apparent clinical applicability. This score needs further validation before any practical conclusions can be reached. Furthermore, we suggest categorization of patients on immunotherapy according to disease control after six months of treatment to be a simple and

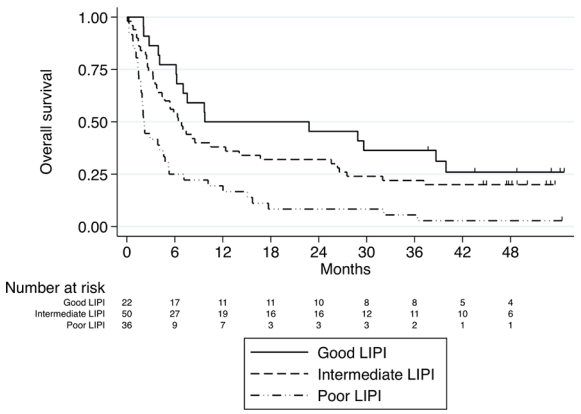

FIGURE 4 | Kaplan–Meier curves for overall survival stratified according to LIPI score. Median OS for poor, intermediate and good LIPI was 2.1 months, 6.4 months, and 9.8 months, respectively, P<0.001.

useful tool for similar studies. DC6 reflects the survival benefit of responding patients as well as of those with prolonged stability, and marks them as having a good chance for durable response and prolonged survival. Thus, this parameter should be further investigated as a surrogate factor for OS in ICI studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was approved by the institutional ethics committees of each of the participating centers. (Sheba MC: IRB #8993-11; Rabin MC: IRB #0391-14; Shamir MC: IRB #0062-17). The studies involving human participants were reviewed and approved by Sheba Medical Center ethics committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SD: Conceptualization, Methodology, Investigation, Writing - original draft. YL: Formal analysis, Data curation, Writing - Review & Editing. ED: Conceptualization, Methodology, Investigation, Writing - Review & Editing. EH: Investigation, Writing - Review & Editing. DU: Conceptualization, Methodology, Writing - Review & Editing. NP: Conceptualization, Methodology, Writing - Review & Editing. RN: Resources, Writing - Review & Editing. RL: Conceptualization, Methodology, Writing - Review & Editing. AH: Resources, Data curation, Writing - Review & Editing. EB: Resources, Data curation, Writing - Review & Editing. TG: Resources, Formal analysis, Writing - Review & Editing. AO: Conceptualization, Methodology, Writing - Review & Editing. JB: Conceptualization, Methodology, Validation, Supervision, Project administration, Writing - Review & Editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.625668/full#supplementary-material

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frontiers in Oncology

REVIEW
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Viral Infection and Lung Cancer Immunotherapy

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Immunotherapy with immune checkpoint inhibitors (mainly anti-PD1 and anti-PDL1 monoclonal antibodies) became a standard of care in non-small cell lung cancer (NSCLC) patients. Most of the clinical trials excluded patients with hepatitis B (HBV), hepatis C (HCV), and human immunodeficiency virus (HIV) active infection (1–10). Despite the progress in treatment of these infections, they remain an unresolved clinical problem when lung cancer immunotherapy should be initiated in an NSCLC patient. This manuscript summarizes the data from the literature concerning this subgroup of patients including the rationale for immunotherapy initiation depending on the HBV, HCV, or HIV infection status; the risk of adverse events; and the efficacy compared to non-infected patients. One of the crucial questions is how the candidates to immunotherapy should be screened for HBV, HCV, and HIV infections. The year 2020 brought the world a new but dynamic viral problem—severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2). The incorporation of known data in oncology guidelines became a burning need, and then, which group of the infected patients can be treated with immunotherapy despite the infection. Oncologists should also know if these patients should receive antiviral therapy and what are the safe combinations in these settings. We also indicate which of the adverse events should be monitored carefully during checkpoint inhibitor treatment.

Keywords: lung cancer, immunotherapy, HBV, HCV, HIV, SARS-Cov-2

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INTRODUCTION

The landscape of advanced non-small cell lung cancer (NSCLC) with no epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) genomic tumor aberrations treatment has evolved substantially during the past few years (1–10). Immunotherapy was a promising potential intervention in lung cancer. The cancer immunoediting phenomenon comprises the following stages: elimination, equilibrium, and escape. In the elimination stage, immunosurveillance proper priming and effector phase of the host is efficient enough to obtain tumor elimination. In the equilibrium phase, the immune system allows the malignancy presence but the host can still control it, avoiding cancer progression. In the escape phase, the immune system passively allows proliferation and tumor growth without active control mechanism (1). Thus, the ideal

therapeutic intervention would transform the immune escape to elimination phase. Therapeutic strategies that allow achievement of equilibrium phase are not curative, but possibly lead to overall survival (OS) improvement despite the lack of cancer elimination. As NSCLC cells are moderately immunogenic, equilibrium is an achievable goal for immune checkpoint inhibitors and allows malignancy control in an important proportion of patients (11).

The incorporation of immune checkpoint inhibitors (ICIs) started when nivolumab showed superiority over docetaxel in second-line treatment in the CA 209-017 and CA 209-057 trials (3, 4). Then, atezolizumab showed OS benefit in second-line treatment over docetaxel in the OAK and POPLAR trials. These trials established ICI as the standard of care in NSCLC patients progressing after platinum-doublet first-line treatment (3–6).

Then, KEYNOTE trials showed that pembrolizumab gives further benefit in first-line treatment in NSCLC patients. First, pembrolizumab in monotherapy showed survival benefit over platinum doublet chemotherapy in treatment-naive patients with programmed death receptor type 1 (PD-L1) expression of at least 50% in the KEYNOTE-024 trial (7). KEYNOTE-407 and KEYNOTE-189 trials demonstrated that the combination of pembrolizumab with platinum doublet chemotherapy led to OS benefit compared to chemotherapy alone in first-line treatment of NSCLC patients with untreated NSCLC, regardless of the PD-L1 status and the histological subtype (8, 9). Thus, treatment-naïve advanced NSCLC patients were treated with pembrolizumab monotherapy if PD-L1 expression was 50% or higher from 2016 or immunochemotherapy in any case from 2018.

The year 2020 brought a new Food and Drug Administration (FDA) registration based on CA 209-227 trial—nivolumab combined with ipilimumab was found superior to chemotherapy alone in an untreated NSCLC patient in whom PD-L1 expression was ≥1%. The most common adverse events (AEs) observed in ≥20% of patients receiving the combination of nivolumab plus ipilimumab, as in other studies using this combination, were the following: (1) general: fatigue, rash, decreased appetite, and musculoskeletal pain; (2) gastrointestinal: diarrhea/colitis, nausea, and hepatitis; (3) respiratory: dyspnea and cough; and (4) dermatological: pruritus (10).

In January 2021, the results of the CheckMate 209-9LA trial were published, showing that in untreated NSCLC patients, nivolumab plus ipilimumab combined with two cycles of platinum-based chemotherapy improve OS versus standard arm. The study demonstrated that addition of ICI therapy shortens chemotherapy duration with no unexpected safety signs (12). During the past months, this trial's results were incorporated into recommendations modifications in this clinical setting.

In the vast majority of trials evaluating ICI in NSCLC patients, the infection with hepatitis B (HBV), hepatis C (HCV), or human immunodeficiency virus (HIV) was an exclusion criterion and none of them included patients with SARS-Cov-2 (3–6, 10, 12). Thus, we cannot drive our clinical decisions on evidence-based medicine as literature does not provide data on scientific evidence of good quality level.

On the other hand, HCV-, HBV-, and HIV-infected patients are at increased risk of developing malignancies, and this population should not be omitted if the most effective treatment option is based on ICI. Precise knowledge of potential risk and specific approach is necessary if such a therapy is to be initiated.

Since 2020, the oncologists have to face the problem of SARS-Cov-2 infection among lung cancer patients. A basic knowledge about clinical presentation, susceptibility, and outcome are substantial to take proper clinical decisions.

This manuscript provides a literature review in these special populations of NSCLC patients treated with ICI. The presented manuscripts are summarized in **Table 1**.

HUMAN IMMUNODEFICIENCY AND HEPATITIS B AND C VIRUS

As previously mentioned, the infection with HIV, HBV, or HCV is one of the most common exclusion criterion for clinical trials incorporating ICI in the treatment plan.

In patients infected with HBV and HCV treated with ICI, the most common concern is potential HBV reactivation and immune hepatitis induced by ICI. Patients infected with HIV are additionally at risk of infectious complications of cancer therapy. Our experience with this patient population started based on case reports, but every year brings new larger evidence in these groups of patients.

In one of the published series, seven patients treated with PD-1 inhibitors nivolumab and pembrolizumab for either metastatic melanoma or metastatic NSCLC with medical history of chronic or past HBV/HCV infection treatment were analyzed retrospectively. One patient showed an increase in alanine transaminase (ALT) grade 2 severity that returned to the normal range after treatment of his HCV infection. In four other patients, ALT elevation grade 1 was noted, with no intervention needed, and in the two remaining patients, no toxicity was seen. Treatment outcome was similar as in the noninfected population. Based on these observations, the authors conclude that patients with NSCLC and metastatic melanoma can be effectively and safely treated with PD-1 inhibitors despite HBV/HCV infection, but a close cooperation with hepatologist is required if eventual antiviral therapy is indicated (13).

In a retrospective analysis from five centers, Shah et al. summarized the treatment course in 50 patients with advanced stage cancer and HBV, HCV, or HIV infection treated with ICI. In the HIV cohort of 21 patients, the rate of immune-related adverse events (irAE) was 24% with 14% if grade ≥3 irAE and no significant changes in viral load and CD4+ T-cell counts. In the HCV/HBV cohort of 34 patients, irAE rate was 44% with 29% grade ≥3. In the HBV/HCV patients with pre- or post-treatment viral load, reactivation was not observed (14).

In 2019, a systematic review aimed to assess the efficacy and safety profile of ICI therapy of advanced cancer patients with

TABLE 1 | Selected manuscripts presenting data concerning ICI therapy in HCV/HBV/HIV infected patients.

Study design	Patient population	Number of patients	Result	First author
Case series	NSCLC and MM with HCV/HBV infection	7	1 patient with ALT G2 elevation improved after anti-HCV treatment initiation, the remaining 6 with no clinically significant hepatic toxicity	Kothapalli A (13)
Retrospective 5 centers analysis	Advanced stage cancers with HCV/HBV/HIV infection	50	HIV–irAE rate 24%, grade ≥3 14%, no changes in viral load or CD4+ count HCV/HBV–irAE rate 44%, grade ≥3 29%—no reactivation observed	Shah NJ (14)
Systematic review	Advanced cancers with HIV infection	73	In 6 out of 70 patients, grade ≥3 irAE occurred In 26 out of 28 patients, viral load remained undetectable CD4+ lymphocytes count increased where data are available Response rates 30% for NSCLC, 27% for MM and 63% for KS	Cook MR (15)
Retrospective study	NSCLC and HCV/HBV infection	19	No hepatic irAE requiring ICI discontinuation Overall response rate—35%	Pertejo-Fernandez A (16)
Retrospective prospective single-institution study	HCC and HBV infection	62	No HBV reactivation in NUC-treated patients In reactivation out of six patients without NUC prophylaxis	Lee PC (17)
Open-label phase 1	Pembrolizumab in HIV infection	33	No unexpected irAE Overall response rate showing feasible efficacy	Uldrick TS (18)
Retrospective cohort study	ICI in HBsAg-positive patients	114	HBV reactivation in 5.3% of patients HBV DNA monitoring and antiviral prophylaxis recommended	Zhang X (19)
Systematic review	Advanced cancer with HBV/HCV	89 HBV 98 HCV		Pu D (18)
Review	HBV reactivation preemptive approach in ICI-treated patients		HBV infection screening in all patients initiating ICI. Antiviral prophylaxis recommended in patients at risk of reactivation	Hwang JP (20)

ICI, immune checkpoint inhibitors; HCV, hepatitis C virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; NSCLC, non-small cell lung cancer; MM, malignant melanoma; KS, Kaposi sarcoma; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; NUC, nucleos(t)ide analogs.

coexisting HIV infection. The authors identified 73 patients from 11 case reports and 2 case series. In this group, 62 patients were treated with anti-PD-1 agents, 6 with anti-CTLA-4, 4 with the combination of both anti-PD-1/anti-CTLA-4, and 1 with sequential ipilimumab and nivolumab. In only 6 of 70 patients were irAE of grade ≥3 observed, with good tolerance among others. In 26 of 28 patients with undetectable viral load pre- and posttreatment, their HIV remained undetectable. Importantly, CD4 T-cell counts increased in patients where data were available. Reported objective response rates were 30% for NSCLC, 27% for melanoma, and 63% for Kaposi sarcoma. Based on the presented results, ICI treatment in HIV-, HBV-, or HCV-infected patients was safe and effective with no new toxicity data (15, 16).

In order to assess pembrolizumab safety and efficacy in HIV-infected patients with a CD4+ count of at least 100 cells/μl and treated with antiretroviral therapy (ART) for at least 4 weeks, an open-label, phase 1 study was conducted in 33 patients (6 with Kaposi sarcoma, 5 with non-Hodgkin lymphoma, and the remaining 19 with non-AIDS-related cancers). In the cohort, 12 irAE occurred, namely, hypothyroidism (6), pneumonitis (3), rash (2), and aminotransferase elevation (1)—thus typical toxicity profile. All patients were on ART, and uncontrolled HIV infection was not noted in any subject. CD 4 T-cell counts increased during the study, but this finding was not statistically significant. The obtained response rates confirm that pembrolizumab is an effective and feasible therapy in HIV-positive cancer patients (18).

Important analysis results were recently published by Lee et al. (17) The retrospective prospective, single-institution study aimed to "review and follow-up consecutive 62 patients with chronic hepatitis B or resolved HBV infection who had received ICIs" for hepatocellular carcinoma (HCC)—thus a population that is high risk for hepatic irAE due to frequent underlying liver disfunction. No HBV reactivation occurred in the 35 patients with HBV DNA ≤100 IU/ml on nucleos(t)ide analog therapy (NUC) as well as in 19 patients in whom NUC was initiated with the ICI treatment. In six patients receiving ICI with no NUC protection, one developed HBV reactivation, while in three a greater than 1 log decrease in HBV viral load was observed. These data are similar to those in the CheckMate-040 trial, where 3 of 51 HBV-HCC patients (6%) presented a 1 log decline of HBV surface antigen (HBsAg) (17). Moreover, in patients receiving NUC, hepatic irAE occurred at the same level of about 10% of patients irrespective of viral load level. This small, but important real-life study provides evidence that ICI therapy can be safely administered even with detectable viral load, but as none of the patients experienced HBV reactivation while on NUC prophylaxis, this strategy during ICI therapy is strongly recommended.

Another retrospective cohort study enrolled 114 HBsAg-positive cancer patients treated with ICIs. HBV reactivation was diagnosed in 5.3% of patients with an undetected HBV DNA at baseline. HBV reactivation prophylaxis was given in only one of these six patients. None of the events was fatal. The authors found that the lack of antiviral prophylaxis was the only significant risk factor of HBV reactivation (OR 17.50, $p = 0.004$). The authors concluded that HBsAg-positive patients are at risk of HBV reactivation during ICI therapy and should be monitored for HBV DNA and given reactivation prophylaxis (19).

A few months ago, we could see the results of a retrospective study designed to assess the safety and efficacy of ICI in patients

with history of HBV or HCV infection and NSCLC. The study population consisted of 19 patients with NSCLC and history of past or chronic HBV (16 cases; 2 of these had HCV co-infection) or chronic HCV infection (5 cases), who received an anti-PD-1 monoclonal antibody. An overall response rate was 35%. The liver function test elevation from baseline was mild and quite rare with no irAE of grade 3–4 requiring ICI discontinuation or steroids to treat hepatic toxicity. No cases of HBV reactivation or HCV flare were observed on ICI therapy, and no changes in viral load occurred. Thus, treatment of NSCLC patients with ICI appears quite safe in the population of HCV- or/and HBV-infected patients with efficacy measures consistent with those in the noninfected population of NSCLC patients (16).

A large systematic review was published in 2020 by Pu et al. It included data from 8 case reports, 4 case series, and 2 trials with 89 patients infected with HBV and 98 with HCV infection who received ICI (ipilimumab, nivolumab, pembrolizumab, atezolizumab, durvalumab, avelumab, and tremelimumab) for advanced stage cancer.

There were no treatment-related deaths, but some hepatic events occurred: In 3.4% of HBV-infected patients, and 17.3% of HCV-infected patients, grade 3 or 4 transaminase elevation was seen. Moreover, 2.8% without antiviral treatment experienced a virus load increase, and in 1.9%, virus-related hepatitis was diagnosed. In 18.6% with hepatocellular cancer, 32.4% with melanoma, and 16.7% of NSCLC patients, objective response was achieved. The authors conclude that ICIs are safe and effective in advanced cancer patients with HBV or HCV infection, but still reactivation of viral hepatitis are possible in an unclear mechanism. They recommend to initiate and continue antiviral therapy during ICI treatment if indicated (21).

An important review was published in 2021 with a recommendation to screen all the patients before ICI administration for serologic tests of HBV infection, including HbsAg, anti-Hbc, total IgG, and anti-HBs in order to identify all patients at risk of HBV reactivation to whom antiviral prophylaxis is strongly advised (20). This recommendation involves changes in practice and requires efforts in the field of oncologists' education. It was shown that even in non-Hodgkin lymphoma and chronic lymphocytic leukemia treated with anti CD20 monoclonal antibodies (a high-risk population for HBV reactivation), the adherence to appropriate HBV screening guidelines is not sufficient, which shows the need of intensification of educational strategies in the global oncohematologic medical community (22).

Further important data for HIV-infected patients with malignancies were provided in the phase 2, open label, non-randomized DURAVAST trial. The trial included 20 patients with malignancies in which ICI therapy was approved or data proving anti-PD1 or anti-PD-L1 activity were published, with no available standard therapy at study enrollment. All the patients were on combination antiretroviral therapy and had no detectable viral load. The CD4+ and CD8+ lymphocyte count and viral load were monitored during durvalumab therapy (at a dose of 1500 mg every 28 days), and no significant changes in these HIV infection activity parameters were observed. Moreover, the study showed a disease control rate of 50%, with 25% of partial responses with no new safety signals in this specific population. The authors conclude that, if indicated, the durvalumab monotherapy should be available to patients with controlled HIV infection (23).

To summarize, HBV, HCV, and HIV infection in NSCLC patients can be treated with ICI if indicated in most of the cases (24, 25). As evidence-based data from prospective clinical trials are still lacking in these populations, ICI therapy initiation requires viral load baseline assessment and close monitoring of specific hepatic irAE in HCV/HBV-infected patients and additionally CD4+ T cell count in HIV patients. Moreover, in treatment planning, oncologists should take into account potential interactions of ICI and antiviral therapy if needed. The occurrence of irAE is especially dangerous in HIV patients in whom immunosuppressive drugs could deteriorate the infection control with increased risk of opportunistic infections. That is why a closer monitoring of irAE occurrence is needed in this patient group.

In the authors' opinion, the modification of HBV/HCV/HIV-infected patients requiring ICI therapy should include:

a. HBV-infected patients
- Reactivation prophylaxis in every patient at risk at least 7 days before ICI initiation
- AST, ALT, and total bilirubin concentration assessment before each ICI administration—if any of them increases, viral load should be monitored
- If reactivation is diagnosed, ICI therapy should be delayed until liver tests return to normal and ICI rechallenge should be discussed with hepatologist

b. HCV-infected patients
- Anti-HCV therapy initiation should be considered in every case, with assessment of viral load at day 15. If decreased, ICI can be safely initiated (17, 21). If anti-HCV treatment is not effective at day 15, ICI therapy is still possible, but safety should be discussed with a hepatologist
- AST, ALT, and total bilirubin concentration assessment before each ICI administration—if any of them increases, viral load should be monitored
- If reactivation is diagnosed, ICI therapy should be delayed until liver tests return to normal and ICI rechallenge should be discussed with a hepatologist

c. HIV-infected patients
- Every patient should receive anti-HIV treatment
- CD4+ lymphocyte count and viral load should be assessed before ICI initiation
- Monitoring of CD4+ lymphocyte count and viral load should be monitored by an infectious disease specialist as in standard practice
- Organ-specific anti-HIV drug toxicity should be monitored before each ICI administration in order to avoid cumulation of toxicity mechanisms

According to published data, there is no need to assess ICI efficacy more frequently than in the rest of the population.

SARS-COV-2 VIRUS

The landscape of lung cancer immunotherapy AEs and clinical course of SARS-Cov-2 infection in lung cancer patients has substantially influenced oncologists' approach to treatment since January 2020. Theoretically, immunotherapy in lung cancer patients could potentially enhance immunologic control and improve COVID-19 and other infection outcome, but it could also lead to increase the risk of COVID-19 complications, especially in the hyperactive immune phase.

Since the pandemic started in China, the first reports originated from Wuhan. In March 2020, a group of Chinese researchers published a retrospective analysis of three hospitals' database in Wuhan; clinical data were collected from medical records from patients with confirmed COVID-19 (26).

The experience from 14 hospitals included 105 cancer patients (20.95% with lung cancer) and a matched control group, all with COVID-19. The authors demonstrated higher rates of death, intensive care unit (ICU) admission, having at least one severe or critical symptom, and higher risk of the need of mechanical ventilation in the cancer patients' group, especially in the metastatic setting. It was observed that patients who received immunotherapy tended to have high rates of death [two (33.33%) of six patients] and high chances of developing critical symptoms [four (66.67%) of six patients]—the numbers were very small and a significant difference could not be shown (27).

The data from the systematic review of 31 studies show 181,323 patients with COVID-19, of whom 23,736 patients with cancer confirmed that they are at increased risk of mortality and morbidity. The mortality was highest in hematological malignancies (OR 2.43) followed by lung cancer (OR 1.8) with no association between a particular type of oncologic therapy (28).

Luo et al. were the first authors to address the question whether PD-1 blockade therapy affects COVID-19 severity in lung cancer patients. In the analyzed population, 41 (59%) patients previously received PD-1 blockade in four categories: ever received PD-1 blockade, most recent dose within 6 months, most recent dose within 6 weeks, and first dose within 3 months. Overall, there was no significant difference in severity regardless of PD-1 blockade exposure. Peak IL-6 level among hospitalized patients was similar based on receipt of PD-1 blockade. In this single-institution cohort, over half of our patients with lung cancers and COVID-19 required hospitalization and almost a quarter died, which is consistent with findings from Hubei province (27, 29).

One of the latest published reports used a global database (TERAVOLT) in order to establish what are the effects of SARS-Cov-2 infection in thoracic malignancies patients (26). Data were obtained from 200 patients (151 with NSCLC) from eight countries; most of the patients were in active treatment at the time of SARS-Cov-2 infection. As in the general population, the most common presenting symptoms were fever, dyspnea, and cough with the most frequent complications being pneumonia or pneumonitis and acute respiratory distress syndrome. Again, as previously reported in the first Chinese observations (27), a high mortality rate of 33% was confirmed in this patient population. Despite the fact that 76% of the chest malignancy population required hospitalization, there was a low rate of admission to ICUs of 9% with mechanical ventilation for 6% of patients only. Of the 66 patients who died, 79% of deaths were attributed to COVID-19 complications only and only 1 (2%) to complications to cancer therapy. Although univariate analysis showed that age above 65 years, active or past smoking history, treatment with chemotherapy alone, and the presence of comorbidities were associated with higher risk of death, in the multivariate analysis, only smoking history was associated with increased risk of death during SARS-Cov-2 infection in thoracic malignancy patients (3.18; 1.11–9.06). The study found no evidence that the type of systemic therapy affected survival; nevertheless, patients treated with TKIs were at decreased risk for hospitalization (30).

The most recent manuscript was dedicated to identify the patient-specific and cancer-specific features that impact severity of COVID-19, as clinical decisions should be driven by this knowledge. The study was based on a single-institution experience, the Memorial Sloan Kettering Cancer Center in New York, and included 102 consecutive lung cancer patients with COVID-19 diagnosis and analyzed the severity of COVID-19 outcome. The authors found that COVID-19 in lung cancer patients is associated with severe clinical course, with 62% requiring hospitalization and a 25% rate of fatal outcome. As in the general population, patient-specific risk factors including smoking status and chronic obstructive pulmonary disease (odds ratios for severe COVID-19 2.9, 95% CI 1.07–9.44 comparing the median [23.5 pack-years] to never and 3.87, 95% CI 1.35–9.68, respectively) correlated with severe outcome (hospitalization, ICU stay, intubation and invasive mechanical ventilation intubation, and/or transition to do not intubate or death). What is encouraging in taking decisions to refer lung cancer patients to ICUs is the fact that 25% of patients initially requiring intubation recovered from COVID-19, as well as the majority of the studied population. The examined impact of cancer therapy on the COVID-19 clinical course and outcome remains a critical question that should drive real-life clinical decisions during the pandemic. In the studied population, recent PD-1 blockade with or without chemotherapy was not linked with increased severity of COVID-19 as well as the comparison of chemotherapy or TKI. The authors did not find an improvement in severity of clinical course of hydroxychloroquine in the studied population (31).

A single-institution retrospective analysis included 820 cancer patients in whom SARS-Cov-2 infection was diagnosed. The observed rates of respiratory failure or death were 36% and 32% for metastatic lung cancer patients who did and did not receive immunotherapy, respectively. The authors admit that immunotherapy use, thoracic cancer, smoking history, and metastatic solid cancer are highly associated; it is difficult to understand which factors are responsible for worse COVID-19 outcomes and need larger analyses in cancer-specific cohorts (32).

The prospective observational study provided more optimistic evidence—the study included 800 patients with active cancer and confirmed SARS-Cov-2 infection by RT-PCR. The clinical course was mild in 52% of patients.

Although a high mortality rate of 28% was observed, the risk of death was associated with age (odds ratio [OR] 9.42, $p < 0.001$), male gender (OR 1.67, $p = 0.003$) and presence of comorbidities with an important role of hypertension (OR 1.95, $p < 0.001$) and cardiovascular disease (OR 2.32) but not with chemotherapy (even administered up to 4 weeks before COVID-19 diagnosis), immunotherapy, radiotherapy, hormone, or targeted therapy. Thus, the impact of cancer diagnosis and/or treatment itself on COVID-19 clinical course and especially the risk of death was not confirmed (33). A small prospective study was designed in Italy to evaluate the incidence and clinical course of COVID-19 in 53 cancer patients treated with ICI. It was observed that influenza-like illness occurred in 8 of them, but only 3 with lung cancer had a confirmed diagnosis of SARS-Cov-2 infection. The low-resolution computed tomography revealed interstitial pneumonia in all 3 cases with 30%, 50%, and 40% of affected lung volume, respectively. All these patients were hospitalized with respiratory failure diagnosis, and two of them died due to acute respiratory distress syndrome—elderly males with cardiovascular comorbidities; the third patient recovered from COVID-19 (34). This study shows that once COVID-19 in ICI-treated cancer patients has a severe course, it is associated with respiratory complications and high mortality, especially if additional risk factors such as age, gender, and comorbidities increase the risk.

As more data were published in 2019 and 2020, a meta-analysis including 3,581 cancer patients with COVID-19 could be planned. The authors found that infected patients who recently received anti-cancer treatment (including surgery, chemotherapy, targeted therapy, immunotherapy, and radiotherapy) were not at higher risk of COVID-19 exacerbation or death. The cancer treatment-related factors with a negative impact on COVID-19 clinical course were as follows: (1) chemotherapy administered within 28 days before infection—associated with increased risk of death (OR 1.45, $p = 0.015$, $p = 0.015$ for interaction) and (2) immunotherapy associated with the risk of exacerbation (OR 2.53, $p = 0.006$, $p = 0.170$ for interaction) (35).

Thus, as reported above, we have different signals concerning the impact of chemotherapy or immunotherapy on COVID-19 endpoints, and in each case, decisions have to be made in a personalized manner in non-vaccinated patients. As the COVID-19 pandemic is still ongoing, clinicians have to consider the potential risk in patients initiating ICI therapy. Important expert opinions support ICI initiation in a metastatic setting in patients without COVID-19. The adjuvant setting prolonging progression-free survival only in ICI therapy is not equally justified in the authors' opinion due to increased risk of healthcare resources—an important risk factor of SARS-Cov-2 infection transmission. Treatment delay can also be advised in low volume indolent malignancy (36). We do not have enough published data to predict COVID-19 clinical course in vaccinated patients; thus, special attention should be made before clear recommendations are issued.

Mid-2020 supported the oncological community with the first recommendations concerning lung cancer diagnosis and treatment in the COVID-19 era. Authors are consistent and bring a similar approach to advanced-stage NSCLC patients. It is clearly emphasized that oncologists "should still follow the principles of providing the best possible care and palliative management of our patients to improve overall survival and maintain quality of life". As we all agree that it is expected to reduce the oncology centers' visit frequency, patients can benefit from trials results that have shown that less frequent anti-PD-1 antibody administration does not influence their efficacy and safety (37, 38). These data allow to prolong intervals between immunotherapy administration and decrease the number of visits in a medical institution, which is one of the most important measures in terms of risk of SARS-Cov-2 infection.

COVID-19 brought a new challenge for oncologists—the differential diagnosis of COVID-19 and immune-related adverse events in the course of immunotherapy. As shown in the literature, both immune-related pneumonitis and SARS-Cov-2-related pneumonia have similar clinical manifestations, with the most frequent symptoms occurring in both populations being cough, dyspnea, fever, hypoxia, and weakness. Moreover, the occurrence of gastrointestinal symptoms is also likely a symptom of both COVID-19 and irAE in the course of ICI treatment (39–41). Radiological differences between these two etiologies do not give enough tools to make a certain differential diagnosis (39, 41). The obvious minimal diagnostic panel must include SARS-Cov-2 infection test and its result defines further treatment. Thus, if the infection is confirmed, the patient should be treated as having COVID-19, but if it is negative, the therapy should cover immune-related pneumonitis without any delay. The exclusion of SARS-Cov-2 infection should be made based on approved testing methods, i.e., molecular testing with or without antigen test in justified cases. It is important to avoid the synergy in drug and infection lung injury mechanisms, which could be life threatening, especially in NSCLC patients who have multiple additional risk factors for adverse pneumonia outcome. It is also advised to check SARS-Cov-2 status before ICI rechallenge after the toxicity is resolved for the abovementioned reasons.

Since the late 2020, anti-SARS-Cov-2 vaccines have become available. As a sufficient number of doses and healthcare resources were not sufficient, governments all over the world had to establish which populations should be vaccinated as a priority. Thus, the populations in which COVID-19 has an unfavorable clinical course compared to the general population had to be determined. Based on important literature review, it was confirmed that cancer patients should be vaccinated as early as possible due to the high risk of COVID-19 complications and mortality, especially higher in patients with hematological malignancies and lung cancer in advanced stages or those undergoing oncological treatment as frequent contact with healthcare workers is an additional risk factor for SARS-Cov-2 infection (42).

In May 2021, the first results showing the safety of the two doses of the BNT162b2 mRNA vaccine in 134 ICI-treated cancer patients were compared to the control group. Similar rates of side effects were noted in both groups (pain at the injection site, muscle pain, fatigue, headache, fever, chills, and gastrointestinal

or flu-like symptoms). The study did not show new irAE or exacerbation of the previously noted side effects. Moreover, in patients with irAE, the vaccine side effects were not more frequent and had a mild clinical presentation (43). These data seemed encouraging as available literature for influenza vaccine showed higher incidence of grade 3 or 4 irAE in vaccinated patients receiving nivolumab or pembrolizumab compared to patients who were not vaccinated. In addition, some series reported an increased influenza syndrome rate in vaccinated patients undergoing ICI treatment compared to unvaccinated subjects. Published studies show that ICI efficacy is not impaired in influenza vaccinated patients; even better cancer control results were reported in the literature (44). We should not extrapolate results obtained for influenza vaccines to those obtained for the BNT162b2 mRNA vaccine as the first one is based on attenuated viruses while the anti-SARS-Cov-2 vaccines are based on a different technology, which results probably in a different pattern of efficacy and irAE occurence. Lately, we were provided evidence that patients with solid tumors undergoing COVID-19 vaccination demonstrate a high anti-spike immunoglobulin G antibody (IgG-Ab) positivity of 98% and 97% in patients treated with ICI. It was observed that mRNA-based vaccines are associated with higher IgG-Ab titers than adenoviral ones (45).

As SARS-Cov-2 infection is a new phenomenon, it is strongly advised to follow updated recommendations available, for example, on American Society of Clinical Oncology or European Society of Medical Oncology websites (46, 47).

To summarize the data we have at the end of 2020, lung cancer patients are more susceptible to SARS-Cov-2 infection and they are at higher risk of developing COVID-19 complications such as pneumonia/pneumonitis and ARDS, of being admitted to the ICU, of using mechanical ventilation, and, unfortunately in about one-third of cases, of dying. We do not have clear indications that patients undergoing ICI, subjected to systemic therapy, are at a different risk of contracting COVID-19 or have a different prognosis than patients treated with other systemic therapies. In 2021, when SARS-Cov-2 vaccines are becoming more and more accessible for cancer patients, it is strongly advised to perform the vaccination in order to avoid COVID-19 in cancer patients. In the non-vaccinated patients, national or local policies concerning SARS-Cov-2 molecular screening before immunotherapy initiation differ substantially between countries and even institutions. Despite that, it seems justified to screen every lung cancer patient for SARS-Cov-2 infection before therapy initiation and every subsequent dose, especially in the non-vaccinated population. If the patient is SARS-Cov-2-positive (even if asymptomatic), the delay of any cancer therapy initiation until full recovery (at least 2 weeks following resolution of symptoms) is the only known reasonable approach (36). The question how often testing should be repeated remains open. Also, testing of family members or people living within the same home is a reasonable option as many healthy adults and children can be infected but asymptomatic with high risk of transmission to the lung cancer patient in whom the clinical course can be severe or even fatal.

AUTHOR CONTRIBUTIONS

EK was responsible for literature search and review, publications choice, data assembly, proper language corrections, manuscript writing and final approval. IC was responsible for manuscript writing, proper language corrections and final approval. KW-K was responsible for literature search and review, publications choice, data assembly, proper language corrections, manuscript writing and final approval. All authors contributed to the article and approved the submitted version.

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