MULTI-DIMENSIONAL BIOMARKERS AND RESISTANCE MECHANISM OF TARGETED THERAPY AND IMMUNOTHERAPY IN LUNG CANCER

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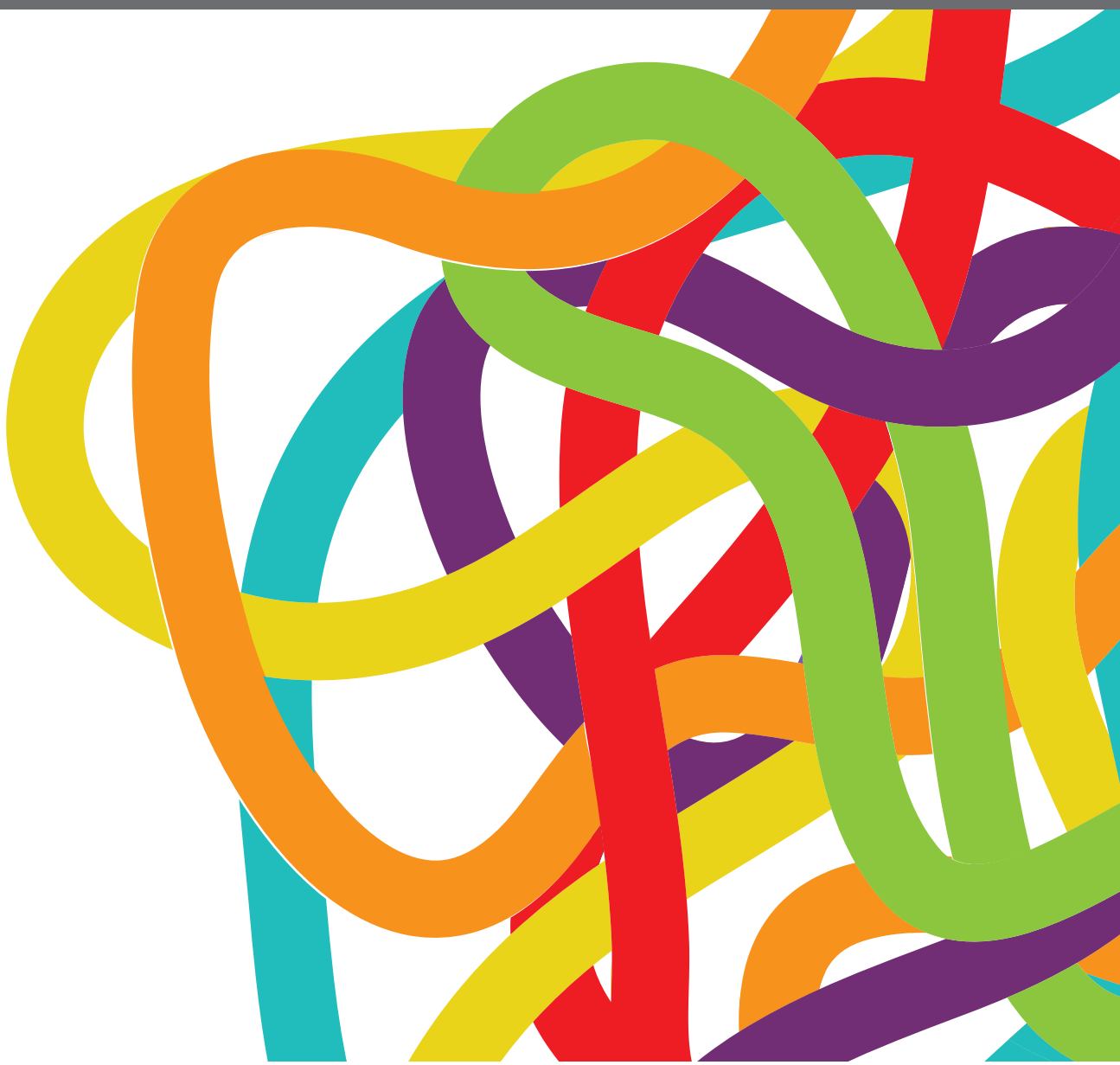

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MULTI-DIMENSIONAL BIOMARKERS AND RESISTANCE MECHANISM OF TARGETED THERAPY AND IMMUNOTHERAPY IN LUNG CANCER

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Progression Patterns, Treatment, and Prognosis Beyond Resistance of Responders to Immunotherapy in Advanced Non-Small Cell Lung Cancer

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Introduction: Immune checkpoint inhibitors (ICIs) have changed the management of non-small cell lung cancer (NSCLC). However, resistance is inevitable. The disease progression patterns, sequential treatment, and prognosis beyond ICI resistance are not completely understood.

Methods: We retrospectively analyzed stage IV NSCLC patients who underwent ICI treatment at Zhejiang Cancer Hospital between January 2016 and January 2020 and who suffered disease progression after at least stable disease on immunotherapy for more than 3 months (at least two cycles). Oligoprogression and systematic progression were defined as previous reports. The main outcome measures were progression-free survival (PFS), second PFS (PFS2), and overall survival (OS). Survival curves were plotted using the Kaplan-Meier method. The Cox proportional hazards model was used for multivariate analysis.

Results: Totally 1,014 NSCLC patients were administered immunotherapy. Of them, 208 NSCLC patients were included in this retrospective study. The estimated PFS, PFS2 and OS were 6.3 months (95% CI 5.6–7.0 months), 10.7 months (95% CI 10.1–12.7 months), and 21.4 months (95% CI 20.6–26.4 months), respectively. After resistance, 55.3% (N = 115) patients developed oligoprogression, and 44.7% (N = 93) systemic progression. For patients with systemic progression, chemotherapy (N = 35, 37.6%), best supportive care (N = 30, 32.3%), and antiangiogenic therapy alone (N = 11, 11.8%) were the major strategies. A combination of local radiotherapy (N = 38, 33.0%) with continued ICIs was the most common treatment used in oligoprogression group, followed by continued immunotherapy with antiangiogenic therapy (N = 19, 16.5%) and local radiotherapy only (N = 17, 14.9%). For patients with oligoprogression, continued immunotherapy plus local radiotherapy can lead to a significantly longer PFS2 (12.9 *vs.* 10.0 months; $p = 0.006$) and OS (26.3 *vs.* 18.5 months, $p = 0.001$). The PFS2 and OS of patients with oligoprogression were superior to those of patients with systemic progression (PFS2: 13.1 *vs.* 10.0 months, $p = 0.001$; OS: 25.8 *vs*. 19.1 months, $p = 0.003$).

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Conclusions: The major progression pattern after acquired resistance from immunotherapy is oligoprogression. Local radiotherapy with continued immunotherapy beyond oligoprogression in responders was feasible and led to prolonged PFS2 and OS in advanced NSCLC patients.

Keywords: non-small cell lung cancer, immunotherapy, treatment beyond progression, oligoprogression, local radiotherapy

INTRODUCTION

Lung cancer is one of the leading causes of cancer-related mortality worldwide and in China, in which approximately 80% of cases are non-small cell lung cancer (NSCLC). The 5-year overall survival (OS) rate of advanced NSCLC patients is no more than 5%. Recently, the development of immune checkpoint inhibitors (ICIs) targeting cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein-1 (PD-1) or its ligand programmed cell death ligand-1 (PD-L1) has rapidly increased (1). Immune checkpoint blockade has demonstrated impressive effects in advanced NSCLC and prolonged OS (2–4). Thus, ICIs are now widely used in clinical practice and have changed the treatment options and outcomes of advanced NSCLC.

Of note, the tumor response patterns of immunotherapy were found to be different from those of chemotherapy and targeted therapy. Delayed response or stabilization after disease progression (pseudoprogression) has been observed in tumors treated with ICIs, including NSCLC (5). These novel findings have led to the development of immune-based response criteria (6–8), helping in the selection of patients who could benefit from treatment beyond progression (TBP). Many subgroup analyses of clinical trials have been performed to investigate the potential benefit of continuing immunotherapy beyond progression (9–12). In addition, the data of the expanded access program (EAP) and retrospective analyses have also confirmed the benefit of TBP with immunotherapy in NSCLC patients in real-life clinical practice (13, 14). These results indicated that advanced NSCLC patients with pseudoprogression after immunotherapy have a subsequent response and survival benefit from TBP with immunotherapy.

However, acquired resistance is inevitable, and it is uncertain whether patients could also benefit from TBP with immunotherapy plus chemotherapy or other treatment strategies after confirmed disease progression. No prospective studies have focused on the treatment and prognosis after acquired resistance to immunotherapy. Moreover, the disease progression patterns beyond ICIs resistance are not completely understood. For patients who were previously treated with immunotherapy and later showed tumor progression, currently, many patients have fewer treatment options. In clinical practice, at the time of confirmed disease progression, some patients discontinue immunotherapy and initiate a new strategy, such as chemotherapy, antiangiogenesis treatment, local radiotherapy, or best supportive care, while other patients insist on continuing immunotherapy and plus a new strategy.

Although immunotherapy can bring a significant long-term survival benefit in the management of NSCLC, tumors often relapse, known as acquired resistance. The common relapse patterns are unclear. The aim of this retrospective study was to provide detailed information on the effectiveness of ICIs treatment as well as progression patterns, sequential therapy, second progression-free survival (PFS2) and OS after ICIs acquired resistance in patients with advanced NSCLC in real-world routine Chinese clinical practice.

MATERIALS AND METHODS

Patient Eligibility

We reviewed the medical records of NSCLC patients from January 2016 to January 2020 who were administered ICI treatment at Zhejiang Cancer Hospital (N = 1014). A total of 208 stage IV NSCLC patients were identified from a screened population of 1041 patients and enrolled in this study. The inclusion criterias were as follows: 1) patients had pathologically or cytologically proven primary stage IV NSCLC; 2) all the patients benefited from prior immunotherapy with a progression-free survival (PFS) of more than 3 months; 3) patients completed tumor response evaluation for ICI at least once; progressive disease (PD) was confirmed using chest computed tomography (CT), brain magnetic resonance imaging (MRI), and bone scan as well as ultrasound examination and/or CT of the abdomen; 4) patients had at least one measurable lesion and an Eastern Cooperative Oncology Group performance status (PS) score of 0 to 2; 5) patients had epidermal growth factor receptor (EGFR) mutation negative and anaplastic lymphoma kinase (ALK) negative disease; and 6) patients had complete medical records.

Diagnosis of Oligoprogressive Disease

Oligoprogressive disease is a concept about only a few sites of patients progressed. However, in clinical practice, how to identify oligoprogressive disease remains challenged. Oligoprogressive disease was considered to satisfy the following conditions: 1) one to several distant recurrences (usually one) in one to several organs (usually one); 2) primary site controlled; 3) one to several distant recurrences can be treated with local therapy; 4) no other distant recurrences other than those in 3) (15, 16). In some prospective studies and retrospective reviews, progression patterns were also documented, and oligoprogressive disease was identified as following: 1) progression in the primary site alone, or 2) an asymptomatic solitary site of extra-cranial

progression, or 3) three or fewer sites of progression with more than six sites before therapy, or 4) five or fewer sites were progressing (17–21). In our study, oligoprogression was defined as ≤ 2 sites and ≤ 2 lesions of progression and can be treated with local therapy. Systematic progression was defined as ≥ 3 sites and ≥ 3 lesions (usually ≥ 5) of progression.

Follow-Up

All patients were evaluated for tumor response, PFS, PFS2, and OS. The follow-up rate was 100%. The last follow-up date was July 31, 2020.

Statistical Analysis

OS was defined as the time from the first cycle of immunotherapy to the date of death or the date of the last follow-up visit for patients who were still alive. PFS was defined as the time from the first cycle of immunotherapy to the first disease progression. PFS2 is defined in the EMA guidance as "time from randomization to objective tumor progression on next-line treatment or death from any cause. In some cases, time on next-line therapy may be used as proxy for PFS" (22). In our study, PFS2 was defined as the time from the first cycle of immunotherapy to the second progression or death. PFS and OS were calculated using the Kaplan-Meier method, and between-treatment differences were assessed by the stratified log-rank test (10% significance level). Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated based on a stratified Cox model. A p-value of less than 0.05 was regarded as statistically significant. All statistical tests were analyzed using the computer software SPSS version 22.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient Characteristics

A total of 1,041 patients were diagnosed with NSCLC and treated with immunotherapy from January 2016 to January 2020 at Zhejiang Cancer Hospital. Patients who received less than two cycles of ICIs, who were lost to follow-up and who did not complete the tumor response assessment were excluded from the study. Patients who had PD as the best response and those who had disease progression at the first assessment of ICI treatment were also excluded from our study. Of the 1,041 patients, 208 (20%) who had a PFS of more than 3 months and later confirmed disease progression were included in the analysis. Among them, 115 (55.3%) patients had oligoprogression, and 93 (44.7%) had systemic progression. The median age of the patients was 61.0 years (range: 32–82 years). The predominant histology of the tumors was squamous cell carcinoma (126/208, 60.6%). A total of 126 patients (126/208, 60.6%) had a smoking history of >= 20 packs of cigarettes/year. Thirty-four (16.3%) patients presented with baseline brain metastasis at the initiation of ICI treatment, and 30 (14.4%) patients had baseline liver metastasis. ICIs were used as first-line treatment in 69 (33.2%) patients, as second-line treatment in 94 (45.2%) patients, and as third-line or later treatment in 45 (21.6%) patients. Sixty-four (30.8%) patients achieved partial response (PR), and 144 (69.2%) had stable disease (SD). A total of 143 (68.8%) patients were treated with ICIs as monotherapy. A greater proportion of patients (68.8%) who achieved PR from immunotherapy developed oligoprogression than systemic progression (31.2%). The patient characteristics are shown in **Table 1**.

Analysis of the PFS and OS of All the Patients

In total, 1,041 NSCLC patients were administered immunotherapy. Of these, 208 NSCLC patients were included in this retrospective study. The estimated median PFS (mPFS), PFS2, and OS were 6.3 months (95% CI 5.6–7.0 months), 10.7 months (95% CI 10.1–12.7 months), and 21.4 months (95% CI 20.6–26.4 months), respectively (**Figure 1**). Several factors were analyzed to predict PFS with ICIs. In multivariable analysis, pathology [squamous cell carcinoma/adenocarcinoma, HR = 0.68, 95% CI (0.48–0.96); $p = 0.026$], response to ICIs [PR/SD, HR = 1.82, 95% CI (1.28–2.59); $p = 0.001$] and monotherapy or combination therapy [HR = 0.67, 95% CI (0.48–0.96); $p = 0.027$] were independent risk factors for PFS (**Table S1**).

Among all the patients experiencing first PD, the estimated median PFS2 was 10.7 months (95% CI 10.1–12.7 months) (**Figure 1**). The univariate analysis showed that no factors were associated with PFS2. In multivariable analysis, response to ICIs (PR/SD; HR = 1.68; 95% CI 1.16–2.43; $p = 0.006$) was the only independent predictive factor for longer PFS2 (**Table S1**).

The estimated median OS (mOS) was 21.4 months (95% CI 20.6–26.4 months) (**Figure 1**). Multivariate Cox analysis revealed that pathology [squamous cell carcinoma/adenocarcinoma, HR = 0.51, 95% CI (0.32–0.82); $p = 0.005$], response to ICIs [PR/SD, HR = 1.9, 95% CI (1.15–3.12); $p = 0.012$] and monotherapy or combination therapy [HR= 0.55, 95% CI (0.34–0.88); $p = 0.014$] were independent risk factors for OS (**Table S1**).

Progression Patterns and Sites Beyond Immunotherapy Resistance

The progression patterns and sites of the 208 patients who experienced first disease progression (1st PD) beyond ICIs are shown in **Figure 2** and **Table 2**. Oligoprogression was defined as ≤ 2 sites and ≤ 2 lesions of progression and can be treated with local therapy. Systematic progression was defined as ≥ 3 sites and ≥ 3 lesions (usually ≥ 5) of progression. After resistance to ICIs, 55.3% (N = 115) of patients developed oligoprogression, and 44.7% (N = 93) developed systemic progression (**Figure 2**). Ninety (90/208, 43.3%) patients developed PD at one site. A greater proportion of patients (68.8%) who achieved PR from immunotherapy developed oligoprogression than systemic progression (31.2%) (**Table 1**). The progression sites included the lung (N = 116, 55.8%), lymph node (N = 73, 35.1%), liver (N = 30, 14.4%), brain (N = 21, 10.1%), pleura (N = 41,19.7%), bone (N = 25, 12%), adrenal gland (N = 6, 2.9%), and subcutaneous nodule (N = 2, 1.0%). A total of 85.7% of patients who experienced brain progression exhibited a pattern of oligo-organ progression (**Figure 2**, **Table 2**).

TABLE 1 | Baseline characteristics of included patients and its correlations with progression model (N=208).

		Total N (%)	Oligo-progression (N=115)		Systemic progression (N=93)		P value
			n	%	n	%	
Age	<65	134 (64.4%)	74	55.2	60	44.8	1
	>=65	74 (35.6%)	41	55.4	33	44.5	
Sex	Male	171 (82.2%)	100	58.5	71	41.5	0.0672
	Female	37 (17.8%)	15	40.5	22	59.5	
Smoking	<20 packs of cigarettes/year	82 (39.4%)	39	47.6	43	52.4	0.0869
	>=20 packs of cigarettes/year	126 (60.6%)	76	60.3	50	39.7	
ECOG	0	29 (13.9%)	14	48.3	15	51.7	0.2187
	1	169 (81.3%)	93	55	76	45	
	2	10 (4.8%)	8	80	2	20	
Pathology	Squamous cell carcinoma	126 (60.6%)	68	54	58	46	0.6702
	Adenocarcinoma	82 (39.4%)	47	58.3	35	42.7	
Brain metastases	Yes	34 (16.3%)	22	64.7	12	35.3	0.2611
	No	174 (83.7%)	93	53.4	81	46.6	
Liver metastases	Yes	30 (14.4%)	20	66.7	10	33.3	0.2337
	No	178 (85.6%)	95	53.4	83	46.6	
Thoracic Radiotherapy	Yes	70 (33.7%)	41	58.6	29	41.4	0.5559
	No	138 (66.3%)	74	53.6	64	46.4	
Brain Radiotherapy	Yes	18 (8.7%)	10	55.6	8	44.4	1
	No	190 (91.3%)	105	55.3	85	44.7	
Lines of ICI therapy	1	69 (33.2%)	39	56.5	30	43.5	0.8295
	2	94 (45.2%)	53	56.4	41	43.6	
	3	45 (21.6%)	23	51.1	22	48.9	
Evaluation of efficacy	PR	64 (30.8%)	44	68.8	20	31.2	0.0161
	SD	144 (69.2%)	73	50.7	71	49.3	
Immunotherapy	Monotherapy	143 (68.8%)	80	55.9	63	44.1	0.8805
	Combination	65 (31.2%)	35	53.8	30	46.2	

ECOG PS, Eastern Cooperative Oncology Group performance score; ICIs, immune checkpoint inhibitors; PR, partial response; SD, stable disease.

Sequential Therapy Beyond Immunotherapy Resistance

The sequential therapies beyond immunotherapy resistance are summarized in **Table 3**. For the patients with systemic progression, chemotherapy (N = 35, 37.6%), best supportive care (N = 30, 32.3%) and antiangiogenic therapy alone (N = 11, 11.8%) were the major treatment strategies. A combination of local radiotherapy (N = 38, 33.0%) on the basis of continued ICI treatment was the most common treatment strategy used in patients with oligoprogression, followed by continued immunotherapy with antiangiogenic therapy (N = 19, 16.5%) and local radiotherapy only (N = 17, 14.9%). Among all patients experiencing 1st PD with oligoprogression, 79 (68.7%) chose to continue immunotherapy beyond progression. In addition, 71 (61.7%) patients with oligoprogression chose local radiotherapy. Only 22 (19.1%) patients with oligoprogression chose systemic chemotherapy.

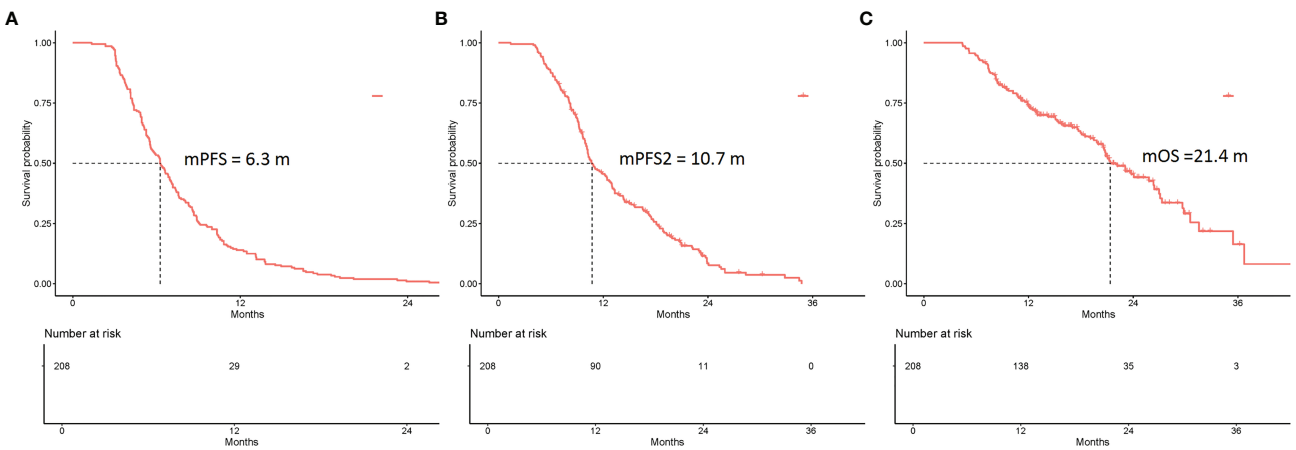

FIGURE 1 | Kaplan-Meier curves of the PFS, PFS2, and overall survival (OS) of all eligible patients (N = 208). **(A)** The mPFS was 6.3 months (95% CI 5.6–7.0 months). **(B)** The mPFS2 was 10.7 months (95% CI 10.1–12.7 months). **(C)** The mOS was 21.4 months (95% CI 20.6–26.4 months).

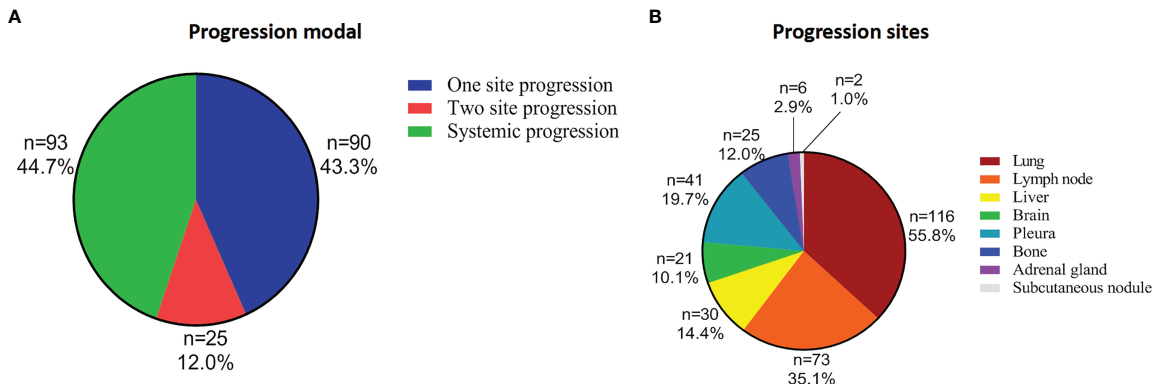

FIGURE 2 | Progression patterns and sites beyond immunotherapy resistance. **(A)** 115 (55.3%) patients developed oligoprogression, and 93 (44.7%) developed systemic progression. Ninety (90/208, 43.3%) patients developed progressive disease at one site. **(B)** The progression sites included the lung (N = 116, 55.8%), lymph node (N = 73, 35.1%), liver (N = 30, 14.4%), brain (N = 21, 10.1%), pleura (N = 41.19.7%), bone (N = 25, 12%), adrenal gland (N = 6, 2.9%), and subcutaneous nodule (N = 2, 1.0%).

PFS, PFS2, and OS Analyses According to Progression Patterns

The PFS, PFS2, and OS of patients with oligoprogression were superior to those of patients with systemic progression (**Figure 3**, **Table S2**). The estimated mPFS were 6.4 and 5.7 months for patients with oligoprogression and patients with systemic progression, respectively; the difference was statistically significant (p = 0.009). The estimated mPFS2 were 13.1 and 10.0 months for patients with oligoprogression and patients with systemic progression, respectively (p = 0.001), and the corresponding mOS were 25.8 and 19.1 months (p = 0.003).

PFS2 and OS Analyses According to Sequential Therapy for the Entire Cohort

For the entire cohort, continued ICI treatment beyond 1st PD after ICI treatment can lead to a significantly longer PFS2 (12.9 *vs.* 10.0 months; p = 0.006) and OS (26.3 *vs.* 18.5 months; p = 0.001) (**Figure S1**). The median duration of ICI treatment was 7.5 months. When separating the patients into two groups according to the ICI treatment length (i.e., ICI >7.5 or ≤ 7.5 months), the mPFS2 and mOS were significantly different. The longer ICI (>7.5 months) treatment group showed superior mPFS2 and mOS compared with the shorter ICI (≤ 7.5 months) treatment group. The estimated mPFS2 values were 16.6 and 8.3 months for the longer and shorter ICI treatment groups, and the mOS were 29.8 and 12.7 months, respectively (p < 0.0001) (**Figure S2**).

Among the 208 patients, 38 (18.3%) patients received continued ICI plus local therapy after resistance. Among these 38 patients, 100% had oligoprogression. In multivariable analysis, continued ICI plus local therapy was a predictive factor for longer PFS2 (p = 0.001) and OS (p = 0.00) (**Table S3**, **Figure 4**). The estimated mPFS2 values were 15.0 and 10.3 months (p = 0.05), and the mOS were 26.4 and 20.8 months (p = 0.02) in patients receiving continued ICI plus local therapy (2 + 4) and patients receiving other strategies, respectively (**Figure 4**).

Among the 208 patients, 66 (31.7%) received antiangiogenic therapy after 1st PD. Forty-five (68.2%) patients had oligoprogression. In multivariable analysis, patients who received antiangiogenic therapy showed longer PFS2 (p = 0.00) and OS (p = 0.001) (**Table S3**, **Figure 5**). The estimated mPFS2 were 16.6 and 10.0 months (p = 0.00), and the mOS were 31.5 and 20.5 months (p = 0.00) in patients receiving antiangiogenic therapy and patients who did not receive antiangiogenic therapy, respectively (**Table S3**, **Figure 5**).

PFS2 and OS Analyses According to Sequential Therapy in Systemic Progression Cohort

In systemic progression cohort (N = 93), 30 (32.3%) patients received best supportive care. Addition of systemic treatment showed a significantly longer PFS [10.4 *vs.* 9.0 m; HR = 0.53, 95% CI (0.34–0.84); p = 0.007] and OS [23.8 *vs.* 10.2 m; HR = 0.3.95%

TABLE 2 | Progression sites beyond immunotherapy resistance.

Disease site	Total N (%)	Oligo-progression	Systemic progression	P value
Lung	116 (55.8%)	50 (43.1%)	66 (56.9%)	0.7249
Lymph node	73 (35.1%)	36 (49.3%)	37 (50.7%)	0.4202
Liver	30 (14.4%)	15 (50.0%)	15 (50.0%)	0.5662
Brain	21 (10.1%)	18 (85.7%)	3 (14.3%)	0.0001
Pleura	41 (19.7%)	0 (0.0%)	41 (100.0%)	<0.0001
Bone	25 (12%)	16 (64.0%)	9 (36.0%)	0.0577
Adrenal gland	6 (2.9%)	3 (50.0%)	3 (50.0%)	1
Subcutaneous nodule	2 (1.0%)	2 (100.0%)	0 (0.0%)	0.1980

TABLE 3 | Sequential therapy beyond immunotherapy resistance.

Strategy	Total N=208 N (%)	Oligo-progression N=115	Systemic progression N=93	P value
0	31 (14.9)	1 (3.2/0.9)	30 (96.8/32.3)	<0.0001
1	13 (6.3)	2 (15.4/1.7)	11 (84.6/11.8)	0.0033
2	17 (8.2)	17 (100.0/14.9)	0 (0.0)	<0.0001
3	40 (19.2)	5 (12.5/4.3)	35 (87.5/37.6)	<0.0001
1+2	3 (1.4)	3 (100.0/2.6)	0 (0.0)	0.2548
1+3	9 (4.3)	8 (88.9/7.0)	1 (11.1/1.1)	0.0443
1+4	28 (13.5)	19 (67.9/16.5)	9 (32.1/9.7)	0.1601
2+4	38 (18.3)	38 (100.0/33.0)	0 (0.0/)	<0.0001
3+4	16 (7.7)	9 (56.3/7.8)	7 (43.7/7.5)	1
1+2+4	13 (6.3)	13 (100.0/11.3)	0 (0.0)	0.0007
ICI halt	113 (54.3)	36 (31.9/31.3)	77 (68.1/82.8)	<0.0001
ICI maintain	95 (45.7)	79 (83.2/68.7)	16 (16.8/17.2)	<0.0001
Anti-angiogenic (yes)	66 (31.7)	45 (68.2/39.1)	21 (31.8/22.6)	0.0114
Anti-angiogenic (no)	142 (68.3)	70 (49.3/60.9)	72 (50.7/77.4)	0.0114
Radiation therapy (yes)	71 (34.1)	71 (100.0/61.7)	0 (0.0)	<0.0001
Radiation therapy (no)	137 (65.9)	44 (32.1/38.3)	93 (67.9/100.0)	<0.0001
Chemotherapy (yes)	65 (31.2)	22 (33.8/19.1)	43 (66.2/46.2)	<0.0001
Chemotherapy (no)	143 (68.8)	93 (65.0/80.9)	50 (35.0/53.8)	<0.0001

0, best supportive care; 1, anti-angiogenesis; 2, local radiotherapy; 3, chemotherapy; 4, ICI maintain.

CI (0.17–0.54); p < 0.001] than best supportive care (**Figure S3A**). When further dividing patients into three sub-groups according to different treatment strategies as following: ICI plus anti-angiogenesis or chemotherapy (4 + 1/3), chemotherapy only (3), anti-angiogenesis only (1), the mPFS2 were 10.0, 10.5, and 11.9 months [HR = 1.1, 95% CI (0.58–2.09); p = 0.9], and the mOS were 23.1, 23.8, and 12.4 months [HR = 1.36, 95% CI (0.49–3.73); p = 0.6], respectively (**Figure S3B**).

PFS2 and OS Analyses According to Sequential Therapy in Oligoprogression Cohort

In oligoprogression cohort (N = 115), 51 (44.3%) patients treated with continued ICI and local radiotherapy with/without anti-angiogenesis. When compared with patients treated with other strategies, the mPFS2 were 15.6 and 12.2 months [HR = 1.5, 95% CI (0.99–2.27); p =**0.053**], and the mOS were 26.4 and 20.8 months [HR = 2.24, 95% CI (1.24–4.05); p = 0.006], respectively (**Figure S4A**). When further divided patients into four sub-groups according to different treatment strategies as following: ICI plus local therapy (a1), ICI plus anti-angiogenesis or chemotherapy (a2), local therapy only (a3), and anti-angiogenesis or chemotherapy (a4), the estimated mPFS2 were 15.6, 13.0, 9.2, and 19.2 months [HR = 0.84, 95% CI (0.42–1.7); p < 0.001], and the mOS were 26.4, 23.1, 10.8 and NR months [HR = 0.69, 95% CI (0.2–2.35); p < 0.001], respectively (**Figure S4B**). Thus, subgroup analyses suggested that OS benefit was observed in the continued ICI and local radiotherapy group.

DISCUSSION

The introduction of ICIs has notably expanded the available therapeutic options for patients with advanced NSCLC.

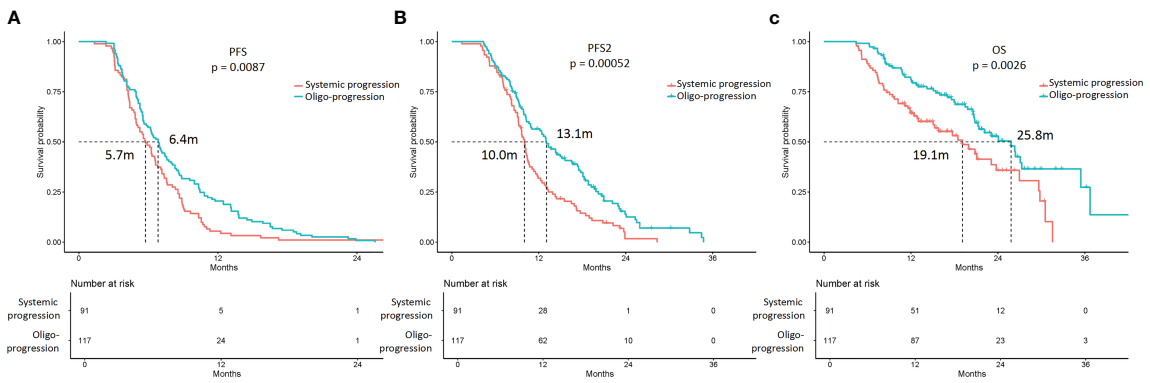

FIGURE 3 | Kaplan-Meier curves of the PFS, PFS2, and overall survival (OS) of patients with oligoprogression and systemic progression. **(A)** The mPFS were 6.4 and 5.7 months in patients with oligoprogression and systemic progression, respectively (p = 0.009). **(B)** The mPFS2 were 13.1 and 10.0 months in patients with oligoprogression and systemic progression, respectively (p = 0.001). **(C)** The mOS were 25.8 and 19.1 months in patients with oligoprogression and systemic progression, respectively (p = 0.003).

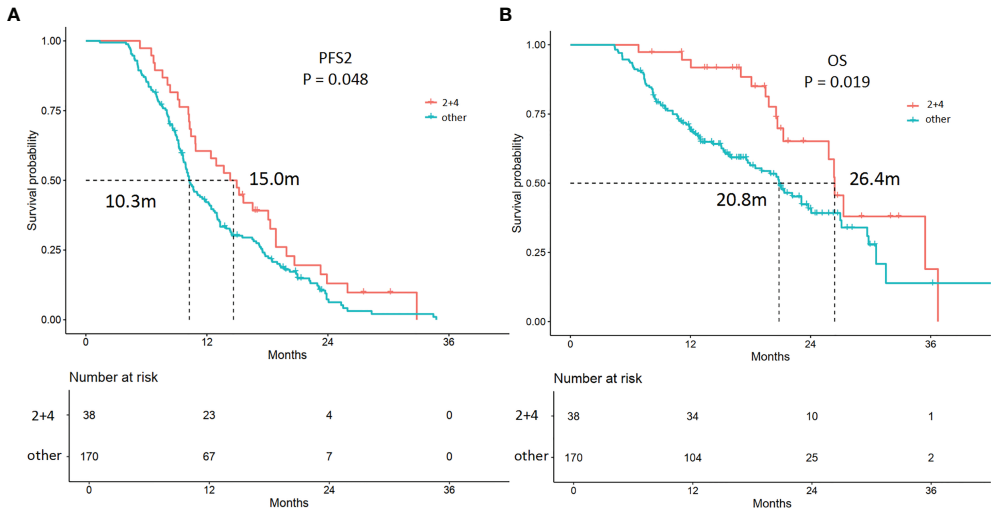

FIGURE 4 | Kaplan-Meier curves of the PFS2 and overall survival (OS) of patients receiving different treatments. **(A)** The mPFS2 were 15.0 and 10.3 months in patients receiving continued ICI plus local therapy (2 + 4) and patients treated with other strategies (p = 0.048), respectively. **(B)** The mOS were 26.4 and 20.8 months in patients receiving continued ICI plus local therapy (2 + 4) and patients treated with other strategies (p = 0.019), respectively.

However, there is no standard treatment for these patients after confirmed disease progression or acquired resistance, and their prognosis remains unclear. Our retrospective study provided first-hand data on the disease progression patterns and sites, sequential treatment strategies, and prognosis beyond ICIs acquired resistance in patients with advanced NSCLC in routine Chinese clinical practice at Zhejiang Cancer Hospital.

According to subgroup analyses from prospective trials, continued ICIs beyond disease progression are applicable in approximately 20–50% of patients who experience PD. Continued ICIs lead to subsequent tumor shrinkage or stabilization in 25–80% of PD patients. Moreover, approximately 5–30% of patients may achieve greater and durable survival benefits compared with patients who stop ICIs and change anticancer therapy (9–14, 23–25). In contrast, the innovation of our research is that we excluded patients with pseudoprogression and analyzed acquired drug resistance in NSCLC patients who benefited from immunotherapy for 3

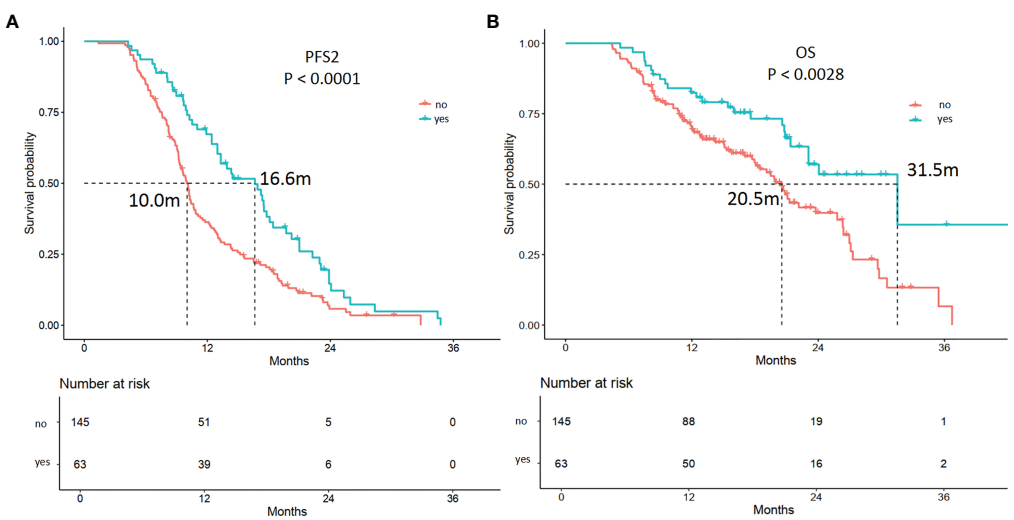

FIGURE 5 | Kaplan-Meier curves of the PFS2 and overall survival (OS) of patients receiving antiangiogenic therapy or not. **(A)** The mPFS2 were 16.6 and 10.0 months in patients receiving antiangiogenic therapy (yes) or not (p = 0.00), respectively. **(B)** The mOS were 31.5 and 20.5 months in patients receiving antiangiogenic therapy (yes) or not (p = 0.00), respectively.

months or more. After resistance from ICIs, 55.3% (N = 115) of patients developed oligoprogression, and 44.7% (N = 93) developed systemic progression. Combination with local radiotherapy (N = 38, 33.0%) on the basis of continued ICIs is the most common treatment used in patients with oligoprogression, followed by continued immunotherapy with antiangiogenic therapy (N = 19, 16.5%). There were 79 (68.7%) patients with oligoprogression who chose to continue ICIs after progression. For patients with oligoprogression beyond 1st PD after ICIs treatment, continued immunotherapy plus local radiotherapy can lead to a significantly longer PFS2 (12.9 *vs*. 10.0 months; $p = 0.006$) and OS (26.3 *vs*. 18.5 months, $p = 0.001$). Currently, established treatment modes after immunotherapy failure are lacking. The continuation of immunotherapy with local radiotherapy beyond progression may be a good choice for patients with oligoprogression as the acquired resistance model. This result must be further validated in population-based clinical research prospectively.

The identification of patients most likely to benefit from continued ICIs beyond progression remains a challenge. Several studies showed that TBP patients had better PSs both at baseline and at progression and had a higher response rate or disease control rate before progression than non-TBP patients (9, 10, 24, 26–28). The present study shows that a greater proportion of patients (68.8%) who achieved PR from immunotherapy before the first progression are more likely to develop oligoprogression. The PFS2 and OS of patients with oligoprogression were superior to those of patients with systemic progression (PFS2: 13.1 *vs*. 10.0 months, $p = 0.001$; OS: 25.8 *vs*. 19.1 months, $p = 0.003$).

Regarding to the frequency of oligoprogressive disease in NSCLC patients under treatment with immunotherapy, Stephan Rheinheimer reported the rate was about 10% to 20% and Antony Mersiades reported the rate was 11% using slightly different criteria. Other studies in melanoma also confirmed the lower rate of oligoprogressive disease after immunotherapy (29, 30). It seems that our conclusion is contrary to their findings. As in our study, totally 1,014 NSCLC patients were administered immunotherapy in our center from January 2016 to January 2020, and screened. Of them, NSCLC patients with imaging evidence of disease progression who benefited from prior immunotherapy with a PFS less than 3 months were excluded from our study. Moreover, most of them were systemic progression patients. In other words, we only included patients with PR and SD (responders) after immunotherapy. This could be the major reason that our conclusion is different to their findings.

Similar evidences were obtained from EGFR mutant NSCLC patients with oligoprogressive disease. A lot of studies suggested that indicating addition of local therapy showed prolonged survival benefit than EGFR-TKI alone in EGFR-mutant NSCLC patients with oligoprogressive disease, including intracranial metastases, primary lesion progression, and liver metastasis (31–34). It is also evident that radiotherapy could kill cancer cells while triggering the release of pro-inflammatory mediators, increasing tumor infiltrating immune cells, and modulating neoantigen expression simultaneously (35). Thus, radiotherapy could enhance immunostimulatory effects and is increasingly viewed as a promising combination strategy with ICIs (36–38).

Nonetheless, there are still no approved criteria for selecting patients who would benefit from continued ICIs treatment beyond disease progression. Patients with better PSs or oligoprogression are more likely to receive ICIs beyond progression. Moreover, the choice of continuing ICIs after resistance is encouraged by the absence of effective treatment strategies. The addition of localized radiotherapy should be considered a useful tool to improve local tumor control, enhancing ICIs efficacy.

The present study possesses intrinsic limitations due to its retrospective design. In addition, the data were collected from a single center, which also influences the clinical applications of our results.

In conclusion, our data suggest that continuing immunotherapy beyond initial progression in addition to local radiotherapy is feasible and effective, especially in oligoprogression patients. Continuing ICIs beyond progression is associated with longer survival in selected patients according to clinical judgment. Future investigations are warranted to identify patients who are most likely to respond after progression according to predictive biomarkers, patient and disease characteristics, and the type of and response to previous treatments both at baseline and at progression. These findings will enhance the personalized approach to clinical decision-making when considering ICIs as a therapeutic choice and continuing immunotherapy beyond progression to maximize its potential benefit.

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 Ethics Committee of Zhejiang Cancer Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YX and YF contributed to the study conception and design. HL collected the patient samples and interpreted the data. HL performed the statistical analysis. YX was a major contributor in writing 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.2021.642883/full#supplementary-material

Supplementary Figure 1 | Kaplan-Meier curves of the PFS2 and OS of patients who halted or maintained ICI treatment. **(A)** Continued ICI (ICI maintenance) beyond the first disease progression after ICI treatment can lead to a significantly longer PFS2 than stopping ICI treatment (ICI halt) (12.9 *vs* 10.0 months; $p = 0.006$). **(B)** Continued ICI (ICI maintenance) beyond the first disease progression after ICI treatment can lead to a significantly longer OS than stopping ICI treatment (ICI halt) (26.3 vs. 18.5 months; $p = 0.001$).

Supplementary Figure 2 | Kaplan-Meier curves of the PFS2 and OS of patients with longer or shorter ICI treatment. The longer ICI (>7.5 months) treatment group showed superior mPFS2 **(A)** and mOS **(B)** compared with the shorter ICI (≤ 7.5 months) treatment group; the mPFS2 **(A)** were 16.6 and 8.3 months, and the mOS **(B)** were 29.8 and 12.7 months, respectively ($p < 0.0001$).

Supplementary Figure 3 | OS between different therapy groups in systemic progression cohort. (A) The mOS were 23.8 and 10.2 months ($p < 0.001$) in patients receiving systemic treatment (Yes) or best supportive care (No), respectively. **(B)** Subgroup analysis of OS in patients with different treatment strategies: ICI plus anti-angiogenesis or chemotherapy (4 + 1/3), chemotherapy only (3), anti-angiogenesis only (1), the mOS were 23.1, 23.8 and 12.4 months ($p = 0.6$), respectively.

Supplementary Figure 4 | OS between different therapy groups in oligoprogressive cohort. **(A)** The mOS were 26.4 and 20.8 months ($p = 0.006$) in patients treated with continued ICI and local radiotherapy with/without anti-angiogenesis (a1) and other strategies (others). (B) Subgroup analysis of OS in patients with different treatment strategies: ICI plus local therapy (a1), ICI plus anti-angiogenesis or chemotherapy (a2), local therapy only (a3), and anti-angiogenesis or chemotherapy (a4), the mOS were 26.4, 23.1, 10.8 and NR months ($p < 0.001$), respectively.

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SYSTEMATIC REVIEW
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Relating Gut Microbiome and Its Modulating Factors to Immunotherapy in Solid Tumors: A Systematic Review

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Background: Gut microbiome is proved to affect the activity of immunotherapy in certain tumors. However, little is known if there is universal impact on both the treatment response and adverse effects (AEs) of immune checkpoint inhibitors (ICIs) across multiple solid tumors, and whether such impact can be modulated by common gut microbiome modifiers, such as antibiotics and diet.

Methods: A systematic search in PubMed followed by stringent manual review were performed to identify clinical cohort studies that evaluated the relevance of gut microbiome to ICIs (response and/or AEs, 12 studies), or association of antibiotics with ICIs (17 studies), or impact of diet on gut microbiome (16 studies). Only original studies published in English before April 1st, 2020 were used. Qualified studies identified in the reference were also included.

Results: At the phylum level, patients who had enriched abundance in *Firmicutes* and *Verrucomicrobia* almost universally had better response from ICIs, whereas those who were enriched in *Proteobacteria* universally presented with unfavorable outcome. Mixed correlations were observed for *Bacteroidetes* in relating to treatment response. Regarding the AEs, *Firmicutes* correlated to higher incidence whereas *Bacteroidetes* were clearly associated with less occurrence. Interestingly, across various solid tumors, majority of the studies suggested a negative association of antibiotic use with clinical response from ICIs, especially within 1-2 month prior to the initiation of ICIs. Finally, we observed a significant correlation of plant-based diet in relating to the enrichment of "ICI-favoring" gut microbiome ($P = 0.0476$).

Conclusions: Gut microbiome may serve as a novel modifiable biomarker for both the treatment response and AEs of ICIs across various solid tumors. Further study is needed to understand the underlying mechanism, minimize the negative impact of antibiotics on ICIs, and gain insight regarding the role of diet so that this important lifestyle factor can be harnessed to improve the therapeutic outcomes of cancer immunotherapy partly through its impact on gut microbiome.

Keywords: gut microbiome, modulating factors, immunotherapy, solid tumors, diet, microbiota, antibiotics, cancer immunotherapy

INTRODUCTION

Immunotherapy such as using immune checkpoint inhibitors (ICIs) targeting PD-1/L1 and CTLA-4 has revolutionized our management of various cancer types including lung cancer (1, 2). However, only a subset of patients derive the benefit, which can be further limited by AEs especially immune-related AEs (irAEs) (3). The gut microbiome, due to its close interaction with immune system, has gained increasing attention for its potential role in cancer immunotherapy (4, 5). This is supported by several preclinical models (6, 7), as well as correlative studies at the human level including ours (8). However, several key questions remain to be addressed: (1) whether there is shared feature of gut microbiome that links to ICI response and AEs across various solid tumors; (2) whether antibiotics can affect cancer immunotherapy. This is important considering there are controversial results (6, 9–11), and antibiotic is such an inevitable gut microbiome modifier in the clinical setting; (3) whether diet, as one of the most important lifestyle factors, will have impact on cancer immunotherapy. We aim to investigate existing evidence that could help address these questions at the human level using a systematic review.

METHODS

This systematic review focused on bacterial gut microbiome. Different search keywords and their combination were used to extract relevant clinical studies from PubMed to address each proposed question. This was followed by a stringent manual selection to include only relevant studies, including those identified in the references. To explore the relationship between gut microbiome and clinical outcomes from ICIs, we used search keywords "gut microbiome" AND "cancer" AND "immunotherapy." To determine the impact of antibiotics on ICIs, we used keywords "antibiotics" AND "immunotherapy" AND "microbiome" AND "cancer." To investigate the impact of diet on gut microbiome, we used "diet" AND "gut microbiome" AND "healthy adult" with series of keyword refinements as detailed below. Only original clinical studies in human subjects written in English, with the publishing date before Apr 1st, 2020 (**Supplementary Table 1**) were included in this review to draw meaningful conclusion at the human level. Various key information such as gut microbiome data, clinical outcome (e.g., therapeutic response and AEs), timing and duration of antibiotic use, and diet were extracted and used to address separate but coherent questions with details below. Descriptive statistics was used to summarize the study findings. Fisher's test was used for the comparisons between 2 groups, and a $P < 0.05$ was considered statistically significant.

Abbreviations: AEs, adverse effects; ICIs, immune checkpoint inhibitors; irAEs, immune-related AEs; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; RCC, renal cell carcinoma; HCC, hepatocellular carcinoma; FMT, fecal microbiota transplantation; SCFA, short chain fatty acids.

RESULTS

Common Features in Gut Microbiome Correlate With the Treatment Response and AEs of ICIs Across Various Solid Tumors

Using search keywords "gut microbiome" AND "cancer" AND "immunotherapy," a total of 240 articles were retrieved from

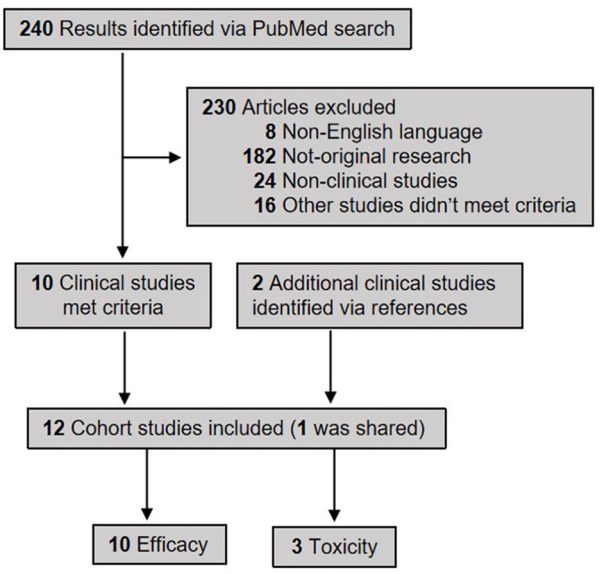


FIGURE 1 | The PRISMA flow diagram of selecting publications to study the correlation of gut microbiome with the efficacy and adverse effects of ICIs across various solid tumors. In total 10 studies were included for the analysis of gut microbiome in correlating with the therapeutic efficacy of immunotherapy, and three studies for toxicity (adverse effects).

PubMed. With stringent manual screening and inclusion of two additional studies from the references, a total of 12 clinical studies were identified that meet our criteria to study the role of gut microbiome in cancer immunotherapy (**Figure 1**). The vast majority are prospective studies. Among them, 10 studies (6, 9, 12–19) had response/efficacy data and three studies (17, 20, 21) had AEs data using ICI therapy, and one study had both (17). Of note, the documented AEs in that three studies (17, 20, 21) were virtually all irAEs. The types of solid tumor involved include melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), renal cell carcinoma (RCC) and hepatocellular carcinoma (HCC). There were 433 cancer patients (age range, 21-92 years-old) from four countries: USA (four studies), France (three studies), China (two studies), and Japan (one study) included in studies relevant to therapeutic response/efficacy; and 86 subjects (age range, 28-85 years-old) from the countries of USA, France and China included in studies relevant to the AEs of ICI treatment.

We extracted the taxa data of gut microbiome and plotted on phyloT. As shown in **Figure 2**, at the phylum level, it is clear that the enrichment of *Firmicutes* and *Verrucomicrobia* are correlated with better clinical outcome (labeled in green; related to better treatment response; and/or longer survival), whereas increased abundance in *Proteobacteria* was clearly associated with poor response (labeled in red). Although enrichment of *Bacteroidetes* correlated to poor response in some studies, opposite association and contradictory findings (labeled in gray) were also noticed in some other studies. Similarly, a mixed association of *Actinobacteria* to ICI treatment response was noticed.

However, regarding the potential link of gut microbiome to the AEs from ICIs, we noticed that the enrichment of *Firmicutes* interestingly correlated to higher incidence of AEs (essentially all irAEs, colored in red). This is reminiscent of clinical observations that patients who develop ICI AEs seem to have better treatment response (22). In contrast, *Bacteroidetes*, which is believed to be associated with less response, also correlated to less AEs (labeled in green, **Figure 3**).

The Potential Impact of Antibiotics on the Therapeutic Effect of ICIs

Noticing the association of gut microbiome with ICI treatment response, we questioned if antibiotics, as potent modifiers of gut microbiota, could potentially affect the treatment response from ICIs. Using search keywords "antibiotics" AND "immunotherapy" AND "microbiome" AND "cancer," we identified 17 eligible studies (**Supplementary Figure 1**) including two prospective (23, 24) and 15 retrospective studies (9–11, 25–36). There were in total 2,593 participants with various solid tumors including lung cancer, melanoma, RCC, HCC, colorectal cancer, head and neck cancer, bladder cancer, gastric cancer, esophageal cancer, cervical cancer, and others. Among them, 29.9% (775) of them received antibiotics treatment, 15 out of 17 received broad-spectrum antibiotics while two did not report the types of antibiotics.

As shown in **Figure 4A**, majority of these studies supported the hypothesis that the use of antibiotics has negative impact on the clinical outcome in patients receiving ICI treatment. However, there were also a few studies that suggested no obvious association or impact. Interestingly, two prospective studies (23, 24) and one retrospective study (25) provided seemingly different results (negative vs. no impact) when different timing of antibiotic exposure was put into consideration, suggesting that the timing and possibly the duration of antibiotics during ICI treatment are potentially important and will need further studies to clarify its impact.

In order to validate this hypothesis, we isolated the effect of the timing and duration of antibiotic exposure from all studies. **Supplementary Figure 3** showed individual studies that exhibited either negative (labeled with black bars) or no association (labeled in gray bars) with ICI treatment. Among them, two studies (23, 24) were prospective (labeled with *). Across all studies, it clearly demonstrated that only antibiotic exposure within 2 months prior to the initiation of ICIs universally exhibited negative impact on treatment response of ICIs (**Figures 4B,C**), except one study (10) (**Supplementary Figure 3**).

Diet Could Potentially Affect the Efficacy of Cancer Immunotherapy

Using search keywords "diet" or "nutrition," "microbiome," "cancer" and "immunotherapy," and their combinations, we were not able to extract sufficient number of clinical studies that directly link diet to cancer immunotherapy, including those published in abstract format (37), which is suggestive of an unmet need in this area. Since gut microbiome impacts cancer immunotherapy, we then investigated whether diet will have effect on gut microbiome that could potentially affect cancer immunotherapy. Based on **Figure 2** and published data, here we define "ICI-favoring" diet as those that enrich *Firmicutes* or *Verrucomicrobia*, or reduce the abundance of *Proteobacteria*, or increase α diversity in gut microbiota, and the "ICI-unfavoring" diet as those that have the opposite effects.

To minimize confounding factors (especially various disease status), we used search terms "diet" AND "gut microbiome" AND "healthy adult" and included only clinical studies in healthy participants that have detailed diet and gut microbiome information (**Supplementary Figure 2**). We identified 16 eligible clinical studies (38–53) that included in total 771 subjects. Among them, 428 were females and 343 were males. Their age ranged 18–72.4 years and BMI ranged 19–36.6 kg/m^2. These clinical studies were conducted in five countries including USA, China, Germany, UK and Belgium. We broadly categorized diet into plant-based diet which mainly contained whole grain, brassica vegetables, walnut and almond, etc; and animal-based diet which used red meat, animal fat and cheese, etc. There are only four studies using animal-based diet (40, 41, 47, 53). Although they also contained non-animal-based diet component,

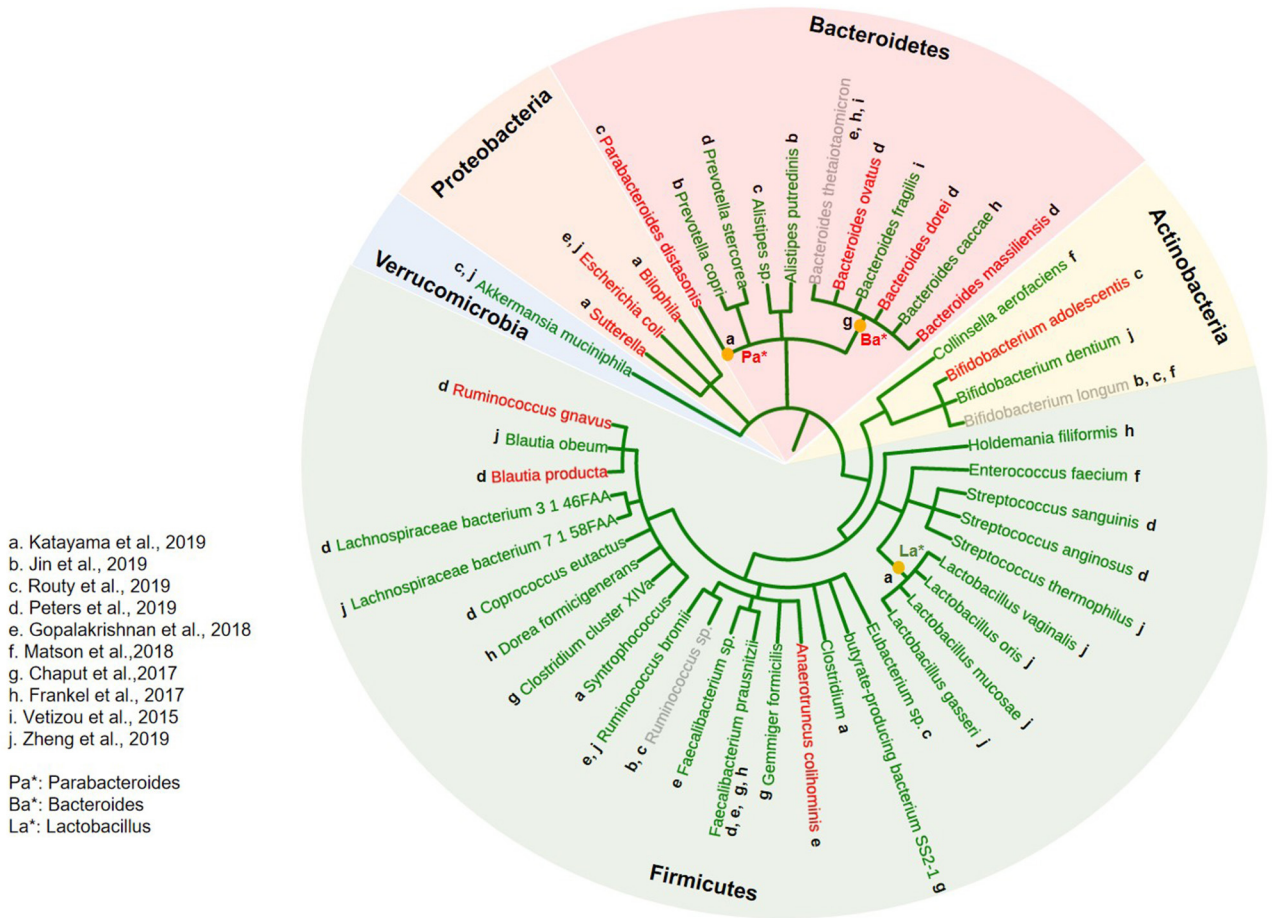


FIGURE 2 | Correlation of gut microbiome to the treatment response of ICIs across various solid tumors. A phylogenetic tree was constructed using the phyloT software (https://phylot.biobyte.de) to capture and categorize all bacterial taxa reported to be associated with the treatment response from ICI in clinical studies across various solid tumors, ranking from phylum to species inside-out. Bacteria correlated to better response were labeled in green, and poor response in red. Those with mixed reports were labeled in gray. The lowercase alphabetical letters next to each bacterium indicate the individual studies from which bacterial taxa information was derived. The asterisks (*) indicate identified bacteria taxa at the genus level.

we were able to precisely derive data that are only relevant to animal-based diet.

Figure 5A in each category, depicts increase or decrease in relative abundance of *Firmicutes* or *Verrucomicrobia* or *Proteobacteria* or α diversity with demonstration of corresponding plant-based diet (labeled as solid dot) and animal-based diet (labeled as hollow circle), respectively. Using above defined "ICI-favoring" and "ICI-unfavoring" criteria, we found that three animal-based diet studies were "ICI-unfavoring" and none were "ICI-favoring." Among the 12 plant-based diet studies, we found five were "ICI-favoring" and 1 was "ICI-unfavoring." In summary, plant-based diet is found to be significantly associated with "ICI-favoring" gut microbiome, whereas animal-based diet is the opposite (**Figure 5B**, $p = 0.0476$). Diet studies that have mixed association, for example a reduced abundance in both the *Firmicutes* and *Proteobacteria* as shown in study n (51) in **Figure 5A**, were not included in the statistical analysis. We have also looked into various dietary patterns such as Mediterranean diet, Western diet, high-fiber diet, etc., however we were able to identify only very few relevant studies for us to draw meaningful conclusions.

DISCUSSION

Despite the great success of cancer immunotherapy using ICIs, their therapeutic benefits are limited by either various resistance mechanisms (54) or irAEs (3). Gut microbiome, due to its proven role in cancer development and immune regulation, has gained increasing expectation as a potential armamentarium to further improve cancer immunotherapy. It is speculated that gut microbiota could potentially affect the efficacy of ICIs through the modulation of immune checkpoints expression; dendritic cell function; lymphocyte homing, circulation and recruitment; as well as the production of critical metabolites such as short chain fatty acids (SCFA), etc. (55, 56). Furthermore,

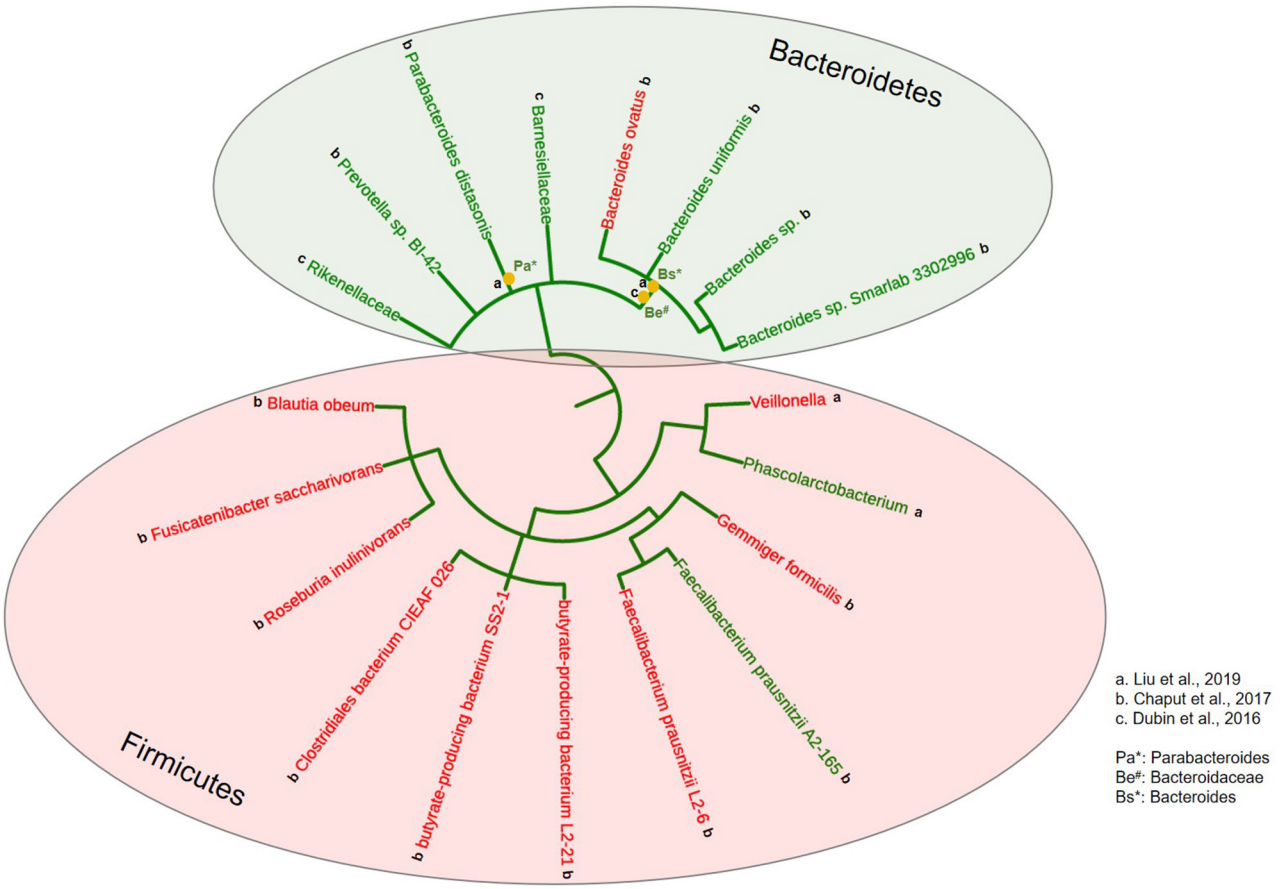


FIGURE 3 | Correlation of gut microbiome to the toxicity of ICIs across various solid tumors. A phylogenetic tree was constructed using the phyloT software (https://phylot.biobyte.de) to capture and categorize all bacterial taxa reported to be associated with the adverse effects from ICI treatment in clinical studies across various solid tumors, ranking from phylum to species inside-out. Bacteria correlated to less toxicity were labeled in green, and more toxicity in red. The lowercase alphabetical letters next to each bacterium indicate the individual studies from which bacterial taxa information was derived. The asterisks (*) indicate identified bacteria taxa at the genus level.

gut microbiota could influence host systemic immunity via cytokine secretion, primed lymphocyte circulation and antigen cross-reactivation induced tissue targeting (56). In consistent with this, we have recently shown that certain gut microbiota correlates significantly to ICI response in NSCLC patients (8), which echoes the findings from other groups (9, 15, 16), as well as preclinical mouse models (7). More importantly, a very recent phase 1 trial has demonstrated fecal microbiota transplantation (FMT) promoted response in ICI-refractory melanoma patients, which was associated with favorable changes in both the gut and tumor microenvironment (57). However, to better harness gut microbiome for clinical applicability, we need to understand whether there is shared gut microbiome feature across various solid tumors treated with ICIs, and whether common gut microbiome modifiers could have impact on ICI therapy.

Using series of systematic review, we first noticed that the enrichment of *Firmicutes* clearly correlated with better ICI response across various solid tumors. This is consistent with a previous report from Gopalakrishnan et al. whose work covered both solid and hematologic tumors (58). In addition, the reciprocal changes in abundance of *Verrucomicrobia* and *Proteobacteria* respectively, was found associated with better ICI response. Although further mechanistic studies are warranted to explain such observations, some speculated that the positive association of *Firmicutes* could in part due to their critical role in producing SCFA, a metabolite that has regulatory effect on inflammation and T cell differentiation (59–61). This is especially true for the fermentation of fiber to SCFA as the necessary enzymatic processes involved, which are largely dependent upon bacteria within the *Clostridia* class in the *Firmicutes* phylum (62). In agreement with this, a recent clinical study demonstrated that elevated fecal SCFA concentration significantly correlates with better clinical outcome from anti-PD-1 treatment across various solid tumors (63). This may also explain the positive correlation of mucin-degrading bacteria *Akkermansia muciniphila* (phylum *Verrucomicrobia*) to ICI response since it produces SCFA (both propionate and acetate) (64, 65). The negative association of *Proteobacteria* with ICI response is likely due to its close link to

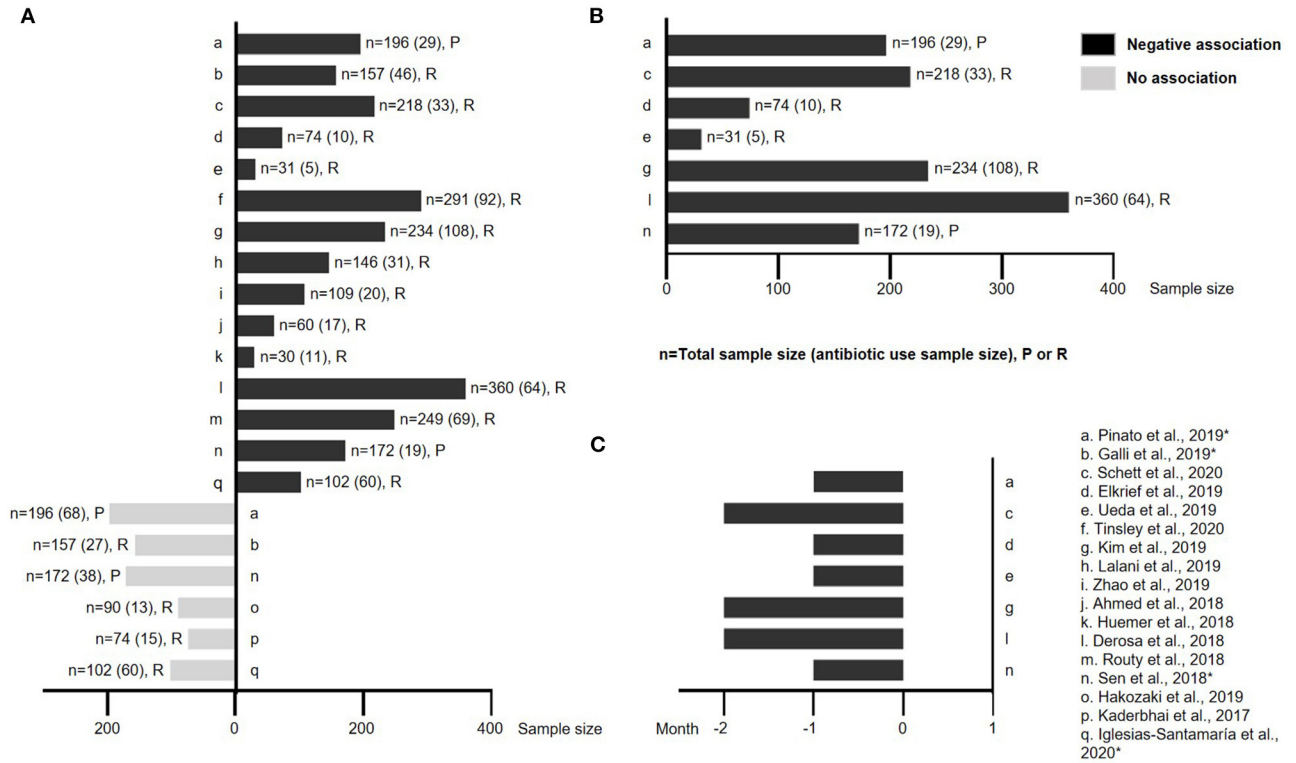
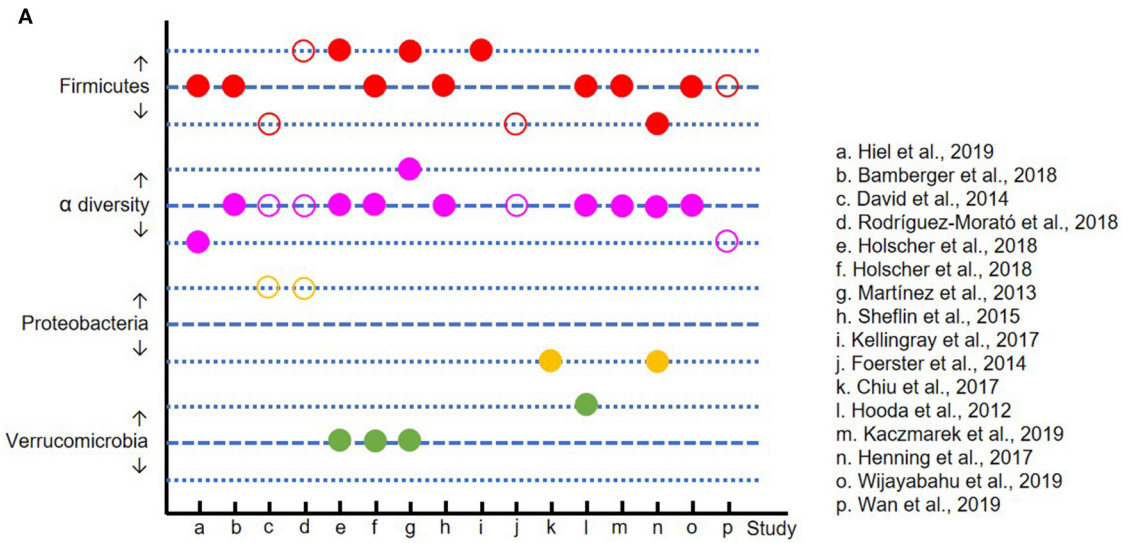


FIGURE 4 | The impact of antibiotic exposure on ICI treatment across various solid tumors. **(A)** A schematic illustration showing studies with either negative or no association between antibiotic use and clinical outcome from ICI treatment. The study name, sample size and retrospective vs. prospective nature are all labeled. **(B)** Studies (including both retrospective and prospective) that have antibiotic use within 2 months prior to the initiation of ICI treatment were universally associated with poor clinical outcome. **(C)** Detail timing and duration of antibiotic use for studies shown in **(B)**. **n**: sample size; **P**: prospective study; **R**: retrospective study; *: mixed results based on the timing of antibiotic use.

dysbiosis (66), which could account for the immune dysfunction in some non-responders to ICI therapy (5, 9, 15).

Although there are studies correlating phylum *Bacteroidetes* with poor response from ICIs (12, 17), we also observed several other studies were suggestive of a positive impact (6, 13, 18). In fact, an earlier preclinical study demonstrated a cause and effect role of certain *Bacteroidetes* (e.g., *B. thetaiotaomicron* or *B. fragilis*) in enhancing the therapeutic effect of anti-CTLA-4 agent (6). In addition, *Bacteroidetes* was found to digest insoluble fibers and mucins and provide SCFA and other metabolites to *Firmicutes*, suggesting its supporting role (67). This is consistent with a recent study using 11 bacteria strain mixture (11-mix: 7 *Bacteroidetes*, 3 *Firmicutes* and 1 *Fusobacteria*): when inoculated into germ-free mice, the 7 *Bacteroidetes*-mix failed to induce IFNγ^+ CD8 T cells, whereas the 4-mix (3 *Firmicutes* and 1 *Fusobacteria*) displayed a significantly better induction capacity. However, the 4-mix alone was not sufficient to achieve the full inductive effect of the 11-mix for a maximal anti-cancer immunity (68). Interestingly, in our study, *Bacteroidetes* enrichment clearly correlated with less ICI-induced toxicity whereas *Firmicutes* abundance was obviously linked to increased incidence of irAEs. While such finding is reminiscent of our clinical observation that patients who experience greater irAEs tend to have better response from ICIs (22), it also supports the concept of using appropriate mix of *Firmicutes* and *Bacteroidetes* to enhance immunotherapy response yet mitigate irAEs (68).

As the ICI-favoring *Firmicutes* are the dominant gut microbial phyla, it is not surprising to see a negative impact on ICIs with the use of broad-spectrum antibiotics as *Firmicutes* will likely be affected most. In addition, antibiotics can induce dysbiosis (69). Our study has demonstrated that the timing and/or duration of antibiotics are critical, which probably explains the discrepancy observed in different studies, as certain period of time is required for gut microbiome to recover after antibiotics exposure. Interestingly, a recent study in healthy adults found that it took about 1.5 months for the gut microbiota of the subjects to recover to near-baseline composition after 4-day intervention with a cocktail of three antibiotics: meropenem, gentamicin and vancomycin (70). This finding is a perfect match to what we have observed in this study that antibiotics exposures within 2 months prior to the initiation of ICIs were universally associated with poor clinical outcome (23, 24, 26–28, 30, 35). In consistent with this, using a more quantitative analysis, Wilson B et al. found through their meta-analysis that the negative



B

	ICI-favoring	ICI-unfavoring	Total
Animal-based	0	3	3
Plant-based	5	1	6
Total	5	4	9
		***p* =0.0476**	

FIGURE 5 | The impact of dietary intervention on gut microbiome. To minimize the confounding factors, only studies on healthy adults were included. **(A)** The alterations of gut microbiome after dietary intervention are displayed in 3 lines, which represent increase, no change and decrease in each category (red: *Firmicutes*; purple: α diversity; orange: *Proteobacteria*; and green: *Verrucomicrobia*). Solid and hollow circles represent plant- and animal-based diet, respectively. **(B)** A Fisher's exact test to compare the effect of plant- vs. animal-based diet on the enrichment of "ICI-favoring" vs. "ICI-unfavoring" gut microbiota ($P = 0.0476$).

impact of antibiotics on the overall survival of patients with solid malignancies treated with ICI was greatest when antibiotic exposures was within 42 days prior to the initiation of ICI (71). Since antibiotic use is quite common among cancer patients, it will be interesting to see whether narrow-spectrum antibiotics could have selective effect on ICI response, especially considering the vast majority of *Firmicutes* bacteria are Gram-positive. In addition, if the use of antibiotics is inevitable, it will be important to understand whether the use of pre- and probiotics will have protective value under this situation.

Since diet is considered as a pivotal determinant of gut microbiota community among various host-endogenous and host-exogenous factors, we sought to determine its impact on ICIs. Despite the lack of direct evidence, we did observe that plant-based diet enriched "ICI-favoring" gut microbiome, represented as increased *Firmicutes* or *Verrucomicrobia* or α diversity, or reduced abundance of *Proteobacteria*. Such finding is consistent with a recent study on melanoma patients demonstrating that the response to immunotherapy can be influenced by dietary manipulation (37)—patients who consumed a high-fiber diet (plant-based) were about five times as likely to respond to anti–PD-1 treatment compared to patients who consumed a low-fiber diet (37). Further studies are warranted to clarify the potential value of diet/nutrition in both the treatment response and irAEs of cancer immunotherapy. In addition, it will be critically important to understand how particular diet affects gut microbiota and its metabolites before it can be better harnessed to modulate gut microbiome and its impact on cancer immunotherapy.

This study is based on systematic literature review, and therefore it is retrospective in nature. In addition, it is subject to selection bias, for example only original articles in English that are published on PubMed were used.

CONCLUSIONS

There is shared feature of gut microbiome that correlates with the outcome of immunotherapy across various solid tumors, which can be potentially affected by antibiotics and diet. Further mechanistic studies are warranted to clarify their role to better harness gut microbiome for the improvement of cancer immunotherapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JZ conceived and designed the study, analyzed the data, guided essay writing, and provided critical revisions for this manuscript. CheH conducted database searches, extracted and analyzed the data, drew the figures, and provided the initial draft and prepared the manuscript. ML, BL, and HZ contributed to vital assistance in analyzing data and generating the figures. QD, XF, KM, ChaH, PN, FW, WS, SU, and CZ provided the consultation and critical revision of the manuscript for important intellectual content. All authors 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.2021.642110/full#supplementary-material

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Robust Prediction of Immune Checkpoint Inhibition Therapy for Non-Small Cell Lung Cancer

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Background: The development of immune checkpoint inhibitors (ICIs) is a revolutionary milestone in the field of immune-oncology. However, the low response rate is the major problem of ICI treatment. The recent studies showed that response rate to single-agent programmed cell death protein 1 (PD-1)/programmed cell death-ligand 1 (PD-L1) inhibition in unselected non-small cell lung cancer (NSCLC) patients is 25% so that researchers defined several biomarkers to predict the response of immunotherapy in ICIs treatment. Common biomarkers like tumor mutational burden (TMB) and PD-L1 expression have several limitations, such as low accuracy and inadequately validated cutoff value.

Methods: Two published and an unpublished ICIs treatment NSCLC cohorts with 129 patients were collected and divided into a training cohort (n = 53), a validation cohort (n = 22), and two independent test cohorts (n = 34 and n = 20). We identified six immune-related pathways whose mutational status was significantly associated with overall survival after ICIs treatment. Then these pathways mutational status combined with TMB, PD-L1 expression and intratumor heterogeneity were incorporated to build a Bayesian-regularization neural networks (BRNN) model to predict the ICIs treatment response.

Results: We firstly proved that TMB, PD-L1, and mutant-allele tumor heterogeneity (MATH) were independent biomarkers. The survival analysis of six immune-related pathways revealed the mutational status could distinguish overall survival after ICIs treatment. When predicting immunotherapy efficacy, the overall accuracy of area under curve (AUC) in validation cohort reaches 0.85, outperforming previous predictors in either sensitivity or specificity. And the AUC in two independent test cohorts reach 0.74 and 0.80.

Conclusion: We developed a pathway-model that could predict the efficacy of ICIs in NSCLC patients. Our study made a significant contribution to solving the low prediction accuracy of immunotherapy of single biomarker. With the accumulation of larger data sets, further studies are warranted to refine the predictive performance of the approach.

Keywords: immunotherapy, non-small cell lung cancer, biomarkers, immune pathway, neural network, prognosis

INTRODUCTION

Immunotherapy is emerging as a beneficial tool for cancer treatment by activating the immune system to produce antitumor effects (1). Recently, the most advanced approach to therapeutically utilize the antitumor activity is via immune checkpoint inhibitors (ICIs) (2). Immune checkpoint inhibitors work by releasing a natural brake on patient's immune system so that immune cells called T cells to recognize and attack tumors (3). Among the ICIs, programmed cell death protein 1(PD-1)/programmed cell death-ligand 1(PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitors showed promising therapeutic outcomes, and some have been approved for numerous cancer therapy, such as melanoma, renal cell carcinoma (RCC), and non-small cell lung cancer (NSCLC) (4, 5). However, ICIs are not universally effective for all patients, and many patients fail to respond to ICIs due to intrinsic resistance or have an initial response followed by disease progression due to acquired resistance (6). For example, response rates to single-agent PD-1/PD-L1 inhibition in unselected patients with melanoma, NSCLC, and RCC are 40% (7, 8), 25% (8, 9), and 19% (10), respectively (11). To identify patients who are more likely to respond to PD-1/PD-L1 blockade as well as other immunotherapeutics, researchers defined several biomarkers to predict the response of immunotherapy in cancer treatment. The commonly used biomarkers include tumor mutational burden (TMB) and PD-L1 expression (11, 12). Patients with a higher TMB or higher PD-L1 expression have a higher likelihood of immunotherapy response. Another novel statistical value, mutant-allele tumor heterogeneity (MATH), has been documented that is not only as a measure of intratumor genetic heterogeneity but also can be used as a biomarker to predict the response of treatment for patients (13–16). In addition, recent studies have shown that some pathways, such as IFN-gamma, NF-κb, and Wnt, are cancer-related immune-regulation pathway, which may be potential indicators to explore the effect of immunotherapy (17–20).

Nevertheless, it has been documented that the available biomarkers have several limitations (21, 22), such as low accuracy, and inadequately validated cutoff value, and previous studies only use one or two of them independently in immunotherapy prediction (23). Therefore, we developed a pathway-model that included TMB, PD-L1, MATH, and immune-related pathway to predict the efficiency of ICIs, especially in NSCLC, which is the leading cause of cancer-related morality worldwide (24). The pathway-model did not only have a high accuracy in published cohorts but also be proven to have an effective prediction ability in GloriousMed cohort with 20 NSCLC patients. This study made a significant contribution to solving the low prediction accuracy of immunotherapy of single biomarker.

MATERIALS AND METHODS

GloriousMed Cohort

Twenty patients with non-small cell lung cancer treated with PD-1/PD-L1 inhibitors in The Second Xiangya Hospital, Central South University who had genomic profiling of whole exome sequencing (WES) before treatment were included in our GloriousMed cohort (**Supplementary Table S1**).

TMB was defined as the total number of somatic mutations per exome in megabases. PD-L1 staining was evaluated centrally by IHC using 22C3 antibody and an automated staining procedure developed by Dako. The percentage of PD-L1 expression was scored by a qualified pathologist in samples with a minimum of 100 viable tumor cells.

Objective response was assessed by investigator-assessed RECIST 1.1 criteria every 6 weeks (two cycles of ICB administration). The complete response (CR) or partial response (PR) was considered as responders, whereas patients with stable disease (SD) or progressive disease (PD) were considered as non-responders.

All patients collection and usage were in accordance with the principles of the Declaration of Helsinki and approved by the Institution Review Board of The Second Xiangya Hospital, Central South University. The written informed consent for sample acquisition was obtained from all patients. All data were deidentified.

Public Cohorts

Three independent public cohorts including Hellmann cohort (25), Rizvi cohort (26), and Samstein cohort (27) were also used in this study. The data for the three independent cohorts were retrieved from published articles (**Supplementary Table S2**). Hellmann cohort included 75 NSCLC patients treated with combined PD-1 and CTLA-4 blockade. Rizvi cohort included 34 NSCLC patients that treated with pembrolizumab. The Samstein cohort contained 1,662 patients received immunotherapy from 11 different cancers.

WES Sequencing

DNA was extracted from FFPE-fixed tumor tissue using QIAamp DNA FFPE Tissue Kit (Qiagen), and Genomic DNA (gDNA) was extracted from white blood cells using the Blood Genomic DNA Mini Kit (Cwbiotech). Integrated DNA Technologies's xGen Exome Research Panel v1.0 according to the standard procedures (IDT) were used to capture whole exome. For each sample, 200 to 500 ng FFPE DNA or 500 ng gDNA was then used for library preparation and quantification guided by KAPA Hyper Prep protocols (KAPA). Libraries were then purified by AMPure XP (Beckman) and quantified by Qubit™ dsDNA HS Assay Kit (Thermo Fisher). Final library was sequenced on the Illumina Novoseq6000 (PE150). Sequencing adapters were trimmed by Trimmomatic from the raw data (28). The reads after adapter trimming were then aligned with the human reference genome hg19 by BWA (29). Duplicated reads were removed by Picard. Mapped reads were also realigned to the genome by Genome Analysis Tool Kit. Somatic mutations were called by Mutect2 with a paired workflow. Variants were then annotated by ANNOVAR and self-development code (30). An in-house script was used to verify the human identity concordance of paired samples. Somatic mutations were filtered with the following rules: (1) base quality value ≥20; (2) mutation reads depth ≥10; (3) variant allele frequency ≥5%; (4) reads supporting variation <4 and frequency <2% in normal, tumor abundance/normal abundance ≥8;

(5) no strand bias (GATK parameter FS > 60 for SNP and FS >200 for indel); (6) discard synonymous mutations.

Quantitative and Statistical Analyses

TMB and PD-L1 expression of Hellmann cohort and Rizvi cohort were retrieved from published articles. MATH was calculated through R package maftools for GloriousMed, Hellmann and Rizvi cohorts (31). Correlation among TMB, MATH, and PD-L1 expression (%) were examined by the Pearson rank correlation method. Correlation between TMB or MATH and grouped PD-L1 expression were examined by the Wilcoxon signed-rank test.

The overall survival (OS) was defined from the start of ICIs treatment until death due to any cause. And the progression-free survival (PFS) was defined as the time from the start of ICIs treatment until disease progression. Of notes, the Samstein cohort merely published OS data and Rizvi cohort provided PFS data. The Kaplan-Meier method was used to estimate OS or PFS, and the log-rank test was used to compare the survival curves. All tests with a p value ≤ 0.05 were considered statistically significant.

Immune-Related Pathway Selection

The detailed profiles of genes involved in HRR, MMR, BER, JAK, MAPK, PI3K, NF-κB, and Wnt pathways were listed in **Supplementary Table S3**. At first, mutational status of aforementioned six immune-related pathways in every sample was classified into two categories: the first one assigned with 0 (no non-synonymous mutation) and the second with 1 (at least one non-synonymous mutation). Then, DDR pathway mutation status of each sample was classified into three groups based on the mutational status of HRR, MMR, and BER. "N" represented no mutation in HRR, MMR, or BER, "C" was stood for co-mutation between HRR and MMR or BER, and "S" was other cases. In addition, the mutational status of PI3K, JAK, and NF-κB were integrated as one variable by summing the mutational status.

Model Construction

Three models were constructed, one model with TMB, PD-L1 expression, MATH, and immune-related pathways, called "pathway-model"; a second with TMB, PD-L1 expression, and MATH, called "tri-model"; the last one, called "bivariate-model", with TMB and PD-L1 expression (**Table 1**). Both TMB and MATH were z-score normalized. PD-L1 expression was stratified as 0% (Z), 1%-49% (L), ≥50% (H), or unknown (N). And immune-related pathways were processed according to Immune-Related Pathway Selection. All of the models were trained via Bayesian Regularized Neural Networks (BRNN) algorithm using corresponding variables with 2 layers and default hyperparameters from R package caret (32), and the resampling method "boot" was used to choose the optimal model. The cutoff value of single-factor variable, TMB, PD-L1 expression and MATH was estimated by BRNN algorithm as well. Fifty-three patients of the Hellmann cohort were used as the training set, and remaining 22 patients were validation set. Rizvi cohort and GloriousMed cohort were processed as above description and were used as testing cohort.

Model Performance Evaluation

Receiver operating characteristic (ROC) curves were constructed with the predictor estimated from each of the previous models and single-factor variables with roc function of R package pROC (33). Benefit probability of each patient was extracted from prediction results, and DCB/NDB information was provided by the cohorts. Differences between DCB and NDB with benefit probability were examined by the Wilcoxon signed-rank test.

Comprehensive Analysis of TCGA LUAD and LUSC Cohorts

The clinical information, RNA expression, mutational status and protein array of The Cancer Genome Atlas Lung Adenocarcinoma (TCGA LUAD) and Lung Squamous Cell Carcinoma (LUSC) patients were retrieved from TCGA database. The patients with EGFR exon 18–21 mutations and ALK gene fusions were filtered to avoid make a disturbance for the analysis. In the signature score analysis, the expression of genes in a signature was normalized in the form of fragments per kilobase of exon model per million mapped fragments (FPKM). Then, a principal component analysis (PCA) was performed, and PC1 was extracted to serve as gene signature score (34). The 18 signatures and their gene sets were summarized from published papers (34–38). The significantly differential expression analysis was based on DESeq2 (39). The row counts of LUAD and LUSC patients were used as input for DESeq2. The differential expression genes were defined as the genes with absolutely log2Foldchange ≥ 1 and p-value ≤ 0.05. The oncoplot of top 30 mutated genes were drawn by using R package maftools (31).

RESULTS

TMB, PD-L1 Expression, and MATH Are Independent Variables

The previous studies documented that higher TMB or PD-L1 expression correlated with better outcomes as compared with lower TMB or PD-L1 expression (11, 12, 25, 40). However, in 70 of 75 patients from Hellmann cohort who had all three biomarkers data, correlation between TMB and PD-L1 expression was not significant (R=-0.14, p-value=0.24). TMB of some patients was more than 10 but PD-L1 expression was less than 25% (**Figure 1A**). The results might reveal the biomarkers were not consistent in response prediction of ICIs treatment. In the meantime, the novel biomarker MATH was not significantly correlated with PD-L1 expression (R = −0.2, p-value = 0.099) or TMB (R = 0.14, p-value = 0.24) as well (**Figures 1B, C**). We further explored the correlation between stratified PD-L1 expression and TMB or MATH by stratifying PD-L1 expression as 0% (Z), 1% to 49% (L), ≥50% (H), and unknown (N). Neither MATH nor TMB showed a significant difference with any PD-L1 expression groups (**Figures 1D, E**). The Rizvi and GloriousMed cohort showed the consistent correlation results as well (**Supplementary Figure 1**). This lack of correlation suggested that TMB, PD-L1 expression,

TABLE 1 | Models and variables.

Model	Variable
Bivariate-model	TMB and PD-L1 expression
Tri-model	TMB, PD-L1 expression and MATH
Pathway-model	TMB, PD-L1 expression, MATH and immune-related pathways

TMB, tumor mutational burden; PD-L1, programmed cell death-ligand 1; MATH, mutant-allele tumor heterogeneity.

and MATH are independent predictive measures of response to ICIs treatment, and a robust model should be constructed to unify these variables.

Mutational Status of Immune-Related Pathway Can Act as Candidate Biomarkers

A prior study has shown that co-mutation information of DNA damage response (DDR) pathway can be used as a predictor of response to immune checkpoint blockade, and the mutation of the DDR solved the problem of difficulty in determining an optimal TMB threshold (22). This finding provided a new way to predict the response of immunotherapy. Besides DDR pathway, we selected six pathways, homologous recombination repair (HRR), Janus kinase (JAK), mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Wnt, through literature survey, which are associated with tumor immunity or immunotherapy escape (41, 42). We also collected the mutational status of these pathways from Samstein cohort treated with ICIs (27) and explored its correlation with the overall survival (OS). The results showed that patients with mutations in any of six pathways had better survival than those without mutation (**Figure 2**). Furthermore, the results also revealed the selected pathways could be used as biomarkers to distinguish the prognosis for ICIs treatment.

Pathway Model Is the Best Model to Predict the Efficiency of ICIs Treatment

We extracted 70% patients from Hellmann cohort, which totally included 75 NSCLC patients, as training data set (25) and the rest 30% patients were used to validate the models. Three different

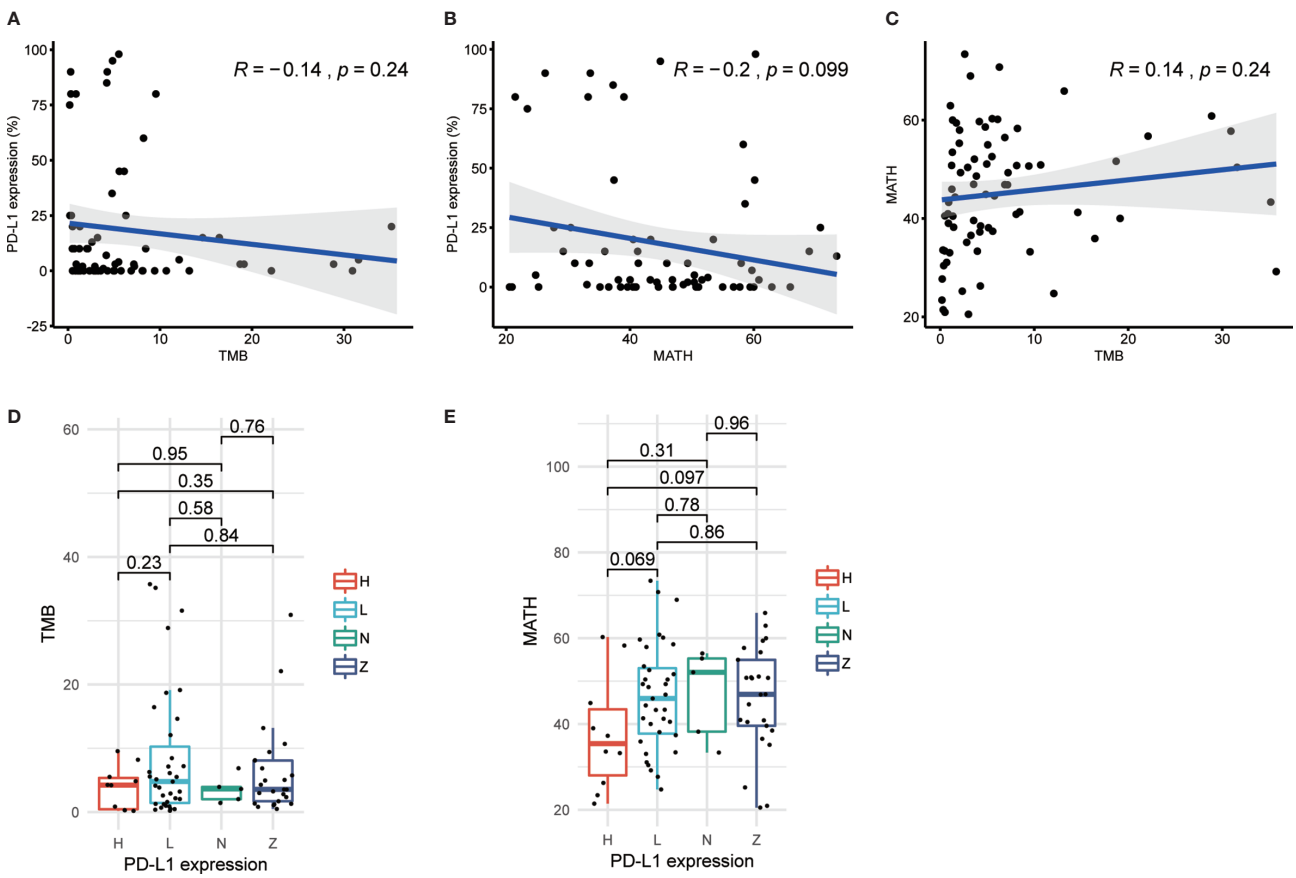

FIGURE 1 | Tumor mutational burden (TMB), programmed cell death-ligand 1 (PD-L1) expression and mutant-allele tumor heterogeneity (MATH) are independent from each other in Hellmann cohort. **(A)** Scatterplot between TMB and PD-L1 expression (%). **(B)** Scatterplot between MATH and PD-L1 expression (%). **(C)** Scatterplot between TMB and MATH. **(D)** Boxplot of TMB and PD-L1 expression. **(E)** Boxplot of MATH and PD-L1 expression. The R value of **(A–C)** represents Pearson correlation coefficient.

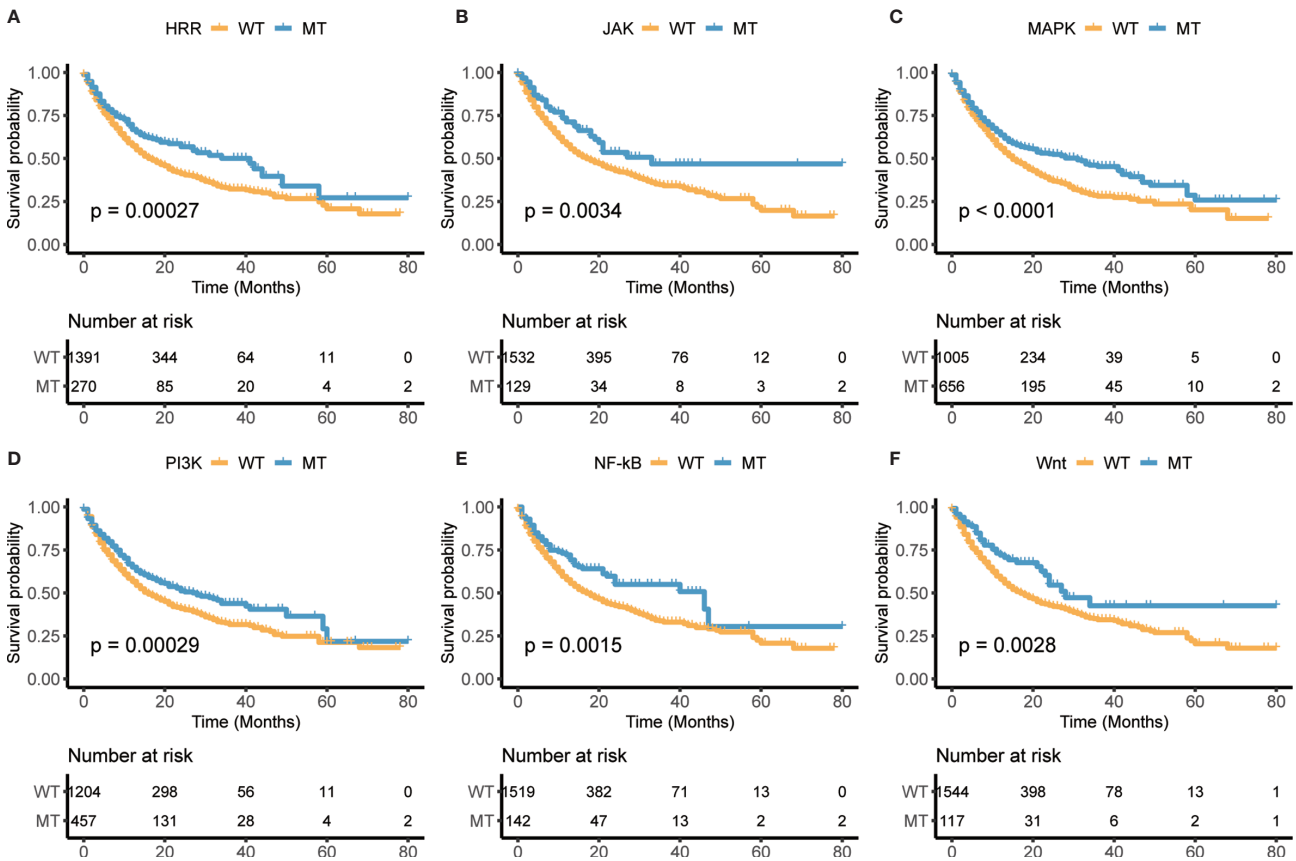

FIGURE 2 | The mutational status of selected immune-related pathways are significantly associated with overall survival (OS) in Samstein cohort. **(A)** Homologous recombination repair (HRR). **(B)** Janus kinase (JAK). **(C)** Mitogen-activated protein kinase (MAPK). **(D)** Phosphoinositide 3-kinase (PI3K). **(E)** Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). **(F)** Wnt.

models were trained by using the training data set with different variables and were adjusted with clinical benefit as outcomes (**Table 1**). The pathway-model contains seven variables, including TMB, PD-L1 expression, MATH and the mutational status of six immune-related pathways (**Figure 3**). The mutational status of JAK, MAPK, and PI3K was integrated into one variable to improve the prediction accuracy. ROC curves based on the predictor for each of the three models estimated on Hellmann cohort (22 patients) were available and the results showed that the pathway-model was more predictive than other two models (AUC is 0.87, 0.83, and 0.59 for pathway-, tri-, and bivariate-model). The AUC of pathway-model was higher than single-factor variables containing TMB, PD-L1 expression, and MATH as well (AUC is 0.56, 0.49, and 0.69 for TMB, PD-L1 expression, and MATH) (**Figure 4A** and **Table 2**). We also checked the prediction benefit probability, a quantitative output generated from the model which represents the likelihood of immunotherapy response, of each patient compared with real clinical benefit information among three models. The benefit probability generated from pathway-model and tri-model are significantly higher in DCB group than in NDB group (p-value is 0.0024 for pathway-model and 0.0066 for tri-model), however, the median benefit probability of pathway-model (0.70) was higher than tri-model (0.46). The difference of benefit probability was not significant in other models and single factors (**Figure 4B**).

We further tested the predictive ability of pathway-model in Rizvi cohort (26), consisting of 34 NSCLC patients treated with pembrolizumab, with all predictive variables and clinical benefit information available. The results showed that pathway-model could more accurately predict the clinical benefit of ICIs than other two models and single-factor variables (AUC is 0.74 for pathway-model, 0.67 for tri-model, 0.68 for bivariate-model, 0.63 for TMB, 0.72 for PD-L1 expression, and 0.55 for MATH) (**Figure 4C** and **Table 2**). The benefit probability of patients in DCB and NDB groups was significantly different as well (p-value is 0.0017, **Figure 4D**). The survival analysis indicated that the high benefit probability group also showed a better PFS (**Figure 4E**).

Pathway Model Can Precisely Predict the Response of ICIs Treatment in GloriousMed Cohort

Finally, we tested pathway-model in GloriousMed cohort with 20 NSCLC patients, who were treated by PD-1/PD-L1 inhibitors (**Supplementary Table S1**). The accuracy of pathway-model was

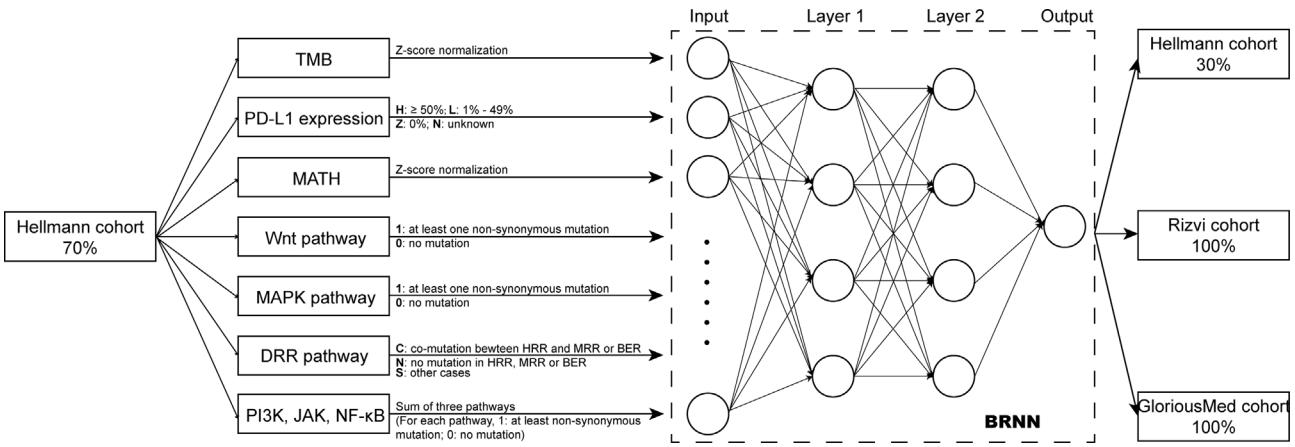

FIGURE 3 | Overview of the model design. Pathway-model was constructed and trained by 70% Hellmann cohort. Then, the predictor was tested in one validation cohort (the remaining 30% of Hellman cohort) and two independently testing cohorts (100% of Rizvi cohort and 100% of GloriousMed cohort).

much higher than tri-model and bivariate-model (AUC is 0.80 for pathway-model, 0.47 for tri-model and 0.64 for bivariate-model) (**Figure 5A** and **Table 2**). Even though, the benefit probability was not significantly different between DCB and NDB group (p-value is 0.08 for pathway-model), all DCB patients have a predictive benefit probability higher than 0.5 (**Figure 5B**). Thus, pathway-model can be generalized in clinical to improve the prediction accuracy of the response to immunotherapy.

Comprehensive Analysis With TCGA NSCLC Cohort Imply that High Benefit Probability Patients Is Associated With Immune Response

We predicted the benefit probability of TCGA LUAD and TCGA LUSC cohorts without EGFR exon 18-21 mutations and ALK gene fusions patients in immunotherapy with pathway-model and classified patients to two groups at the median cut-point. Then, we calculated signature scores of 18 gene sets with principle component analysis (PCA) method. In TCGA LUAD cohort, thirteen signatures are significantly different between high benefit probability group and low probability group (**Figure 6A**). In consideration of TMB, and mutational status of DDR and Wnt pathways are included in prediction model, the benefit probability difference in DDR, WNT target, DNA repair–related signatures and cell cycle were expected. The signature score of CD 8 T effector and Immune Checkpoint were higher in high probability group than in that of low group, while the signature score of EMT3 and FGFR3 related was lower in high probability group (**Figure 6A**). However, in LUSC cohort, we did not find significant difference between high and low benefit probability groups as LUAD cohort (**Figure 6D**).

Furthermore, we analyzed the differential expression genes between high benefit probability groups and low group in LUAD and LUSC respectively (**Figures 6B, E**, **Supplementary Table S4**). There are 153 differential expression genes (106 up-regulated) in LUAD, including *AFP* and *G6PC*, which related to P53 downstream pathway and FOXO pathway. In LUSC, there are 120 differential expression genes (50 up-regulated) including *FGF3* and *DLK1*, which related to FGFR pathway and NOTCH pathway. Apart from that, part of the top 30 mutated genes, such as *KRAS* and *PTPRD*, have different mutation pattern between high benefit probability group and low group, as well as between LUAD and LUSC (**Figures 6C, F**).

Above all, the comprehensive analysis of TCGA LUAD and LUSC cohorts imply that high benefit probability patients from pathway-model is associated with immune response.

DISCUSSION

Immune checkpoints inhibitors (ICIs), such as PD-1 and PD-L1, have revolutionized the treatment of many cancers, including NSCLC. However, how to select patients most likely to benefit from immunotherapy is the current leading challenge in the field. Previous ICIs-related studies preferred to use several single biomarkers, respectively, to predict the prognosis of immunotherapy (25, 26). Our study constructed a robust pathway-model based on deep learning approach, which included two common biomarkers, TMB, PD-L1 expression, a recent developed intratumor heterogeneity evaluation value MATH and potential marker-immune-related pathways. To the best of our knowledge, this is the first study to combine mutational status of pathways and common biomarkers for efficacy of prediction in NSCLC. Not only the ROC curves but also the significant difference of benefit probability from our predictor between DCB and NDB showed that our model had high accuracy in both training and test NSCLC data sets. The comparison among our pathway-model, tri-model, bivariate-model, and single-factor variables showed that our pathway-model had the highest accuracy in predicting the response to ICIs treatment. We found that tri-model with MATH had a lower AUC than bivariate-model without MATH in Rizvi and

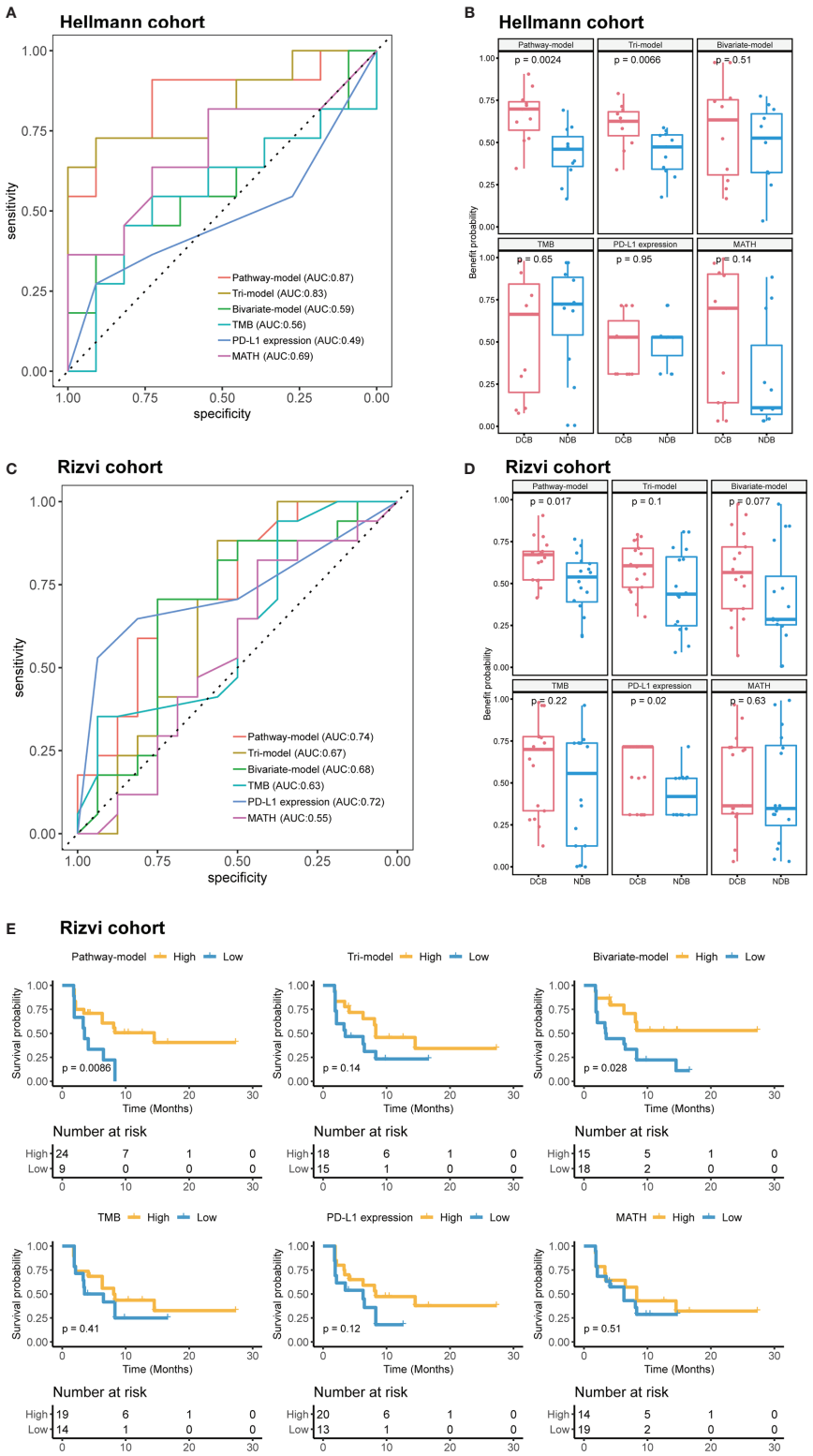

FIGURE 4 | The performance comparison different models and single-factor variables of in validation cohort (Hellmann cohort) and independent test cohort (Rizvi cohort). **(A)** Receiver operating characteristic (ROC) curves of different models. **(B)** Benefit probability and risk of patients in different response groups. **(C)** ROC curves of different models. **(D)** Benefit probability and risk of patients in different response groups. **(E)** Survival analysis based on different models and single-factor variables, time was progression-free survival (PFS). Patients of **(A, B)** were from Hellmann cohort, and patients of **(C–E)** were from Rizvi cohort.

TABLE 2 | Performance of models in three cohorts.

	Pathway-Model	Tri-model	Bivariate-model	TMB	PD-L1	MATH
Hellmann cohort	0.87	0.83	0.59	0.56	0.49	0.69
Rizvi cohort	0.74	0.67	0.68	0.63	0.72	0.55
GloriousMed cohort	0.80	0.47	0.64	0.65	0.78	0.46

TMB, tumor mutational burden; PD-L1, programmed cell death-ligand 1; MATH, mutant-allele tumor heterogeneity.

GloriousMed cohort. However, there is no denying that MATH did not improve the efficacy in distinguishing DCB and NDB patients in Rizvi and GloriousMed cohort in tri-model compared with bivariate-model. And pathway-model with MATH is the most stable model compared to other models and single factor variables. A recent study has shown that the integration of TMB and MATH forms a predictive marker for the response of ICIs treatment in melanoma (16), and another study has also revealed that intratumoral heterogeneity (MATH is an indicator of intratumoral heterogeneity) can be used as a biomarker to predict the response of ICIs treatment in NSCLC (15). Moreover, we found that the common biomarkers were not significant correlation according to the Pearson correlation coefficient, and the accuracy of each single-factor variable was lower than the pathway-model or tri-model. It might indicate there was a great synergy among these biomarkers. When we grouped the patients at the median of benefit probability generated from pathway-model, the PFS time was significantly different between high and low group, specifically patients with high benefit probability were more likely to have longer PFS time. These results suggested that besides the ability of response prediction of ICIs treatment, benefit probability is also associated with the prognosis of NSCLC patients. In addition to, the prediction results of GloriousMed cohort prove that our pathway-model can effectively predict the benefit probability of ICIs treatment and can be generalized in clinical to provide some reference during the treatment.

Furthermore, the enrichment analysis of 18 immune-related gene sets in TCGA LUAD and LUSC cohort suggested that our model might reveal the possible mechanism of the immune phenotype of tumors. Previous studies have proven that CD8 cell play a central role in immunity to cancer through their capacity to kill malignant cells, EMT-related genes may contribute to tumor immune escape, and FGFR mutated cases have a more deserted immune phenotype than the wild type (43–46). Our immune infiltration analysis also showed that the high benefit probability group of LUAD cohort had higher CD8 T effector scores. However, the significant difference of signature scores between high benefit probability group and low group were only found in TCGA LUAD cohort, but not in TCGA LUSC cohort. It is implied that the underlying immune response

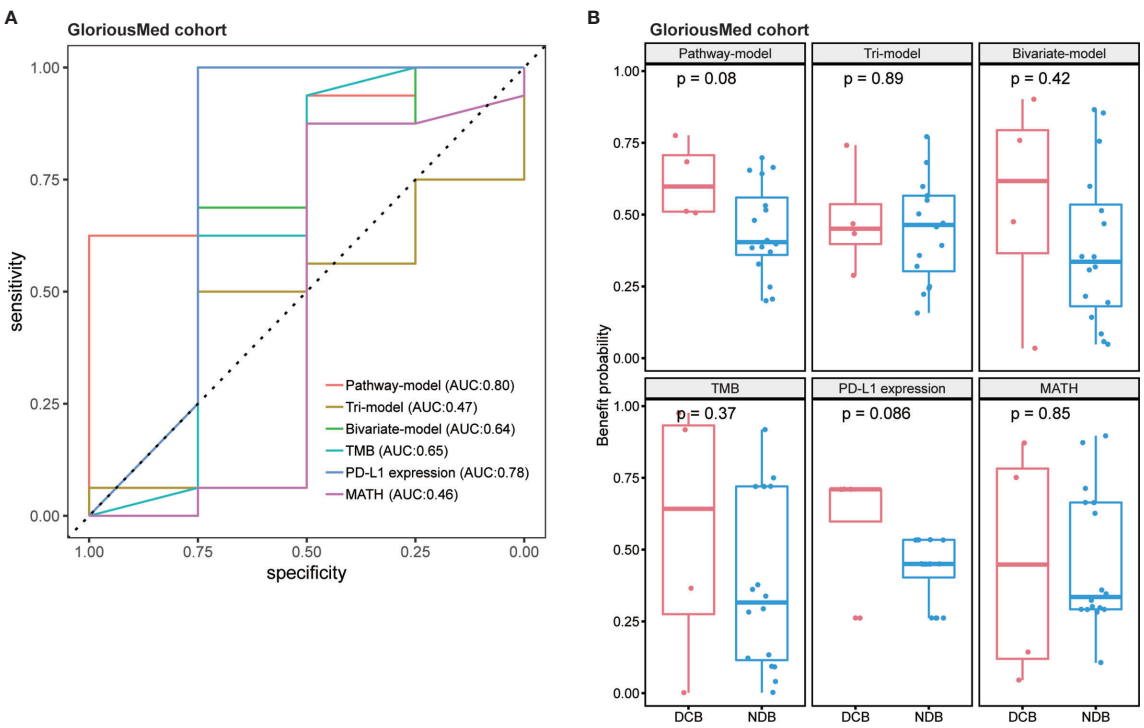

FIGURE 5 | The performance comparison of different models in GloriousMed cohort. **(A)** Receiver operating characteristic (ROC) curves of different models. **(B)** Benefit probability and risk of patients in different response groups.

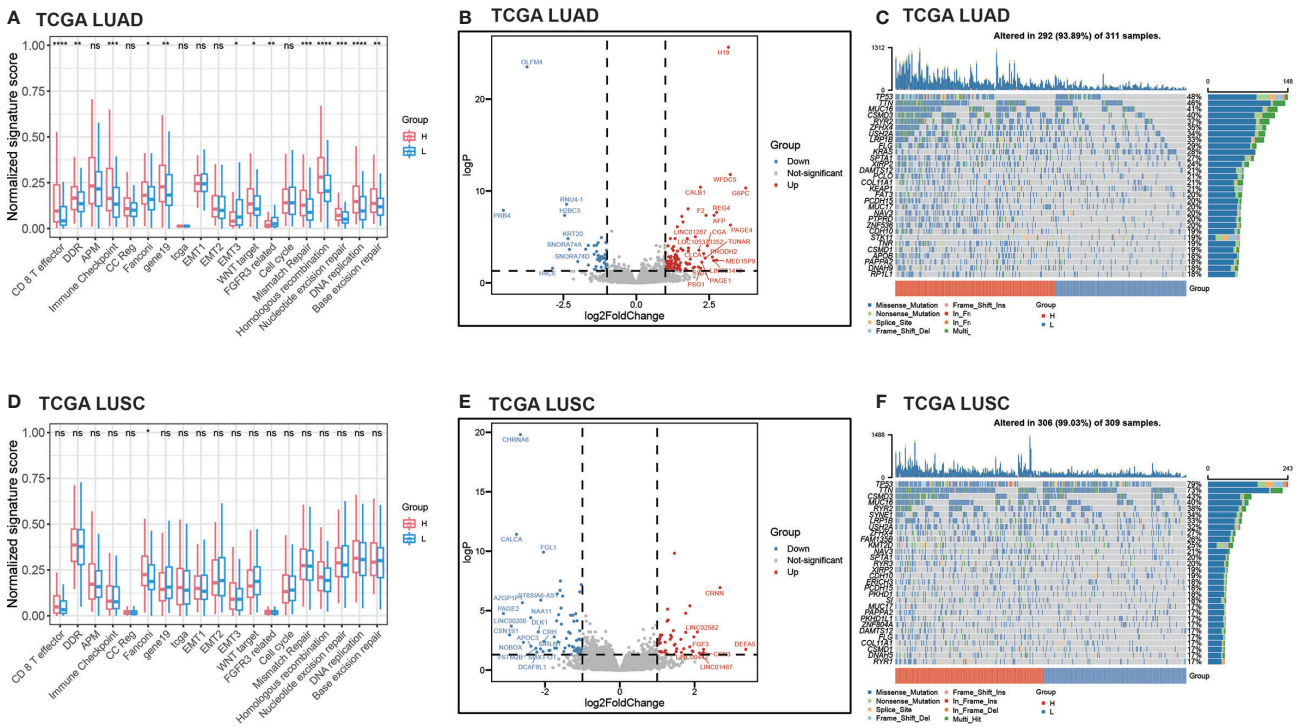

FIGURE 6 | The comprehensive analysis between high benefit probability group and low benefit probability group in The Cancer Genome Atlas Lung Adenocarcinoma (TCGA-LUAD) and Lung Squamous Cell Carcinoma (LUSC) cohort. High and low group were stratified based on median of benefit probability of the patients through pathway-model. **(A)** The significant signature scores of 18 gene sets in LUAD cohort. **(B)** Differential expression genes in LUAD cohort. **(C)** Oncoplot of top 30 mutated genes in LUAD cohort. **(D)** The significant signature scores of 18 gene sets in LUSC cohort. **(E)** Differential expression genes in LUSC cohort. **(F)** Oncoplot of top 30 mutated genes in LUSC cohort.

mechanism may be different between LUAD and LUSC. The differential expression genes in LUAD and LUSC are not complete same. P53 downstream pathway and FOXO pathway may be enriched in LUAD due to the up-regulation genes *AFP* and *G6PC*. P53 signaling pathway has been known as an important pathway in immune response, for example, it can function in immune cells including myeloid and T cells (47). Previous study has shown that FOXO pathway can be a target in tumor drug development (48). In LUSC, two differential expression genes, *FGF3* and *DLK1* are related two different pathways, FGFR pathway and Notch pathway. The enrichment of FGFR pathway implies a desert-immune subtype and high tumor purity of LUSC (45). Notch pathway can control the fate of various T cell type and myeloid cells that down-regulated DLK1 might influence the immune cells (49). The different regulated pathways between LUAD and LUSC may be one of the reasons of different immune response mechanism. In LUAD cohort, the mutation ratio of *KRAS*, an oncogene which leads to immune escape in the tumor microenvironment (50), and *PTPRD*, which affects the tumor proliferation (51), were higher than LUSC also suggests the difference immune response mechanisms. All above inference is based on naïve treatment public cohort, the exact mechanism would still to be explored with treatment samples. Except that, the probability of some differential expression genes, such as *MUC2*, *CLCA1*, *REG4*, and *FGF3* can be used as prognostic biomarkers in NSCLC is worth exploring because they have been reported as a biomarkers in other cancers as well (52–55).

There were limitations in our study that should be acknowledged. First, patients in the training cohort were treated with Nivolumab Plus Ipilimumab, and the model generated from which may be distracted in predicting patient in test cohort treated with Pembrolizumab or Tislelizumab due to pharmaceutical and medication differences. Second, the PD-L1 expression was quantified with different antibodies in training and validation cohort. Also, in the exploring cohort in TCGA data set, the PD-L1 expression was quantified using reverse phase protein array. The platform discordant of PD-L1 quantification may impair the power of our prediction model. Besides, due to the limitation of the training data sets, it is difficult to get a satisfactory model. Also, there are other features that are not incorporated into our model due to unavailability in either training or validation cohort, such as immune phenotype, which is known to affect the immunotherapy efficacy. In future studies, we will include more patients and features to guarantee the training process and the clinical practice of the predicting ICIs treatment efficacy in NSCLC patients.

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 Second Xiangya Hospital, Central South University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KX, YiY, JJ, and JW conceived and designed the project. YiZ, DT, and LP prepared and collected the data. ZJ, YuZ, YiZ, and ZZ contributed to analysis and interpretation. KX, ZJ, JJ, and YaY drafted the manuscript. KX, YW, JJ, JW, and YaY performed the quality assessment 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/fimmu.2021.646874/full#supplementary-material

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Novel Biomarkers of Dynamic Blood PD-L1 Expression for Immune Checkpoint Inhibitors in Advanced Non-Small-Cell Lung Cancer Patients

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Background: Immune checkpoint inhibitors (ICIs) have become a high-profile regimen for malignancy recently. However, only a small subpopulation obtains long-term clinical benefit. How to select optimal patients by reasonable biomarkers remains a hot topic.

Methods: Paired tissue samples and blood samples from 51 patients with advanced malignancies were collected for correlation analysis. Dynamic changes in blood PD-L1 (bPD-L1) expression, including PD-L1 mRNA, exosomal PD-L1 (exoPD-L1) protein and soluble PD-L1 (sPD-L1), were detected after 2 months of ICIs treatment in advanced non-small-cell lung cancer (NSCLC) patients. The best cutoff values for progression-free survival (PFS) and overall survival (OS) of all three biomarkers were calculated with R software.

Results: In 51 cases of various malignancies, those with positive tissue PD-L1 (tPD-L1) had significantly higher PD-L1 mRNA than those with negative tPD-L1. In 40 advanced NSCLC patients, those with a fold change of PD-L1 mRNA ≥ 2.04 had better PFS, OS and best objective response (bOR) rate. In addition, a fold change of exoPD-L1 ≥ 1.86 was also found to be associated with better efficacy and OS in a cohort of 21 advanced NSCLC cases. The dynamic change of sPD-L1 was not associated with efficacy and OS. Furthermore, the combination of PD-L1 mRNA and exoPD-L1 could screen better patients for potential benefit from ICIs treatment.

Conclusion: There was a positive correlation between bPD-L1 and tPD-L1 expression. Increased expression of PD-L1 mRNA, exoPD-L1, or both in early stage of ICIs treatment could serve as positive biomarkers of efficacy and OS in advanced NSCLC patients.

Keywords: blood PD-L1, immune checkpoint inhibitors, NSCLC, exosome, biomarker

INTRODUCTION

Immune checkpoint inhibitors (ICIs) treatment has become an increasingly high-profile regimen for malignancies since 2013. Patients with malignancies obtain remarkable survival benefits from ICIs treatment, for example, when antibodies against programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) are compared to traditional chemotherapy in non-small-cell lung cancer (NSCLC) (1, 2). As effective as ICIs treatment can be, only 10–40% of patients obtain dramatic responses (3), and the five-year overall survival (OS) rate of ICIs treatment ranges from 15.5% to 41% in advanced malignancies (4–6). Using single or multiple biomarkers to select patients who could benefit from ICIs was the focus in the current study.

To date, various biomarkers, including tumor tissue PD-L1 (tPD-L1) expression, tumor mutation burden (TMB), tumor neoantigen burden (TNB), high microsatellite instability (MSI-high), deficient mismatch repair (dMMR), tumor-infiltrating lymphocytes (TIL), T-cell receptor clonality, effector T-cell gene signature, DNA damage and repair genes (DDR), intestinal microbiota, etc. have been demonstrated to be associated with a better response rate and prolonged survival (7–10).

In the tumor microenvironment (TME), the PD-L1 protein is expressed on the surface of tumor cells (TCs) or immune cells (ICs). Its binding to PD-1 leads to the impairment of the antitumor function of T cells, similar to a blockade in the flow of a pipeline. Anti-PD-1/anti-PD-L1 therapy could move the blockade away and restore the flow (11). Hence, the detection of pretreatment PD-L1 protein expression on TCs or ICs by immunohistochemistry (IHC) is the most frequently used predictive biomarker in clinical practice. Previous studies KEYNOTE 024 and IMpower 110 have demonstrated that NSCLC patients with higher tPD-L1 expression could obtain better clinical benefits, including objective response rate (ORR), progression-free survival (PFS) and OS (12, 13). In addition, the dynamic changes in tPD-L1 expression help distinguish responders from non-responders (14, 15). However, in the CHECKMATE-026 study (16), the nivolumab subgroup did not have a PFS benefit compared with the platinum-based chemotherapy subgroup in patients with 5% or higher tPD-L1 expression. Hence, tPD-L1 expression is a controversial predictive biomarker in the clinic. There are several reasons. First, there is heterogeneity of PD-L1 protein expression in the TME. The PD-L1 protein in the TME includes constitutive expression from the activation of some oncogenic pathways or chromosomal abnormalities (17, 18), and inducible expression by the activation of NF-κB or IFN-γ secreted by infiltrating lymphocytes (19, 20). Second, previous treatment had an effect on tPD-L1 expression. A study demonstrated that radiotherapy upregulated tPD-L1 expression (21), while EGFR-TKIs downregulated tPD-L1 expression (22). Third, there is no standard measure of tPD-L1 expression, for the inconsistency and subjectivity between different detection kits. In conclusion, tPD-L1 expression may not be a robust predictive biomarker.

Liquid biopsy is an emerging assay to obtain tumor-related molecular information. The sample sources of liquid biopsy included cerebrospinal fluid, saliva, pleural effusion, blood, ascites, urine, etc. Compared to tissue biopsy, liquid biopsy is noninvasive and convenient, which could help obtain multiple biopsies to monitor the molecular changes during ICIs treatment. In addition, liquid biopsy could help to reduce the effect of tumor heterogeneity. Some blood biomarkers, such as blood TMB (bTMB) (23), derived neutrophil/(leukocyte minus neutrophil) ratio (24), circulating exosomal PD-L1 (exoPD-L1) protein expression (25), soluble PD-L1 (sPD-L1) (26) have been explored to predict efficacy of ICIs treatment. However, these studies showed controversial results in different research centers.

To explore the value of bPD-L1 in ICIs treatment, the current study was designed to detect multi-modal bPD-L1 expression (including PD-L1 mRNA, exoPD-L1 and sPD-L1), evaluate the correlation between tPD-L1 and bPD-L1, and monitor the dynamic changes in early stage of ICIs treatment.

MATERIALS AND METHODS

Study Design and Patients

Paired tumor tissue samples and blood samples, as well as clinicopathologic features were obtained from 51 various malignant tumor patients (ClinicalTrials.gov, NCT02890849). Repeated blood biopsies from forty other advanced NSCLC patient with anti-PD-1/anti-PD-L1 therapy were collected at baseline and at two months after the first intravenous transfusion (ClinicalTrials.gov, NCT03073902). In addition, blood samples from ten healthy donors (HDs) were collected. All patients and HDs provided informed consent. All tissue samples underwent overnight fixation in 10% phosphate-buffed formalin and then were processed and embedded in paraffin blocks for further analysis. All blood samples were centrifuged for 10 minutes at 2000 × g to obtain plasma and then stored at -80°C for further analysis. This study was approved by the ethics committee of the Xinqiao Hospital of Army Medical University (2016-No.054-01, 2017-No.011-01). The best objective response (bOR) to anti-PD-1/anti-PD-L1 antibody treatment was determined by iRECIST (27) and included complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). PFS was defined as the time from the first dose of ICIs treatment to PD. OS was defined as the time from the first dose of ICIs treatment to death for any reason.

PD-L1 IHC Staining and Scoring

PD-L1 IHC staining was conducted on 3 μm thick sections of formalin-fixed paraffin embedded (FFPE) tumor blocks according to the VENTANA SP142 PD-L1 immunohistochemistry assay (Ventana, AZ, USA). The score of tPD-L1 expression on both TCs and tumor-infiltrating ICs was evaluated by digital image analysis software (Aperio membrane v9 and Aperio Genie Classifier, LEICA CAMERA AG, Wetzlar, Germany). The scoring criteria used were from a previous study (28) (TC3, ≥50%; TC2, ≥ 5 to 50%; TC1, ≥1 to < 5%; TC0, <1%; IC3, ≥10%; IC2, ≥5 to < 10%;

IC1, ≥1 to < 5%; and IC0, < 1%). Additionally, all patients were divided into three groups according to tPD-L1 expression (TC0/IC0, TC1~2/IC1~2 and TC3/IC3).

Measurement of Plasma PD-L1 mRNA

Total RNA was extracted using TRIzol Reagent (Invitrogen, Invitrogen, CA, USA), according to the manufacturer's instructions. After the concentration and purity of the total RNA were determined, reverse transcription was performed using a PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). PLACON (**Supplementary Figure 1**), a self-designed plasma external control rewarded as China patent of invention (201810102695.2), was used for amplification and comparison to detect plasma PD-L1 mRNA. The relative expression level of plasma PD-L1 mRNA in tumor patients was calculated by referring to the average expression level of plasma PD-L1 mRNA in 10 HDs samples. The formula is $y=2^{-(\Delta CTx-\Delta CT0)}$. The following primer was used: PD-LI (Forward: 5'-GCTA TGGTGGTGCCGACTAC-3', Reverse: 5'-TTGGTGG TGGTGGTCTTACC-3').

Isolation of Exosomes From Plasma

Stored plasma samples were thawed in a water bath at 25°C. Exosomes were isolated from 200 μL of patient plasma using a Exosome Isolation Kit (Wayen Biotechnologies, Shanghai, China), according to the manufacturer's instructions. Then, isolated exosome samples were immediately stored at -80°C until further analysis.

Verification of Isolated Exosomes

We randomly selected one isolated exosome sample for verification. First, the size distribution of the isolated exosomes was determined through Nanosight Tracking Analysis (NTA) by utilizing ZetaView (Particle Metrix, Germany). Second, exosome morphology was analyzed by using transmission electron microscopy (TEM) (Tecnai G2 spirit BioTwin, FEI, USA). Third, exosomal proteins were subjected to SDS-PAGE followed by Western blotting (WB). The nitrocellulose membranes were blocked with 5% nonfat milk for 60 minutes at room temperature and incubated overnight at 4°C with the corresponding primary antibodies at dilutions recommended by the suppliers, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hour. The blots were developed with enhanced chemiluminescence (ECL) PierceTM detection reagents (Thermo Scientific). CD63, CD9, and calnexin were used as exosome markers. Finally, immunoreactive proteins were visualized using a chemiluminescence detection system (FluorChem HD2, USA).

Measurement of exoPD-L1

Exosomal PD-L1 protein was measured with a Simoa^{TM} PD-L1 Reagent Kit (Quanterix Corp, Lexington, MA). In short, all isolated exosome samples were loaded at a mass of 280 μg and then diluted with sample diluent to 130 μL for single-well detection. Standard samples were added to a 96-well plate. After the completion of the sample preparation, beads, detector, and SBG were loaded into the reagent holder, and RGP was loaded into the tube holder. Then, the sample was transferred to the Simoa Disc, using oil to seal the sample so that the signal was only in the well. Finally, pictures were taken, and the concentration was analyzed on a Simoa HD-1 platform (Quanterix Corp).

Measurement of Soluble PD-L1

Soluble PD-L1 expression in plasma was determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, USA), according to the manufacturer's instructions. The expression level of each sample was calculated according to standard curves.

Statistical Analysis

All experiments repeated three times, and the mean value of each sample was reported. The difference in PD-L1 mRNA and sPD-L1 expression in different subgroups was calculated by using independent-samples t-test. The difference in tPD-L1 expression and bOR in different subgroups was calculated by using Pearson's chi-square test or Fisher's exact test. Univariate and multivariate analyses were performed to identify independent factors of efficacy and OS. Survival analyses were performed by the Kaplan-Meier method and the log-rank test. SPSS version 23.0 (IBM, Armonk, NY, USA) was used for performing these statistical analyses. The "survival" and "survminer" packages from R software (version 3.5.2) were used for calculating the best cutoff point of each biomarker, conducting statistical calculations, and drawing Kaplan–Meier curves. A two-sided P value < 0.05 was considered statistically significant.

RESULTS

Clinicopathologic Features, tPD-L1 Expression and bPD-L1 Expression in 51 patients With Various Malignancies

Fifty-one patients with various malignancies were enrolled, including 33 NSCLC patients. Of these patients, 26 were less than 60 years old, 31 were male, 21 had a smoking history, and 33 had metastatic disease (**Table 1**). In 33 NSCLC patients, male patients had a higher PD-L1 mRNA expression than female patients. Patients with a smoking history had higher PD-L1 mRNA expression than those without a smoking history (**Supplementary Figure 2A**). No differences were found between patients younger than 60 years and older than 60 years or between patients with metastasis and without metastasis. The expression levels of tPD-L1 and sPD-L1 showed no significant differences in each subgroup (**Supplementary Figures 2B, C**). There was a trend that patients with positive tPD-L1 expression had higher PD-L1 mRNA expression (**Figure 1A**). However, the expression of sPD-L1 did not correlate with the PD-L1 mRNA expression (**Figure 1B**).

In the overall population, the PD-L1 mRNA expression was higher in both the TC3/IC3 group (P=0.036,

TABLE 1 | Baseline clinicopathological features of 51 patients with diverse malignancies.

Clinicopathologic feature	Number of patients (%)
Age (years)	
<60	26 (51%)
≥60	25 (49%)
Gender	
Male	31 (61%)
Female	20 (39%)
Smoking history	
Yes	21 (41%)
No	30 (59%)
Tumor type	
NSCLC	33 (65%)
Others	18 (35%)
Distant metastasis	
Yes	33 (65%)
No	18 (35%)

NSCLC, non-small cell lung cancer.

Supplementary Figure 3A) and the TC1~2/IC1~2 group (P=0.026, **Supplementary Figure 3A**) than in the TC0/IC0 group. There was also a trend that the TC3/IC3 group had a higher PD-L1 mRNA expression than the TC1~2/IC1~2 group (P=0.083, **Supplementary Figure 3A**). For sPD-L1, only the TC1~2/IC1~2 group had significantly higher expression than the TC0/IC0 group (P=0.023, **Supplementary Figure 3B**). No differences were found between the other groups. In addition, no significant differences in tPD-L1 and bPD-L1 expression were found between subgroups (**Supplementary Figure 4**).

Dynamic Changes in bPD-L1 in 21 NSCLC Patients Treated With ICIs

Multimodal bPD-L1 expression detection, including PD-L1 mRNA, exoPD-L1, and sPD-L1, were performed in 21 advanced NSCLC patients treated with ICIs. Fifteen patients had increased PD-L1 mRNA expression at 2 months compared to baseline, while the other six patients had decreased PD-L1 mRNA expression (**Figure 2A**); the fold change ranged from 0.11 to 55.72 times. Almost all patients but three had increased exoPD-L1 expression levels (**Figure 2B**), and the fold change ranged from 0.40 to 113.76 times. For sPD-L1 expression, nine patients had increased sPD-L1 expression, while the other twelve patients had decreased sPD-L1 expression (**Figure 2C**); the fold change ranged from 0.54 to 4.72 times. An overview of the fold changes of all three kinds of bPD-L1 expression is shown in **Figure 2D**.

Dynamic Changes in PD-L1 mRNA Expression to Predict Efficacy and OS in the Expanded 40 NSCLC Cohort

To explore the role of dynamic changes in PD-L1 mRNA expression in predicting efficacy and OS, we expanded the sample size into 40 advanced NSCLC patients. According to iRECIST, 8 patients had PD; 11 had PR; 21 had SD; and no patients had CR. Blood PD-L1 mRNA expression levels at baseline and at 2 months were detected. The best cutoff value for fold change of PD-L1 mRNA expression was 2.04. The median PFS was 4.2 months (95% confidence interval [CI] 0.2-8.2 months) in patients with a fold change < 2.04. It was 10.0 months (95% CI 3.6-10.4 months) in patients with a fold change ≥ 2.04. The hazard ratio (HR) was 0.373 (fold change ≥ 2.04 vs. fold change < 2.04, 95% CI 0.174-0.797, P=0.011) (**Figure 3A**). The median OS was 7.0 months (95% CI 3.6-10.4 months) in patients with a fold change < 2.04 and 19.0 months (95% CI 9.1-28.9 months) in patients with a fold change ≥ 2.04 (HR 0.281, 95% CI 0.119-0.666, P=0.004) (**Figure 3B**). The bOR rate was 10.5% in patients with a fold change < 2.04 compared with 42.9% in patients with a fold change ≥ 2.04 (P=0.022) (**Figure 3C**).

Dynamic Changes in exoPD-L1 and sPD-L1 to Predict Efficacy and OS in the 21 NSCLC Cohort

To verify the isolated exosomes, TEM, NTA and WB were conducted. As shown in **Supplementary Figure 5A**, the obtained exosomes had a distinctive cup shape. Then, positive marker proteins of exosomes, CD3 and CD69, were found in WB (**Supplementary Figure 5B**). A negative marker protein, calnexin, was not found (**Supplementary Figure 5B**). The size of exosomes ranged from 20 nm to 200 nm, and the average size was 117.5 nm (**Supplementary Figure 5C**).

We conducted efficacy and OS analyses according to fold changes of exoPD-L1 and sPD-L1 expression in the 21 NSCLC cohort. For exoPD-L1, patients with a fold change equals or greater than 1.86 at 2 months compared to baseline had better PFS (9.9 vs. 4.3 months, P=0.001; HR 0.165, 95% CI 0.052-0.525, P=0.002) and OS (13.7 vs. 6.3 months, P=0.004; HR 0.237, 95% CI 0.082-0.684, P=0.008) as well as a higher bOR rate (33.3% vs. 11.1%, P=0.338) (**Figures 4A–C**). For sPD-L1, no best cutoff point was found. The PFS, OS and bOR rates showed no differences (**Figures 4D–F**).

The Combination of PD-L1 mRNA and exoPD-L1 to Predict Efficacy and OS in the 21 NSCLC Cohort

Univariate and multivariate analyses were performed. The results demonstrated that both the dynamic changes of PD-L1 mRNA and exoPD-L1 were independent factors for PFS and OS in the 21 NSCLC cohort (**Supplementary Tables 1** and **2**). Furthermore, we conducted survival analyses by combining the two biomarkers. Better PFS and OS were found in the combined high group compared with the single high group or the combined low group (PFS 11.2 vs. 7.0 vs. 3.2 months, P<0.001; OS 22.0 vs. 13.0 vs. 4.0 months, P<0.001) (**Figures 5A, B**). The bOR rate in the combined high group and single high group was higher than that in the combined low group (33.3% vs. 33.3% vs. 0%, P=0.269) (**Figure 5C**).

DISCUSSION

In the current study, we identified the correlation among tPD-L1, bPD-L1 and clinicopathologic features in 51 patients with

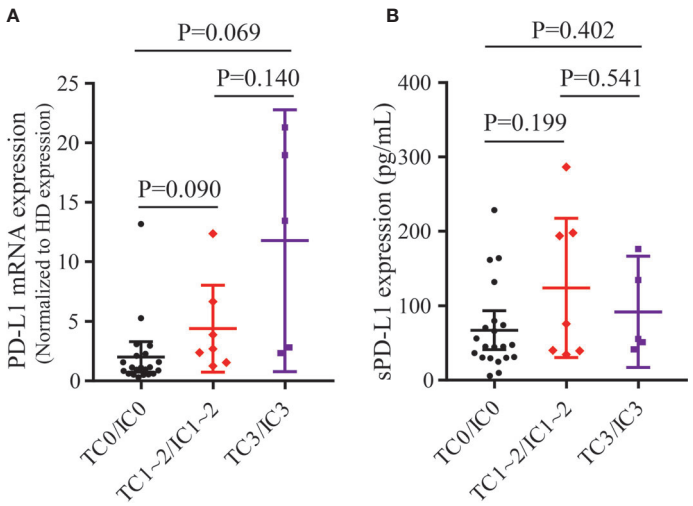

FIGURE 1 | The correlation of tPD-L1 and bPD-L1 in 33 NSCLC patients. **(A)** The correlation of PD-L1 mRNA and tPD-L1. **(B)** The correlation of sPD-L1 and tPD-L1. tPD-L1, tissue PD-L1; bPD-L1, blood PD-L1; sPD-L1, soluble PD-L1; NSCLC, non-small-cell lung cancer. P values were calculated by independent-samples t-test.

various malignancies. Then, we explored the predictive power of multimodal bPD-L1 expression, including PD-L1 mRNA, exoPD-L1 and sPD-L1, in advanced NSCLC patients treated with ICIs.

Our results demonstrated that patients with positive tPD-L1 expression had higher PD-L1 mRNA and sPD-L1 expression in plasma, which demonstrated that bPD-L1 expression had a positive correlation with tPD-L1 expression at the same timepoints. Obviously, the acquisition of blood samples is much more convenient, less expensive, less invasive, therefore helps monitor bPD-L1 changes during ICIs treatment.

Our study first demonstrated that plasma PD-L1 mRNA could predict the efficacy and survival in NSCLC patients with ICIs treatment. The preliminary results of 21 NSCLC patients had been postered in the 2019 World Conference on Lung Cancer (29). Afterwards, we still found the same conclusion in a larger sample size of 40 patients and longer follow-up duration. Noteworthy, a report showed that a decrease of exoPD-L1 mRNA was correlated with response to ICIs treatment (30), which implied the different value of exoPD-L1 mRNA and blood PD-L1 mRNA.

Tumor-derived exosomes are extracellular vesicles with bilayer lipid membranes that carry many bioactive molecules. Tumor-derived exosomes are considered to be a key messenger in tumor progression and metastasis (31). Not surprisingly, the PD-L1 protein was found on the surface of tumor-derived exosomes (32). In vivo and *in vitro* (33), exoPD-L1 suppressed the function of T cells by binding to PD-1. Furthermore, PD-L1-positive exosomes could spread directly from the TME to the whole body to induce systemic immunosuppression. Exosomal PD-L1 exhibits the potential to serve as a biomarker in the clinic. In a cohort of 44 melanoma patients treated with pembrolizumab (25), pretreatment exoPD-L1 expression was lower in responders than in nonresponders. In addition, pretreatment exoPD-L1 expression was positively correlated with circulating IFN-γ expression and overall tumor burden. Correspondingly, patients with an elevated exoPD-L1 expression of fold change over 2.43 had a much higher ORR. In our work, we also found an increased fold change (≥ 1.86) of exoPD-L1 in early stage of ICIs treatment indicated better efficacy and OS in NSCLC patients. In contrast, Cordonnier and colleagues (34) reported that a decrease in exoPD-L1 was associated with better response in melanoma patients. Patients with exoPD-L1 increased > 100 pg/ml had worse PFS and OS. Baseline exoPD-L1 blood levels were not associated with PFS and OS. Noticeable, the results of exoPD-L1 protein expression in this study were different from exoPD-L1 mRNA expression (30).

The source and regulation of sPD-L1 remains unclear. A paper reported that sPD-L1 might be derived from TCs and retained the PD-1-binding domain (35). Plasma sPD-L1 could systemically impair host immunity and promote tumor progression. Zhou et al. (26) reported that higher initial sPD-L1 expression was prone to disease progression in malignant melanoma patients with ICIs treatment, while over 1.5-fold increase of sPD-L1 expression at five months showed a positive correlation with PR. Okuma et al. (36) reported that a higher baseline sPD-L1 expression was negatively associated with OS and ORR in NSCLC patients receiving nivolumab. Costantini et al. (37) demonstrated that high sPD-L1 at 2 months and increase of sPD-L1 concentrations were associated with poor response and absence of clinical benefit in NSCLC patients treated by nivolumab. In the current study, the sPD-L1 change showed no correlation with efficacy and OS, which were different from the previous studies.

Additionally, tPD-L1 expression in the TME increased at early stage of treatment in patients who responded to ICIs

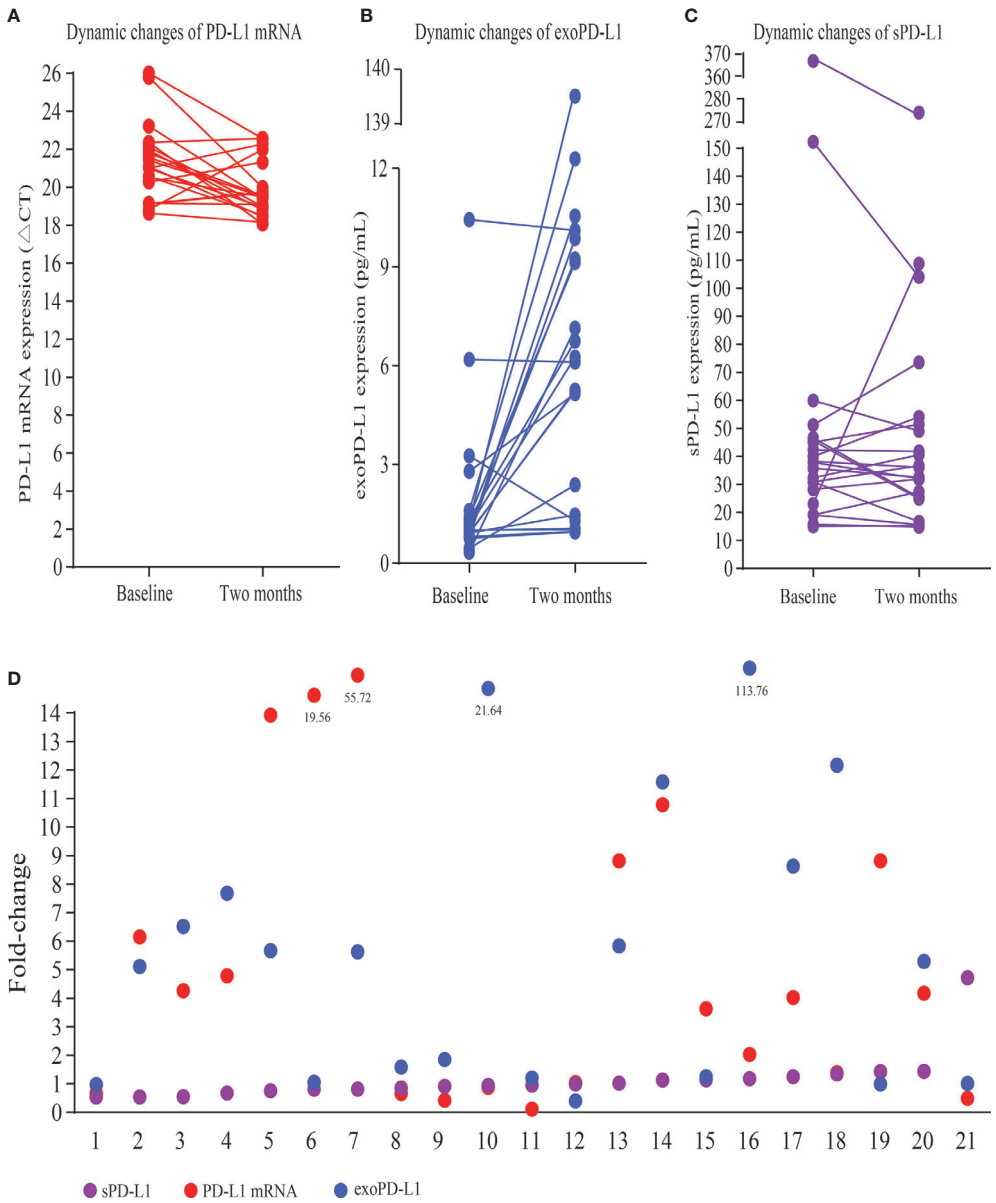

FIGURE 2 | Dynamic changes in multimodal bPD-L1 expression during early treatment. **(A)** Dynamic changes in PD-L1 mRNA (CT values). **(B)** Dynamic changes in exoPD-L1. **(C)** Dynamic changes in sPD-L1. **(D)** An overview of fold changes of the three biomarkers. bPD-L1, blood PD-L1; CT, cycle threshold; exo-PD-L1, exosomal PD-L1; sPD-L1 soluble PD-L1.

(14, 15). These data suggested that in the early stage of ICIs treatment, both tPD-L1 and bPD-L1 expression could be upregulated. The underlying mechanism on higher level of PD-L1 on TCs could be a feedback and T-cell reinvigoration of immune response. Nevertheless, elevated PD-L1 expression couldn't play its role of negative immune regulation because ICIs therapy had blocked the interaction of PD-1 and PD-L1.

Furthermore, our work demonstrated that the combination of blood PD-L1 mRNA and exoPD-L1 could better determine NSCLC subgroups who may benefit from ICIs treatment. Though patients might have a fold change of exoPD-L1 < 1.86, part of them could have a fold-change of PD-L1 mRNA ≥ 2.04. These patients had better efficacy and OS than those with fold changes of PD-L1 mRNA and exoPD-L1 both low. In addition, patients with both a fold change of exoPD-L1 ≥ 1.86 and a fold change of PD-L1 mRNA ≥ 2.04 had the best efficacy and OS outcomes.

Besides the above indexes, bPD-L1 was also found on the surface of circulating tumor cells (CTCs) (38, 39). Nicolazzo et al. (40) monitored PD-L1 expression on CTCs from baseline to 6

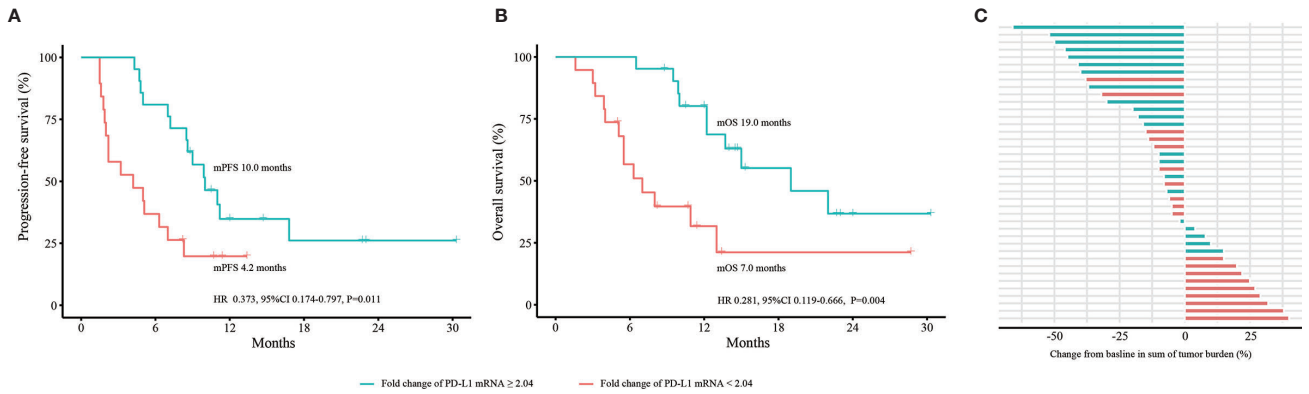

FIGURE 3 | Dynamic change in PD-L1 mRNA expression to predict efficacy and OS in the expanded 40 NSCLC cohort. **(A)** PFS analysis based on fold change of PD-L1 mRNA expression. **(B)** OS analysis based on fold change of PD-L1 mRNA expression. **(C)** bOR of each patient stratified by fold change of PD-L1 mRNA expression. OS, overall survival; PFS, progression-free survival; bOR, best objective response; HR, hazard ratio; CI, confidence interval. P values were calculated by log-rank test.

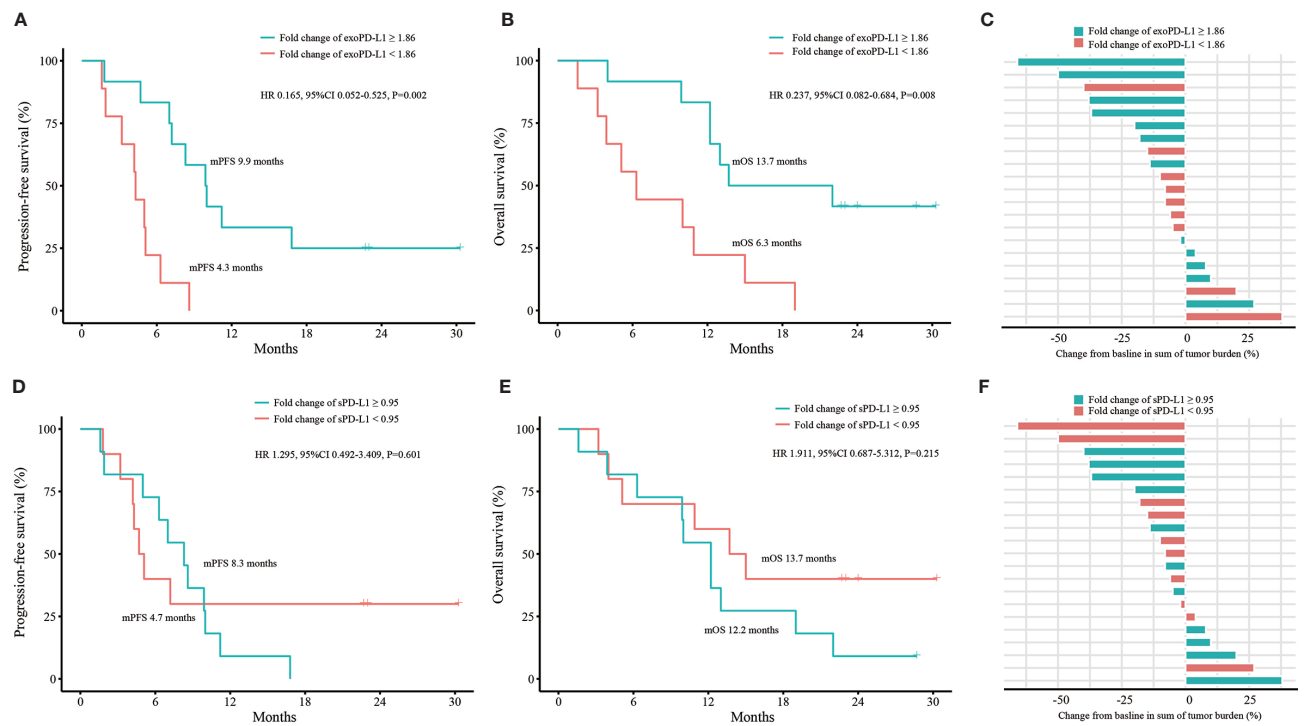

FIGURE 4 | Efficacy and OS analyses based on fold change of exoPD-L1 or sPD-L1 expression in the 21 NSCLC cohort. **(A)** PFS analysis based on fold change of exoPD-L1 expression. **(B)** OS analysis based on fold change of exoPD-L1 expression. **(C)** bOR of each patient stratified by fold change of exoPD-L1 expression. **(D)** PFS analysis based on fold change of sPD-L1 expression. **(E)** OS analysis based on fold change of sPD-L1 expression. **(F)** bOR of each patient stratified by fold change of sPD-L1 expression. OS, overall survival; exoPD-L1, exosomal PD-L1; sPD-L1, soluble PD-L1; PFS, progression-free survival; bOR, best objective response; HR, hazard ratio; CI, confidence interval. P values were calculated by log-rank test.

months in 24 advanced NSCLC patients treated with nivolumab. The results showed that those with continuous PD-L1 expression experienced disease progression, while those with negative PD-L1 expression at 6 months obtained tumor response. Another work (41) got the same results. However, Yue et al. (42) reported the opposite conclusion that that patients with a higher PD-L1^{high} CTCs (abundance over 20%) at baseline had an obvious disease control and longer PFS, and decreased PD-L1^{high}

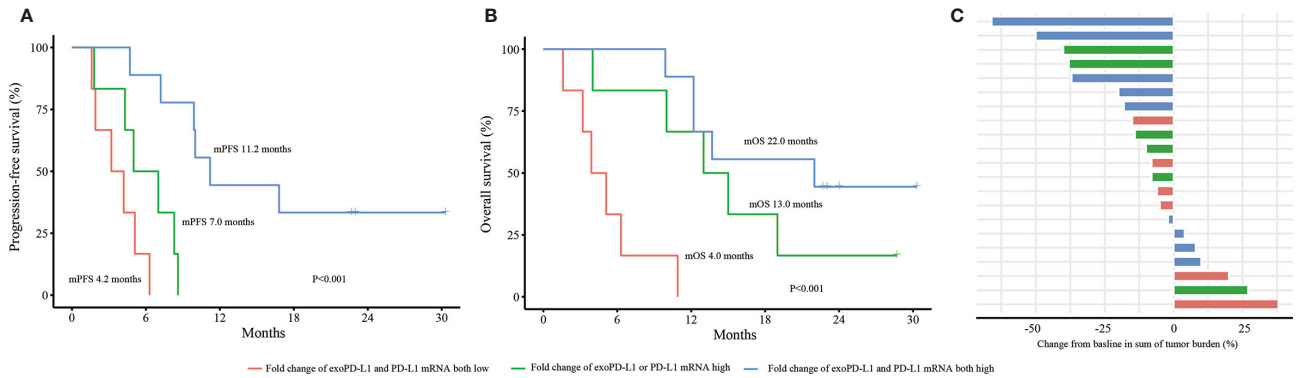

FIGURE 5 | Efficacy and OS analyses based on the combination of PD-L1 mRNA and exoPD-L1 expression in the 21 NSCLC cohort. **(A)** PFS analysis based on the combination of two biomarkers. **(B)** OS analysis based on the combination of two biomarkers. **(C)** bOR of each patient stratified by the combination of two biomarkers. OS, overall survival; exoPD-L1, exosomal PD-L1; PFS, progression-free survival; bOR, best objective response. P values were calculated by log-rank test.

CTCs at 9 weeks were associated with disease control. More research in this field is necessary.

To the best of our knowledge, this is the first report of changes in PD-L1 mRNA and exoPD-L1 to predict the efficacy of ICIs treatment. Dynamic liquid biopsy of multimodal PD-L1 is a good way to occasionally monitor patients during ICIs treatment. Our findings have crucial clinical significance. First, we know which patients would benefit from ICIs treatment and which subgroups would not. Second, we may pay more attention to the potential disease progression in those patients who have lower fold changes in exoPD-L1 and PD-L1 mRNA during early treatment. Some salvage therapy, such as chemotherapy, radiotherapy, or antivascular drug, could be intervened earlier than imaging progress. Third, we built a patent product of external control for blood mRNA detection to make the blood PD-L1 mRNA a standard biomarker to evaluate the clinical benefit of ICIs treatment.

There are some limitations in our work. The sample size is relatively small. In the future, we plan to design a prospective clinical trial to confirm the value of blood PD-L1 biomarker from ICIs treatment in NSCLC patients. We did not recruit early-stage NSCLC patients. Thus, we do not know if bPD-L1 is an efficacy biomarker for neoadjuvant ICIs treatment before surgery, or adjuvant ICIs treatment after surgery. All these questions could be explored and solved in future studies.

CONCLUSIONS

In summary, bPD-L1 expression has a positive correlation with tPD-L1 expression in various malignancies. Upregulated expression of blood PD-L1 mRNA and exoPD-L1 predicted good efficacy and survival for ICIs treatment. In particular, the combination of these two biomarkers could screen better subpopulation. Our viewpoint of dynamic changes of blood PD-L1 mRNA and exoPD-L1 could serve as novel biomarkers in NSCLC patients with ICIs treatment.

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 ethics committee of the Xinqiao Hospital of Army Medical University (2016-No.054-01, 2017-No.011-01). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Contributions to the conception: LuZ, JS. Design of the work: ZC, WZ. The acquisition, analysis, and interpretation of data: KN, XZ, LiZ, ZX, YY. The creation of new software used in the work: FL, LM. Draft the work and substantively revised it: QY, MC, JG, LuZ, JS. All authors contributed to the article and approved the submitted version. All authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.665133/full#supplementary-material

Supplementary Figure 1 | PLACON sequence. We selected a conserved sequence from the genome of *Caenorhabditis elegans*, which we named PLACON. The following primers were used: (Forward: 5'-AGTGCAGGGTCCGAGGTATT-3', Reverse: 5'-CGACTCTACAACGACCGTGA-3'). The PLACON sequence: 5'-CUCG CUAACGACUCUACAACGACCGUGAAUUCAAGCGCCGCUUGGAUGUCCGC-3' **(A)**. Then, we identified that PLACON had good specificity through BLAST (https://www.ncbi.nlm.nih.gov/). No cross-correlation with the human genome was found. Then, we identified the amplification ability of PLACON by comparing it with internal references, including GAPDH and β-actin, in plasma from 8 patients with malignant tumors and cancer cell supernatants. As shown in **(B, C)** the CT value of PLACON was much lower than that of GAPDH and β-actin. In conclusion, PLACON is specific, and it has an obvious amplification advantage. It could be used as an external reference for the quantitative detection of mRNA in plasma. CT, cycle threshold.

Supplementary Figure 2 | The differences of tPD-L1 and bPD-L1 between subgroups in 33 NSCLC patients. **(A)** Comparison of PD-L1 mRNA expression between subgroups. **(B)** Comparison of sPD-L1 expression between subgroups. **(C)** Comparison of tPD-L1 expression between subgroups. tPD-L1, tissue PD-L1; bPD-L1, blood PD-L1; NSCLC, non-small cell lung cancer; sPD-L1, soluble PD-L1. P values were calculated by independent-samples t-test **(A, B)** and Pearson's chi-square test or Fisher's exact test **(C)**.

Supplementary Figure 3 | The correlation of tPD-L1 and bPD-L1 in 51 patients with various malignancies. **(A)** The correlation of PD-L1 mRNA and tPD-L1. **(B)** The correlation of sPD-L1 and tPD-L1. tPD-L1, tissue PD-L1; bPD-L1, blood PD-L1; sPD-L1, soluble PD-L1; NSCLC, non-small-cell lung cancer. P values were calculated by independent-samples t-test.

Supplementary Figure 4 | The differences of tPD-L1 and bPD-L1 between subgroups in 51 patients with various malignancies. **(A)** Comparison of PD-L1 mRNA expression between subgroups. **(B)** Comparison of sPD-L1 expression between subgroups. **(C)** Comparison of tPD-L1 expression between subgroups. tPD-L1, tissue PD-L1; bPD-L1, blood PD-L1; sPD-L1, soluble PD-L1. P values were calculated by independent-samples t-test **(A, B)** and Pearson's chi-square test or Fisher's exact test **(C)**.

Supplementary Figure 5 | Verification of exosomes. **(A)** Exosome morphology detected by TEM. **(B)** Positive markers (CD9, CD63) and a negative marker (calnexin) of exosomes detected by WB. **(C)** Size analysis of exosomes through NTA. TEM, transmission electron microscopy; WB, western blotting; NTA, nanosight tracking analysis.

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Predictive Value of *KDM5C* Alterations for Immune Checkpoint Inhibitors Treatment Outcomes in Patients With Cancer

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Lysine (K)-specific demethylase 5C (*KDM5C*) plays a significant role in the tumor cell proliferation, invasion, drug resistance and the regulation of tumor-related gene expression. Here, we aimed to investigate its predictive value in patients with cancers received immune checkpoint inhibitors (ICIs). We explored the predictive value of *KDM5C* alterations and the association between *KDM5C* alteration and immune landscape by using published cohort with clinical outcome and sequenced data from online database. The frequency of *KDM5C* alterations was 2.1% across 48045 tumor samples with different cancers from 185 studies. *KDM5C* alterations were correlated with markedly inferior overall survival (OS, 53 vs. 102 months, $P<0.0001$) than those without. However, in ICI-treated group, patients with *KDM5C* alterations had a substantially prolonged OS than the wild-type group (not reached vs. 18 months, $P=0.0041$). The predictive value of *KDM5C* alterations for ICI treatment outcome was not observed in patients with microsatellite-stable tumors ($P=0.2875$). Intriguingly, patients with non-small-cell lung cancer and *KDM5C* alterations receiving ICI had the better progression-free survival than wild type group (13.2 vs. 3.2 months, $P=0.0762$). Mechanistically, *KDM5C* altered tumors had dramatically higher TMB level and was associated with significantly higher level of CD8+ T cell infiltration and T effector signature. In conclusion, *KDM5C* alterations was correlated with enhanced tumor immunogenicity and inflamed anti-tumor immunity, thus resulting in better treatment outcome in cancer patients receiving ICIs.

Keywords: immune checkpoint inhibitors, biomarker, *KDM5C*, outcome, prognosis

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INTRODUCTION

Immune checkpoint inhibitors (ICIs) targeting cytotoxic T lymphocyte antigen-4 (CTLA-4), or programmed cell death protein 1 (PD-1) and its ligand (PD-L1) interaction have shifted the treatment paradigms and significantly improve the overall survival (OS) in diverse cancers (1–4). Nevertheless, ICIs could only benefit a minority (~20%) of unselected population (5). Herein, there is an urgent need to develop novel predictive biomarkers for the majority of patients, who could not benefit from ICIs treatment. The mutational landscape of tumor cells is a direct reflection of tumor

immunogenicity and could dictate the extent and phenotype of immune infiltrates (6–8). Understanding the relationship between tumor genomic alterations and response to ICIs could lay a foundation for the development of novel predictive biomarkers and therapeutic strategies to improve the clinical benefit (8).

Lysine (K)-specific demethylase 5C (*KDM5C*) is a histone demethylase that specifically removes methyl residues from tri-, di-, and monomethylated lysine 4 on histone H3 lysine 4 (H3K4), thus resulting in suppressing gene transcription by reducing H3K4 trimethylation levels (9–11). Previous studies reported that genetic alterations of *KDM5C* were common in various types of cancers including breast, colon, ovarian, prostate cancer and so on. It plays a significant role in the tumorigenesis, cancer cell proliferation, invasion, drug resistance and the regulation of tumor-related gene expression (12–14). Moreover, a recent elegant study analyzed the multi-omics data of 823 advanced renal cell carcinoma and found that somatic mutations in *KDM5C* correlate with high angiogenesis and AMPK/fatty acid oxidation gene expression, which was enriched in ICIs beneficial group. These findings revealed the contribution of *KDM5C* to antitumor immune response. Therefore, it is valuable to explore the predictive value of *KDM5C* alterations for ICIs treatment outcome in multiple cancers.

Here, we performed this pan-cancer analysis to investigate *KDM5C* alterations frequency and their predictive significance for ICIs treatment outcomes across cancer types. We also evaluated the relationship between *KDM5C* alteration and immune infiltrates and signatures by using online database to unravel the potential mechanism.

MATERIALS AND METHODS

Data Collection and Pan-Cancer Analysis

We downloaded the sequenced data and collected clinical information from several online database as shown in **Figure 1**. For determination of the frequency of *KDM5C* alterations among different types of solid tumors, the genomic alterations and clinical characteristics were identified from the cBioPortal online database (https://www.cbioportal.org) (15, 16). *KDM5C* alterations were recorded as all kinds of nonsynonymous mutations including mutations, missense, frame-shift, splice site, nonstop, nonsense, and translation start site changes. Non-redundant publications were identified. If two or more studies reported the same cohort, only the study with the largest sample size and latest information was included. To avoid the selection bias and limitation of small sample size, we excluded the records of cancer type with patients less than 100. Analysis of TMB normalization, clinical cohort and treatment outcomes were summarized in Supplemental Material.

TUMOR IMMUNOGENICITY AND IMMUNE LANDSCAPE ANALYSIS

To delineate the immune microenvironment features of tumors with *KDM5C* alterations, we calculated and compared immune infiltrates, immune signatures and immune-related gene expression between *KDM5C* altered and wild type group by using RNA-seq data from TCGA. The correlation between

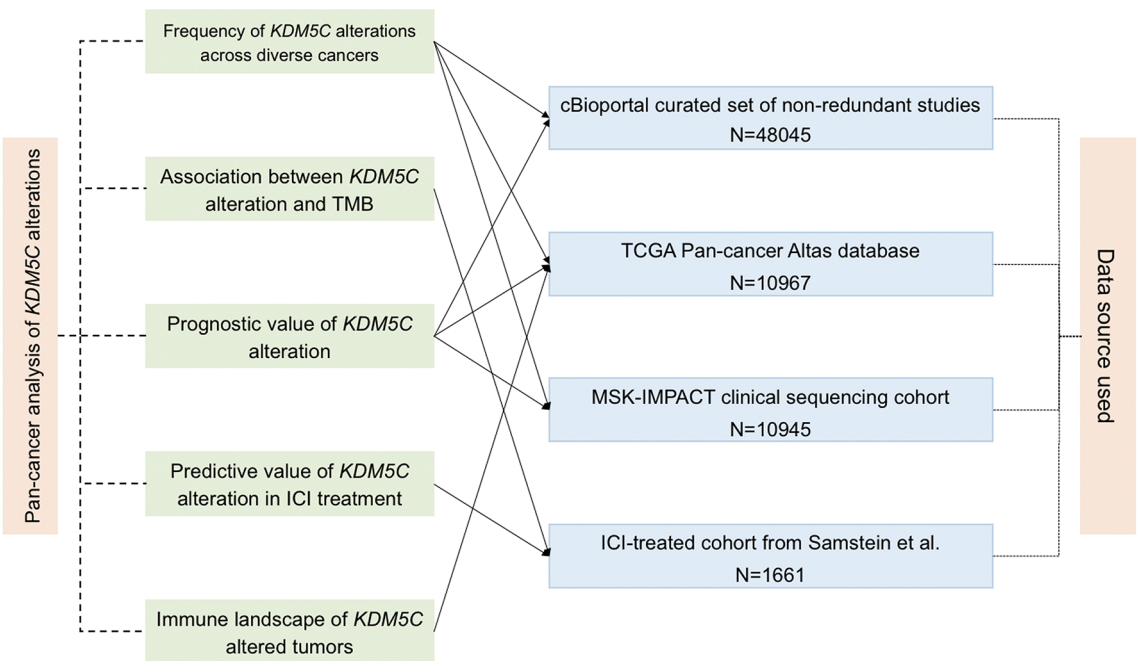

FIGURE 1 | Flowchart of the sequenced data and clinical cohort. The connected solid line between analysis aim (middle left) and data source (middle right) means the used cohort by this analysis.

KDM5C expression and immune checkpoints expression in different cancers was evaluated by using online database, named Tumor Immune Estimation Resource (TIMER). The statistical methods were listed in this website (https://cistrome.shinyapps.io/timer/) and their previous publications (17, 18). The abundance of tumor infiltrating leukocytes, including $CD8^+$ T cells, $CD4^+$ T cells, regulatory T cells (Tregs), dendritic cells, B cells, macrophage, myeloid-derived suppressor cells (MDSC), NK cells, mast cell, neutrophils, endothelial cells and cancer-associated fibroblasts (CAFs), was estimated by using different bioinformatic algorithm and compared between *KDM5C* altered and wild type group.

Statistical Analysis

The association between *KDM5C* status and clinical features were evaluated by using fisher's exact test. $\chi 2$ test were performed to test whether the sampling distribution was equal for two groups. The continuous variables were analyzed by ANOVA and Tukey's multiple comparison tests. The differences of TMB, tumor-infiltrating immune cells, immune signatures, or immune-related gene expressions between *KDM5C* altered and wild type tumors were tested by using Mann-Whitney U test. We conducted two-tailed Mann-Whitney U tests for comparison of the nonparametric data set. Survival outcomes were measured with OS, or progression-free survival (PFS) according to the accessibility for each cohort. Kaplan-Meier curves with two-sided log-rank tests and Cox proportional hazards model with calculated hazard ratios (HRs) and 95% confidence intervals (CIs) were adjusted for available confounding factors to determine the different clinical outcomes between *KDM5C* altered and wild-type groups. Two-sided $P<0.05$ was considered significant. All statistical analyses were performed using the SPSS statistical software, version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Overview of Pan-Cancer Analysis

We identified a cohort of 45614 cancer patients with 48045 sequenced tumor samples. This cohort was consisted of 271 cancer studies and 47 cancer types. The prevalence of *KDM5C* alterations was 2.1%, with patients with esophagogastric cancer having the highest levels of *KDM5C* alterations (11.5%, 118/1023). We then investigated the prevalence and spectrum of *KDM5C* alterations in two representative cohorts (TCGA cohort, N = 10967; MSK-IMPACT cohort, N = 10945). In TCGA cohort, endometrial carcinoma had the highest levels of *KDM5C* alterations (9.6%, 56/586; **Figure 2A**). In MSK-IMPACT cohort, renal cell carcinoma had the highest levels of *KDM5C* alterations (9.4%, 34/361; **Figure 2B**). Most detected *KDM5C* alterations were copy number alterations (either amplifications or deep deletion) in TCGA cohort (**Figure 2A**), while most were *KDM5C* somatic mutations in MSK-IMPACT cohort (**Figure 2B**).

Association Between *KDM5C* Alterations and Clinical Outcomes

Next, we evaluated the association between *KDM5C* alterations and clinical outcomes. We firstly found that patients with *KDM5C* alterations showed a significantly shorter OS (53 vs. 102 months; HR = 1.31, 95% CI 1.17-1.58, $P < 0.0001$; **Figure 3A**) than those without in 45614 cancer patients by merging 271 non-redundant studies from the cBioPortal online database. Subgroup analyses showed that *KDM5C* alterations were correlated with numerically shorter OS in TCGA (68 vs. 80 months; $P = 0.4336$; **Figure 3B**) and MSK-IMPACT cohort (23 vs. 26 months, $P = 0.5220$; **Figure 3C**).

In the ICI treatment cohort (19), we firstly identified 1661 patients with different cancers receiving ICI therapy and 73 of

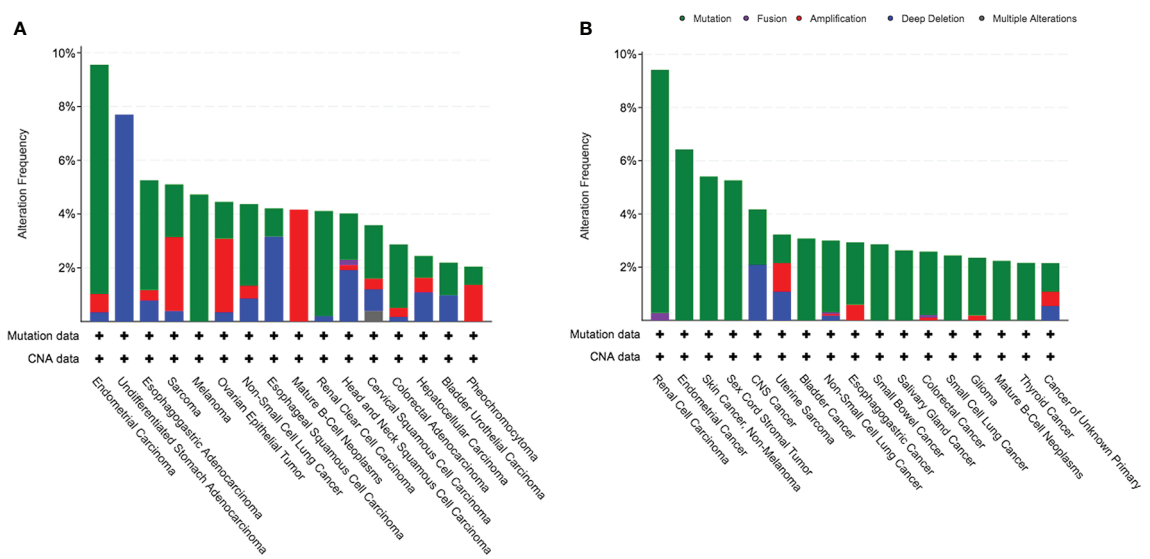

FIGURE 2 | Prevalence of *KDM5C* alterations in different cancers. **(A)** TCGA cohort; **(B)** MSK-IMPACT cohort.

them with *KDM5C* alterations. Clinicopathological features, including age, sex, sample type, drug type and tumor purity, were well balanced between altered and wild type group (**Supplemental Table S1**). Patients with *KDM5C* alterations had a significantly prolonged OS than those in wild-type group (not reached vs. 18 months; HR = 0.56, 95% CI 0.46-0.86, P = 0.0041; **Figure 3D**). Importantly, we compared the overall survival of patients who received ICI with those who did not in KDM5C mutant group. As shown in the following figure A, we found that patients received ICI treatment had markedly longer overall survival than those received chemotherapy in KDM5C mutant group (HR = 0.584, P = 0.0168; **Supplemental Figure S2A**). However, in KDM5C wild type group, patients received ICI treatment had analogous overall survival with those received chemotherapy (HR = 0.949, P = 0.1067; **Supplemental Figure S2B**). Although *KDM5C* alterations were associated with higher level of TMB and mutation count, multivariate analysis revealed that *KDM5C* alterations was associated with substantially longer OS than wild type independent of TMB (HR = 0.60, 95% CI 0.40-0.91, P = 0.015; **Supplemental Table S2**). Notably, we did not observe the association between *KDM5C* alterations and better OS in patients with microsatellite-stable (MSS) solid tumors (12 vs. 21 months; HR = 1.62, 95% CI 0.50-5.63, P = 0.2875; **Figure 3E**). Interestingly, in non-small-cell lung cancer (NSCLC) treated with ICI, patients with *KDM5C* alterations had markedly longer progression-free survival (PFS) than other alterations and wild type groups (13.2 vs. 3.2 months; HR = 0.52, 95% CI 0.34-1.05, P = 0.2875; P = 0.0762; **Figure 3F**).

Association Between *KDM5C* Alteration and TMB Level

Previous publications revealed the close relationship between ICIs treatment outcomes and TMB/mutation counts. Thus, it is valuable to evaluate the relationship between *KDM5C* alterations and TMB level/mutation counts. In MSK-IMPACT cohort (20), we found that mutation count of patients with *KDM5C* alterations was significantly higher than those without these alterations (10 vs. 4, P < 0.0001; **Supplemental Figure S1**). This was validated in the ICI-treated cohort that included 1661 patients (mutation count of *KDM5C* alterations vs. wild type: 15 vs. 6, P < 0.0001; **Figure 4A**). Notably, cancers with *KDM5C* alterations also had the higher TMB level than those without these alterations (12 vs. 6 mut/Mb, P < 0.0001; **Figure 4B**). Co-occurring of genetic mutations in cancers with *KDM5C* alterations were not uncommon in both early-stage and advanced stage cohort (**Figures 4C, D**) and some of them are prevalent driver genes (e.g., *LRP2, KMT2C, PBRM1, NOTCH1, FAT1, SETD2, NSD1*, etc.), while their clinical significance remained undetermined.

Immune Feature Analysis of *KDM5C* Altered Tumors

To depict the tumor immune microenvironment of *KDM5C* altered tumors, we compared the immune infiltrates and anti-tumor immunity between *KDM5C* altered and wild type tumors. As we previously mentioned, *KDM5C* altered tumors had significantly higher TMB level than those with wild type,

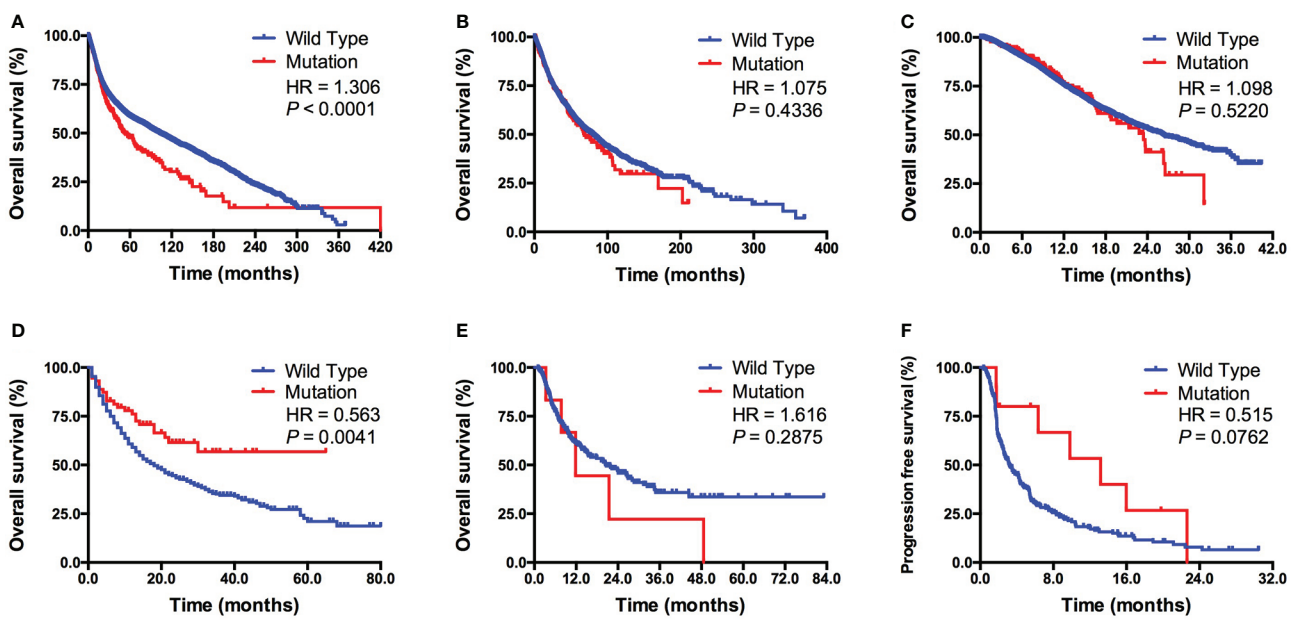

FIGURE 3 | Association between *KDM5C* alterations and clinical outcome. **(A)** Prognostic value of *KDM5C* alterations in all cancers; **(B)** Prognostic value of *KDM5C* alterations in TCGA cohort; **(C)** Prognostic value of *KDM5C* alterations in MSK-IMPACT cohort; **(D)** Predictive value of *KDM5C* alterations in ICI treated cohort; **(E)** Predictive value of *KDM5C* alterations in patients with microsatellite-stable solid tumors; **(F)** Predictive value of *KDM5C* alterations in patients with non-small-cell lung cancer patients received ICI treatment.

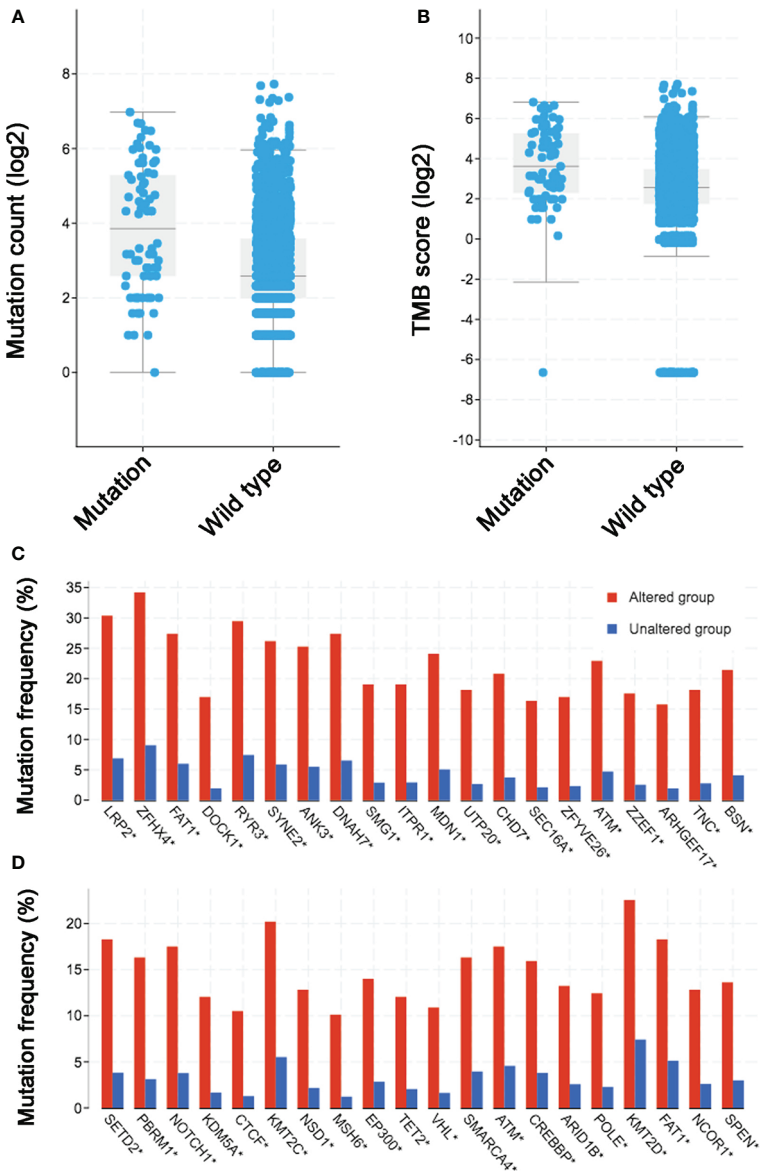

FIGURE 4 | Association between *KDM5C* alterations and mutation count/tumor mutation burden (TMB) across diverse types of cancer. **(A)** The association between mutation count and *KDM5C* alterations in immune checkpoint inhibitor (ICI) treated cohort; **(B)** The association between TMB and *KDM5C* alterations in ICI treated cohort; **(C)** Co-occurring of genetic mutations in cancers with *KDM5C* alterations versus wild type in TCGA cohort; **(D)** Co-occurring of genetic mutations in cancers with *KDM5C* alterations versus wild type in MSK-IMPACT cohort.

suggesting the potential enhanced tumor immunogenicity of *KDM5C* altered tumors. We then surveyed the relationship between *KDM5C* alterations and common immune infiltrates including $CD8^+$ T cells, $CD4^+$ T cells, Tregs, dendritic cells, B cells, macrophage, MDSC, NK cells, mast cell, neutrophils, endothelial cells and CAFs across different cancer types (**Figure 5** and **Supplemental Figures S3–S8**). The results showed that tumor-infiltrating $CD8^+$ T cells, were generally more abundant in the *KDM5C* altered colon adenocarcinoma and uterine corpus endometrial carcinoma when compared with those in the wild type tumors (**Figure 5A**). Whereas other immune infiltrates had similar abundance in *KDM5C* altered and wild type group (**Supplemental Figures S3–S8**). Moreover, *KDM5C* altered colon adenocarcinoma and uterine corpus endometrial carcinoma had dramatically higher level of antitumor T effector signature (**Figure 5B**). We also evaluated the association between *KDM5C* expression and several inhibitory (e.g., CD160, CD96, CSF1R, CTLA-4, TIM-3, IDO1, IL10, LAG3, PD-1, PD-L1, PD-L2, TFGB1, TGFBR1, TIGIT, VEGFA) and stimulatory (e.g., CD27, CD28, CD40, CD40LG, CD70, CD80, CD86, CXCL12, CXCR4, ICOS, ICOSLG, MICA, MICB, TNFRSF14, TNFRSF17, TNFRSF18, TNFRSF4,

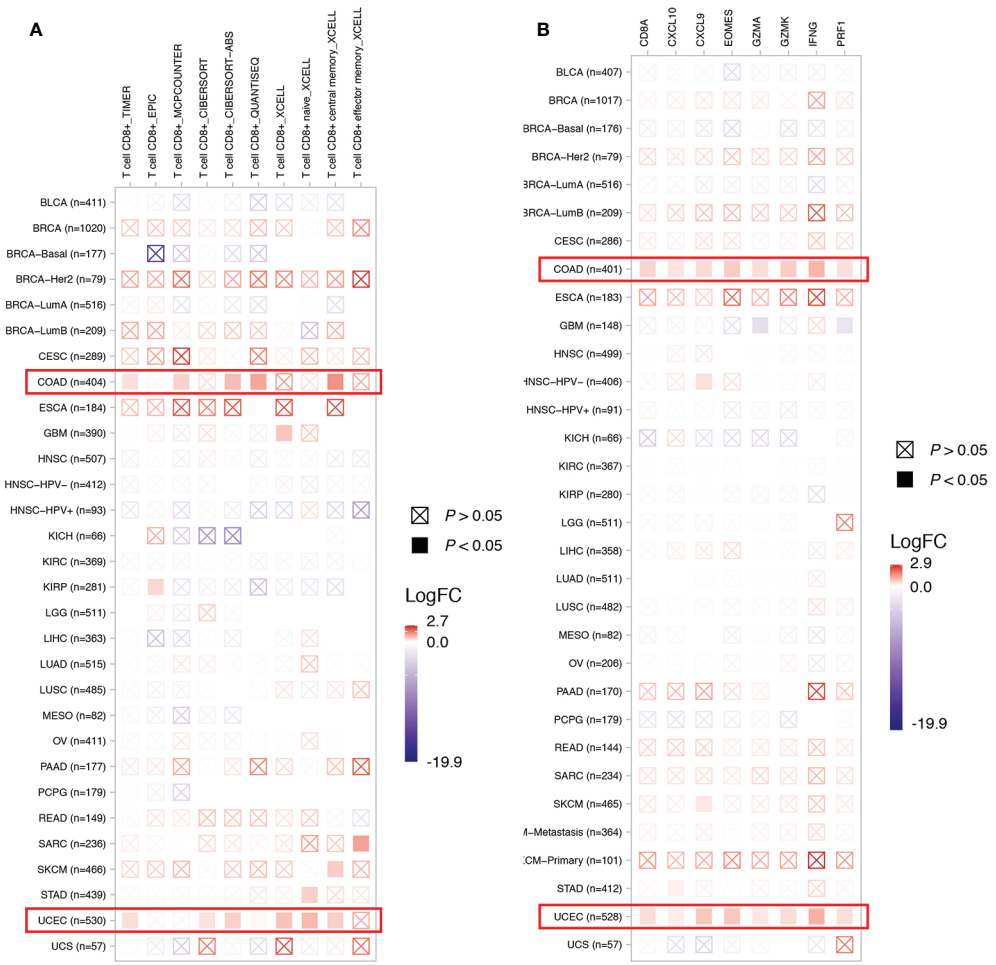

FIGURE 5 | Association between *KDM5C* alterations and CD8+ T cell abundance **(A)** and T effector signature **(B)**. BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous-cell carcinoma and endocervical adenocarcinoma; COAD, Colon adenocarcinoma; ESCA, esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, Liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous-cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; READ, rectum adenocarcinoma; SKCM, Skin cutaneous melanoma; STAD, Stomach adenocarcinoma; UCEC, Uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma.

TNFRSF9, TNFSF9, TNFSF13) immune checkpoints expression in various cancers. Intriguingly, we also found the significantly higher expression level of these immune checkpoints in *KDM5C* altered colon adenocarcinoma and uterine corpus endometrial carcinoma (**Figures 6A, B**).

DISCUSSION

To our knowledge, this study firstly reported the frequency of *KDM5C* alterations and its pan-cancer predictive value to ICI treatment in various cancers. *KDM5C* alterations were a negative prognostic marker in whole group but it might be utilized to predict survival benefit from ICI treatment across diverse cancers. Although *KDM5C* altered tumors had significantly higher TMB level, multivariate analysis showed that *KDM5C* alterations was associated with significantly longer OS independent of TMB. Moreover, we did not observe the association between *KDM5C* alterations and prolonged OS in patients with MSS solid tumors, suggesting that it may not be suitable for predicting ICI treatment outcome in MSS solid tumors. Mechanistically, *KDM5C* altered tumors was found to be markedly correlated with enhanced tumor immunogenicity and immunosupportive features of anti-tumor microenvironment.

In this pan-cancer analysis, the frequency of *KDM5C* alterations was 2.1% in a cohort of 45614 cancer patients, with esophagogastric cancer, endometrial carcinoma and renal cell carcinoma having the highest levels of *KDM5C* alterations, which was similar to previous publications (21, 22). Interestingly, we found a positive association between co-occurrence of *KDM5C* alterations and some common

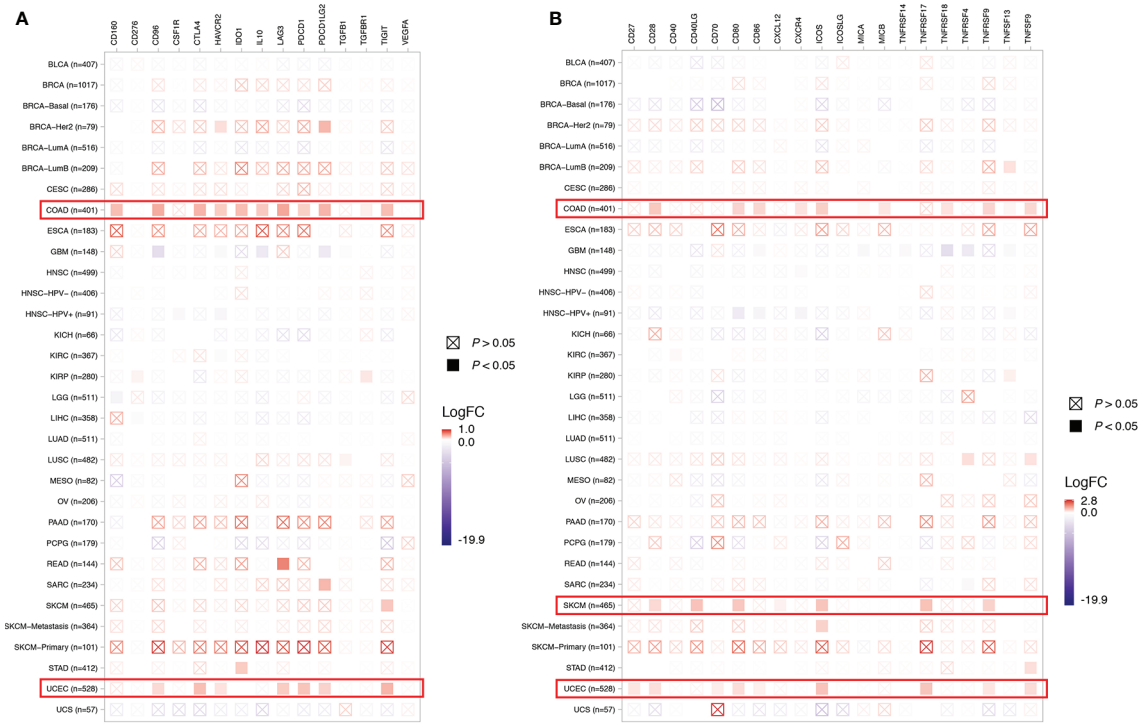

FIGURE 6 | Association between *KDM5C* alterations and several inhibitory **(A)** and stimulatory **(B)** immune checkpoints expression across different cancer types. BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous-cell carcinoma and endocervical adenocarcinoma; COAD, Colon adenocarcinoma; ESCA, esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, Liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous-cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; READ, rectum adenocarcinoma; SKCM, Skin cutaneous melanoma; STAD, Stomach adenocarcinoma; UCEC, Uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma.

epigenetic regulatory genes including *PBRM1, KMT2C, SETD2, NSD1*, etc. In spite of the unclear biological function of these co-mutations, several previous studies have shown that these diver genes are very important tumor suppressor genes in renal cell cancers and could contribute to the aggressive phenotype, therapeutic efficacy and/or prognostic value (23–29). Therefore, it would be valuable to unravel the biological and molecular mechanisms, and impact on clinical outcome of this co-occurrence for specific cancer types in future studies.

Previous studies reported *KDM5C* is required for proper DNA replication at early origins and its alterations could lead to genomic instability in sporadic renal cancer (30, 31). We thus evaluated the association between *KDM5C* alteration and TMB level. As expected, our results showed that *KDM5C* altered tumors had significantly higher TMB level than wild type ones in two independent cohorts, indicating that *KDM5C* alterations could be considered as predictive biomarkers for ICI treatment. Having noticed this relationship, we then investigated both predictive and prognostic significance of *KDM5C* alterations. In whole group, patients with *KDM5C* alterations had a significantly shorter OS than those with wild type, suggesting that *KDM5C* alterations could not confer an intrinsic survival benefit to treatment-naïve patients receiving ICI treatment. In ICI-treated cohort, patients with *KDM5C* alterations had a substantially prolonged OS. Moreover, subgroup analyses showed the association between *KDM5C* alterations and OS was independent of TMB in patients receiving ICI. More interestingly, in NSCLC treated with ICI, we found patients with *KDM5C* alterations had the significantly longer PFS than wild type groups. Collectively, *KDM5C* alterations could be considered as a potential pan-cancer predictive biomarker for ICI treatment, especially for NSCLC.

As a histone demethylase, *KDM5C* could suppress gene transcription by reducing H3K4 trimethylation levels (9–11). *KDM5C* plays a significant role in the tumorigenesis, cancer cell proliferation, invasion, metastasis and drug resistance (12–14). Recently, an elegant study analyzed the multi-omics data of 823 advanced renal cell carcinoma and found that somatic mutations in *KDM5C* correlate with high angiogenesis and AMPK/fatty acid oxidation gene expression, which was enriched in ICIs beneficial group (32). These findings suggested that *KDM5C* altered tumor would have specific immune microenvironment features. In this study, we observed that *KDM5C* altered tumors had markedly higher TMB and were associated with anti-antitumor immune signatures, indicating that *KDM5C* altered tumors would possess the enhanced tumor immunogenicity and

relatively immunosupportive microenvironment, supporting its predictive value to ICI treatment.

Pan-cancer universality of immunotherapy targeting PD-1 and PD-L1 interaction challenges us to rethink the investigation and development of predictive biomarkers. To date, MSI-high (MSI-H) is the only pan-cancer biomarker approved by the FDA with a relatively low frequency (~4%) (33, 34). MSI-H is common in digestive cancer including colorectal cancer and gastric cancer, while *KDM5C* alterations were more common in endometrial and renal cell carcinoma, indicating the predictive value of MSI-H and *KDM5C* alterations is not overlapped. Notably, *KDM5C* alterations could not predict the clinical outcome in patients with MSS solid tumors receiving ICI, which need future investigation. Collectively, the pan-cancer predictive significance of *KDM5C* alterations and its complementation to MSI-H in ICI therapy are anticipated.

There are several limitations that should be acknowledged. First, the origin of included cohorts was diverse, which could result in the selection bias and inconsistency of data quality. Combining different groups of patients with distinct histologies without meta-analysis could lead to the methodological pitfalls. Second, the *KDM5C* altered cohort included both gain (e.g., amplifications) and loss (e.g., deletions) of function alterations whether they could cause the same survival or ICI response difference compared to WT remained undetermined. Without adjustment per histology and type of *KDM5C* alterations, these results should be interpreted with caution. Third, due to the unavailable PD-L1 expression results from online database, we could not evaluate the relationship between *KDM5C* alterations and PD-L1 expression. Last but not least, in patients with MSS tumors, only six patients had *KDM5C* alterations. The association between *KDM5C* alterations and prolonged OS in MSS tumors needs further exploration.

In summary, the present study firstly provides the evidence that *KDM5C* alterations were associated with enhanced tumor immunogenicity and inflamed anti-tumor immunity, which result in prolonged OS in cancer patients treated with ICIs. The predictive value of *KDM5C* alterations were independent of tumor mutational burden and microsatellite status, suggesting that *KDM5C* alterations could be considered as a potential pan-cancer predictive biomarker for ICI treatment. In the future, we still need to investigate the exact molecular mechanism and large-scale, prospective studies are also warranted.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cbioportal.org.

ETHICS STATEMENT

Ethical approval was waived since we used only publicly available data and materials in this study. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

X-JC and LZ designed this study. X-JC and AR collected the clinical and sequenced data. X-JC performed the statistical analyses. X-JC, LZ and E-DZ drafted the manuscript. LZ and E-DZ provided critical comments, suggestions and revised the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.664847/full#supplementary-material

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Characteristics of Pan-Cancer Patients With Ultrahigh Tumor Mutation Burden

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Background: Tumor mutation burden has been proven to be a good predictor for the efficacy of immunotherapy, especially in patients with hypermutation. However, most research focused on the analysis of hypermutation in individual tumors, and there is a lack of integrated research on the hypermutation across different cancers. This study aimed to characterize hypermutated patients to distinguish between these patients and non-hypermutated patients.

Methods: A total of 5,980 tumor samples involving 23 types of solid tumors from the in-house database were included in the study. Based on the cutoff value of tumor mutation burden (TMB), all samples were divided into hypermutated or non-hypermutated groups. Microsatellite instability status, PD-L1 expression and other mutation-related indicators were analyzed.

Results: Among the 5,980 tumor samples, 1,164 were selected as samples with hypermutation. Compared with the non-hypermutated group, a significant increase in the mutation rates of DNA mismatch repair genes and polymerase genes was detected in the hypermutated group, and there was an overlap between high TMB and high microsatellite instability or high PD-L1. In addition, we found that EGFR, KRAS and PIK3CA had a high frequency of both single nucleotide variation and copy number variation mutations. These identified mutant genes were enriched in the oncogenic signaling pathway and the DNA damage repair pathway. At the same time, the somatic cell characteristics and distribution of the two groups were significantly different.

Conclusions: This study identified genetic and phenotypic characteristics of hypermutated tumors and demonstrated that DNA damage repair is critically involved in hypermutation.

Keywords: pan-cancer, hypermutation, tumor mutation burden, mismatch repair, polymerase

INTRODUCTION

The fact that many different cancers share common genomic characteristics (1) and respond well to relevant inhibitors has led researchers to perform integrated studies involving multiple types of cancers. Comparison of tumor types analyzed by The Cancer Genome Atlas (TCGA) through the Pan-Cancer Atlas can further supplement and summarize the completed TCGA results (2). The integration of these data sets provides a comprehensive picture of somatic mutations (3, 4), copy number changes (5, 6), mutational signatures (7), and other genetic variations in tumors, furthering the understanding of cancer mechanisms.

Tumor mutation burden (TMB) is defined as the total number of somatic gene coding errors, base substitutions, and gene insertions or deletions detected per million bases (8). The number of somatic mutations in different types of cancers ranges from 0.01 mut/Mb to more than 400 mut/Mb. Tumor antigenicity increases with increased TMB and is a prerequisite for PD1/PDL1 antibody efficacy. In recent years, TMB has been proven to be a good predictor for the efficacy of immunotherapy in multiple clinical trials (9, 10). Retrospective analysis of the CheckMate 568 clinical trial revealed that among patients with advanced/metastatic NSCLC, those with a TMB of 10 mut/Mb or higher had higher objective response and progression-free survival rates than those with a TMB of less than 10 mut/Mb (11). Similar results were observed in the KEYNOTE-028 trial (12).

Hypermutation refers to a cellular mechanism that causes the genome to be mutated at a frequency at least 100,000 to millions of times higher than the background mutation rate. It mainly involves point mutations (single base substitution), as well as occasional base insertion or deletion. Many types of cancers, such as colorectal cancer (13) and gastrointestinal cancer (14,) are classified into two molecular pathological groups: hypermutation and non-hypermutation. Recently, several longitudinal observational studies conducted comparisons of glioma and prostate cancer before and after treatment and found hypermutation differences in the genomes of patients, in particular when the tumor recurs (15–17). In the case of hypermutation, an increasing number of mutations in hypermutant cells may result in decreased fitness, rendering the cells less aggressive and more susceptible to treatment (18). Therefore, hypermutation plays an essential role in tumor occurrence and progression and can improve therapeutic efficacy. However, to date, most research has focused on the analysis of hypermutation in individual tumors, and there is a lack of integrated research on the hypermutation of different cancers.

Here, we performed a comprehensive pan-cancer classification of 5,980 tumor samples involving 23 types of solid tumors from the in-house database (Genecast Biotechnology Co., Ltd). This study aimed to identify the differences in characteristics of the genome mutation profile between patients with hypermutation and those with non-hypermutation (low group). The findings may have significance in guiding clinical practice.

MATERIALS AND METHODS

Genomic and Clinical Data

Genomic and clinical data from 23 different types of solid tumors were gathered from the in-house database (Genecast Biotechnology Co., Ltd). The in-house database was built based on the information collected from clinical samples that was sequenced by a customized 543-gene panel, which covered 1.7 Mb of the genome. The filtering criteria for the samples used in this study were as follows: 1). Samples that were sequenced from January 1, 2019 to December 31, 2019; 2). Samples that were tested by a 543-gene panel; 3). Tissue samples; 4). Patients who aged >25 years old; 5) samples that were collected at the earliest time. The exclusion criteria were as follows: 1). Samples with TMB=0; 2). Tumors with <20 samples; 3). Metastatic samples. The following types of solid tumors were included in the study: non-small cell lung cancer (adenocarcinoma, LUAD, n=2384; squamous cell carcinoma, LUSC, n=456; others, NSCL, n=554), stomach cancer (STAD, n=534), colon cancer (COAD, n=476), rectal cancer (READ, n=344), esophageal carcinoma (ESCA, n=184), liver hepatocellular carcinoma (LIHC, n=162), cholangiocarcinoma (CHOL, n=123), pancreatic cancer (PAAD, n=120), breast cancer (BRCA, n=111), head and neck squamous cell carcinoma (HNSC, n=95), small cell lung cancer (SCL, n=80), ovarian cancer (OV, n=60), cervical squamous cell carcinoma (CESC, n=47), glioblastomas (GBM, n=47), nasopharyngeal cancer (NASO, n=38), skin cutaneous melanoma (SKCM, n=35), bladder cancer (BLCA, n=29), kidney cancer (LICH, n=28), soft tissue sarcoma (SARC, n=28), uterine corpus endometrial carcinoma (UCEC, n=25), and gastrointestinal stromal cancer (GIST, n=20). The cutoff value for hypermutation (8.561943) was determined by the segmented linear regression analysis in R language (19, 20). Among 5,980 Chinese patients, 1,164 (19.5%) had a significantly higher TMB than the others and were identified as patients with hypermutation.

Identification of Mismatch Repair and Polymerase Gene Mutations

After analyzing the population frequency in the database, as well as the cosmic database and dbSNP database, we screened for nonsynonymous mutations in the exon region or cleavage region of DNA mismatch repair (MMR) genes (MSH2, MLH1, MSH6, and PMS2) and polymerase genes (POL; POLE and POLD1). Manual review was performed to determine the final mutation set. Samples containing more than one mutation in the 6 genes were identified as MMR/POL mutation samples. The Wilcoxon test was used to compare the distribution of TMB between the mutation (MUT) group and wild-type (WT) group, while the difference in the proportion of samples with MUT or WT between the Hypermutation group and Low group was analyzed by using Fisher's test.

Analysis of Microsatellite Instability (MSI)

For each microsatellite locus, all spanning reads (covering at least 2 bp in both the 5' and 3' directions) were extracted from the realigned BAM file. Following deduplication, the length of the

mononucleotide repeat in each deduped alignment was counted and tallied by length. The baseline reference value was calculated by using 30 normal blood samples and was used to assess the instability of microsatellite loci. Finally, the fraction of unstable loci out of the total number of loci analyzed was calculated for each experimental sample. Based on the fraction value, samples were classified into the MSI-H group and MSS/MSI-L group. A fraction value of 0.3 was set as the cutoff value for defining an unstable locus as an MSI-positive locus. The Wilcoxon test was used to compare the distribution of TMB between the MSI-H group and MSS/MSI-L group, while the difference in the proportion of samples with MSI-H or MSS/MSI-L between the Hypermutation group and Low group was analyzed by using Fisher's test.

Detection of PD-L1 Expression

The expression of PD-L1 on the surface of tumor cells (TCs) and tumor-infiltrating immune cells (ICs) was assessed by IHC staining using anti-PD-L1 (SP142) rabbit monoclonal primary antibody (Roche, Indianapolis, IL, USA). PD-L1 expression was described as a continuous variable based on the percentage of tumor cells with a certain staining intensity (21). Samples were also classified into the negative (N), low-positive (positive 1, P1), medium-positive (positive 2, P2), and high-positive (positive 3, P3) groups according to the expression level of PD-L1. The Wilcoxon or Kruskal-Wallis test was used to compare the distribution of TMB in the high and low PD-L1 groups, while the difference in PD-L1 expression between the Hypermutation group and the Low group was analyzed by using Fisher's test.

Identification of Single Nucleotide Variation (SNV)

Sequencing reads were processed through an in-house pipeline. The pipeline included Trimmomatic (v.0.39) for read adapter trimming and quality filtering, BWA (v.0.7.17) for mapping reads to the hg19 reference genome, the Picard toolkit (v.2.1.0) for sorting and making duplicates, and the Genome Analysis ToolKit (v.3.7) for read realignment. VarDict (v.1.5.1) was introduced for SNV calling, and compound heterozygous mutations were merged with FreeBayes (v.1.2.0). The generated candidate mutations were annotated using the ANNOVAR software tool and then filtered by using the ExAC, COSMIC, and dbSNP databases. Manual curation was performed to generate the final somatic SNV/InDel data set. The differences between the two groups of variation were evaluated by Fisher's test.

Identification of Copy Number Variation (CNV)

The GC content, target region length, and read count were corrected. Thereafter, the copy number and gene specificity score (GCS) was calculated using 30 normal blood samples as a control. GCS represents the degree of gene level difference between the tested sample and control. CNV was determined by a joint statistical significance test on GCS and the absolute value of the copy number.

Pathway and Mutational Signature Analysis

We identified genetic mutations in 10 major cancer pathways and 8 repair pathways in the samples, counted the number of mutations in each pathway for each cancer population and calculated the mutation frequency of each cancer population. The mutational signature was determined based on these somatic SNVs/InDels using maftools (v.2.4.10). The Wilcoxon test was used to compare the distribution and difference of the somatic signature among or between the Hypermutant and Low groups.

RESULTS

TMB Screening-Based Detection of Hypermutation

As shown in **Figure 1A**, 1,164 patients from a cohort of 5,980 patients with pan-cancer in the in-house dataset were selected as those with hypermutation based on the calculated cutoff value of TMB. The median value of the calculated TMB for each group is shown in **Figure 1B**. Notably, the TMB values of GBM and UCEC were much higher in the Hypermutation group (**Figure 1C**). The age was older and male proportion was higher in the Hypermutation group than in the Low group ($p<0.05$) (**Table S1**).

MSI Status and PD-L1 Expression of Patients With Hypermutation

We next evaluated the impact of TMB on MSI and PD-L1 expression using statistical methods to identify events associated with TMB in solid tumors. MSI, especially high MSI (MSI-H), is closely associated with the occurrence and progression of many tumors. In all samples, the MSI-H samples had a significantly higher TMB than the MSS/MSI-L samples (**Figure 2A**). No difference in TMB was observed between the MSI-H and MSS/MSI-L groups due to the low frequency of MSI in LUSC, HNSC, LICH, PAAD, SKCM, LUAD and other solid tumors. In contrast, there were significant differences in TMB values among UCEC ($P<0.05$), COAD ($P<0.001$), READ ($P<0.001$), NSCL ($P<0.01$), STAD ($P<0.001$), CHOL ($P<0.01$) and NASO ($P<0.05$), indicating that TMB was elevated in MSI-H samples (**Figure 2B**). Moreover, analysis of Hypermutation and Low samples revealed that Hypermutation samples were more prone to MSI-H (**Figures 2C, D**).

Similar to the analysis of MSI-H characteristics, studies on PD-L1 expression showed that the P2/P3 group displayed a significantly higher overall TMB than the N group, albeit in only six types of tumors, including COAD ($P<0.01$), READ ($P<0.05$), NSCL ($P<0.01$), STAD ($P<0.01$), SARC ($P<0.05$), and LUAD ($P<0.0001$) (**Figures 3A, B**). In addition, a comparison of the difference between Hypermutation and Low samples suggested that there was a correlation between high TMB and high expression of PD-L1 (**Figures 3C, D**).

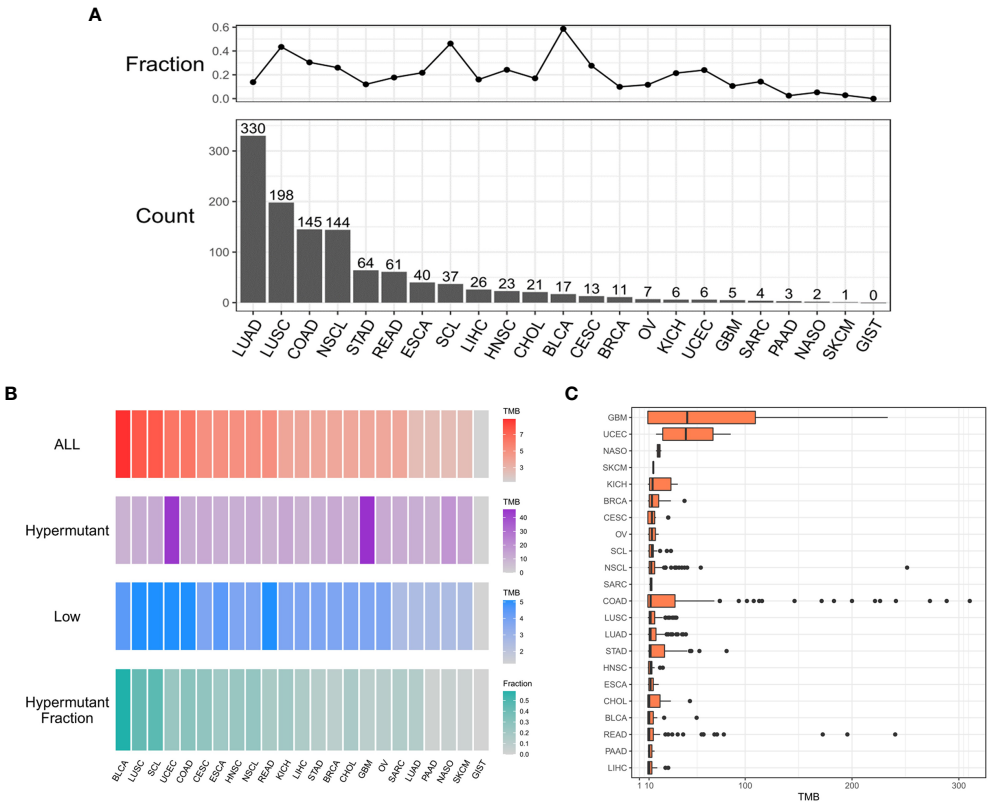

FIGURE 1 | The distribution of cancer types. **(A)** Histogram of the number of samples with hypermutation per cancer type. The numbers were sorted from highest to lowest. The line graph shows the proportion of the number of samples with hypermutation to the total number of samples for each cancer type. **(B)** The median value of TMB in the three sample groups (ALL, Hypermutation, and Low) for each cancer type and the number of Hypermutation samples for each cancer type as a proportion of the total number of samples for that cancer type. **(C)** The distribution of TMB in samples with hypermutation for each cancer type.

Mutational Characteristics of Patients With Hypermutation

While MSI is caused by a defect in MMR genes, POLE or POLD1 mutations serve as immunotherapeutic indicators of all types of tumors except for those with MSI-H. Therefore, we first examined the distribution of TMB in the MUT group and WT group at the global and carcinoma-specific hypermutation level. As shown in **Figures 4A, B**, samples with MMR and/or POL mutations had a higher TMB than the MUT group. The TMB values of COAD, GBM and UCEC were higher than those of other cancer types, and there were significant differences between COAD and UCEC in the MUT and WT groups (P <0.0001). Combined with the data from the Low group, a redescription of the mutation landscape for the two types of samples revealed that Hypermutation samples harbored more MMR/POL mutations than the MUT group (**Figures 4C, D**).

We next investigated whether the TMB level affects tumor mutation and CNV burden by quantifying the mutation rate and percentage of CNV in each group. **Table 1** lists CNVs with significant differences between the Hypermutation group and the Low group. Three genes (EGFR, KRAS and PIK3CA) in the top 10 list of mutated genes were identified as having significantly differential CNVs (**Figure 5**). In addition, TP53 was found to be the gene with the highest mutation frequency, with missense mutation as the main mutation type.

Statistical analysis of genetic mutations in 10 major cancer pathways and 8 repair pathways in the two types of samples revealed that mutations in the Hypermutation group were mainly enriched in p53 and RTK-RAS cancer-related pathways as well as the homologous recombination and MMR pathways. The mutation frequency of each pathway differed between the two samples (**Figure 6**). To better understand pathways globally dysregulated in the setting of TMB, we further performed a somatic signature analysis in the Hypermutation and Low groups. As shown in **Figure 7**, a total of five signatures, including defective DNA MMR and defects in polymerase POLE, displayed significant differences between the two groups (P <0.0001).

DISCUSSION

In this study, we delineated the distribution of cancer types in patients with hypermutation and identified an association

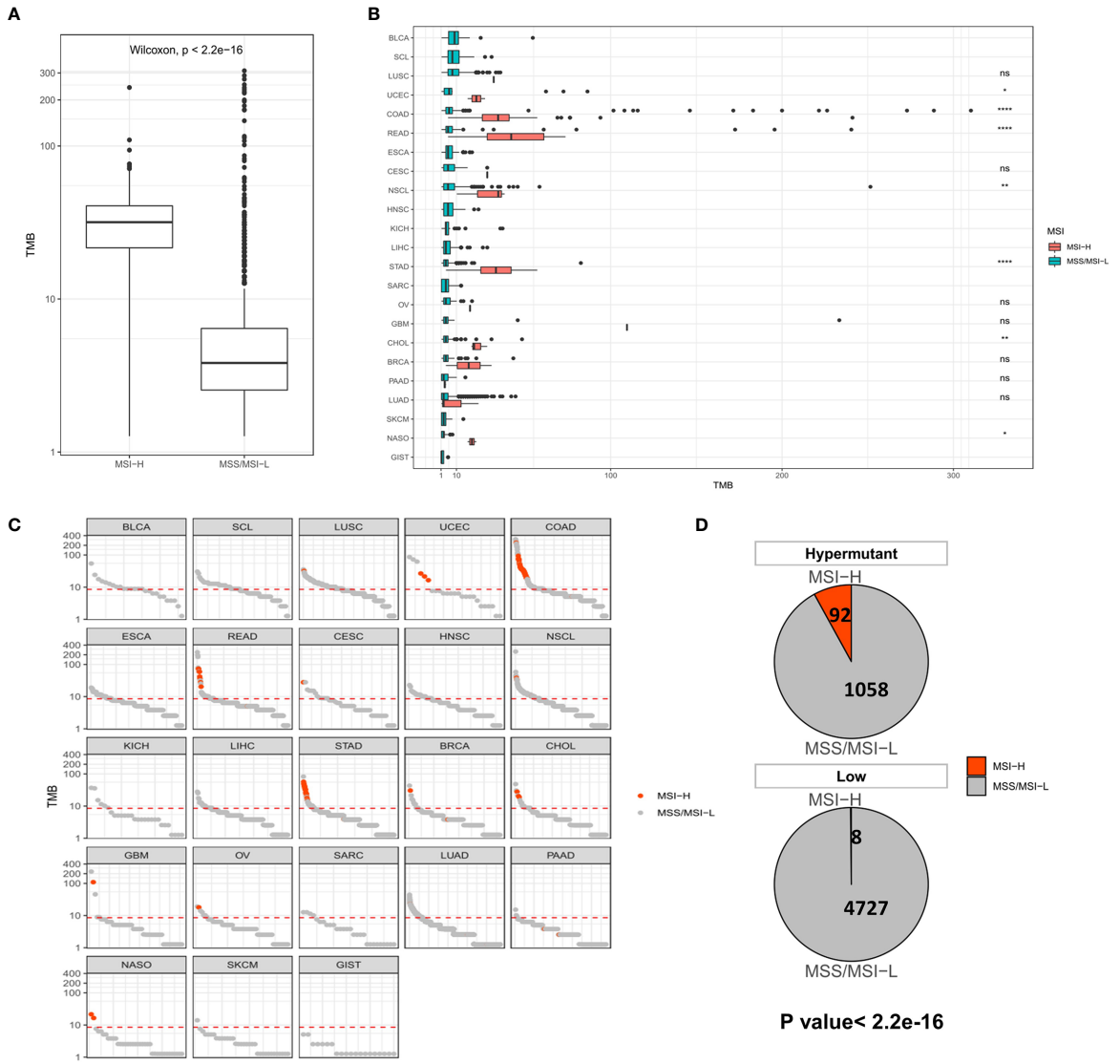

FIGURE 2 | **(A)** Comparison of TMB distribution between MSI-H and MSS/MSI-L groups. **(B)** TMB distribution in different cancer types grouped according to high and low MSI. (**C**) TMB value of each sample in each cancer type. Orange and gray represent MSI-H samples and MSS/MSI-L samples, respectively. **(D)** The number and proportion of MSI-H and MSS/MSI-L samples in Hypermutation and Low groups. *P* <0.05 was considered a significant difference, **P* < 0.05, ***P* < 0.01, *****P* < 0.0001, NS, Not Significant.

between hypermutation and MSI status, PD-L1 expression and MMR/POL gene mutations. This finding is similar to Gong's report, which suggested that POLE mutations and MSI tumors (hypermutation phenotype) may increase the expression of immune checkpoint genes, including PD-1, PD-L1 and CTLA-4 (22). Moreover, the present study showed that a comprehensive dissection of high-frequency CNVs, related pathways and somatic signatures, as well as the identification of high-frequency SNVs, are required to identify hypermutation cases with unique characteristics.

Known immune efficacy markers can be roughly divided into two categories: the first is related to tumor neoantigen load, including molecular markers such as MSI or TMB elevation, while the second is related to the tumor T cell inflammatory microenvironment, including core gene markers for PD-L1 protein expression, tumor lymphocyte infiltration and CNV (23). These two types of markers reflect the overall picture of tumor immune efficacy. A combination of two or more methods to determine the immune status of the tumor microenvironment is an effective and universal approach for predicting the efficacy of immune checkpoint inhibitors. In investigating the relationship between MSI or PD-L1 and TMB, we emphasized that the effect of MSI or PD-L1 on TMB mutation rates may vary with tumor type and may be influenced by other endogenous and

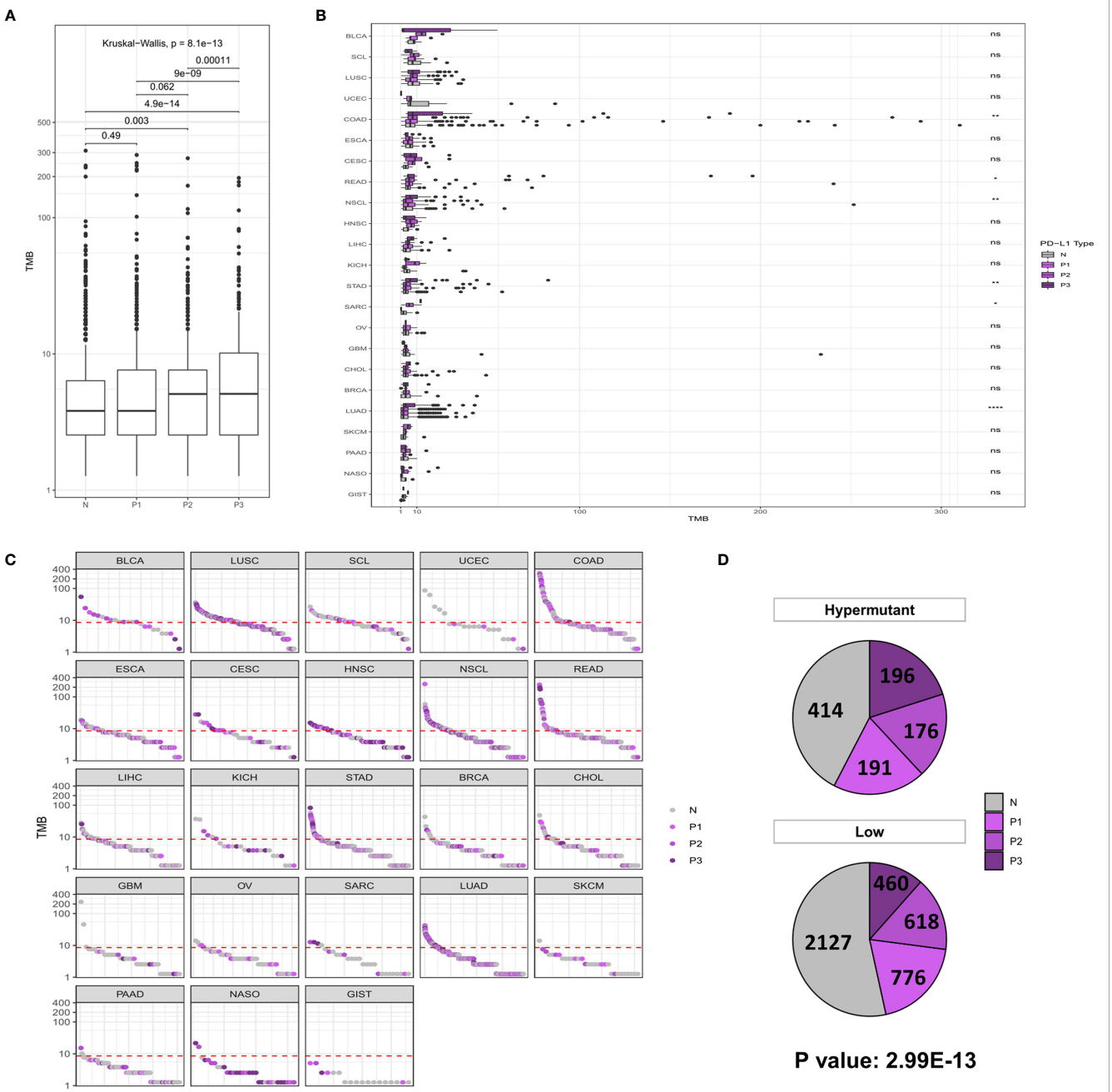

FIGURE 3 | **(A)** Comparison of TMB distribution among N, P1, P2, and P3 groups. **(B)** The TMB distribution of different cancers in the population is shown according to PD-L1 high and low groups. **(C)** TMB value of each sample in each cancer type. Shades of purples indicate the different levels of PD-L1 expression, while grays indicate PD-L1 negative samples. **(D)** The number and proportion of PD-L1 high and low samples in Hypermutation and Low groups. $P < 0.05$ was considered a significant difference, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. NS, not significant.

exogenous tumor factors, such as tumor-infiltrating lymphocytes and microbial flora. It has been documented that a high proportion of hypermutation cooccurs with MSI-H or high PD-L1 expression in colorectal and ovarian cancers (24–26). Interestingly, MMR/POL mutations have been shown to be associated with higher TMB in pan-cancer patients. MSI is caused by MMR defects due to the inactivation of one of the four main MMR genes, MSH2, MLH1, MSH6, and PMS2, and is characterized by extensive polymorphism in microsatellite sequence length as a result of DNA polymerase slippage (27). Furthermore, studies have shown that tumors with MMR defects can also contain other DNA repair defects, such as POLD or POLE

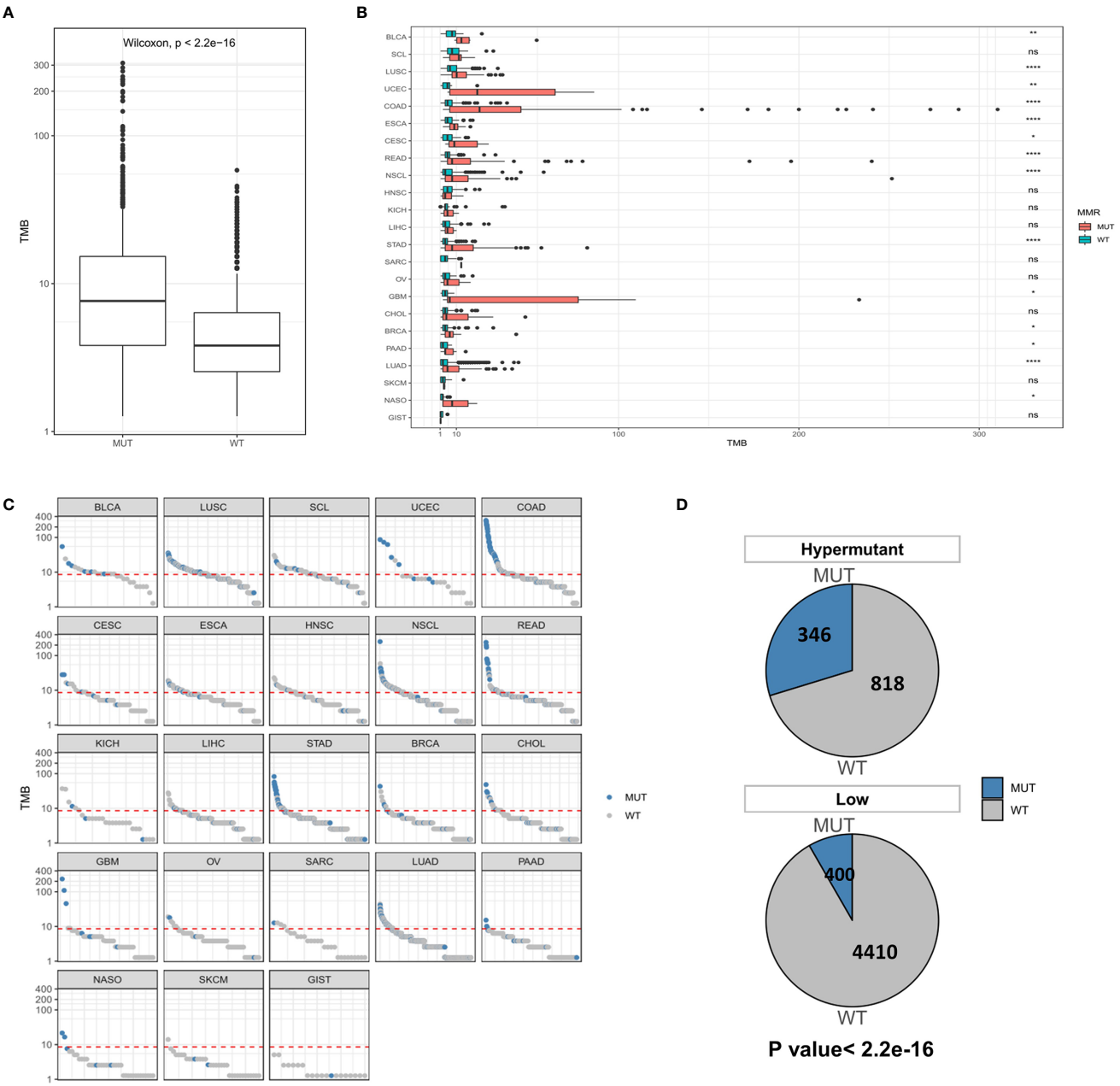

FIGURE 4 | **(A)** Comparison of TMB distribution between the MUT group and WT group. **(B)** The distribution of TMB in the MUT group and WT group for each cancer type. **(C)** TMB value of each sample in each cancer type. Blue and gray represent samples in the MUT group and those in WT group, respectively. **(D)** The number and proportion of MUT and WT samples in Hypermutation and Low groups. $P < 0.05$ was considered a significant difference, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. NS, not significant.

mutations, and several immune checkpoint ligands, including PD-1, PD-L1, CTLA-4, LAG-3 and IDO, are also highly expressed in the tumor microenvironment of these patients (28, 29). Therefore, MMR and/or POL mutations may underlie the complex interaction between MSI or PD-L1 expression and TMB.

In the present study, we further demonstrated that patients with hypermutation had a much higher frequency of MMR and/or POL mutations than those with non-hypermutation. On the one hand, we observed that among the eight pathways of the DNA damage response system, the homologous recombination and MMR pathways were the most frequently mutated in the tumor samples. Notably, the correlation between MMR and homologous recombination pathways has been reported in colon cancer and rectal cancer (30). On the other hand, we showed in the somatic signature that hypermutant tumors have defects in both MMR genes and the POLE polymerase gene. Similarly, one study looked

TABLE 1 | The number and proportion of samples with CNV mutation in the Hypermutation and Low groups.

CNV	Class	Total	Var_num	Var_per (%)	Novar_num	Novar_per (%)	P value	Odds ratio
ALK_GAIN	Hypermutation	633	5	0.79	628	99.21	0.0290771	4.916107
	Low	1857	3	0.16	1854	99.84		
CDK4_GAIN	Hypermutation	633	31	4.90	602	95.10	5.13E-06	0.4267569
	Low	1857	200	10.77	1657	89.23		
EGFR_GAIN	Hypermutation	633	68	10.74	565	89.26	0.001678634	0.642541
	Low	1857	293	15.78	1564	84.22		
KRAS_GAIN	Hypermutation	633	21	3.32	612	96.68	0.02631793	0.5784877
	Low	1857	104	5.60	1753	94.40		
MET_GAIN	Hypermutation	633	21	3.32	612	96.68	0.03308558	0.5904969
	Low	1857	102	5.49	1755	94.51		
PIK3CA_GAIN	Hypermutation	633	103	16.27	530	83.73	5.70E-08	2.148361
	Low	1857	154	8.29	1703	91.71		

Var_num, Number of variation; Var_per, Percentage of variation; Novar_num, Number of no variation; Novar_per, Percentage of no variation; P value and odds ratio were tested using Fisher's analysis.

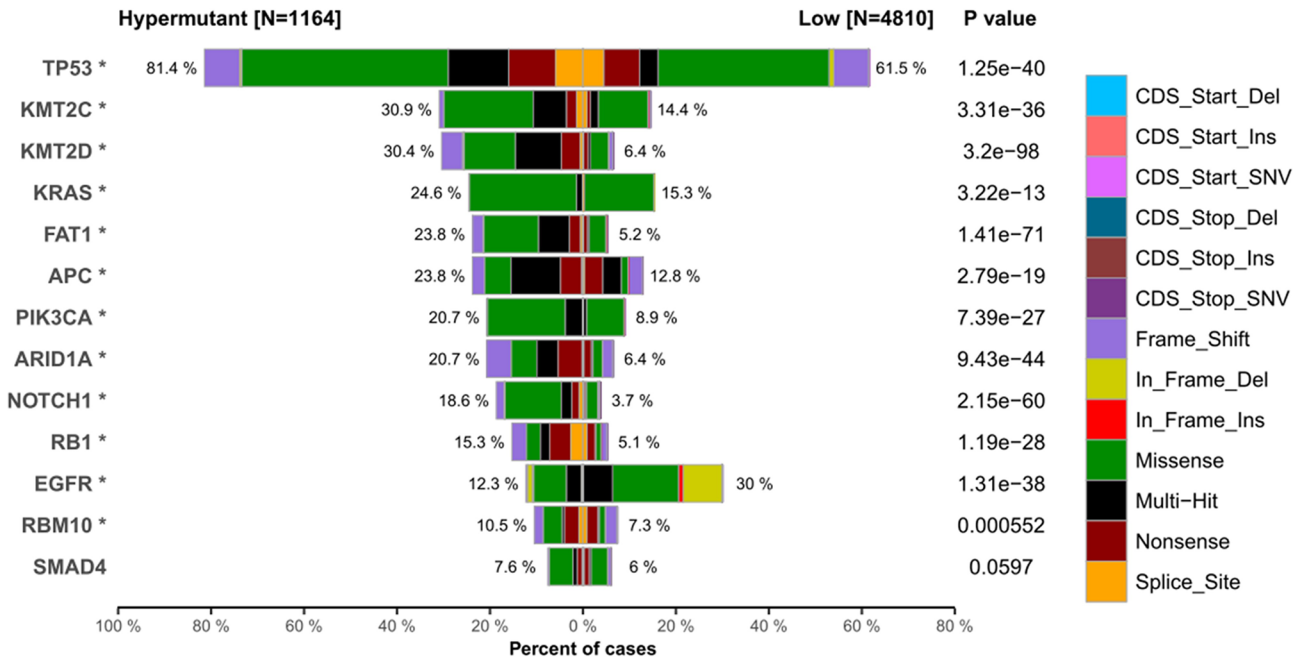

FIGURE 5 | The top 10 high-frequency mutated genes in the Hypermutant and Low groups. *$P < 0.05$.

at TCGA PanCancer studies involving 10,967 samples as of November 2018 and found 92 POLE exonuclease domain mutations in hypermutant tumors (31).

A disruption of DNA repair pathways will increase mutagenesis and genome instability, thereby affecting cancer progression and drug resistance (32). Here, we found that somatic SNVs in hypermutant tumors are mainly enriched in the p53 pathway. This observation may be linked to the high frequency of TP53 mutations. In addition, SNV and CNV frequency was found to be high in EGFR, KRAS and PIK3CA. Studies using new technologies such as liquid biopsy and next-generation sequencing have revealed that the mechanism of anti-EGFR treatment resistance involves acquired mutations in the KRAS and EGFR ectodomain (33), and PIK3CA mutations are closely related to KRAS mutations (34). These data characterized tumors involving specific gene mutations.

CONCLUSION

In this study, we collected data on 5,980 tumor samples involving 23 types of solid tumors and performed a comprehensive analysis on the relationship between hypermutation and gene mutation, MSI, and PD-L1, as well as its clinical significance and

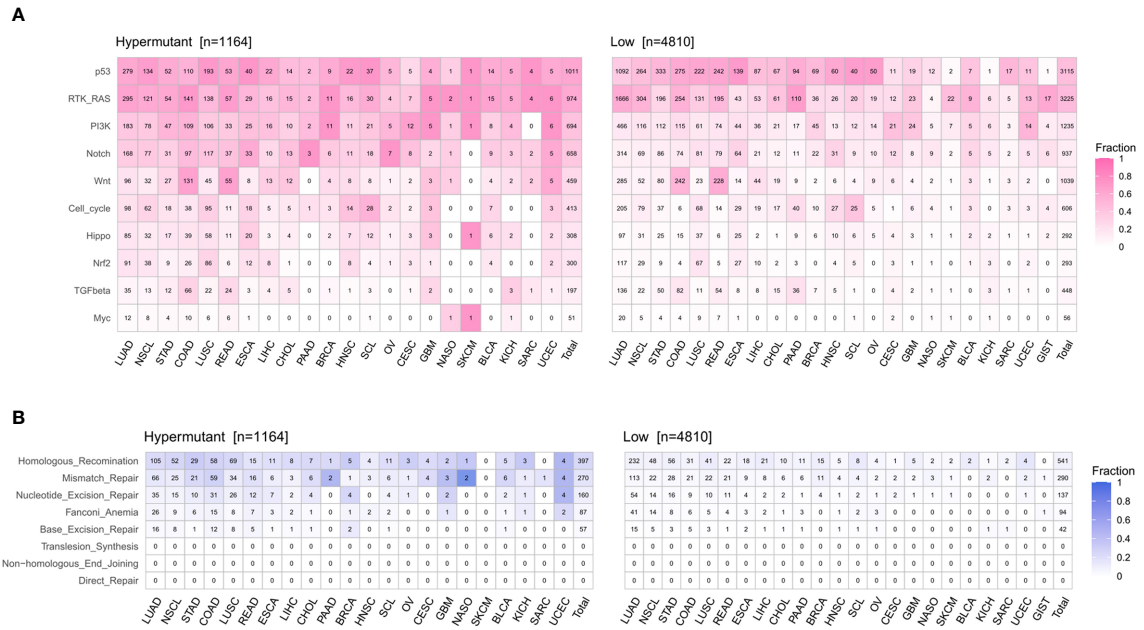

FIGURE 6 | The number and frequency of mutant samples in different pathways in Hypermutant and Low groups for each cancer type. **(A)** The oncogenic signaling pathways were illustrated in pink. **(B)** DNA damage repair pathways were depicted in blue.

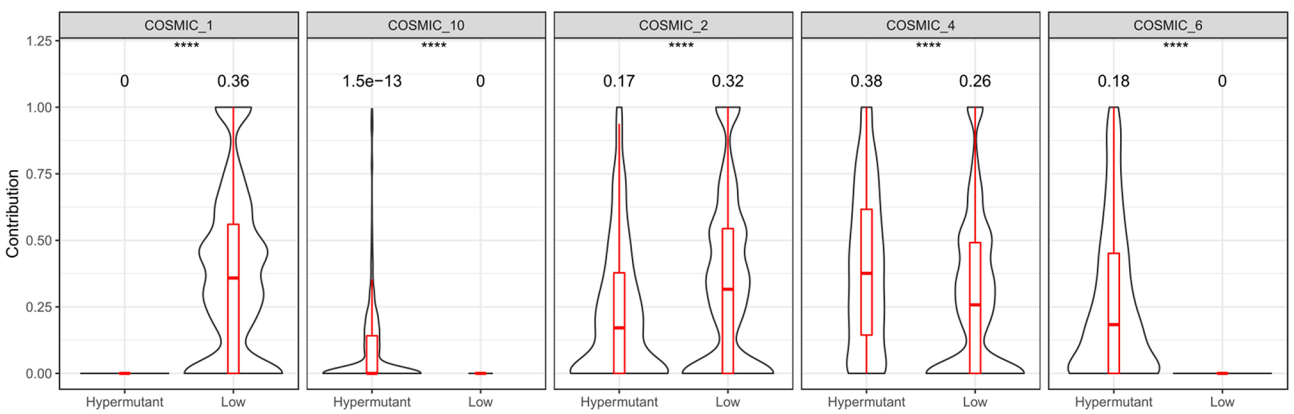

FIGURE 7 | Somatic signature of Hypermutant and Low groups and the proportion of each feature. COSMIC_1: spontaneous deamination of 5-methylcytosine; COSMIC_2: APOBEC Cytidine Deaminase (C>T); COSMIC_4: exposure to tobacco (smoking) mutagens; COSMIC_6: defective DNA mismatch repair; COSMIC_10: defects in polymerase POLE. $P<0.05$ was considered a significant difference, $^{****}P < 0.0001$.

characterized the relationship between genotype and phenotype in hypermutant tumors. The overlap between high TMB and MSI-H or high PD-L1 is most likely attributable to MMR and/or POL mutations. In addition, hypermutant tumors displayed a higher rate of cancerous driver gene changes than tumors with non-hypermutation.

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 below: https://bigd.big.ac.cn/bioproject/browse/PRJCA004800, PRJCA004800.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ruijin Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JZ conceived the study. HY, JJ, and JZ designed the study and wrote the draft manuscript. MS, YS, JLiu, and JW analyzed and interpreted the data. CY and WX collected and interpreted the clinical data. QL and WZ extracted the data and assess the data quality. JLi and XG did literature searches and identified the eligible studies. 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.2021.682017/full#supplementary-material

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The Application of Combined Immune Checkpoint Inhibitor Modalities in Previously Treated Non-Small Cell Lung Cancer Patients and the Associations Thereof With the Lung Immune Prognostic Index

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Background: Immune checkpoint inhibitor (ICI) monotherapy remains the standard of care for patients with previously treated non-small cell lung cancer. However, few reports have compared the clinical benefits of second-line ICIs alone with those of ICIs combined with other therapies, including anti-angiogenesis therapy or chemotherapy.

Methods: Patients with previously treated advanced non-small cell lung cancer who received ICIs were retrospectively reviewed. The progression-free survival (PFS), overall survival, objective response rate, disease control rate, and safety were assessed. Complete blood cell counts and serum lactate dehydrogenase (LDH) levels were measured before and after ICI treatment.

Results: Of 120 patients, 75 were treated with ICI monotherapy, 26 with ICIs plus anti-angiogenic therapy (ICI+A), and 19 with ICIs plus chemotherapy (ICI+C). The objective response rate was significantly higher in the ICI+C group (57.9%) than ICI monotherapy (26.3%) and ICI+A (31.8%) groups. The depth of response was significantly greater in the ICI+C (-35.1%) than ICI+A (–2.04%) and ICI monotherapy (3.963%) groups. ICI+C afforded a better PFS compared with the ICI monotherapy and ICI+A groups (8.5 *vs*. 4.6 and 4.1 months, respectively). Notably, the pre- and post-treatment peripheral neutrophil/lymphocyte ratios and serum LDH levels were negatively correlated with the PFS of the entire cohort. More importantly, the pretreatment lung immune prognostic index (neutrophil/lymphocyte ratio ≥ 4 and LDH level ≥ upper limit of normal) satisfactorily predicted the responses to ICI-based strategies. Adverse events (AEs) occurred in 65.3%,

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92.3%, and 94.7% of patients in the ICI monotherapy, ICI+A, and ICI+C groups, respectively. Grade 3–5 AEs were more common in the combination therapy groups (ICI+A, 19.2%; ICI+C, 21%; ICI monotherapy, 4%).

Conclusion: In second-line settings and beyond, ICIs combined with chemotherapy prolonged survival, with tolerable AEs. Addition of anti-angiogenic agents to ICIs did not afford any additional benefits. Further prospective studies are warranted.

Keywords: immune checkpoint inhibitor (ICI), anti-angiogenic therapy, chemotherapy, non-small cell lung cancer (NSCLC), lung immune prognostic index (LIPI)

INTRODUCTION

Immunotherapy has become the new paradigm for treatment of non-small-cell lung cancer (NSCLC) (from beginning to end) and serves as an important addition to the treatment armamentarium. Monotherapy targeting the programmed death receptor 1 (PD-1) inhibitor or its ligand PD-L1 is the recommended standard of care for patients with previously treated advanced NSCLC (1, 2); such treatment significantly prolongs overall survival (OS) and exhibits a better benefit-to-risk profile compared with docetaxel chemotherapy (3). However, an initial rapid decrease in survival curves, limited objective response rates (ORRs) in entire cohorts, and the poor efficacy toward and risk of hyperprogressive disease in patients with driver gene mutations restrict the applications of immune monotherapies in clinical settings (4–8). Moreover, biomarkers of the response to second-line immunotherapy remain unclear. Currently, PD-L1 expression serves as an inclusion criterion for second-line trials. However, some of the patients who benefited lacked PD-L1 expression (9). Thus, identification of biomarkers other than PD-L1 in patients likely to respond to second-line immune checkpoint inhibitor (ICI) therapy is critical. A previous study devised a lung immunoprognostic index (LIPI), based on a neutrophil-to-lymphocyte ratio (NLR) greater than 3 and a lactate dehydrogenase level (LDH) greater than the upper limit of normal (ULN). The LIPI is an economical, rapid, and easily calculated biomarker predicting the outcomes of ICI-treated patients with advanced and emerging locally advanced NSCLC (10).

Accumulating evidence has confirmed that, in NSCLC patients, the combination of PD-1 inhibitors and chemotherapy in first-line settings improves OS and progression-free survival (PFS) more so than chemotherapy alone (11). In second-line settings, the recent PROLUNG study found that the combination of pembrolizumab and docetaxel was well-tolerated and substantially improved the outcomes of patients with advanced NSCLC, including epidermal growth factor receptor (EGFR)-mutant NSCLC (12). Preclinical and pilot clinical studies have shown that anti-angiogenic drugs, such as bevacizumab and the small molecular agents apatinib and anlotinib, potentiated the efficacy of PD-1 inhibitors by affecting the tumor environment (13).

We performed a multicenter, retrospective study to determine whether the addition of platinum-based chemotherapy or anti-angiogenic agents to PD-1 inhibitors improved the outcomes of patients with previously treated advanced NSCLC. We compared PD-1 inhibitor monotherapy with PD-1 inhibitor plus chemotherapy (ICI+C) or PD-1 inhibitor plus an anti-angiogenic agent (ICI+A) combination therapy. We also explored the predictive value of the LIPI in these contexts.

MATERIALS AND METHODS

Patient Selection

Patients with NSCLC who received second-line immunotherapy between June 1, 2017 and March 1, 2020 at the Second Affiliated Hospital, Zhejiang University School of Medicine and Peking University Cancer Hospital were screened retrospectively. The inclusion criteria were (a) histologically confirmed unresectable stage III or IV NSCLC, (b) treatment with ICI monotherapy, ICI+C, or ICI+A as second-line or later therapy, (c) immunotherapy-naïve status, (d) a minimum of two cycles of therapy, and (e) at least one measurable lesion as defined by the Response Evaluation Criteria in Solid Tumors, ver. 1.1. The data were collected and censored to March 2021. This retrospective study was approved by the Ethics Committee of the Second Affiliated Hospital and was conducted according to the principles of the Declaration of Helsinki of 2013. The need for informed patient consent was waived by the committee given the retrospective nature of the study.

Data Collection and Response Assessment

Complete blood cell counts and LDH levels pretreatment (i.e., within 3 days before the first treatment) and post-treatment (i.e., at 6 weeks after the first treatment) were extracted from electronic medical records. Demographic, clinical, pathological, and molecular data were also collected. The NLR was computed manually. LIPI scores were calculated based on the NLR (> 4 = 1 point) and the LDH level (> UNL = 1 point), with good, intermediate, and poor LIPI scores defined as 0, 1, and 2, respectively.

We measured the ORR, disease control rate (DCR), PFS, and OS. Patients were followed-up using computed tomography or magnetic resonance imaging until disease progression occurred. The best response was defined as a complete response or a partial response achieved at least once throughout the course of therapy, as assessed by dedicated radiologists in each center using the Response Evaluation Criteria in Solid Tumors, ver. 1.1. Toxicity data were obtained from medical records and telephone interviews during follow-up and were graded using

the National Cancer Institute Common Terminology Criteria for Adverse Events, ver. 5.0.

Statistical Analysis

Comparisons were performed using the $\chi 2$ or Fisher's exact test for discrete variables and the unpaired t-test, Wilcoxon sign-ranked test, or analysis of variance for continuous variables. Survival curves were generated using the Kaplan–Meier method and compared using the log-rank test. Hazard ratios were calculated using Cox's proportional hazards models. Multivariate models were used to explore the associations between biomarker levels and survival.

RESULTS

Patient Demographic and Baseline Characteristics

From June 1, 2017 to March 1, 2021, 120 patients with previously treated NSCLC who received ICI monotherapy, ICI+A, or ICI+C were reviewed in terms of eligibility. Of the 120 patients, 92 (76.7%) were men, 36 (30%) were never-smokers, and 13 (10.8%) harbored EGFR or anaplastic lymphoma kinase mutations. Regarding treatment, 75 patients received ICI monotherapy, 26 ICI+A, and 19 ICI+C. All baseline characteristics including sex, age, smoking status, performance status, stage, and the treatment stage (second-line or beyond) were well-balanced among the three groups (**Table 1**). Adenocarcinomas affected 37 patients (49.3%) in the ICI monotherapy group, 11 (57.9%) in the ICI+A group, and 18 (69.2%) in the ICI+C group (**Table 1**). ICI, chemotherapy and anti-angiogenesis agents employed in the trial were listed in **Table 2**.

Responses to Immunotherapy

Dedicated radiologists and physicians independently reviewed all clinical information. The median PFS times were 4.6, 4.1, and 8.5 months in the ICI, ICI+A, and ICI+C groups, respectively (**Figure 1A**). PFS tended to be longer in the ICI+C group, but the OS did not differ among the ICI monotherapy, ICI+A, and ICI+C groups (22.7, 23.2, and not attained, respectively; **Figure 1B**). A swimmer plot summarizing the responsiveness of EGFR mutant patients was shown in **Figure 1C**.

We evaluated the treatment responses. The ORR was significantly higher in the ICI+C group (57.9%) than ICI

TABLE 1 | Patient characteristics.

Characteristic	ICI monotherapy (N = 75)	ICI +Chemotherapy (N = 19)	ICI +Antiangiogenic therapy (N = 26)	P value
Median age, years (range)	62 (26-82)	64 (49-85)	60 (26-85)	0.271
<65 years	52 (69.3%)	12 (63.2%)	16 (61.5%)	0.672
≥65 years	23(30.7%)	7 (36.8%)	10 (38.5%)	
Sex, n (%)				0.320
Male	60 (80.0%)	12 (63.2%)	20 (76.9%)	
Female	15 (20.0%)	7 (36.8%)	6 (23.1%)	
Tumor histology, n (%)				0.086
Squamous	38 (50.7%)	7 (36.8%)	8 (30.8%)	
Adenocarcinoma	37 (49.3%)	11 (57.9%)	18 (69.2%)	
Others	0(0.0%)	1 (5.3%)	0 (0.0%)	
Smoking history, n (%)				0.583
Former	48 (64.0%)	10 (52.6%)	14 (53.8%)	
current	7 (9.3%)	1 (5.3%)	4 (15.4%)	
Never	20 (26.7%)	8 (42.1%)	8 (30.8%)	
Performance status (ECOG), n (%)				
0	1 (1.3%)	3 (15.8%)	1 (3.8%)	0.286
1	69 (92.0%)	10 (52.6%)	24 (92.3%)	
2	4 (5.3%)	6 (31.6%)	1 (3.8%)	
Stage				
III	15(20.0%)	4 (21.1%)	6 (23.1%)	0.946
IV	60(80.0%)	15 (78.9%)	20 (76.9%)	
EGFR/ALK mutations	7(9.3%)	2 (10.5%)	4 (15.4%)	0.105
Previous systemic therapy				
Chemotherapy	74(98.7%)	17 (89.5%)	25 (96.2%)	0.091
EGFR TKI	6(8.0%)	6 (31.6%)	4 (15.4%)	0.024
Anti-angiogenesis therapy	18(24.0%)	8 (42.1%)	8 (30.8%)	0.278
No. of previous systemic treatments				
1	48 12(64.0%)	12 (63.2%)	15 (57.7%)	0.829
≥2	27 (36.0%)	7 (36.8%)	11 (42.3)	
Metastatic site				
Brain	9(12.0%)	4 (21.1%)	1 (3.8%)	0.210
Liver	6(8.0%)	1 (5.3%)	3 (11.5.%)	0.798
Bone	19(25.3%)	3 (15.8%)	3 (11.5.%)	0.285
Lung	32(42.7%)	9 (47.4%)	13 (50%)	0.835
Pleura	18(24.0%)	3 (15.8%)	9 (34.6%)	0.335

TABLE 2 | The summary of ICI, chemotherapy and anti-angiogenesis agents.

Characteristic	ICI monotherapy (N = 75)	ICI +Chemotherapy (N = 19)	ICI +Antiangiogenic therapy (N = 26)
ICI, n (%)			
Nivolumab	30(40%)	1(5.3%)	2 (7.6%)
Pembrolizumab	10(13.3%)	4(21.1%)	1 (3.8%)
Camrelizumab	3(4%)	9(47.3%)	17 (65.4%)
Tislelizumab	18(24%)		6(23.1%)
Sintilimab	10(13.3%)	5(26.3%)	
Atezolizumab	4(5.3%)		
Chemotherapy drugs, n (%)			
Paclitaxel based		13(68.4%)	
Gemcitabine		3(15.8%)	
Pemetrexed		3(15.8%)	
Anti-angiogenesis agents, n (%)			
Bevacizumab			3(11.5%)
Apatinib			14(53.8%)
Arotinib			3(11.5%)
Sitravatinib			6(23.1%)

monotherapy group (26.3%) and ICI+A group (31.8%, P = 0.036, **Figure 2D**). In contrast, the DCR was similar among the three arms (ICI monotherapy *vs.* ICI+A *vs.* ICI+C: 72.2% *vs.* 72.2% *vs.* 89.5%, P = 0.275, **Figure 2E**). The depths of the treatment responses are summarized in **Figure 2**. Of patients in the ICI+C group, two with a complete response had an average depth of response of -35.1%, which was significantly greater than those in the ICI+A group (−2.04%, P = 0.0161) and ICI monotherapy group (3.963%, P = 0.0105). The percentages of patients exhibiting no reduction in tumor size in the ICI monotherapy, ICI+A, and ICI+C groups were 45.1%, 38.1%, and 11.1%, respectively.

Associations of the NLR and LDH Level With Clinical Efficacy

We analyzed the associations of the peripheral absolute neutrophil count, absolute lymphocyte count (LNC), NLR, and LDH level between pretreatment and post-treatment. Therapeutic efficacy was evident in the entire cohort. Pretreatment, the LNC was positively, but the absolute neutrophil count negatively, associated with PFS, indicating a significant negative association between the pretreatment NLR and PFS (r = −0.1962, P = 0.0365, **Figure 3A**). Notably, the correlations of PFS with the LNC and NLR were more pronounced after two cycles of treatment (LNC: r = 0.2106, P = 0.0287; NLR: r = −0.2273, P = 0.0186, **Figure 3B**). Similarly, PFS was negatively associated with the pretreatment LDH level and even more so with the post-treatment level (r = −0.2312,P = 0.0182).

A pretreatment NLR greater than 4 was independently associated with PFS, and a pretreatment LDH level greater than the ULN was marginally associated with PFS in a Cox's proportional hazard model. These two biomarkers were combined to create the LIPI, as reported previously (14). Of 113 evaluable patients, 31 (27.4%) had good LIPI scores (NLR < 4 and LDH level < ULN), 57 (50.4%) intermediate scores (NLR ≥ 4 or LDH level ≥ ULN), and 25 (22.1%) poor scores (NLR ≥ 4 and LDH ≥ ULN). The median PFSs of the patients with poor, intermediate, and good LIPI scores were 4.2, 11.3, and 9.1 months, respectively (P = 0.0119, **Figure 3C**). We generated waterfall plots of the best responses and LIPI scores (**Figure 3D**).

Safety

The different treatment strategies were associated with unique adverse events (AEs) (**Figure 4**). During initial therapy, treatment-related AEs occurred in 65.3% of patients in the ICI monotherapy group, 92.3% in the ICI+A group, and 94.7% in the ICI+C group. Serious (grade 3–5) treatment-related AEs occurred in five-fold more patients in the combination treatment groups (ICI+A, 19.2%; ICI+C arm, 21%) than in the ICI monotherapy group (4%). The most common AEs included fever, fatigue, loss of appetite, nausea, vomiting, and diarrhea. More hematological toxicities were observed in the ICI+C group, whereas hypertension and proteinuria were more common (but not severe) in the ICI+A group. The rates of immune-related AEs (irAEs), such as thyroid dysfunction and pneumonitis, were comparable among the three groups. Notably, one patient in the ICI+C group developed grade 5 pneumonitis.

DISCUSSION

To the best of our knowledge, this is the first study to compare the efficacies of ICI+C, ICI+A, and ICI monotherapy in patients with previously treated NSCLC. We also evaluated the value of the LIPI score as a biomarker. We found that ICI+C treatment significantly improved the ORR and depth of response and tended to improve the PFS of previously treated patients, more so than did ICI monotherapy. Compared with ICI monotherapy, ICI+A as second-line or later therapy did not afford any additional clinical benefits in terms of the ORR, DCR, depth of response, PFS, or OS. Of note, the pre- and post-treatment peripheral NLRs and LDH levels were correlated with the PFS of the whole cohort, and more importantly, the pretreatment LIPI score well-predicted the responsiveness to ICI-based strategies in NSCLC patients undergoing second-line or later therapy.

In the first-line setting, anti-PD-1/PD-L1 monotherapy (2, 15) combined with chemotherapy (16, 17), or chemotherapy combined with bevacizumab (18), significantly improved survival, with favorable safety profiles. However, ICI monotherapy is usually recommended for the second-line setting, in which the ORR is almost 20%, PFS 3.5–4.2 months, and OS 9.2–12.2 months (1, 19, 20). In the phase 2 PROLUNG trail, compared with docetaxel monotherapy, pembrolizumab plus docetaxel improved the ORR from 15.8% to 42.5% and the modified PFS from 3.9 to 9.5 months (12). One retrospective study reported a trend of longer PFS (7.5 *vs.* 3.7 months) and a significant improvement in OS (28.6 *vs.* 15.9 months) in the ICI plus nab-paclitaxel group compared with the ICI monotherapy group. We found that the ICI monotherapy group exhibited an ORR of 26.3% and a PFS of 4.6 months. The median PFS was 9.1 months in the ICI+C group, comparable with that in the PROLUNG trial. In line with previous findings, although statistical significance was not attained, the PFS also tended to be better with combination therapy. However, the OS curves of

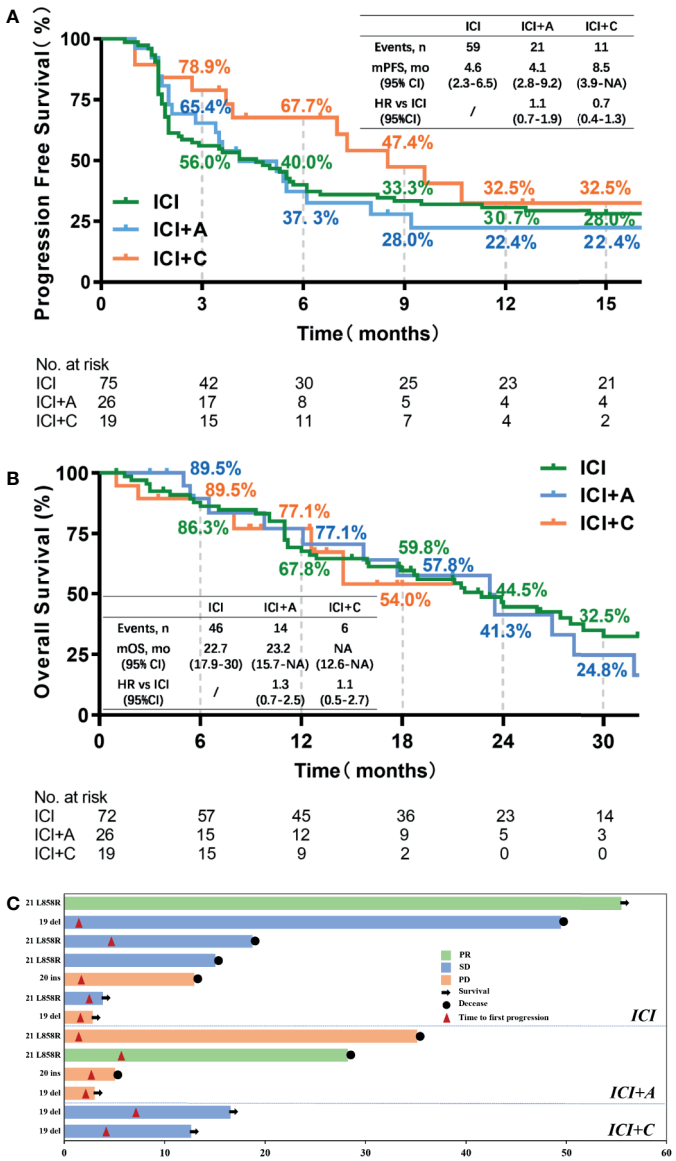

FIGURE 1 | The progression-free survival and overall survival of patients treated with three immune checkpoint inhibitor-based strategies. Kaplan–Meier estimates of the progression-free survival **(A)** and overall survival **(B)** of patients treated with ICI monotherapy (green), ICI plus an anti-angiogenic agent (blue), and ICI plus chemotherapy (orange). Censored data are indicated by ticks. In the analysis of progression-free survival, data from patients who had not progressed and were still alive at the time of analysis were censored at their last assessment. In the analysis of overall survival, data from patients who were considered to be alive at the time of analysis were censored at the last recorded date on which the patients were known to be alive. **(C)** A swimmer plot summarizing the responsiveness of thirteen patients with EGFR mutations. CI, confidence interval; HR, hazard ratio; ICI, immune checkpoint inhibitor; A, anti-angiogenic therapy; C, chemotherapy; PR, partial response; SD, stable disease; PD, progressive disease.

the three groups overlapped extensively. Several studies have shown that anti-angiogenic agents act synergistically with PD-1/PD-L1 inhibitors to improve the low efficacy of ICI monotherapy, with an ORR of ~30% (21, 22). The combination treatments increased infiltration of CD8+ T cells, reduced recruitment of tumor-associated macrophages, reversed inhibition of DC maturation, and promoted the development of an angiostatic and immune system-activating tumor microenvironment (23, 24). In second-line or higher settings, a real–world retrospective study found that a PD-1 inhibitor plus anlotinib was associated with an ORR of 19.3%, DCR of 85.5%, and PFS of 5 months (25). In our present study, the ORR, DCR, and PFS of the ICI+A group were 31.8%, 72.7%, and 4.1 months, respectively. However, our data suggest that the addition of anti-angiogenic agents to ICIs does not translate into improved outcomes. To the best of our knowledge, this is the largest cohort study to compare ICI monotherapy, ICI+C, and ICI+A simultaneously. ICI+C should be considered in second-line and higher settings, but evidence

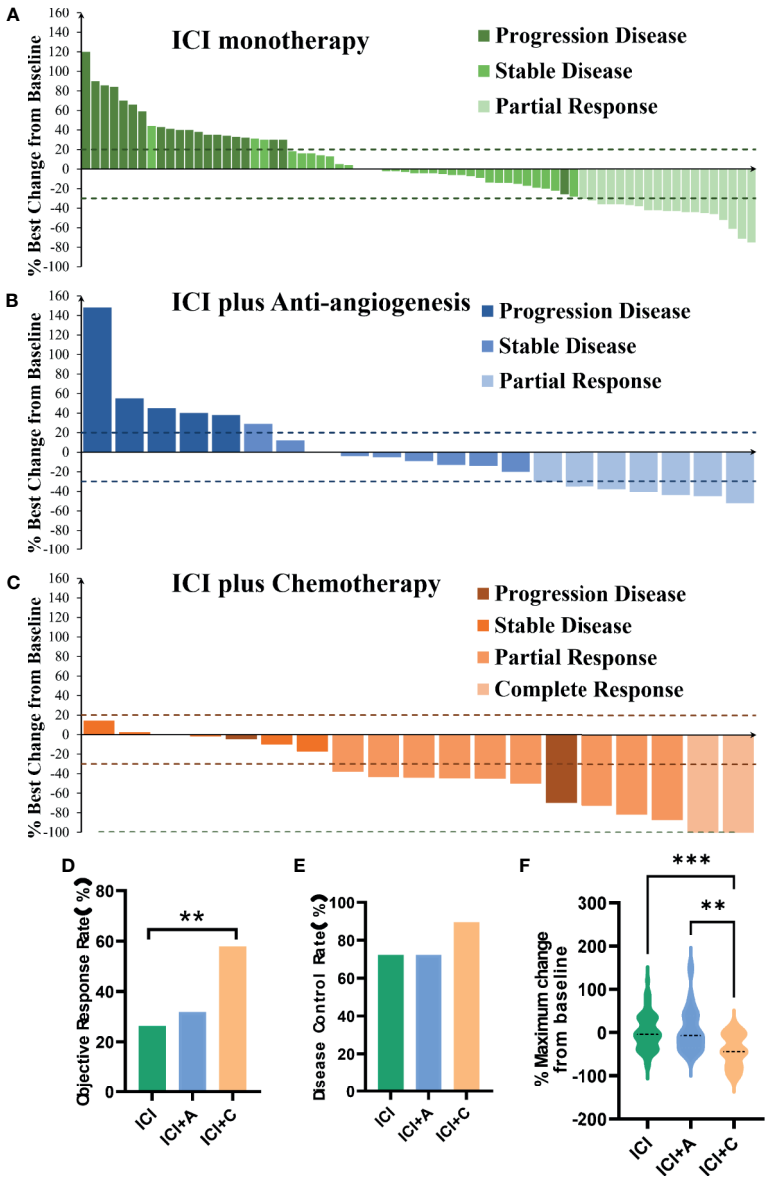

FIGURE 2 | Responses to treatment. Waterfall plots of the treatment response in terms of the greatest change in tumor size compared with the pretreatment size **(A–C)** in patients treated with ICI monotherapy (green panel), ICI plus an anti-angiogenic agent (ICI+A, blue panel), and ICI plus chemotherapy (ICI+C, orange panel). Each bar represents the greatest reduction in the target lesion size in an individual patient. The dashed lines show the cutoffs used to define progressive disease (≥ 20% increase) and a partial response (≥ 30% reduction). The objective response rate **(D)**, disease control rate **(E)**, and maximum change compared with pretreatment values **(F)** in the ICI monotherapy (green), ICI+A (blue), and ICI+C (orange) groups. A complete response, a partial response, stable disease, and progressive disease were estimated using the Response Evaluation Criteria in Solid Tumors criteria, ver. 1.1. ICI, immune checkpoint inhibitor; A, anti-angiogenic agent; C, chemotherapy. **P < 0.01, ***P < 0.001

supporting the combination of an anti-angiogenic agent with an ICI in patients with previously treated NSCLC is lacking.

The lack of significant differences in PFS and OS has several possible explanations. First, the proportions of patients who did not attain the PFS (47.3%) and OS (68.9%) endpoints were higher in the ICI+C group than in the other two groups. We suspect that the significant survival benefit of the ICI+C group reflects the longer follow-up period in this group. Second, different PD-1 inhibitors were used. The selection of PD-1/PD-L1 inhibitors in the real world depends on the clinical evidence, patient's choice, and physician's experience, all of which cause bias.

Inflammation, particularly chronic inflammation, is tightly linked to cancer progression (26). Inflammatory cytokines influence lymphocytes and neutrophils. Many routine blood parameters have been investigated as potential inflammatory

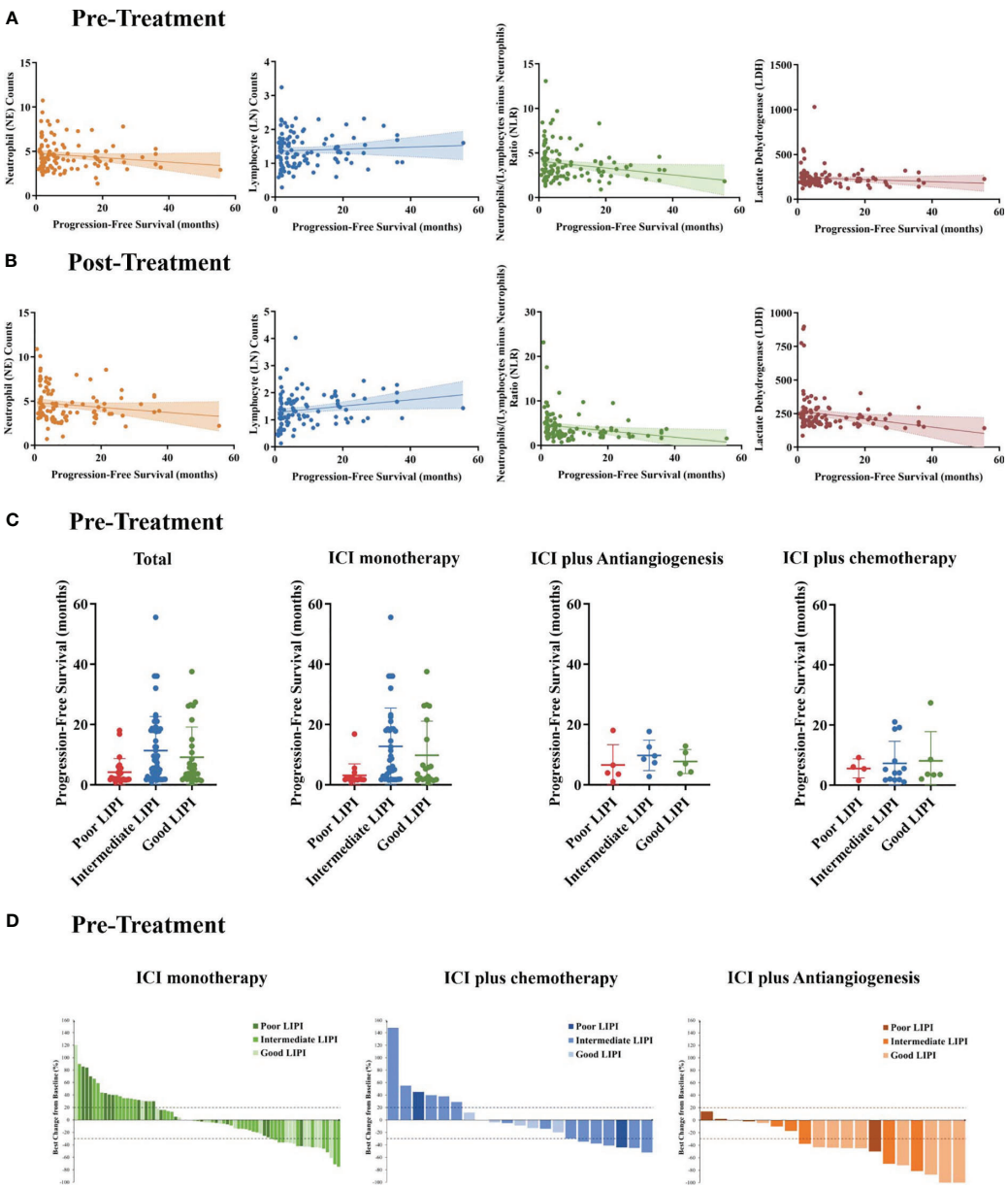

FIGURE 3 | Pre- and post-treatment biomarker measurements. The associations between pre- **(A)** and post-treatment **(B)** neutrophil (NE) counts, Lymphocyte (LN) counts, neutrophil/lymphocyte ratios (NLRs), and the serum lactate dehydrogenase (LDH) level with progression-free survival (PFS). **(C)** PFS stratified by the lung immune prognostic index (LIPI) score in all patients, the ICI monotherapy group, the ICI plus anti-angiogenic agent (ICI+A) group, and the ICI plus chemotherapy (ICI+C) group. **(D)** Waterfall plots showing the best responses and the LIPI scores in the ICI monotherapy (green panel), ICI+A (blue panel), and ICI+C (orange panel) groups. The LIPI is based on an NLR greater than 3 and an LDH level greater than the upper limit of normal. ICI, immune checkpoint inhibitor.

biomarkers, including elevated neutrophil and LDH levels and hypoalbuminemia, all of which are associated with poor cancer outcomes (27). The pretreatment NLR is a well-known prognostic factor in patients with NSCLC (28); however, the value of the post-treatment NLR has not been fully explored. This is the first study to evaluate the effects of both pretreatment and post-treatment parameters on the outcomes of three different ICI-based treatments. Interestingly, we found that the NLR, especially the post-treatment NLR, strongly predicted the outcomes of later-line ICI-based strategies. The LDH level is a classic inflammatory marker in patients with cancer. When the tumor burden is high, an elevated LDH level reflects increased tumor glycolytic activity and tumor necrosis caused by hypoxia (29). The LDH level was inversely related to the response to ICIs and may even trigger hyperprogressive disease. We found that the pretreatment LDH level tended to have a negative association with PFS, and that the post-treatment LDH level was significantly associated with poor PFS, reflecting the potential utility of the LDH level as a biomarker.

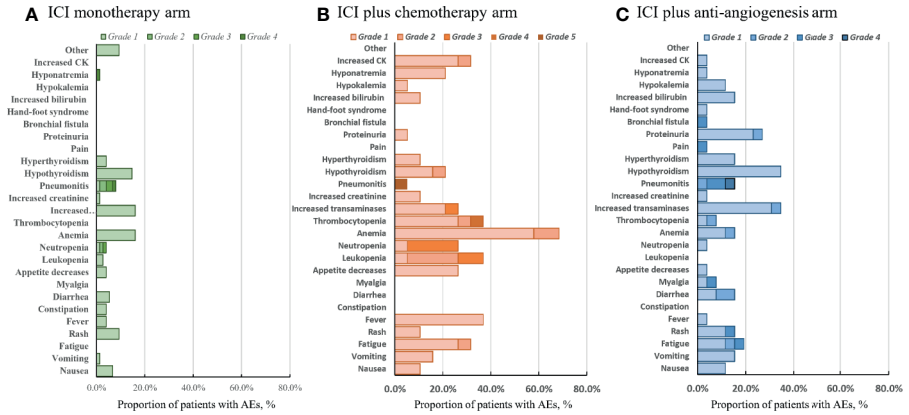

FIGURE 4 | Adverse events. All-cause adverse events of grades 1–5 in the ICI monotherapy **(A)**, ICI plus chemotherapy **(B)**, and ICI plus anti-angiogenic agent **(C)** groups. The color intensity reflects severity.

There are some limitations in our study. First, this was a retrospective study with a relatively small sample size, and the three groups were not completely balanced. The ICI+C group comprised more patients with EGFR mutations compared with the other groups. Second, due to the retrospective nature of our study, the platforms and calculated logics of TMB were varied, also the antibody used for PD-L1 testing were different. Thus, to avoid any artificial effect, we did not analyze these validated biomarkers. Third, several ICIs were used, including nivolumab, pembrolizumab, camrelizumab, tislelizumab, and sintilimab. The effects of each drug may differ. Finally, a longer follow-up time is needed to estimate OS more objectively, especially in the ICI+C group.

In conclusion, ICI monotherapy remains the standard of care in second-line settings. ICI+C combination therapy afforded certain advantages and tolerable AEs. Although addition of an anti-angiogenic agent to an ICI should theoretically afford a synergistic effect, we failed to detect any such effect. Further prospective studies are warranted.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Second Affiliated Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

TZ, XY, JiZ, LX, HL, WL and YX contributed to study conception and design. JiZ, LX, QW, LZ, BZ and YX conducted data collection. TZ, XY, JiZ, LX, QW, RJ, LZ, BZ, JuZ and YX conducted data analysis. TZ, XY, JiZ, LX, QW and YX drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Low Infiltration of CD8+ PD-L1+ T Cells and M2 Macrophages Predicts Improved Clinical Outcomes After Immune Checkpoint Inhibitor Therapy in Non-Small Cell Lung Carcinoma

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Background: Many clinical studies have shown that patients with non-small cell lung carcinoma (NSCLC) can benefit from immune checkpoint inhibitor (ICI) therapy; however, PD-L1 and tumor mutation burden (TMB), which are recommended by the NCCN guidelines, are still insufficient in predicting the response to and prognosis of immunotherapy. Given the widespread use of ICIs, it is important to find biomarkers that can predict immunotherapy outcomes in NSCLC patients, and the exploration of additional effective biomarkers for ICI therapy is urgently needed.

Methods: A total of 33 stage II-IV NSCLC patients were included in this study. We analyzed immune markers in biopsy and surgical tissue resected from these patients before treatment with ICIs. We examined the infiltration of immune cells and expression of PD-L1 in immune cells using fluorescent multiplex immunohistochemistry (mIHC) stained with CD8/CD68/CD163/PD-L1 antibodies.

Results: In this cohort, we observed that the levels of CD8+ T cells, CD8+PD-L1+ T cells, and CD68+CD163+ M2 macrophages in the total region were independent prognostic factors for progression-free survival (PFS) in NSCLC patients treated with ICIs (HR=0.04, P=0.013; HR=17.70, P=0.026; and HR=17.88, P=0.011, respectively). High infiltration of CD8+ T cells and low infiltration of CD8+PD-L1+ T cells throughout the region were correlated with prolonged PFS (P=0.016 and P=0.02, respectively). No statistically significant difference was observed for CD68+CD163+ M2 macrophages. The joint parameters CD8+ high/CD8+PD-L1+ low, CD8+ high/CD68+CD163+ low and CD8+PD-L1+ low/CD68+CD163+ low predicted better PFS than other joint parameters (P<0.01, P<0.01, and P<0.001, respectively), and they also demonstrated

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stronger stratification than single biomarkers. The response rate of patients with high infiltration of CD8+ T cells was significantly higher than that of those with low infiltration (P<0.01), and the joint parameters CD8+/CD8+PD-L1+ and CD8+/CD68+CD163+ also demonstrated stronger stratification than single biomarkers.

Conclusions: This retrospective study identified the predictive value of CD8+PD-L1+ T cells, CD8+ T cells, and CD68+CD163+ M2 macrophages in NSCLC patients who received ICIs. Interestingly, our results indicate that the evaluation of joint parameters has certain significance in guiding ICI treatment in NSCLC patients.

Keywords: immune checkpoint inhibitor therapy, non-small cell lung carcinoma, T cells, macrophages, biomarker

INTRODUCTION

Clinical trials and studies have reported that PD-1/PD-L1 inhibitors can significantly improve the outcomes of advanced non-small cell lung cancer (NSCLC) patients in recent decades (1–6), and PD-1/PD-L1 inhibitors are recommended as the standard first-line therapy for advanced or metastatic NSCLC by the National Comprehensive Cancer Network (NCCN) guidelines (7). However, only approximately 20% of NSCLC patients have prolonged and durable responses to PD-1/PD-L1 inhibitors (2, 8). Therefore, it is important to identify biomarkers that can predict the immunotherapy outcomes of NSCLC patients.

Previous studies have explored the use of PD-L1, tumor mutational burden (TMB) and tumor-associated immune cells (TAICs) to predict the clinical outcome of immunotherapy (3, 9–13), and based on those studies, PD-L1 and TMB have been included in the NCCN guidelines. However, the current work shows that these factors still have limitations in predicting the clinical outcome of immunotherapy. The NCCN guidelines recommend PD-1/PD-L1 inhibitors as the first-line treatment for advanced NSCLC patients with a PD-L1+ cell rate ≥ 1%; nonetheless, some studies have shown that PD-L1 has limitations in predicting the efficacy of immunotherapy. CheckMate227 found that NSCLC patients who received nivolumab plus ipilimumab therapy as first-line therapy had better overall survival (OS) than those who received chemotherapy, and this outcome was independent of PD-L1 expression (10). In addition, the CheckMate 026 results showed that no difference in treatment efficacy was found between the nivolumab and chemotherapy groups in the population with a PD-L1+ cell rate ≥50% (11). The predictive value of TMB was demonstrated in KEYNOTE-01. However, no such predictive value for TMB was found in an exploratory analysis of KEYNOTE-189 and KEYNOTE-407 (14, 15). Therefore, PD-L1 and TMB are still insufficient in predicting the response to and prognosis of immunotherapy, and further study is needed to explore more effective biomarkers for immunotherapy.

It has been reported that the type of immune cells can influence the clinical outcomes of patients with tumors (16, 17). In addition to TMB and PD-L1, multiple immune cell subsets have been assessed to determine their predictive value for immunotherapy outcomes in NSCLC (12, 13, 18). It was reported that the infiltration of CD8+ T cells was associated with ICI efficacy (12, 19–21). Tumor-associated macrophages (TAMs) are important immune cells in the tumor microenvironment (TME), as they mediate tumor progression by regulating TME (22). The M1 and M2 states are two main phenotypes of macrophages, and different types of macrophages predict opposite survival outcomes (23). However, which types of macrophages are associated with the efficacy of immunotherapy in NSCLC remains uncertain. PD-L1 expressed on tumor cells and macrophages is a negative regulator of T cell responses (24). Liu et al. (25) found that high levels of CD68+PD-L1+ immune cells were associated with prolonged OS in NSCLC patients treated with ICIs. However, few studies have reported the relationship between the expression of PD-L1 on T cells and ICI efficacy. It has been reported previously that PD-L1^{high} CD8+ T cells are functional effector cells (26). However, a recent pancreatic cancer study found that PD-L1+ T cells may have negative effects on adaptive antitumor immunity. Since CD8+ T cells play an important role in the immune system in killing cancer cells, studying PD-L1 expression on T cells may help us to understand the prediction of ICI efficacy in NSCLC. However, few articles have paid attention to the effects of the expression of PD-L1 on T cells and macrophage subsets on the efficacy and prognosis of immunotherapy in NSCLC, which require further investigation.

The aim of this retrospective study was to explore the predictive value of multiple immune cell subsets, including CD8+ T cells, CD68+ macrophages, CD68+CD163+ M2 macrophages, CD68+CD163- M1 macrophages, CD8+PD-L1+ T cells, CD68+PD-L1+ macrophages, CD68+CD163+PD-L1+ M2 macrophages, and CD68+CD163-PD-L1+ M1 macrophages, in tumors, the stroma and the total region in the context of NSCLC treatment with immunotherapy by using multiplex immunohistochemical staining (27).

METHODS AND MATERIALS

Patients

We used a retrospective cohort of stage II-IV NSCLC patients from Guangdong Provincial Hospital of Chinese Medicine and Guangzhou Panyu Central Hospital that consisted of 33 patients from May 2016 to April 2019. The detailed clinicopathological characteristics are summarized in **Table 1**. Among the 33 NSCLC patients, 24 patients (72.7%) had lung adenocarcinoma

TABLE 1 | Clinical characteristics.

Category		n(%)		
		Overall (n = 33)	Response (n = 11)	Non-response (n = 22)
Gender	Male	26(78.8%)	9(81.8%)	17(77.3%)
	Female	7(21.2%)	2(18.2%)	5(22.7%)
Age	>=62	16(48.5%)	7(63.6%)	9(40.9%)
	<62	17(51.5%)	4(36.4%)	13(59.1%)
Cancer type	LUAD	24(72.7%)	7(63.6%)	17(77.3%)
	LUSC	9(27.3%)	4(36.4%)	5(22.7%)
Stage	II	1(3.0%)	0(0%)	1(4.5%)
	III	11(33.3%)	6(54.5%)	5(22.7%)
	IV	20(60.6%)	4(36.4%)	16(72.7%)
	NA	1(3.0%)	1(9.1%)	0(0%)
Smoking history	Yes	22(66.7%)	8(72.7%)	14(63.6%)
	No	11(33.3%)	3(27.3%)	8(36.4%)
Treatment	PD-1 inhibitor	26(78.8%)	8(72.7%)	18(81.8%)
	PD-L1 inhibitor	3(9.1%)	1(9.1%)	2(9.1%)
	PD-1 inhibitor + chemotherapy	3(9.1%)	2(18.2%)	1(4.5%)
	PD-1+CTLA-4 inhibitor	1(3.0%)	0(0%)	1(4.5%)
Treatment time	First-line	7(21.2%)	3(27.3%)	4(18.2%)
	Second-line	16(48.5%)	4(36.4%)	12(54.5%)
	≥Third-line	10(30.3%)	4(36.4%)	6(27.3%)

(LUAD) and 9 patients (27.3%) had lung squamous cell carcinoma (LUSC). Among the NSCLC patients, 26 (78.8%) were treated with a PD-1 inhibitor, 3 (9.1%) were treated with a PD-L1 inhibitor, 3 (9.1%) were treated with a PD-1 inhibitor combined with chemotherapy, and 1 (3.0%) received combined PD-1 and CTLA-4 inhibitor therapy. Twenty-two patients (66.7%) had a smoking history. All patients were EGFR/ALK wild-type. Seven patients (21.2%) received first-line immunotherapy, sixteen patients (48.5%) received second-line immunotherapy, and ten patients (30.3%) received ≥ third-line immunotherapy.

Fluorescent Multiplex Immunohistochemistry (mIHC) Analysis

Biopsy tissue and postoperative surgical tissue samples collected before ICI treatment were processed into paraffin blocks and then cut into 4-μm-thick FFPE sections. Staining of the 4-μm FFPE slides was performed by using the Opal Seven-color IHC Kit (NEL797B001KT; PerkinElmer, Massachusetts, USA). The immune markers evaluated included CD8 (ZA-0508, clone SP16; Zsbio; 1:100), CD68 (ZM-0060, clone KP1; Zsbio; 1:400), CD163 (ZM0428, clone 10D6, Zsbio; 1:200), and PD-L1 (CST13684, clone E1L3N, CST, 1:100). Markers were identified and quantified by mIHC. Briefly, sections were cut from tumor tissue, deparaffinized, rehydrated, and washed in tap water before epitope retrieval/microwave treatment (MWT). Endogenous peroxidase activity was blocked using Antibody Diluent/Block (72424205; PerkinElmer, Massachusetts, USA). Protein blocking was performed using Antibody Diluent/Block. One antigen required one round of labeling, including primary antibody incubation, secondary antibody incubation, and TSA visualization, followed by labeling with the next antibody. Slides were scanned using PerkinElmer Vectra (Vectra 3.0.5; PerkinElmer, Massachusetts, USA). The percentage of positively stained cells among all nucleated cells was counted.

Statistical Analysis

Statistical analyses were performed using the R software program (version 3.6.2, https://www.r-project.org/), SPSS (version 22) and GraphPad Prism 8 software. The Kaplan-Meier method was used to analyze the associations between marker expression and progression-free survival (PFS). PFS was defined as the time elapsed between ICI treatment initiation and tumor progression. The statistical significance of differences between survival curves was assessed with the log-rank test. The chi-square test was used to analyze associations between immune marker expression and response. PFS analyses were performed by the Kaplan-Meier estimator and log-rank test. Multivariate/univariate Cox proportional hazard regression models and logistic regression models were utilized to examine the variables that were significant in the univariate analyses and their associations with the outcome. Variables with a P value <0.1 in the univariate analyses were entered into the multivariate analysis. $P < 0.05$ was considered significant in all the analyses.

RESULTS

Infiltration of Tumor-Associated Inflammatory Cells (TAICs) in NSCLC

We examined a retrospective cohort of 33 patients with stage II-IV NSCLC recruited at Guangdong Provincial Hospital of Chinese Medicine, Nanhai People's Hospital and Guangzhou Panyu Central Hospital between May 2016 and April 2019, for whom clinical, treatment and extended follow-up data were retrospectively assembled with medical ethics committee approval. Among these patients treated with ICI therapy, we evaluated the infiltration of CD8+ T cells, CD68+ macrophages, CD68+CD163+ M2 macrophages, CD68+CD163- M1 macrophages, CD8+PD-L1+ T cells, CD68+PD-L1+ macrophages, CD68+CD163+PD-L1+ M2 macrophages, and CD68+CD163-PD-L1+ M1 macrophages using mIHC, as shown in **Figures 1A, B**. The immune landscape of NSCLC is shown in **Figures 1C** and **S1**. The percentages of differentially expressed cells were log-transformed and z-score standardized. Heatmaps of immune cell infiltration in the total (**Figure 1C**), stromal (**Figure S1A**) and tumor regions (**Figure S1B**) were plotted and clustered. In the total, stromal and tumor regions, the degree of infiltration of CD8+ T cell, CD68+ TAMs, and PD-L1 + cells were higher in the response [R, response to immunotherapy, including complete response (CR) and partial response (PR)] subgroup than in the nonresponse [NR, nonresponse to immunotherapy, including stable disease (SD) and progressive disease (PD)] subgroup. There were two examples in which a patient who responded to immunotherapy (**Figure 1A**) had more infiltration of immune cells than those who did not respond to immunotherapy (**Figure 1B**). PFS was longer in the R subgroup than in the NR subgroup (**Figure 1C**). This result suggests that the

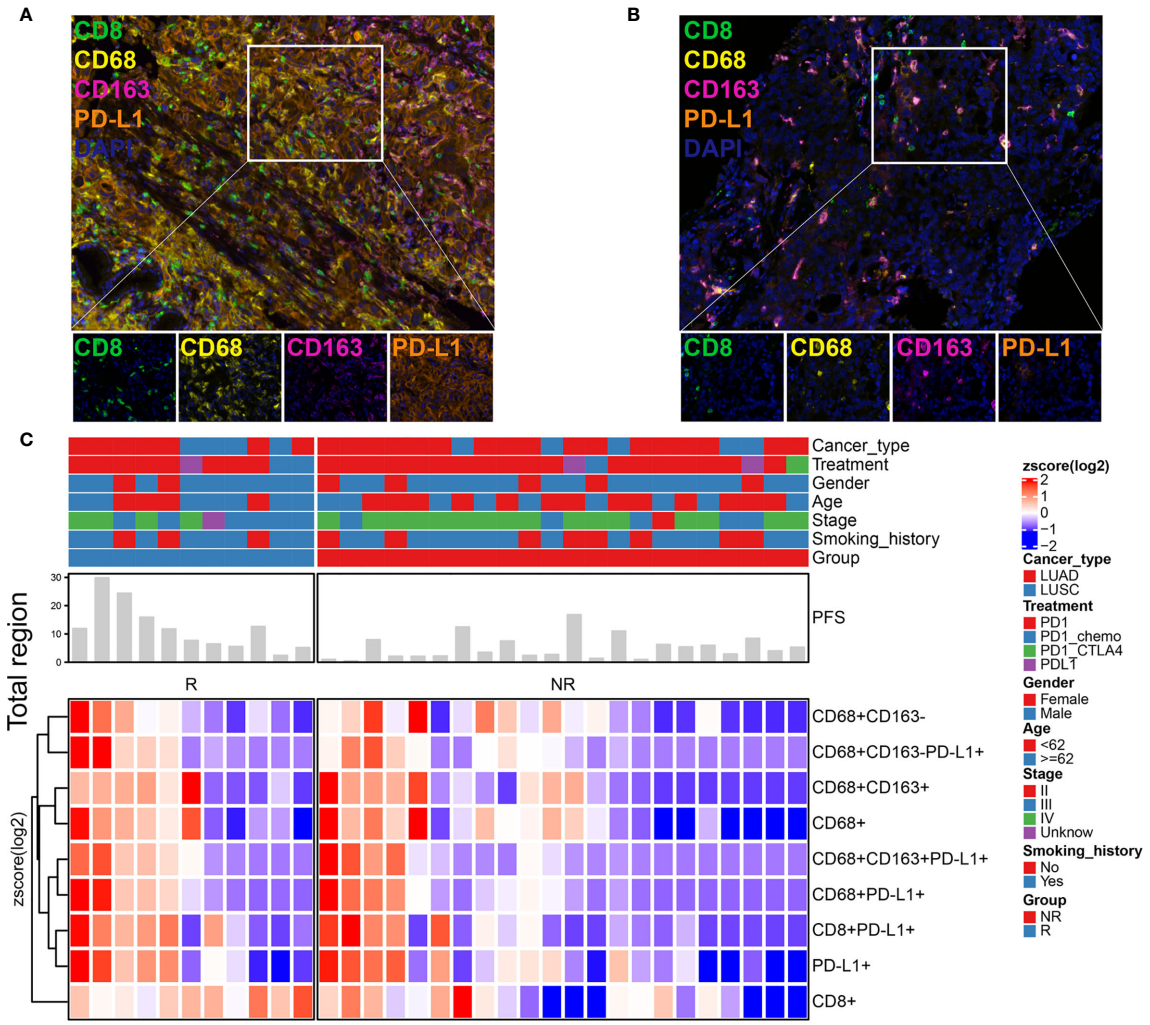

FIGURE 1 | Immune landscape of NSCLC patients treated with immune checkpoint inhibitor (ICI) therapy. Immune cell infiltration was detected using a multiplex immunohistochemistry (mIHC) platform (panel: CD8/CD68/CD163/PD-L1). The mIHC images represent the group that responded to immunotherapy **(A)** and the group that did not respond to immunotherapy **(B)**. Magnification, 200×. The percentages of differentially expressed cells were log-transformed and z-score standardized. Heatmaps of immune cell infiltration in the total region **(C)** were plotted and clustered. They indicated that each patient had a different immune microenvironment, which may lead to different responses to and benefits from immunotherapy. R, response; NR, nonresponse.

difference in the tumor immune microenvironment may be the reason for the differences in the response rate and PFS of patients who received ICI therapy, and the relationship between TAIC infiltration and PFS needs further study.

Infiltration of T Cells and M2 Macrophages Were Independent Prognostic Factors

First, to find an "optimal" cutoff value for each marker, the cutoff values for CD8+ T cells, CD68+ macrophages, CD68+CD163+ M2 macrophages, CD68+CD163- M1 macrophages, CD8+PD-L1+ T cells, CD68+PD-L1+ macrophages, CD68+CD163+PD-L1+ M2 macrophages, and CD68+CD163-PD-L1+ M1 macrophages (high vs low) in the total, stromal and tumor regions were determined using the survminer package in R software according to PFS, and the cutoff points are displayed in **Table 2**.

Second, a forest plot of univariate survival analysis results was produced to examine the variables that were significant in the univariate analysis (**Figure 2**). As shown in **Figure 2**, the levels of CD8+ T cells in the total (hazard ratio [HR]=0.29 [95% CI, 0.10-0.84], P =0.02) and stromal regions (HR=0.22 [95% CI, 0.07-0.66], P < 0.01) were correlated with PFS, and the levels of CD8+ PD-L1+ T cells in the total (HR=4.25 [95% CI, 0.13-15.93], P =0.02) and stromal regions (HR=4.25 [95% CI, 0.13-15.93], P =0.02) also revealed significant associations with PFS. In a multivariate Cox analysis, we included clinical parameters and screened the indicators with $P \leq 0.1$ in the forest plot of the univariate survival analysis results and a correlation coefficient less than 0.8 (**Figure S2**). The multivariate Cox analysis results showed that CD8+ T cells, CD8+PD-L1+ T cells, and CD68+CD163+ M2 macrophages in the total region were independent prognostic factors for PFS in NSCLC patients treated

TABLE 2 | Cut-off points of all markers according to PFS.

Marker	Cut-off point		
	Total region	Stroma region	Tumor region
CD8+	4.70	6.02	1.35
CD68+	0.48	0.56	0.01
PD-L1+	26.24	17.99	36.19
CD8+PD-L1+	4.57	4.95	3.26
CD68+PD-L1+	3.13	3.16	4.09
CD68+CD163-	0.03	0.06	5.06
CD68+CD163+	6.05	3.82	8.28
CD68+CD163+PD-L1+	1.84	1.76	4.28
CD68+CD163-PD-L1+	1.00	1.57	1.33

with ICIs (HR=0.04 (0.0031-0.51), P=0.013; HR=17.70 (1.4066-222.79), P=0.026; and HR=17.88 (1.9539-163.67), P=0.011, respectively; **Figure 3**).

Prognostic Roles of TAICs and Joint TAIC-Based Parameters

Kaplan-Meier curves were generated to study whether the infiltration of CD8+ T cells, CD8+PD-L1+ T cells or CD68+CD163+ M2 macrophages or joint parameters based on these cell types in the total region, which were selected by multivariate Cox analysis, had an impact on PFS. As shown in **Figure 4**, the Kaplan-Meier curves for PFS confirmed that high

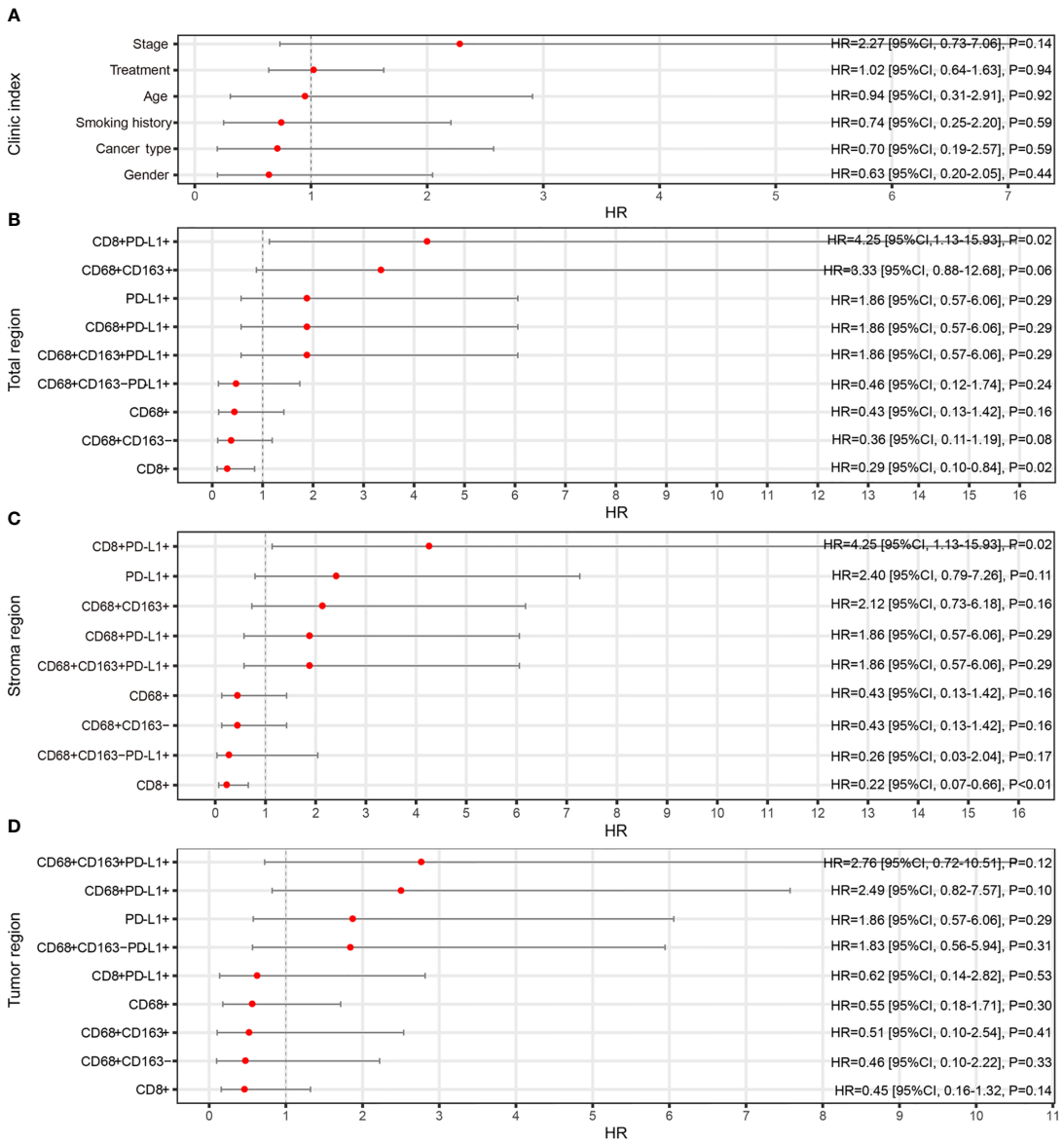

FIGURE 2 | Relationships between TAIC enrichment and progression-free survival (PFS). The forest plot of univariate survival analysis results for clinical indexes **(A)** and enriched TAICs in the total **(B)**, stromal **(C)** and tumor regions **(D)** indicates that high infiltration of CD8+ T cells was a protective factor for prognosis and that high infiltration of CD8+PD-L1+ T cells was a risk factor for prognosis.

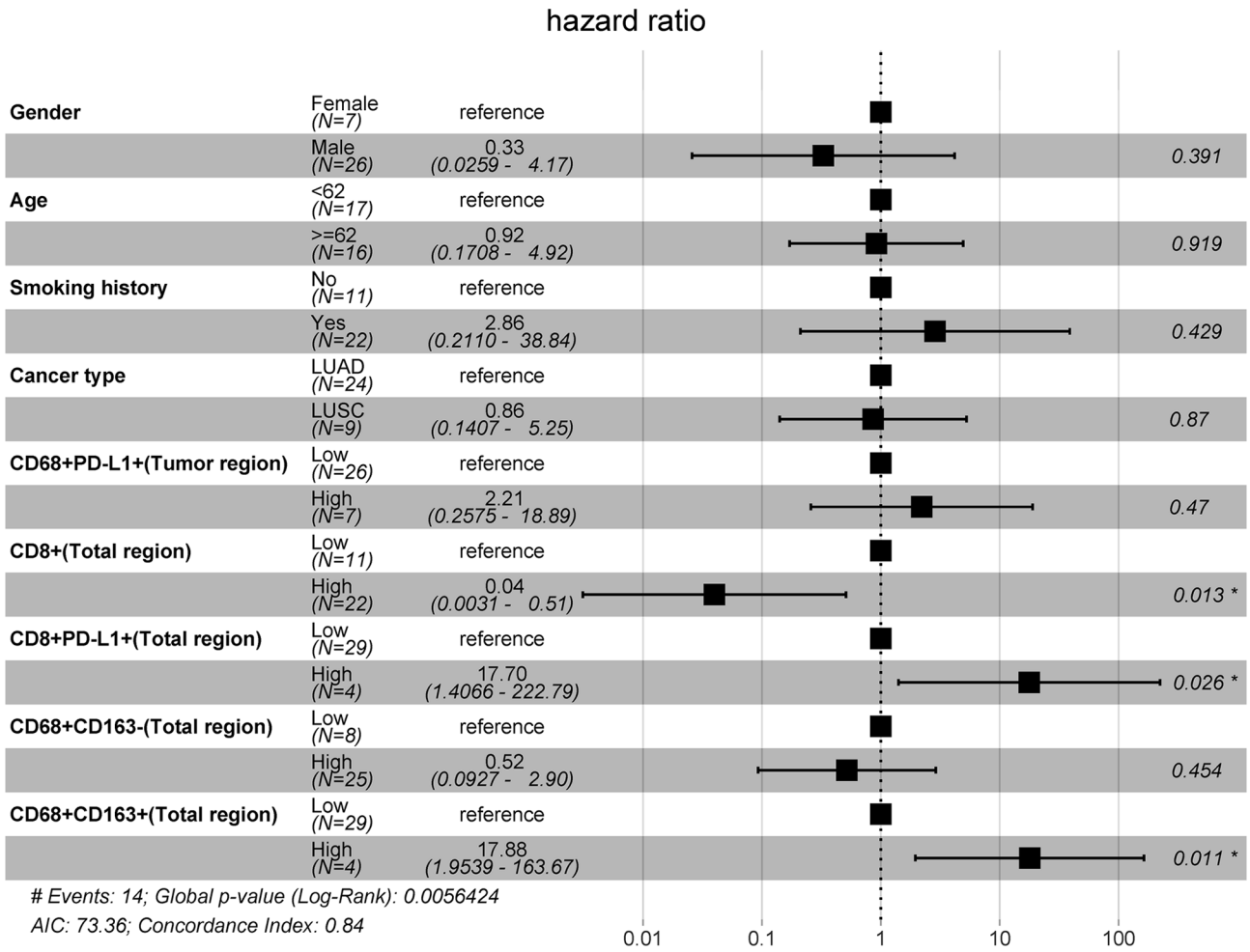

FIGURE 3 | Multivariate Cox model analyses of prognostic factors. The forest plot of multivariate survival analysis results indicates that the levels of infiltrating CD8+, CD8+PD-L1+, and CD68+CD163+ cells in the total region were independent prognostic markers for PFS.

infiltration of CD8+ T cells in the total region was correlated with prolonged PFS (P=0.016), and high CD8+PD-L1+ T cell infiltration in the total region was correlated with shortened PFS (P=0.02). A similar trend was observed for CD68+CD163+ M2 macrophages, although there was no statistically significant difference (P > 0.05). As CD8+ T cells, CD8+PD-L1+ T cells and CD68+CD163+ M2 macrophages in the total region can influence clinical outcomes, we stratified the patients according to joint parameters (CD8+/CD8+PD-L1+, CD8+/CD68+CD163+, and CD8+PD-L1+/CD68+CD163+). Patients with high infiltration of CD8+ T cells and low infiltration of CD8+PD-L1+ T cells in the total region had better PFS than those with any of the other three patterns (P<0.01). Analogously, patients with high infiltration of CD8+ T cells and low infiltration of CD68+CD163+ M2 macrophages in the total region had better PFS than those with any of the other three patterns (P<0.01). A similar result was observed in patients with low infiltration of CD8+ PD-L1+ T cells and low infiltration of CD68+CD163+ M2 macrophages in the total region (P<0.001). This indicates that CD8+ T cells, CD8+PD-L1+ T cells, CD8+/CD8+PD-L1+, CD8+/CD68+CD163+, and CD8+PD-L1+/CD68+CD163+ are potential biomarkers for predicting PFS in NSCLC patients receiving ICI therapy and that the CD8+/CD8+PD-L1+ and CD8+/CD68+CD163+ signatures provide better stratification of PFS than CD8+ T cells, CD8+PD-L1+ T cells, or CD68+CD163+ M2 macrophages.

Infiltration of T Cells and M2 Macrophages Was Correlated With the Response to ICI Therapy

In this study, it was found that the levels of infiltrated CD8+ T cells, CD8+PD-L1+ T cells and CD68+CD163+ M2 macrophages were independent prognostic factors for PFS. However, whether they can also predict the response to ICI therapy needs further study. Receiver operating characteristic (ROC) curve analysis revealed the better predictive performance of the infiltration of CD8+ T cells (AUC=0.76) or CD8+PD-L1+ T cells (AUC=0.62) than that of CD68+CD163+ M2 macrophages (AUC=0.59) (**Figures 5A–C**). Chi-square tests were performed to study the relationships between

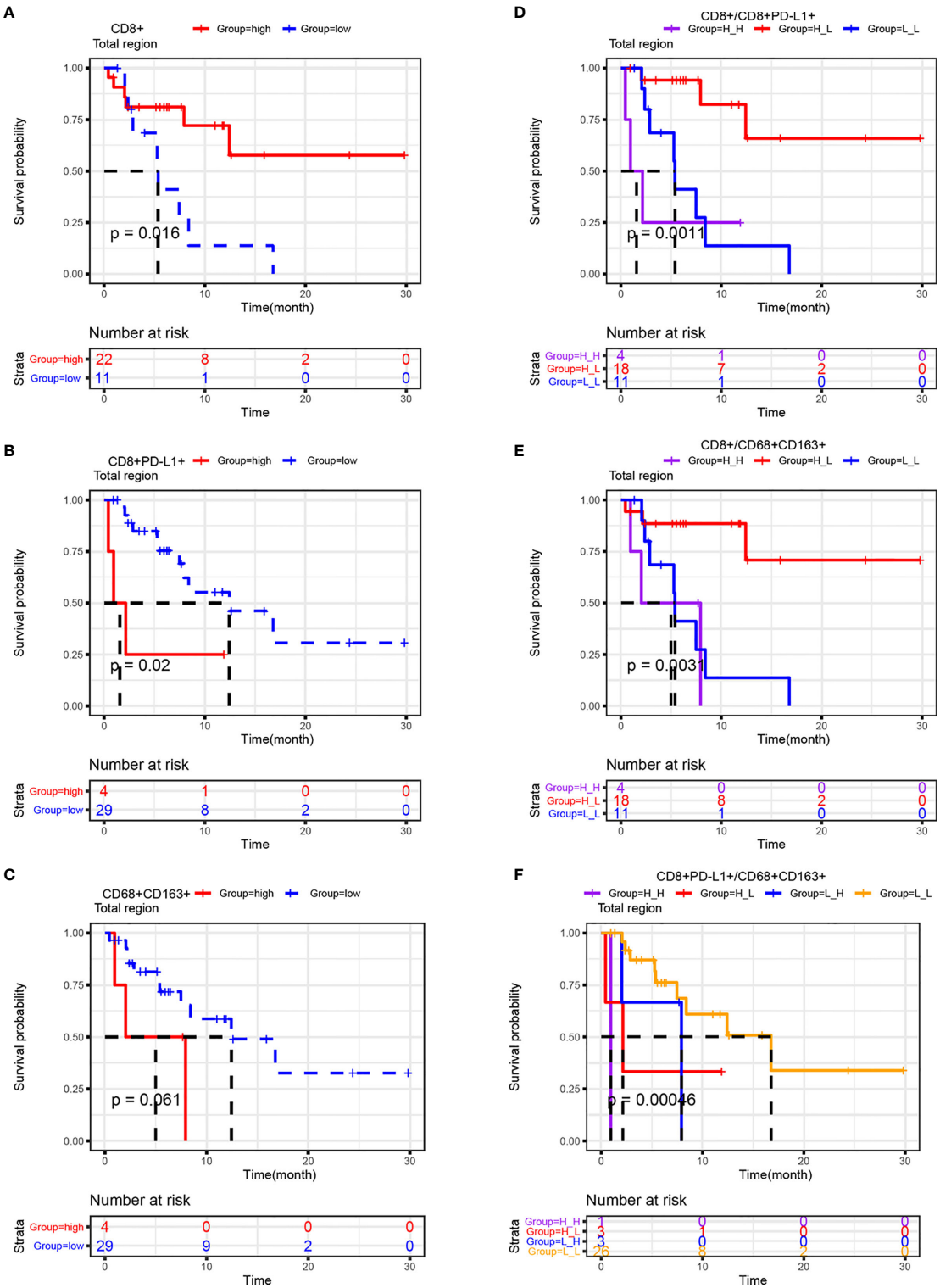

FIGURE 4 | Prognostic roles of CD8+, CD8+PD-L1+, CD68+CD163+ and related joint parameters. Kaplan-Meier analysis of the progression-free survival of NSCLC patients stratified by their CD8+ **(A)**, CD8+PD-L1+ **(B)**, CD68+CD163+ **(C)** or joint parameter results **(D-F)** for the total region. The cutoff point for TAICs (high vs low) was determined using the survminer package in R software. Higher infiltration of CD8+ and lower infiltration of CD8+PD-L1+ T cells were correlated with improved progression-free survival. The results also indicated that CD8+ high/CD8+PD-L1+ low, CD8+ high/CD68+CD163+ low, and CD8+ low/CD68+CD163+ low NSCLC patients had better PFS than the other three corresponding types of patients.

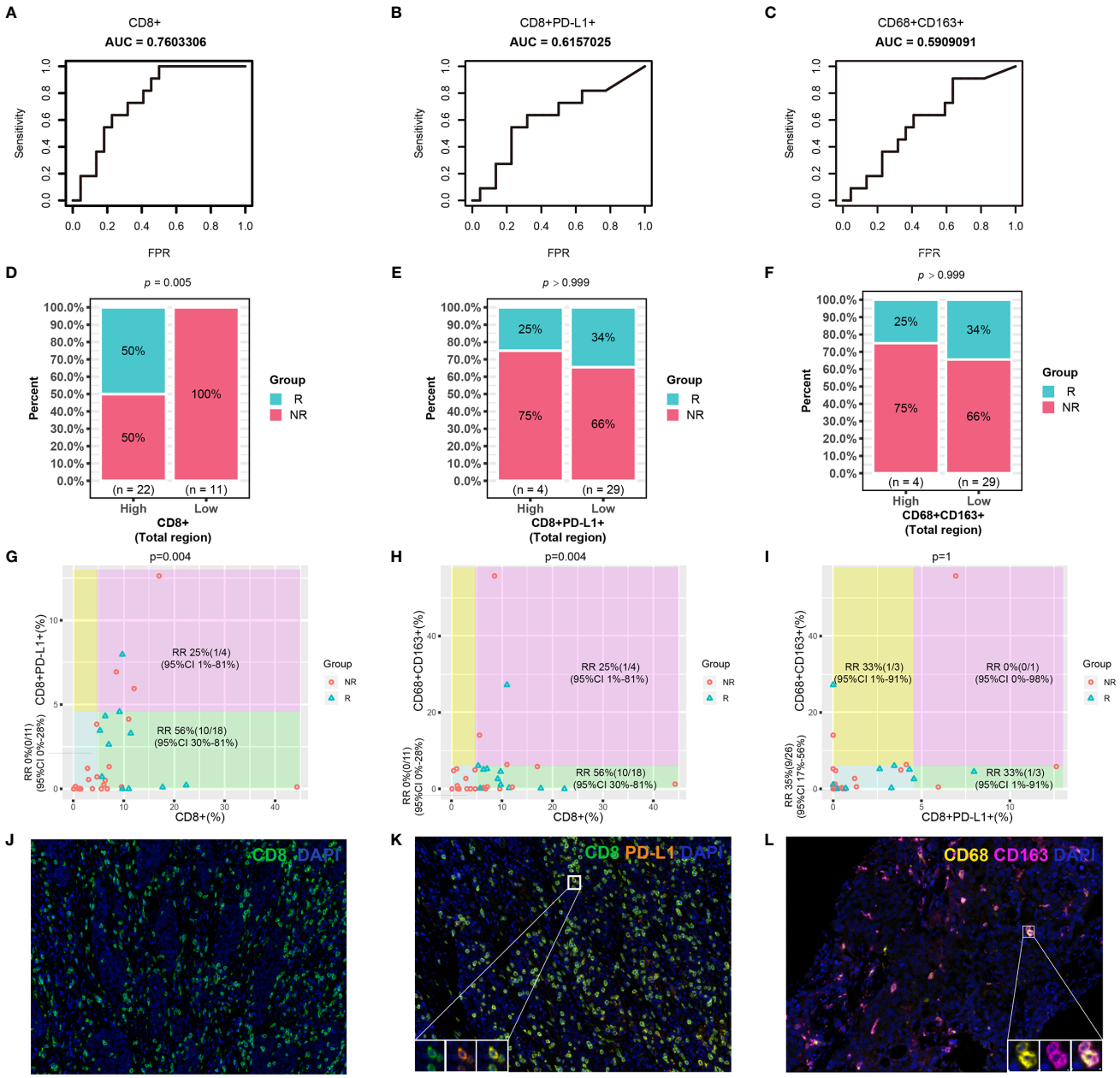

FIGURE 5 | Predictive roles of CD8+, CD8+PD-L1+, CD68+CD163+ and related joint parameters. **(A–C)** ROC curves for CD8+, CD8+PD-L1+, and CD68+CD163+. **(D–F)** Chi-square tests for CD8+, CD8+PD-L1+, and CD68+CD163. **(G–I)** Scatter plot and chi-square test for joint parameters. **(J–L)** Representative mIHC images of CD8+, CD8+PD-L1+, and CD68+CD163+ cell infiltration. NSCLC patients with higher CD8+ and lower CD8+PD-L1+ and CD68+CD163+ cell infiltration were more likely to respond to ICI therapy than patients with other cell infiltration patterns. RR, response rate.

CD8+ T cell, CD8+PD-L1+ T cell, or CD68+CD163+ M2 macrophage infiltration and the response rate of NSCLC patients who received ICI therapy. As shown in **Figures 5D–F**, the results showed that the response rate for ICI treatment in NSCLC patients with high-density infiltration of CD8+ T cells was significantly higher than that in those with low-density infiltration (P<0.01). In the total region, the response rate for ICI treatment was lower in the CD8+PD-L1+ T cell high-density infiltration subgroup than in the low-density infiltration group, but no significant difference was found (P>0.05). A similar trend was found for CD68+CD163+ M2 macrophages. A scatter plot and the chi-square test showed that the response rate in CD8+ high/CD8+PD-L1+ low subsets was 56%, which was significantly higher than that in the other two subsets (P<0.01) (**Figure 5G**). Patients with high CD8 T cell and low CD68+CD163+ M2 macrophage infiltration had a higher response rate than other patients (P<0.01) (**Figure 5H**). However, there were no

significant differences identified by the CD8+PD-L1+/CD68+ CD163+ parameter (**Figure 5I**). There were some examples in which a patient who responded to immunotherapy had high infiltration of CD8+ T cells and low infiltration of CD8+PD-L1+ T cells and CD68+CD163+ M2 macrophages (**Figures 5J–L**). This suggests that NSCLC patients with high infiltration of CD8+ T cells and low infiltration of CD8+PD-L1+ T cells or high infiltration of CD8+ T cells and low infiltration of CD68+CD163+ M2 macrophages were more likely to respond to ICIs than patients with other biomarker patterns. The CD8+/CD8+PD-L1+ and CD8+/CD68+CD163+ signatures provided greater stratification of the response rate than CD8+ T cells, CD8+PD-L1+ T cells, or CD68+CD163+ M2 macrophages alone.

DISCUSSION

This study demonstrates a relationship between TAICs in the tumor immune microenvironment and clinical outcomes in 33 NSCLC patients treated with ICIs. Our data suggest that the levels of infiltrating CD8+ T cells, CD8+PD-L1+ T cells, and CD68+ CD163+ M2 macrophages in the total region were independent prognostic factors for PFS in NSCLC patients treated with ICIs. The joint parameters CD8+/CD8+PD-L1+, CD8+/CD68+CD163+, and CD8+PD-L1+/CD68+CD163+ were also potential indicators for predicting PFS in NSCLC patients receiving ICIs. In addition, the infiltration of CD8+ T cells, the combination of CD8+ and CD8+ PD-L1+ T cells, and the combination of CD8+ T cells and CD68+ CD163+ M2 macrophages were potential indicators for predicting the response to ICIs in NSCLC.

Our study underlines the prognostic and predictive roles of CD8+PD-L1+ T cells, CD8+ T cells, CD68+CD163+ M2 macrophages and related joint parameters in NSCLC. Our study found that NSCLC patients with high CD8+ PD-L1+ T cell infiltration had relatively poor PFS (**Figures 2–4**). Recently, some studies have investigated the relationships between the expression of PD-1 or PD-L1 on TAICs and the prognosis of ICI therapy, and high infiltration of CD8+PD-1+ T cells was found to indicate better OS or an increased response rate (13, 25, 28, 29). Since PD-L1 expressed on tumor cells and macrophages is a negative regulator of T cell responses, blocking the PD-1/PD-L1 axis can improve immune responses against tumors (24); furthermore, it makes sense that in a 62-person cohort of NSCLC patients treated with ICIs, high levels of CD68+PD-L1+ immune cells were associated with prolonged OS (25). However, few studies have reported the relationship between the expression of PD-L1 on T cells and immunotherapy. It has been reported previously that PD-L1^{high} CD8+ T cells express more CD107a and IFN-γ than PD-L1^{low}CD8+ T cells, which indicates that PD-L1^{high} CD8+ T cells are functional effector cells (26). However, the ligation of PD-L1 in T cells can induce IL-10 expression and T cell apoptosis (30). A recent pancreatic cancer study showed that PD-L1+ T cells exerted tumor-promoting tolerance in 3 ways: PD-L1+ T cells could prevent activation, reduce Th1 polarization and promote Th17 differentiation (31). Through the PD-L1–PD-1 axis, PD-L1+ T cells can suppress effector T cells even without endogenous PD-L1 (31). The engagement of PD-L1+ T cells and PD-1+ macrophages can induce M2 macrophages, which have negative effects on adaptive antitumor immunity (31). This may explain why NSCLC patients with high levels of CD8+PD-L1+ T cells have relatively poor PFS. These results indicate that CD8+ PD-L1+ T cells are a risk factor for ICI therapy in NSCLC.

The present work identified the value of CD8+ T cells in predicting PFS and treatment response (**Figures 2–5**). Multiple studies have shown that high infiltration of CD8+ T cells correlates with improved survival in patients treated with ICIs (18, 32, 33). It was reported that a high density of CD8+ cells was associated with a higher median OS time in 163 NSCLC patients who received durvalumab ($P<0.01$) (32). Some studies have also found that high infiltration of CD8+ T cells is correlated with a relatively good response in patients treated with ICIs (12, 19–21). A previous study found that the level of CD8+ T cells was significantly higher in patients achieving CR/PR than in those with SD/PD in melanoma ($P < 0.0001$) (32). Our experimental results confirmed that CD8+ T cells are a good biomarker for predicting the response and survival of patients treated with ICIs.

Our data revealed that NSCLC patients with high CD68+ CD163+ macrophage infiltration had relatively poor PFS (**Figure 3**). Macrophages in the tumor microenvironment are defined as TAMs, which can produce growth factors, cytokines, and other molecules to regulate metastasis (22). The M1 and M2 states are two main phenotypes of macrophages. It has been reported that CD68+CD163- cells and CD68+CD163+ cells are considered M1-like macrophages and M2-like macrophages, respectively (23). Low infiltration of M1-like macrophages and high infiltration of M2-like macrophages are strongly associated with poor disease-free survival (23). M2-like macrophages have repair- and growth-inducing properties that can promote tumor progression, angiogenesis and metastasis (23, 34–36). This may explain the reason why high levels of CD68+CD163+ M2 macrophages were associated with poorer PFS than low levels of CD68+CD163+ M2 macrophages in this cohort.

Because of the prognostic and predictive roles of CD8+PD-L1+ T cells, CD8+ T cells, and CD68+CD163+ M2 macrophages, it was unsurprising to find that NSCLC patients with a CD8+ high/CD8+ PD-L1+ low, CD8+ high/CD68+CD163+ low or CD8+PD-L1+ high/CD8+PD-L1+ low signature had better PFS than patients with the other corresponding signatures (**Figure 4**). We also found that the response rate in NSCLC patients with a CD8+ high/CD8+ PD-L1+ low or CD8+ high/CD68+CD163+ low signature was significantly higher than that in patients with the other corresponding signatures (**Figure 5**). Overall, for PFS, the joint parameters CD8+/CD8+PD-L1+, CD8+/CD68+CD163+ and CD8+PD-L1+/CD8+PD-L1+ demonstrated stronger stratification than the single biomarkers. Regarding the response rate, the joint parameters CD8+/CD8+PD-L1+ and CD8+/CD68+CD163+ also demonstrated stronger stratification than the single biomarkers. Our findings suggest that joint evaluation of multiple biomarkers has certain significance in studying the immune status of tumors and guiding ICI treatment in NSCLC patients.

There were several limitations of this study. First, the analysis of this study was based on 33 NSCLC patients. The predictive values of

CD8+PD-L1+ T cells, CD8+ T cells, CD68+CD163+ M2 macrophages and related joint parameters need to be validated in larger studies. Second, the molecular characteristics of these biomarkers, especially CD8+PD-L1+ T cells, need further study.

In summary, our retrospective study revealed the prognostic and predictive value of CD8+PD-L1+ T cells, CD8+ T cells, and CD68+CD163+ M2 macrophages in NSCLC patients who received ICIs. These biomarkers are likely to predict response and survival. Interestingly, our results indicate that evaluation of joint parameters composed of these biomarkers has certain significance for guiding ICI treatment in NSCLC. Our results warrant validation and further study.

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 Ethics Committee of Guangdong Hospital of Traditional Chinese Medicine. The patients/participants provided their written informed consent to participate in this study. 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: LL, GL, YL, WG, and LY. Methodology: LL, GL, and YL. Software: LL, GL, and YL. Formal analysis: LL, GL, and YL. Investigation: LL, GL, YL, LG, and XZ. Resources: LL and GL. Data curation: LL, WG, GL, YL, and XZ. Writing - original draft preparation: LL, GL, and YL. Writing - review and editing: LL, GL, YL, LG, XZ, HZ, LY, and WG. Visualization: LL, GL, and YL. Supervision: LL, GL, and YL. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.658690/full#supplementary-material

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m5C RNA Methylation Regulators Predict Prognosis and Regulate the Immune Microenvironment in Lung Squamous Cell Carcinoma

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RNA methylation is a novel epigenetic modification that can be used to evaluate tumor prognosis. However, the underlying mechanisms are unclear. This study aimed to investigate the genetic characteristics of 5-methylcytosine (m5C) and N1-methyladenosine (m1A) regulators in lung squamous cell carcinoma (LUSC) and the prognostic value and immune-related effects of m5C regulators. To this end, we selected the public LUSC dataset from the Cancer Genome Atlas and Gene Expression Omnibus. The least absolute shrinkage and selection operator regression model was used to identify prognostic risk signatures. We used the UALCAN and Human Protein Atlas databases to study the expression of target gene mRNA/protein expression. Furthermore, the Tumor Immune Single Cell Hub and the Tumor Immune Estimation Resource were used to evaluate the degree of immune cell infiltration. Most of the m5C and m1A regulators showed significantly different expression between LUSC and normal samples. The m5C regulators were associated with poor prognosis. In addition, a prognostic risk signature was developed based on two m5C regulators, NOP2/Sun RNA methyltransferase 3 (*NSUN3*), and NOP2/Sun RNA methyltransferase 4 (*NSUN4*). Compared with normal lung tissues, the expression of *NSUN3* and *NSUN4* in the LUSC TCGA dataset was increased, which was related to clinicopathological characteristics and survival. *NSUN3* and *NSUN4* were related to the infiltration of six major immune cells; especially *NSUN3*, which was closely related to CD8+ T cells, while *NSUN4* was closely related to neutrophils. Our findings suggest that m5C regulators can predict the clinical prognosis risk and regulate the tumor immune microenvironment in LUSC.

Keywords: RNA methylation, tumor, lung squamous cell carcinoma, 5-methylcytosine, prognosis, tumor immune microenvironment

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide. Each year, 1.8 million people are diagnosed with lung cancer, and 1.6 million people die from the disease (1, 2). Approximately 85% of lung cancer patients have the non-small cell lung cancer (NSCLC) subtype (3). More than half of the patients diagnosed with lung cancer die within one year after diagnosis, and the 5-year survival rate is approximately 17.8%. NSCLC includes three types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Lung squamous cell carcinoma (LUSC), which accounts for about 40% of NSCLC, is closely related to smoking and economic levels (4). Compared with lung adenocarcinoma (LUAD), LUSC has a poor clinical prognosis and lacks targeted drugs. Therefore, a more comprehensive understanding of the molecular mechanism of LUSC progression is essential for the development of new treatment methods.

Known mRNA post-transcriptional modifications include 5-terminal capping, pre-mRNA splicing, polyadenylation, and mRNA export epigenetic mechanisms (5). RNA modification is also controlled by writers, erasers, and readers. Writers are the proteins that add chemical modification to specific sites of RNA molecules, erasers remove chemical modification added by the writers, and readers can recognize and bind RNA modification sites (6). These proteins work together as a complex network that dynamically regulates RNA modification. The internal modifications of mRNA mainly include N6-methyladenosine (m6A), N1-methyladenosine (m1A), 5-methylcytosine (m5C), 5-hydroxymethyl cytosine (hm5C), N7-methylguanosine (m7G), and pseudopurine (C) (7). m6A is the most common post-transcriptional modification method and is enriched in many eukaryotes and prokaryotes. With the application of high-throughput sequencing technology, it was found that m6A was mainly distributed in the coding region, and 30 untranslated regions were significantly enriched upstream of the stop codon (8). Increasing numbers of studies have shown that changes in m6A affected tumor progression, including proliferation, growth, invasion, and metastasis (9). In addition, studies have shown that METTL3-mediated m6A mRNA methylation improved the stability of *YAP* mRNA by regulating the MALAT1-miR-1914-3p-YAP axis and increased the induction of NSCLC drug resistance and metastasis (10).

5-Methylcytosine (m5C) is a widespread mRNA modification discovered in 1925, located in the untranslated regions (UTRs) of mRNA transcripts (11). Previous studies have shown that m5C played an important regulatory role in many aspects of gene expression, including RNA export, ribosome assembly, and translation (12). A recent study has shown that m5C also played an important role under pathological conditions (13), such as in cancer; *NSUN2* and *YBX1* promoted pathogenesis in human bladder urothelial carcinoma by targeting the m5C methylation site in the untranslated region of *HDGF3*. Adding a methyl group to the N1 position of adenosine will form an m1A, which appears mainly upstream of the initiation codon of the first splicing site and has a strong enrichment effect on translation in the 5'UTR (14). However, little is known about the function of m5C and m1A regulators in NSCLC.

In recent years, a large number of studies have proved that the tumor immune microenvironment (TIM) played a vital role in cancer progression and therapeutic efficacy. Li et al. (15) found that there was no significant difference in lymphocyte infiltration between the low and high immune risk groups of non-squamous NSCLC in the Cancer Genome Atlas (TCGA) dataset. In the high-immune risk group, it was found that the level of neutrophil necrosis and infiltration was increased significantly. The inflammatory tumor microenvironment is known to be associated with a poor prognosis. Liu et al. (16) found that in the early clinical stage of LUAD, lack of memory B cells or increased M0 macrophages were related to poor prognosis. In LUSC, T follicle helper cells were associated with good prognosis, while an increase in the number of neutrophils indicated poor prognosis. Previous studies have shown that mRNA post-transcriptional modification was associated with the progression and prognosis of LUSC. However, the relationship between mRNA post-transcriptional modification and the TIM in LUSC remains unclear.

In this study, we used the TCGA database and GEO dataset to conduct an in-depth analysis of m5C and m1A regulators in LUSC tissues and adjacent normal tissues. The purpose of this study was to explore the regulation of m5C and m1A in LUSC. Specifically, differentially expressed genes, clinicopathological characteristics, differences in survival, and impact on the TIM were addressed to provide therapeutic significance for the treatment of LUSC.

MATERIALS AND METHODS

Data Acquisition

Transcriptome analysis of raw data and corresponding clinical information of the LUSC cohort were downloaded from TCGA data portal (http://cancergenome.nih.gov/). A total of 551 research samples were obtained, including 502 LUSC tissues and 49 normal lung tissues, as was the corresponding clinical information (**Table 1**), as the training cohort. Independent gene microarray data from Gene Expression Omnibus (GEO) public datasets (https://www.ncbi.nlm.nih.gov/geo/) served as a validation cohort. Three datasets, GSE3349, GSE3141, and GSE19188, were selected, with a total of 125 samples including 82 LUSC tissues and 43 normal lung tissues.

Selection of Differentially Expressed Genes in the TCGA Database

A total of 9 m1A regulators were obtained from the published literature, including *YTHDF2*, *RRP8*, *ALKBH3*, *YTHDC1*, *TRMT61A*, *YTHDF1*, *ALKBH1*, *TRMT6*, and *YTHDF3*; there were 15 m5C regulators, including *NSUN1*, *NSUN*, *NSUN3*, *NSUN4*, *NSUN5*, *NSUN6*, *NSUN7*, *ALYREF*, *DNMT1*, *DNMT2*, *DNMT3A*, *DNMT3B*, *TET2*, *TRDMT1*, and *YBX1* (**Table 2**). Among them, *NSUN1* and *DNMT2* were not found in the TCGA LUSC data. Extract of the expression matrix and clinical data of m1A and m5C regulators of 502 LUSC samples and 42 normal lung

TABLE 1 | The clinical characteristics of lung squamous cell carcinoma patients in the training cohort.

Variables	No. of Patients	Percentage (%)
Age (years)		
≤65	189	38.3
>65	303	61.5
Unknown	1	0.2
Gender		
Male	364	73.8
Female	129	26.2
T stage		
T1	110	22.3
T2	289	58.6
T3	70	14.2
T4	24	4.9
N stage		
N0	313	63.5
N1	129	26.2
N2	40	8.1
N3	5	1.0
Unknown	6	1.2
M stage		
M0	405	82.2
M1	7	1.4
Unknown	81	16.4
Pathological stage		
I	240	48.7
II	158	32.1
III	84	17.0
IV	7	1.4
Unknown	4	0.8
Total	493	100.0

TABLE 2 | The list of the RNA modifying proteins involve in m1A, m5C.

Regulators	Type
m1A	
TRMT6	"writers"
TRMT61A	"writers"
RRP8	"writers"
ALKBH1	"readers"
ALKBH3	"readers"
YTHDF1	"erasers"
YTHDF2	"erasers"
YTHDF3	"erasers"
YTHDC1	"erasers"
m5C	
TRDMT1	"writers"
NSUN1	"writers"
NSUN2	"writers"
NSUN3	"writers"
NSUN4	"writers"
NSUN5	"writers"
NSUN6	"writers"
NSUN7	"writers"
DNMT1	"writers"
DNMT2	"writers"
DNMT3A	"writers"
DNMT3B	"writers"
ALYREF	"readers"
YBX1	"erasers"
TET2	"erasers"

tissues were obtained from the TCGA database. Then, the R version (4.0.2) of the limma software package was used to identify the m1A regulators and m5C regulators that were differentially expressed between the tumor and the control groups. *P* values <0.05 and |log2 (FC)|>1 were considered to indicate the significance threshold in all tests. In addition, we used heat maps and violin maps to visually show the differential expression of m5C regulators and m1A regulators between the two groups.

GEO Database Verified Differentially Expressed Genes

We first integrated all of the samples in the two datasets, using the sva package in the R computing environment for batch normalization, which increased the number of samples and avoided unreliable results (a total of 72 samples, including 29 LUSC samples and 43 healthy controls). Next, we performed differential analysis (|Log2FC|>2, adjusted *p*-value <0.05) by comparing tumor tissues to normal tissues in the R computing environment using the limma package. Subsequently, heat maps and violin maps were used to visually show the expression differences between the two groups.

Construction of the Protein–Protein Interactions Network

To further screen out the hub genes, we use the Search Tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org/) to analyze the differentially expressed m5C regulators to construct the Protein–Protein Interactions (PPI) network (17). The threshold of PPI network hub genes was the minimum gene interaction score >0.4. This network uses an evidence model to predict the association between proteins based on up to seven different types of evidence (fusion evidence, neighborhood evidence, co-occurrence evidence, experimental evidence, text mining evidence, database evidence, and co-expression evidence).

Construction and Validation of the Prognostic Risk Scoring Model

To evaluate the prognostic value of the m5C regulators, we performed univariate Cox regression analysis (18).Then, we used the following formula to calculate the risk score of the prognostic characteristics in each patient: risk score = coefficient 1 ∗ value1 + coefficient 2 ∗ value 2, where coefficient was determined using the least absolute shrinkage and selection operator (LASSO) algorithm, and the value was the relative expression level of each selected gene. Finally, based on the median risk score, LUSC patients included in the TCGA database were stratified into high-risk and low-risk groups. Survival differences between the two risk groups were evaluated using the Kaplan–Meier (KM) survival curve. According to the risk score formula, we combined GSE3141 with GSE19188 as a validation set to verify the reliability of the risk score model.

UALCAN Database

The UALCAN online database (http://ualcan.path.uab.edu) can analyze the correlation between gene expression and clinicopathological characteristics and survival according to

different subtypes of the disease. Using the UALCAN database, we analyzed the database based on clinicopathological parameters such as age, race, tumor grade, smoking, and *TP53* mutations.

Human Protein Atlas

Immunohistochemistry (IHC) showed a difference in the expression of *NSUN3* and *NSUN4* proteins in human normal lung and LUSC tissues from the Human Protein Atlas (HPA) website (https://www.proteinatlas.org). According to the staining intensity (negative, weak, medium, strong) and the proportion of staining cells (<25, 25–75%, or >75%), immunoreactivity score (IRS) was divided into four levels: 1) No detection; 2) Low; 3) Medium staining; 4) High staining.

CBioPortal Database

We used the cBioPortal platform (http://www.cbioportal.org/) to analyze the prognostic-related m5C regulators changes in LUSC (including missense mutations, fusion, amplification, and deep deletion) in TCGA. All searches were performed according to the online instructions at the cBioPortal.

Gene Enrichment Analysis

Gene Enrichment Analysis (GSEA) was performed in the LUSC cohort to gain insight into the biological pathways of the high-risk and low-risk subgroups defined by 13 gene expression characteristics. GSEA was used to find rich terms predicted to be associated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway in C2. Terms enriched in hub genes with p <0.01 and FDR (Error Found Rate) <0.05 were considered statistically significant.

Tumor Immune Single Cell Hub Database

The Tumor Immune Single-Cell Hub (TISCH) (http://tisch.comp-genomics.org) was a RNA-seq database focused on the tumor microenvironment (TME). TISCH provided detailed cell type annotations at the single-cell level, allowing TME to explore across different cancer types (17). In this study, the TISCH database was used to analyze the heterogeneity of TME in different datasets and different cells.

TIMER Database

The TIMER database (https://cistrome.shinyapps.io/timer/) was used to evaluate the potential correlation between prognostic-related m5C regulators and tumor infiltrating lymphocytes. It can use the immune penetration algorithm to calculate the infiltration abundance of the six immune cells (B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells) in the TCGA database. In addition, it provides three main analysis modules: Immune, Exploration, and Estimation. The Immune module includes clinical outcomes, somatic mutation, and somatic copy number change, enabling users to comprehensively analyze the relationship between immune cell infiltration and multiple factors (19).

Statistical Analysis

One-way analysis of variance was used to compare the expression levels of 13 m5C regulators and nine m1A regulators in 502 LUSC tissues and 49 normal lung tissues. The Spearman test was used to identify the correlation between m5C regulators. The median risk value was used as a cut-off value to divide patients into high- and low-risk groups. Kaplan–Meier method was used to assess the correlation between high- and low-risk groups and survival. Univariate and multivariate COX regression analyses identified whether risk score, gender, race, grade, smoking, *TP53* mutation, *etc.* can be used as independent prognostic factors. All analyses used R v3.6.0 and SPSS v25.0 software. p values <0.05 were considered statistically different.

RESULTS

The Differentially Expressed m5C and m1A Regulators Between LUSC and Normal Control Samples

In this study, 502 LUSC tissues and 49 normal tissues from TCGA were analyzed. The results showed that most of the m5C regulators were differently expressed between LUSC and normal tissues (**Figure 1A**). Nine genes, including *NSUN6* ($p < 0.001$), *NSUN5* ($p < 0.001$), *ALYREF* ($p < 0.001$), *DNMT1* ($p < 0.001$), *DNMT3B* ($p < 0.001$), *NSUN2* ($p < 0.001$), *DNMT3A* ($p < 0.001$), *YBX1* ($p < 0.001$), and *NSUN3* ($p < 0.001$) were significantly up-regulated in LUSC samples ($p < 0.001$) compared to those in normal tissues. In addition, the expression levels of two genes, *TRDMT1* and *NSUN7*, were significantly down-regulated in LUSC tissues (both $p < 0.001$) compared to those in normal tissues. However, the levels of *TET2* and *NSUN4* were not significantly different (**Figure 1C** and **Supplementary Table 1**). The expression of the m1A regulators also differed between LUSC tissues and adjacent normal tissues (**Figure 1B**). *ALKBH3* ($p < 0.001$), *TRMT61A* ($p < 0.001$), *YTHDF1* ($p < 0.001$), *ALKBH1* ($p < 0.001$), and *TRMT6* ($p < 0.001$) in cancer tissues were significantly up-regulated compared to those in normal tissues ($p < 0.001$). The expression of *YTHDF2* in cancer tissues was also up-regulated ($p < 0.05$), while those of *RRP8*, *YTHDC1*, and *YTHDF3* were not significantly different (**Figure 1D** and **Supplementary Table 1**).

Correlation Between m5C, m1A Regulators, and Overall Survival in LUSC Patients

In order to study the correlation between m5C and m1A regulators and the prognosis of LUSC, we used univariate cox regression to analyze the relationship between m5C and m1A regulators and OS in the TCGA database. The results of the m5C regulators showed that *NSUN3* [hazard ratio (HR) = 1.057, 95% confidence interval (CI) = 1.002–1.115, $p = 0.040$] and *NSUN4* (HR = 1.130, 95% CI = 0.998–1.280, $p = 0.052$) were high risks, showing an HR of >1 (**Figure 2A**). Next, we used these two genes to build a prognostic risk model (**Figures 2B, C**). LASSO algorithm was used to calculate the correlation coefficient (**Table 3**). The risk value for each patient with LUSC was calculated as follows: risk score = 0.057 * NSUN3 + 0.122 * NSUN4. After that, according to the median risk value, LUSC patients in the TCGA database were divided into high-risk and low-risk groups, and further survival analysis was performed in

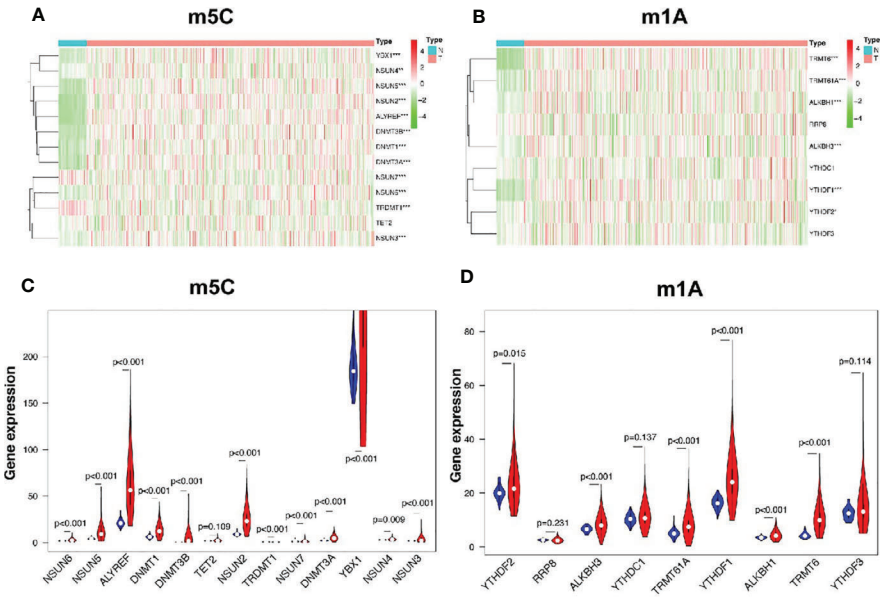

FIGURE 1 | Identification of differential genes between m5C and m1A regulators in LUSC and normal groups. **(A, C)** The heatmap and violin plot visually show the expression differences in m5C regulators between the two groups. (In the tumor group, the expression interval of *YBX1* was large, and only partial data were intercepted.) **(B, D)** The heatmap and violin plot visually show the expression differences in m1A regulators between the two groups. N, normal samples; T, tumor samples; blue violins represent normal samples; red violins represent tumor samples; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

these groups. As shown in **Figure 2D**, the OS in the high-risk group was significantly lower than that in the low-risk group ($p < 0.001$). In order to evaluate the accuracy of prognostic-related risk values for predicting prognosis, we conducted a time-dependent ROC analysis. The area under the ROC curve (AUC) for the 3-year analyses in the training datasets was 0.561 (**Figure 2E**). At the same time, the samples in the validation datasets (n = 85) were also divided into high-risk groups (n = 42) and low-risk groups (n = 43) using the same method. In the validation set, AUC = 0.629 for the 3-year analyses (**Figure 2F**), indicating the risk score model can better predict the prognosis. The results of the m1A regulators showed that only *ALKBH1* was correlated with OS (HR = 0.865, 95% CI = 0.781–0.957, $p = 0.005$), while the rest had $p > 0.05$, and *ALKBH1* was the protective factor of HR <1 (**Supplementary Figure 2**). Therefore, this study focused on the prognostic value of m5C regulators in LUSC.

GEO Database Verified Differentially Expressed Genes in m5C Regulators

Next, we used the GEO database to further verify differentially expressed genes in the m5C regulators. Compared with single array analysis, the integration of multiple arrays can improve the reliability of the results. Therefore, we first integrated all the samples from the two datasets to increase the sample size. The results showed that there were significant differences in the expression of m5C regulators between LUSC and normal tissues (**Figure 3A**). Among them, the expression levels of *NSUN5*, *DNMT1*, *DNMT3B*, *NSUN2*, *DNMT3A*, *YBX1*, *NSUN3*, and *NSUN4* in cancer tissues were significantly higher than those in normal tissues ($p < 0.001$); the expression levels of *NSUN6*, *TRDMT1*, and *NSUN7* in cancer tissues were significantly lower than those in normal tissues ($p < 0.001$). No significant difference was found in the expression levels of *TET2* between the two groups; *ALYREF*, *DNMT2*, and *NSUN1* were not found in the database (**Figure 3B** and **Supplementary Table 2**).

Interaction and Correlation Between m5C Regulators

Next, we further analyzed the interaction between the m5C regulators. As shown in **Figures 3C, D**, *TRDMT1* was the hub gene of the network and interacts with 10 other genes. In the correlation analysis, *TRDMT1* did not show a strong correlation with other genes. However, interestingly, *ALYREF*, *NSUN2*, *NSUN6*, *DNMT1*, and *DNMT3A* had weak to moderate correlations with other genes. *NSUN4* and *YBXI* had the strongest correlation (**Figure 3E**). The above results indicated that there was a certain interaction between m5C regulators.

Prognosis-Related Risk Values in LUSC Were Not Only Related to Clinical Outcome and Clinicopathological Characteristics, but Also an Independent Prognostic Factor in LUSC

Then, we further analyze the risk value and clinicopathological characteristics. **Figure 4A** indicated that the LUSC patients in the high-risk group generally contained a higher proportion of *NSUN3* and *NSUN4* than those in the low-risk group. In addition, significant differences in terms of survival state ($p < 0.05$) were also observed between the high-and low-risk groups. However, no significant differences were found between the two

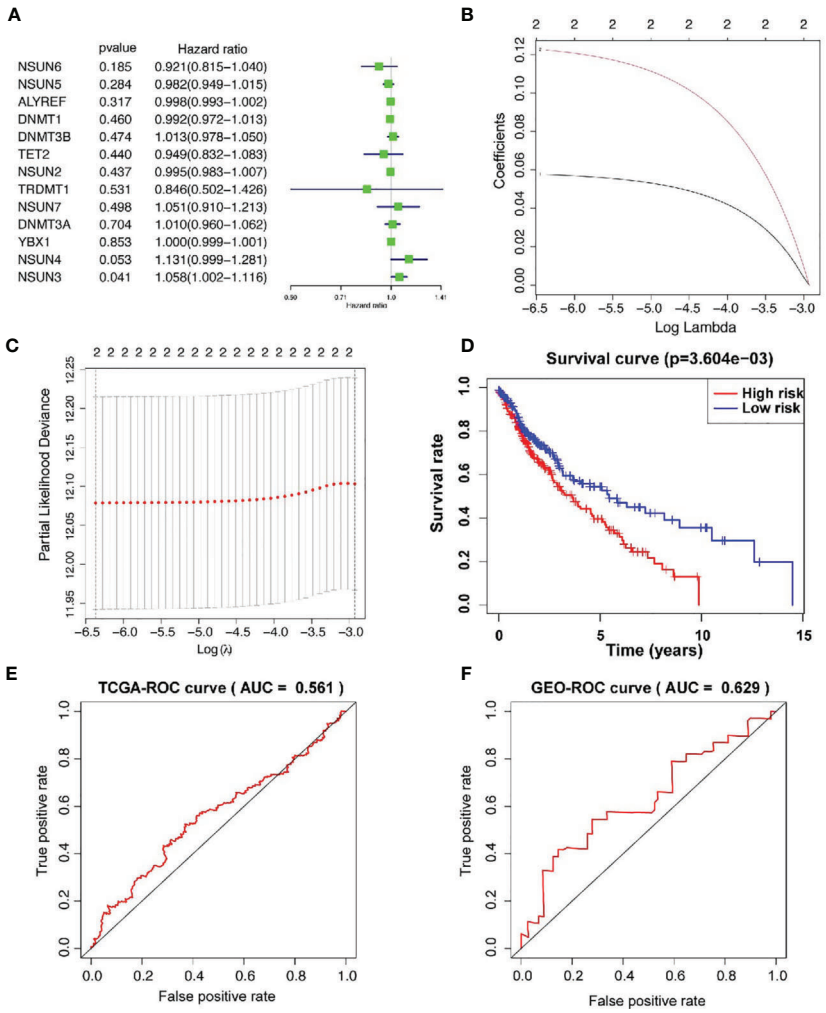

FIGURE 2 | Prognostic risk model construction. **(A)** Univariate Cox regression analysis indicates that *DNMT3B*, *NSUN7*, *DNMT3A*, *NSUN3*, and *NSUN4* are the risk genes for LUSC with the hazard ratio (HR) >1. **(B, C)** The coefficients of genes are obtained using least absolute shrinkage and selection operator (LASSO) algorithm. Ten-fold cross-validation used for tuning parameter selection in the LASSO model **(D)** Kaplan–Meier curves of OS between the high risk and low risk groups. **(E)** The 3-year receiver operating characteristic (ROC) curves of the training cohort. **(F)** The 3-year receiver operating characteristic (ROC) curves of the validation cohort. AUC, area under ROC curve.

TABLE 3 | Genes selected to build risk signature and the corresponding coefficients.

Genes	Coefficients
NSUN3	0.0575621521710856
NSUN4	0.122380087085298

groups for gender, T, N, M, pathological stage, and age. Next, we used univariate Cox and multivariate Cox regression to analyze whether the prognostic risk values in LUSC can be used as an independent prognostic factor. Univariate Cox regression analysis showed that age (HR = 1.024, 95% CI =1.003–1.044, p = 0.019), pathological stage (HR = 1.024, 95% CI =1.003–1.044, p = 0.019), T (HR =1.267, 95% CI =1.032–1.556, p = 0.023), and risk score (HR =1.732, 95% CI =1.262–2.377, p = 0.000) were significantly correlated with OS (**Figure 4B**). However, there was no significant correlation between M, N, gender, and OS. Multivariate Cox regression analysis showed that only the age (HR = 1.029, 95% CI =1.008–1.051, p = 0.007) and risk score (HR =1.763, 95% CI =1.285–2.420, p < 0.001) could be used as independent prognostic factors for LUSC (**Figure 4C**). These results indicated that the prognosis-related risk values of m5C regulators have the potential to predict prognosis in LUSC patients.

Expression Levels of *NSUN3* and *NSUN4* in LUSC Patients and Prognostic Analysis

To further analyze *NSUN3* and *NSUN4* expression between LUSC tissues and normal lung tissues, we explored their expression in the UALCAN databases. As shown in **Figure 5A** and **Supplementary Figure 2A**, compared with normal tissues (N = 52), the expression

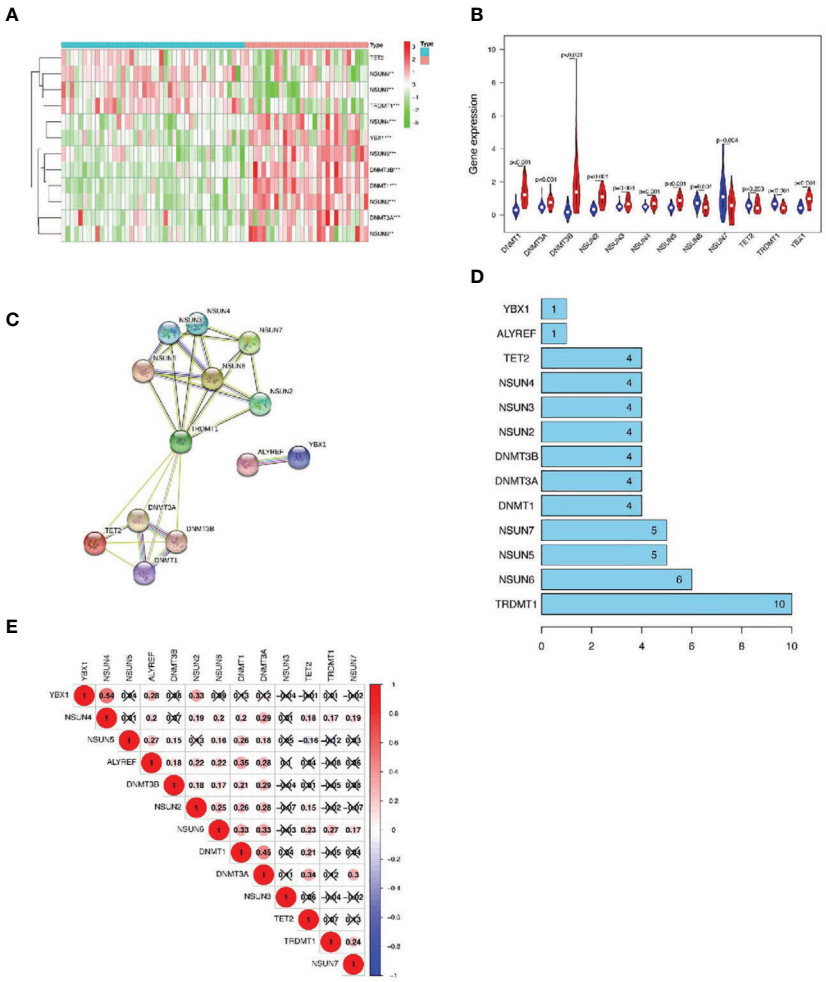

FIGURE 3 | The GEO database verifies the differentially expressed genes (DEGs) in m5C regulators and the interaction and correlation between m5C regulators. **(A, B)** Comparison of m5C regulators between tumor and normal groups in GEO datasets. **(C, D)** The protein–protein interaction (PPI) network shows the interaction between differentially expressed genes among m5C regulators. **(E)** The Pearson correlation analysis of the m5C regulators. *$p < 0.05$,**$p < 0.01$,***$p < 0.001$.

of *NSUN3* in LUSC tissues (N = 503) was significantly up-regulated ($p < 0.05$). With respect to gender, the expression of *NSUN3* in men was higher than that in women ($p < 0.05$). In terms of stage, the expression of *NSUN3* was higher in stages I–IV than in normal tissues, and there was statistical significance in stages I–III compared to the normal tissues ($p < 0.05$). However, stage IV was not statistically significant, possibly because the sample size was too small (N = 7), leading to a potential bias. For *TP53* mutation, compared with *TP53* wild patients, the expression level of *NSUN3* in the *TP53* mutant was higher. However, with respect to race, smoking history, and survival, there was no significant difference in the expression of *NSUN3* among the different groups. As shown in **Figure 5B** and **Supplementary Figure 2B**, *NSUN4* expression was significantly up-regulated in LUSC (N = 503) compared with normal tissue (N = 52) ($p < 0.05$). In terms of gender, the expression level of *NSUN4* was higher in females, but this difference was not statistically significant. With regard to stage, the expression level of *NSUN4* in stage I–III cancer tissues was higher than that in normal tissues, but there was no significant difference. According to the survival analysis curve, the OS in patients with high *NSUN4* expression was significantly shorter than that in patients with low *NSUN4* expression ($p < 0.05$). It may be due to the uneven distribution of sample size, but there were no significant differences in the expression of *NSUN4* in different races, smoking, or *TP53* mutations. These results suggested that *NSUN3* and *NSUN4* were closely related to clinicopathological features and may be the oncogenes in LUSC.

Protein Expression Difference, Gene Alteration Types and Enrichment Analysis of *NSUN3* and *NSUN4*

In addition, we attempted to detect the expression levels of *NSUN3* and *NSUN4* in LUSC using the HPA database. IHC detection showed (**Figure 6A**) that *NSUN3* was not present in

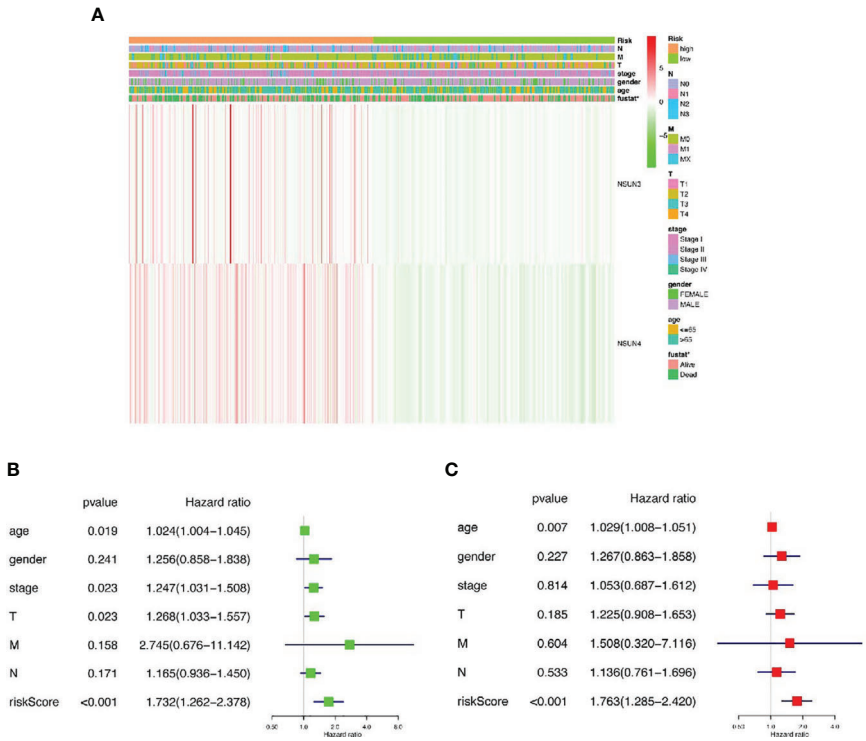

FIGURE 4 | The relationship between risk score and clinical outcome, pathological characteristics, and prognostic value of LUSC. **(A)** Expression differences in clinicopathological characteristics and risk scores between high and low risk groups from The Cancer genome Atlas (TCGA) dataset. **(B)** Univariate Cox regression analysis of clinicopathological characteristics and risk score. **(C)** Multivariate Cox regression analysis of clinicopathological characteristics and risk score. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

normal tissues, but low levels of expression (as assessed *via* staining intensity) were observed in cancer tissues with no significant difference between the two; *NSUN4* protein was not expressed in normal lung tissues but was moderately expressed in LUSC tissues. Using the cbiopotals database, we found that in the TCGA database of 1,176 samples, *NSUN3* and *NSUN4* gene changes included missense mutation, fusion, amplification, and deep deletion. The mutation frequencies in *NSUN3* and *NSUN4*

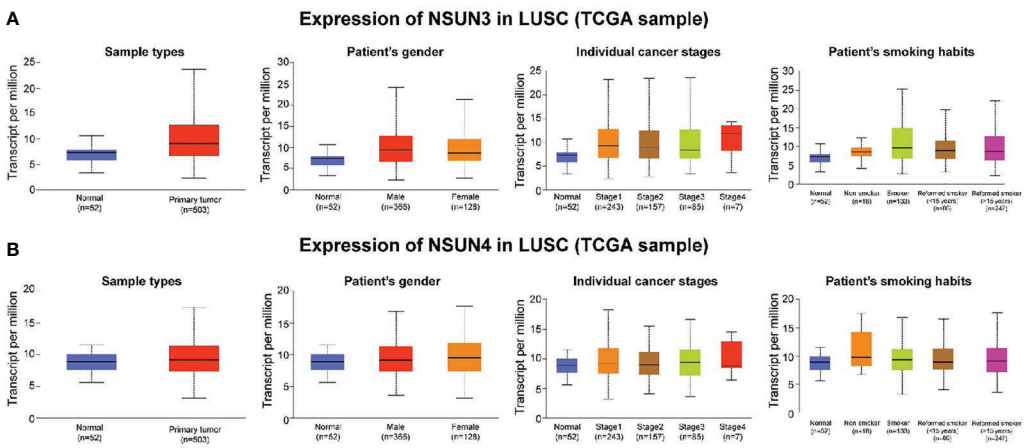

FIGURE 5 | Association between *NSUN3* and *NSUN4* expression and clinicopathological parameters in patients with LUSC (UALCAN). **(A)** Expression of *NSUN3* in normal and LUSC tissues based on sample types, patients' gender, cancer stage, and smoking habits. **(B)** Expression of *NSUN4* in normal and LUSC tissues based on sample types, patients' gender, cancer stage, and smoking habits.

were 11 and 1.1%, respectively. *NSUN3* mainly proliferates in TCGA, TCGApan, and TCGA pub data; NSUN4 mainly proliferates in TCGA, TCGApan, and mainly mutation in TCGA pub data (**Figure 6B, C**).To identify the signaling pathways activated by the differential expression of *NSUN3* and *NSUN4* in LUSC, GSEA was performed. Single-gene GSEA analysis showed that the high expression of *NSUN3* was correlated with the *p53* signaling pathway (NSE = 1.47, $p < 0.05$) and cell cycle signaling pathway (NSE = 2.04, $p < 0.001$). Meanwhile, the high expression of *NSUN4* was correlated with the cell cycle signaling pathway (NSE = 2.07, $p < 0.001$), *mTOR* signaling pathway (NSE = 1.84, $p < 0.001$) and *p53* signaling pathway (NSE = 1.83, $p < 0.001$) (**Figure 6D**).

Relationship Between m5C Regulators and Tumor Immune Microenvironment in LUSC

The tumor microenvironment (TME), including immune cells, inflammatory cells, and stromal cells (**Table 4**), played an important role in tumor genesis, development, metastasis, recurrence, and drug resistance. Therefore, we used the TISCH database to analyze the degree of invasion of the risk-related genes *NSUN3* and *NSUN4* in TME-related cells. The 18 tumor tissues of GSE124765 contain six LUSC tissues, and the six tumor tissues of GSE139555 contain two LUSC tissues. Immune cells such as neutrophils, macrophages, dendritic cells (DCs), and Tregs had low to moderate infiltration. *NSUN3* had the highest infiltration level in Tregs, and *NSUN4* had the highest infiltration degree in mast cells, followed by monocyte/macrophagecells (**Figure 7A**). As can be seen from the figures, the infiltration degree of *NSUN4* in immune cells was higher than that of *NSUN3*. Using the TISCH database, GSE124765 was divided into 25 cell clusters and 12 types of cells; the pie chart (**Figure 7B**) showed the number of cells in each cell type. The distribution and number of various TME-related cells can be visually observed. The GSE124765 dataset had the largest number of mononuclear macrophages (7,032), followed by CD4+ T cells (6,757). In the database, *NSUN3* was the top

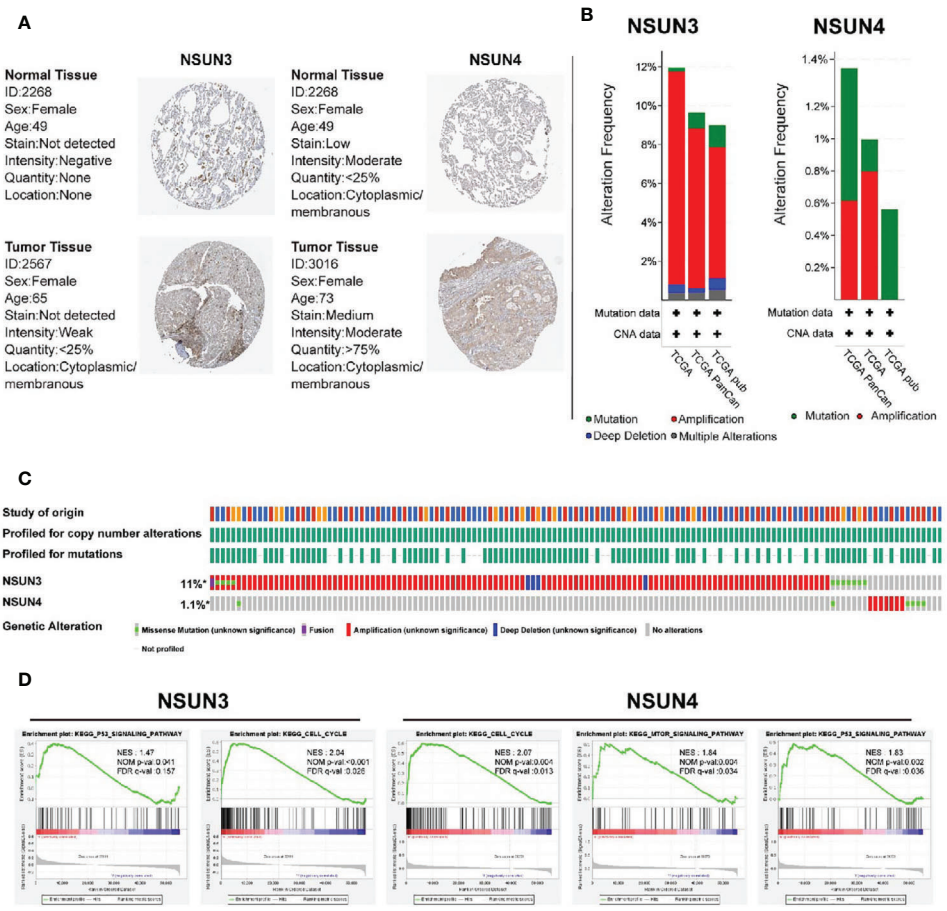

FIGURE 6 | Immunohistochemical analysis (HPA), change frequency analysis (cbiopotal), and gene set enrichment analysis (GSEA) of *NSUN3* and *NSUN4* in LUSC. **(A)** The protein expressions of *NSUN3* and *NSUN4* in LUSC tissues and normal tissues using the HPA. **(B, C)** The frequency of gene changes in *NSUN3* and *NSUN4* in LUSC in three independent studies. **(D)** Enrichment pathway of *NSUN3* and *NSUN4* high expression group in C2KEGG. NES, normalized enrichment score; NOM, nominal; FDR, false discovery rate. Gene sets with NOM p-val < 0.05 and FDR q-val < 0.25 are considered as significant.

TABLE 4 | The tumor microenvironment includes immune cells/inflammatory cells, stromal cells, and malignant cells.

Immune cells	Conventional CD4 T Cells (CD4Tconv)
	Regulatory T Cells (Treg)
	Proliferative T cells (Tprolif)
	CD8 T Cells (CD8+ T)
	Exhausted CD8 T Cells (CD8Tex)
	Natural Killer Cells (NK)
	B Cells (B)
	Plasma
	Dendritic Cells (DC)
	Monocytes or Macrophages (Mono/Macro)
	Mast Cells (Mast)
	Neutrophils
Stromal cells	Endothelial Cells (Endothelial)
	Fibroblasts (Fibroblasts)
Malignant cells	Malignant Cells (Malignant)

gene in plasma cells of the GSE124765 dataset ($p < 0.001$). It can be seen from **Figure 7C** that *NSUN4* had a higher degree of infiltration in TME-related cells than *NSUN3*, which was consistent with the results shown in **Figure 7A**. In short, the above results indicated that m5C regulators were related to LUSC, especially immune cells.

Immune Cell Infiltration Analysis Showed That the Expression of m5C Regulators Were Correlated With Immune Cells

In order to further analyze the correlation between m5C regulators and immune cells, we used the TIMER database to analyze the correlation between *NSUN3 and NSUN4* and the degree of infiltration of six immune cells (**Figure 8A**). Interestingly, except for the negative correlation between *NSUN3* and the degree of CD4+ T cell infiltration, *NSUN3* and *NSUN4* were positively correlated with the degree of infiltration of most immune cells, and the expression levels of two genes were positively correlated with tumor purity ($p < 0.05$). We then analyzed the relationship between *NSUN3* and *NSUN4* somatic cell copy number variation and the degree of infiltration of the six immune cells (**Figure 8B**). Compared with normal *NSUN3* somatic cells, the expression of CD8+ T cells in each mutant group was down-regulated, and in the arm level gain and high amplification mutation group, the expression of CD8+ T cells was significantly down-regulated ($p < 0.05$). Compared with normal NSUN4 somatic cells, the expression of neutrophils in each mutation group was down-regulated, and the expression of neutrophils in the arm level deletion group was significantly down-regulated ($p < 0.05$), and the expression of CD4+ T cells in the arm level deletion group was significantly down-regulated ($p < 0.05$). Next, we combined m5C regulators with immune cells for survival analysis (**Figure 8C**) and found that in the low *NSUN3* expression group, the survival period of CD8+ T cell infiltration degree was shorter than that in the CD8+T cell infiltration degree group ($p < 0.05$), while the combined analysis of the *NSUN3* high expression group and CD8+ T cell infiltration degree showed no significant difference in survival between the groups. In the *NSUN4* high expression group, the survival period of neutrophils with a high degree of infiltration was shorter than that of neutrophils with a low degree of infiltration ($p < 0.05$), and the combined analysis of *NUSN4* expression level and CD4+T cell infiltration degree showed no significant difference in survival. Therefore, it can be seen from the above analysis that in LUSC, *NSUN3* was closely related to CD8+ T cells, and *NSUN4* was closely related to neutrophils.

DISCUSSION

More than 150 modifications of RNA have been found, of which m6A, m5C, and m1A are the main ones. They play an important role in regulating gene expression and cell fate. Abnormalities in RNA modification lead to the occurrence of a series of diseases, including cancer (20).Liu et al. found that m6A regulators were significantly differently expressed in LUSC, and METTLE3 and HNRNPC were significantly related to the prognosis of LUSC (8). In this study, we found that m1A and m5C regulators were significantly differently expressed in LUSC. The m5C regulators *NSUN3* and *NSUN4* were highly expressed in LUSC and significantly related to its prognosis. We then used *NSUN3* and *NSUN4* data and constructed a prognostic risk model and used it to divide the patients into high-risk and low-risk groups. We also analyzed the relationship between m5C regulators and the TIM. As far as we know, this is the first in-depth analysis of the role of m5C regulators in LUSC and the first discovery that m5C regulators are associated with tumor immune infiltration.

N1-methyladenosine (m1A) is an important post-transcriptional modification of RNA that was first documented more than 50 years ago (21). Zhao et al. found that, in gastrointestinal cancer, the highly expressed m1A regulatory factor ALKBH3 was associated with poor prognosis and metastasis. After knocking down *ALKBH3*, the expressions of *ErbB*, *mTOR*, and *AKT1S1*, the hub genes of the *ErbB* and *mTOR* pathways, were down-regulated, indicating that *ALKBH3* was related to the mTOR pathway and had an adverse effect on the prognosis of gastrointestinal cancer (22).Studies have shown that the m1A demethylase *ALKBH3* can act on the 5'UTR near the initial translation site of the cytokine macrophage colony stimulating factor (CSF-1) to promote the invasion of breast cancer and ovarian cancer cells, indicating that the regulation of m1A RNA methylation can lead to functional changes in cancer cells. In this study, the expression of m1A regulators was significantly different between LUSC tissues and adjacent normal tissues. However, in the univariate Cox regression analysis, the m1A regulators had no significant correlation with prognosis, which may be due to inherent biases in TCGA database, the nature of LUSC, and the normal sample size distribution, all of which could have caused bias; furthermore, this study is the first one to assess the relationship between m1A regulators and LUSC, and more *in vivo* and *in vitro* studies are needed for further verification.

m5C regulators are closely related to cell growth and development. Previous studies (23, 24) have reported that the m5C regulators *NSUN2* was the downstream target gene of the oncogene *MYC*. Upregulation of *MYC* promotes cell cycle

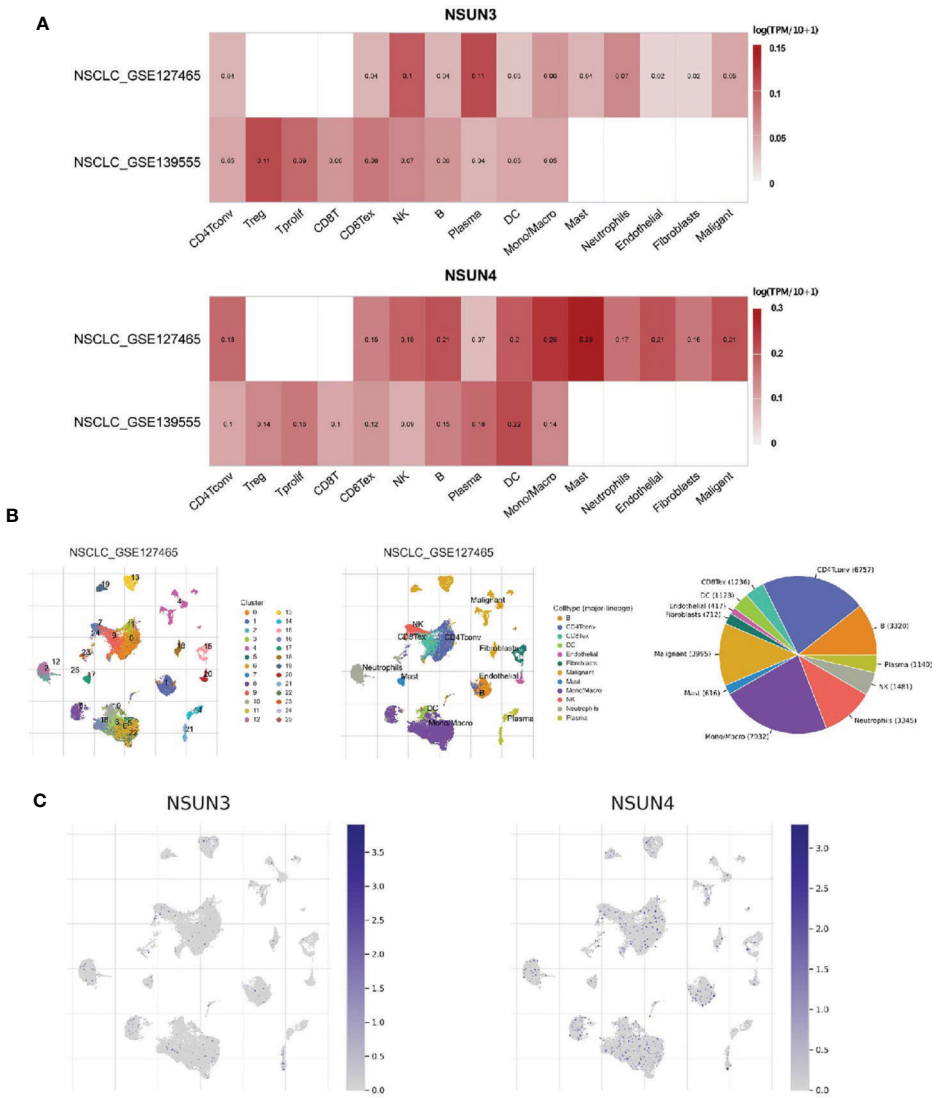

FIGURE 7 | Expression of m5C regulators in tumor microenvironment-related cells (TISCH). **(A)** The expression levels of *NSUN3* and *NSUN4* in the tumor microenvironment-related cells of lung squamous cell carcinoma in the GEO dataset. **(B)** Annotation of all cell types in GSE127465 and the percentage of each type of cell. **(C)** The proportion of *NSUN3* and *NSUN4* in GSE127465 in various types of cells.

progression and upregulation of *NSUN2*, with the highest expression observed in the S phase. In breast cancer, the expression of *NSUN2*was significantly associated with the clinical stage, tumor type, pathological differentiation, estrogen receptor, progesterone receptor, and Ki-67 expression levels (24). This shows that *NSUN2* is a powerful and clinically significant biomarker in breast cancer and can be used as a potential therapeutic target for breast cancer. In NSCLC, *NSUN1* has been identified as a prognostic marker (25),but it was done mainly in the context of the LUAD research. In this study, m5A regulators were significantly differentially expressed between LUSC and normal tissues. The m5C regulators *NSUN3* and *NSUN4* were significantly correlated with prognosis as risk factors, and these two genes were used to construct a prognostic risk model. The survival rate of the low-risk group was higher ($p < 0.05$), thus indicating the use of this risk value as an independent prognostic factor. This study is the first in-depth study on the m5C regulators in LUSC, and the results need to be further verified in *in vitro* and *in vivo* studies. *NSUN3* is required for the deposition of m5C on the anticodon loop of the mitochondrial transfer RNA methionine (mt-tRNAMet). The mutation of m5C in mt-tRNAMet results in a lack of 5-formylcytosine (fC) at the same tRNA position, indicating that *NSUN3* is required for efficient mitochondrial translation (26). *NSUN4*, which forms a complex with MTERF4, is necessary for mitochondrial ribosome biogenesis. Mitochondrial translation is disrupted after gene knockout in *NSUN4*-deficient mice (27), and research on *NSUN3* and *NSUN4* in cancer is limited.

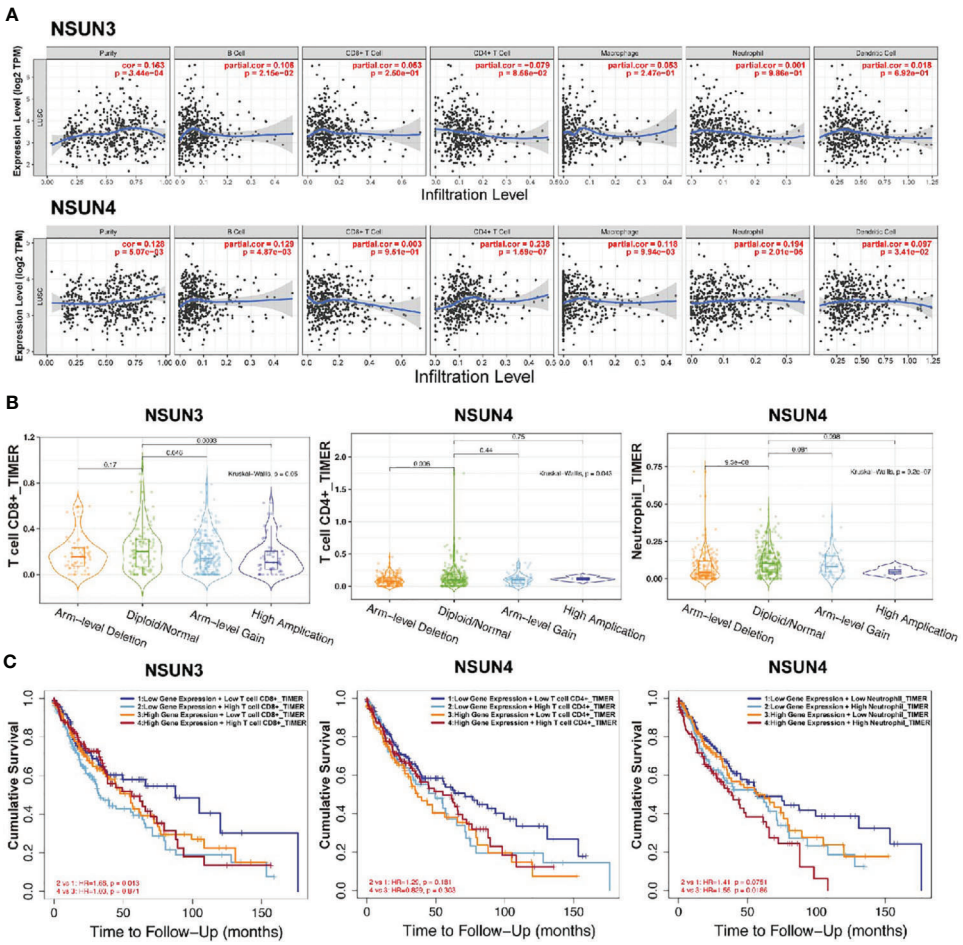

FIGURE 8 | Correlation analysis (TIMER) between m5CRNA methylation regulators and the infiltration levels of the sixmajor immune cells. **(A)** Correlation analysis of the infiltration levels of *NSUN3* and *NSUN4* and the six major immune cells after adjusting for purity. **(B)** Correlation analysis between somatic copy number alterations of *NSUN3* and *NSUN4* and the level of immune cell infiltration. **(C)** Survival analysis of combined *NSUN3* and *NSUN4* expression levels and immune cell infiltration levels.

In this study, we found that the mRNA expression level of *NSUN3* in LUSC tissues was significantly upregulated compared with that in normal tissues and was closely related to clinicopathological features. However, no significant difference in IHC was found in HPA data, suggesting that *NSUN3* itself may regulate its protein expression through post-transcriptional modification. The mRNA expression level of *NSUN4* in LUSC tissues was significantly upregulated compared with that in normal tissues, the expression level of *NSUN4* was higher in women, and the OS in patients with high *NSUN4* expression was shorter. However, compared with *NSUN3*, the differences in clinicopathological features of *NSUN4* were not significant, which may be related to the differences in the number of TCGA LUSC tissues and normal tissues. Previous studies have also shown that *NSUN3* mutations affected mitochondrial translation. This study found that 11% of LUSC tissues had mutations in *NSUN3* while only 1.1% of LUSC tissues had mutations in *NSUN4*. These mutations included missense mutation, fusion, amplification, and deep deletion, and mainly affected proliferation. Gene mutations can cause phenotypic changes and are important pathogenic factors for tumors. Therefore, the analysis of m5C regulators mutation plays an important role in understanding the occurrence and development of LUSC and has therapeutic guiding significance.

Through GSEA, we found that the up-regulation of *NSUN3* and *NSUN4* were closely related to the *p53* signaling pathway, cell cycle signaling pathway, and *mTOR* signaling pathway.*p53* is a tumor suppressor gene, which plays a major role in inhibiting tumor angiogenesis (28). *p53* can maintain the cell cycle at the G1/S regulatory point, thereby activating DNA repair proteins, initiating apoptosis, or inducing growth stagnation (29). *p53* mutations occur in approximately 50% of NSCLC cases (30). In addition to the loss of tumor suppressor function, *p53* mutation can also promote malignant progression and enhance cell invasion and metastasis (31, 32). Studies have shown that *p53* mutations had a synergistic effect with the oncogene Kras, which

could shorten the incubation period of LUAD and increase the ability of metastasis, while somatic mutations in *p53* are the most common co-mutations in *EGFR*-mutated LUAD (54.6–64.5%) (33). The *p53* mutation is related to the increased expression of PD-L1 in tumor cells in the inflammatory tumor immune microenvironment and *KRAS* mutation in NSCLC. Some of these events could be due to the activation of the nuclear factor kB (NF-κB) pathway due to mutations in *p53*, leading to enhanced cellular immunogenicity (34, 35). Previous studies have reported that cell cycle-related proteins are closely related to tumor progression in NSCLC (36, 37). The rapamycin (*mTOR*) signaling pathway is involved in various cell functions. Rapamycin (*mTOR*) is a serine/threonine kinase that regulates cell growth, survival, metabolism, autophagy and senescence. Dysregulation of the *mTOR* pathway is more common in squamous cell lung cancer than in adenocarcinoma, and patients with mutant *EGFR* always show abnormal PI3K/AKT/mTOR activation, which leads to resistance to clinical treatment with EGFR-tyrosine kinase inhibitor (EGFR-TKI) (38). However, the correlation between *NSUN3* and *NSUN4* in LUSC and the *p53* signaling pathway, cell cycle signaling pathway, and *mTOR* signaling pathway has been reported for the first time, and its regulatory mechanism needs to be further clarified.

In recent years, the TIM has received extensive attention in tumor research. The tumor immune escape mechanism is an early event of malignant precancerous lesions progressing to invasive cancer (39). In NSCLC, LUSC has a higher degree of tumor-related neutrophil infiltration than LUAD. Neutrophils are immunosuppressive factors, and the degree of infiltration is inversely proportional to the degree of CD8+ Tcell infiltration (40). Jiang et al. (41) performed whole-exome sequencing of 189 cases of surgically resected LUSC and found that tumors with mutations in *KEAP1* or *NFE2L2* had a higher level of oxidative stress, which may cause CD8+ tumor-infiltrating lymphocytes and other immune cells to be destroyed and DNA damage levels to be increased, leading to an increase in somatic mutations in tumor cells. Rizvi et al. (42) also found that in NSCLC patients treated with anti-programmed cell death (PD)-1and anti-programmed death-Lig and 1 (PD-L1), TMB was not associated with PD-L1 expression. In this study, we used the TISCH and TIMER databases to analyze the correlation between m5A regulators in LUSC and the six major immune cells in the tumor immune microenvironment, and found that *NSUN3* and *NSUN4* were expressed to a certain extent in immune cells. *NSUN4* was stronger than *NSUN3*, but both had a certain correlation with the six major immune cells. Furthermore, *NSUN4* had the strongest correlation with CD4+T cells and tumor-associated neutrophils, which is consistent with the results shown in previous studies. The m5A regulators were related to the TIM, but the specific regulatory mechanism needs further study.

However, there are still some shortcomings in this study. First, there were fewer studies on LUSC compared to LUAD. The uneven distribution of LUSC samples (N = 503) and normal tissue samples (N = 52) in the TCGA database resulted in subsequent impacts on the results related to m5A regulators and clinical pathology. Second, we did not find a large sample of data in the GEO database. The two data sets GSE3349 and GSE19188 used in this study had a small sample size and it had certain limitations. Third, predicted differentially expressed genes and prognostic risk score models failed to be validated *in vitro* and *in vivo*. Fourth, although the TIMER database (2.0) can perform correlation analysis on differential genes and immune cells in cancer, it failed to correlate clinicopathological features with the degree of immune cell infiltration. Therefore, more in-depth research is needed to overcome these problems.

In short, the current research on LUSC is far behind that on LUAD. Our research showed that there were significant differences in the expression of m5C and m1A regulators in LUSC and adjacent tissues, and we have developed prognostic risk markers using m5C regulators and found that m5C regulators could affect the TIM. Therefore, m5C regulators are expected to become prognostic markers in LUSC and provide strategies for the treatment of this disease.

CONCLUSION

In summary, we found that m5C regulators could predict the clinical prognostic risk in LUSC patients and regulate the TIM, thus possessing the potential to become new prognostic indicators in LUSC patients.

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**.

AUTHOR CONTRIBUTIONS

JP and YX designed this experiment, JP and ZH were responsible for literature review, data collection, analysis and writing, and YX was responsible for modification. All authors contributed to this 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.2021.657466/full#supplementary-material

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Pan-Cancer Analysis Identifies Liver Metastases as Negative Predictive Factor for Immune Checkpoint Inhibitors Treatment Outcome

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This study aimed to investigate the predictive value of liver metastases (LM) in patients with various advanced cancers received immune-checkpoint inhibitors (ICIs). First, clinical and survival data from a published cohort of 1,661 patients who received ICIs therapy were downloaded and analyzed. Second, a retrospective review of 182 patients with advanced non-small-cell lung cancer (NSCLC) who received PD-1/PD-L1 monotherapy was identified. Third, a meta-analysis of published trials was performed to explore the impact of LM on the efficacy of anti-PD-1/PD-L1 based therapy in advanced lung cancers. Pan-cancer analysis revealed that patients with LM had significantly shorter overall survival (OS) than those without LM (10 *vs.* 20 months; $P < 0.0001$). Subgroup analysis showed that the presence of LM was associated with markedly shorter OS than those without LM in ICI monotherapy group ($P < 0.0001$), but it did not reach the statistical significance in ICI-based combination therapy ($P = 0.0815$). In NSCLC, the presence of LM was associated with significantly inferior treatment outcomes in both pan-cancer and real-world cohort. Interestingly, ICI-based monotherapy and combination therapy could simultaneously prolong progression-free survival (PFS) and OS than chemotherapy in patients without LM. However, ICI-based monotherapy could not prolong PFS than chemotherapy in patients with LM while ICI-based combination therapy could dramatically prolong both PFS and OS. Together, these findings suggested that the presence of LM was the negative predictive factor in cancer patients received ICIs monotherapy, especially in NSCLC. ICI-based combination therapy might overcome the intrinsic resistance of LM to ICIs while the optimal combinatorial strategies remain under further investigation.

Keywords: pan-cancer, liver metastases, immune checkpoint inhibitor, prognosis, treatment outcome

INTRODUCTION

The liver is a large and very vascular glandular organ of human beings, which secretes bile and causes important biological changes in many of the substances contained in the blood (1, 2). It is also the main sites of distant metastases in patients with advanced cancers including melanoma, gastrointestinal cancer, breast cancer, as well as lung cancer (3, 4). Approximately 15–40% of patients with advanced cancers would be diagnosed with liver metastases (LM) during his/her lifetime (5, 6). Patients with LM often have an unsatisfactory prognosis (7). To make matters worse, several previous publications revealed that the presence of LM was a negative predictive factor for molecular targeted therapy in patients with driver gene mutations (e.g. EGFR) (8), indicating that alternative treatment strategy is warranted.

Immune-checkpoint inhibitors (ICIs) targeting cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed cell death 1 (PD-1) and its ligand (PD-L1) interaction have shifted the treatment landscape of advanced cancers and significantly improved the overall survival (OS) (9–12). Currently, ICI is one of the key and standard treatment strategies for various solid tumors. Nevertheless, several recent studies reported that patients with LM cannot benefit from ICI monotherapy (13, 14). Osorio et al. analyzed 761 individual lesions from 214 patients with non-small-cell lung cancer (NSCLC) and 290 lesions from 78 patients mismatch repair deficiency (MMRD) carcinoma treated with PD-1 monotherapy and found that LM had the least responses (15). However, other studies reported that LM did not compromise the survival benefit of patients received ICIs (16, 17). These contrary findings indicated that the predictive value of LM for ICIs treatment remains further investigation.

Therefore, we performed this pan-cancer analysis to investigate the predictive value of LM in patients with various advanced cancers received ICIs. We also analyzed a real-world cohort and conducted a systematic review with meta-analysis to explore the impact of LM on the efficacy of anti-PD-1/PD-L1 based treatment in advanced lung cancers.

METHODS

Data Identification and Pan-Cancer Analysis

To investigate the impact of LM on ICIs treatment outcome, we downloaded the pan-cancer clinical and survival data from a recently published cohort of 1,661 patients treated with ICIs therapy from the cBioPortal online database (https://www.cbioportal.org) (18–20). Firstly, we analyzed the predictive significance of LM in all included patients with various cancers. Then, we explored the predictive value of LM for ICIs treatment outcomes in several common types of solid tumors including melanoma, colorectal cancer and NSCLC. We also compared the tumor mutational burden (TMB) level between patients with and without LM. Similar to previous study, TMB was defined as the total number of nonsynonymous mutations including somatic, coding, base substitution, and indel mutations per megabase (mut/Mb) of genome examined.

Patients' Selection in a Real-World Cohort

To further assess the impact of LM for ICI treatment outcome in NSCLC, we performed a retrospective review of the patients diagnosed with advanced NSCLC who received anti-PD-1/PD-L1 monotherapy from January 1, 2016 to November 1, 2020 in two medical centers. The major inclusion criteria were (i) histological or pathological confirmation of advanced NSCLC, (ii) radiological confirmation of LM including magnetic resonance imaging (MRI) and/or enhanced computed tomography (CT), and (iii) evaluable for treatment response assessment. Firstly, patients with initial diagnosis of stage IV NSCLC were identified. Then, patients with LM and sufficient clinical information were selected. Other distant metastases were detected by using whole body positron emission tomography (PET) or PET/CT, cranial and thoracic CT/MRI, abdominal ultrasound or bone scan. All of them had received anti-PD-1/PD-L1 antibodies as monotherapy, regardless of treatment lines. The dose of each type of anti-PD-1/PD-L1 antibodies was used according to the recommended dose from drug instructions or phase II/III trials. This study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by the ethics committee of each medical center.

Data Collection

The major clinicopathological parameters including age, sex, smoking history, Eastern Cooperative Oncology Group performance status (ECOG PS), lung cancer histology (WHO classification) (21), sites of metastasis, therapeutic regimens and treatment lines were collected. Smoking status, ECOG PS and age were recorded at the time of initial diagnosis. A never smoker was defined as a person who had smoked less than 100 cigarettes during his/her lifetime. Which anti-PD-1/PD-L1 antibodies were selected according to clinical treatment guidelines or by the investigators' or patients' discretion. Response including complete response (CR), partial response (PR), stable disease (SD) and disease progression (PD) was assessed using Response Evaluation Criteria in Solid Tumors version 1.1. Progression-free survival (PFS) was assessed from the date the patient began ICI treatment to the date of PD or death of any cause. Patients who had not progressed were censored at the date of their last follow-up. OS was calculated from the beginning of immunotherapy to the date of death of any cause. Patients who were still alive or lost contact were censored at the date of last scan. The last follow-up was December 1, 2020.

Meta-Analysis of Published Trials

We performed a publication search of the PubMed/Medline, EMBASE, Google Scholar, Cochrane Library, and Web of Science databases through December 31, 2020, using "lung cancer" and "PD-L1" and "liver metastasis" and their related words. Data on the relationship between liver metastasis and OS or PFS in NSCLC patients treated with anti-PD-1/PD-L1 based treatments were collected from publications meeting the eligibility criteria. The details of our methodology are described in the **Supplemental Material**.

Statistical Analysis

Clinicopathologic characteristics were descriptively summarized by number and percentages. The categorical variables were compared by using chi-square test, or Fisher's exact test when needed. The continuous variables were analyzed by ANOVA and/or Tukey's multiple comparison tests. The difference of baseline features between different treatment groups was compared with the χ^2 test. PFS was defined as the time from the date of initiation of ICIs based treatment to the date of systemic progression or death and was censored at the date of last tumor assessment (when carried out). OS was calculated from the date of ICIs based treatment start to the date of death of any cause or last follow-up. Kaplan–Meier curves with two-sided log-rank tests and Cox proportional hazards model with calculated hazard ratios (HRs) and 95% confidence intervals (CIs) were used to determine the survival difference. All *P* values were two-sided and considered significant at $P < 0.05$. All statistical analyses were performed using the SPSS statistical software, version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Pan-Cancer Analysis

We identified a cohort of 1,661 cancer patients with 11 cancer types. Among them, 139 (8.4%) cases had LM. Baseline features of included patients were listed in **Table 1**. Totally, 1,034 (62.3%) male patients were included, and 739 (44.5%) cases had age ≥65 years. Most of them received PD-1/PD-L1 inhibitors treatment (78.7%). There was a significantly higher rate of patients received ICI-based combination therapy in patients with LM than those without LM ($P = 0.018$).

Patients with LM had significantly shorter OS than those without LM (10 *vs.* 20 months; HR = 1.70, $P < 0.0001$; **Figure 1A**) in all included patients. Intriguingly, TMB level was comparable between patients with and without LM (5.6 *vs.* 6.1, $P = 0.2782$; **Figure 1B**). Subgroup analysis showed that patients with LM also had markedly inferior OS than those without LM (9 *vs.* 17 months; HR = 1.79, $P < 0.0001$; **Figure 1C**) in ICI monotherapy group. However, the presence of LM was associated with inferior OS in ICI combination therapy without statistical significance (not reached *vs.* 41 months; HR = 1.66, $P = 0.0815$; **Figure 1D**). Interestingly, in patients treated with PD-1/PD-L1 monotherapy, the presence of LM was associated with significantly shorter OS (9 *vs.* 16 months; HR = 1.79, $P < 0.0001$; **Figure 1F**). Whereas the presence of LM was associated with inferior OS in CTLA-4 monotherapy but it did not reach the statistical significance (13 *vs.* 42 months; HR = 2.01, $P = 0.0752$; **Figure 1E**) mainly due to small sample size. We also investigated the predictive value of LM in several specific types of tumors. The presence of LM was associated with obviously worse OS in colorectal cancer ($P = 0.0289$; **Supplemental Figure S1A**) and NSCLC ($P = 0.0449$; **Supplemental Figure S1C**) group than those without LM, but it did reach the statistical significance in melanoma cohort ($P = 0.0668$; **Supplemental Figure S1B**). Multivariate analysis revealed that LM was significantly associated with worse OS ($P < 0.001$; **Table 2**). Additionally, ICIs based combination therapy and high tumor purity was significantly associated with longer OS ($P < 0.001$, $P = 0.042$, respectively; **Table 2**).

TABLE 1 | Baseline characteristics of the study population.

Variables	All	Liver metastasis	No. liver metastasis	*P* value
Total	1,661	139	1,522	
Age at diagnosis				
<65 years	922	80	842	0.612
≥65 years	739	59	680	
Gender				
Male	1,034	83	951	0.519
Female	627	56	571	
Cancer type				
Bladder Cancer	215	13	202	—
Breast Cancer	44	6	38	
Cancer of Unknown Primary	88	13	75	
Colorectal Cancer	110	26	84	
Esophagogastric Cancer	126	9	117	
Glioma	117	0	117	
Head and Neck Cancer	139	8	131	
Melanoma	320	31	289	
Non-Small Cell Lung Cancer	350	31	319	
Renal Cell Carcinoma	151	2	149	
Skin Cancer, Non-Melanoma	1	0	1	
Drug type				
Combination	255	31	224	0.018
CTLA-4 inhibitor	99	10	89	
PD-1/PD-L1 inhibitor	1,307	98	1,209	

CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death protein 1; PD-L1, programmed cell death protein ligand 1.

Baseline Features of Included Patients in Real-World Cohort

To further assess the predictive value of LM in patients with advanced NSCLC, we identified a total of 182 NSCLC patients received PD-1/PD-L1 monotherapy from January 1, 2016 to November 1, 2020 in two medical centers. Around 23 (18.0%) of them were initially diagnosed with LM. The clinical characteristics of the study population were summarized in **Table 3**. In total, 146 (80.2%) male patients were included, and the mean age was 61 years. Most of them were smokers (58.8%) and had performance status of ECOG 1-2 (91.2%). Adenocarcinoma is the most common histological type (58.8%). Some 53 (29.1%) patients received PD-1/PD-L1 monotherapy as first-line therapy.

The Predictive Value of LM in Real-World Cohort

Survival analyses using the Kaplan–Meier method and log-rank test showed significantly shorter PFS in patients with LM received PD-1/PD-L1 monotherapy compared to patients without LM (3.3 *vs.* 5.6 months; HR = 1.77, $P = 0.0119$; **Figure 2A**). Patients with LM also had significantly shorter OS than those without LM (8.2 *vs.* 17.6 months; HR = 1.83, $P = 0.0408$; **Figure 2B**). The objective response rate (ORR) was

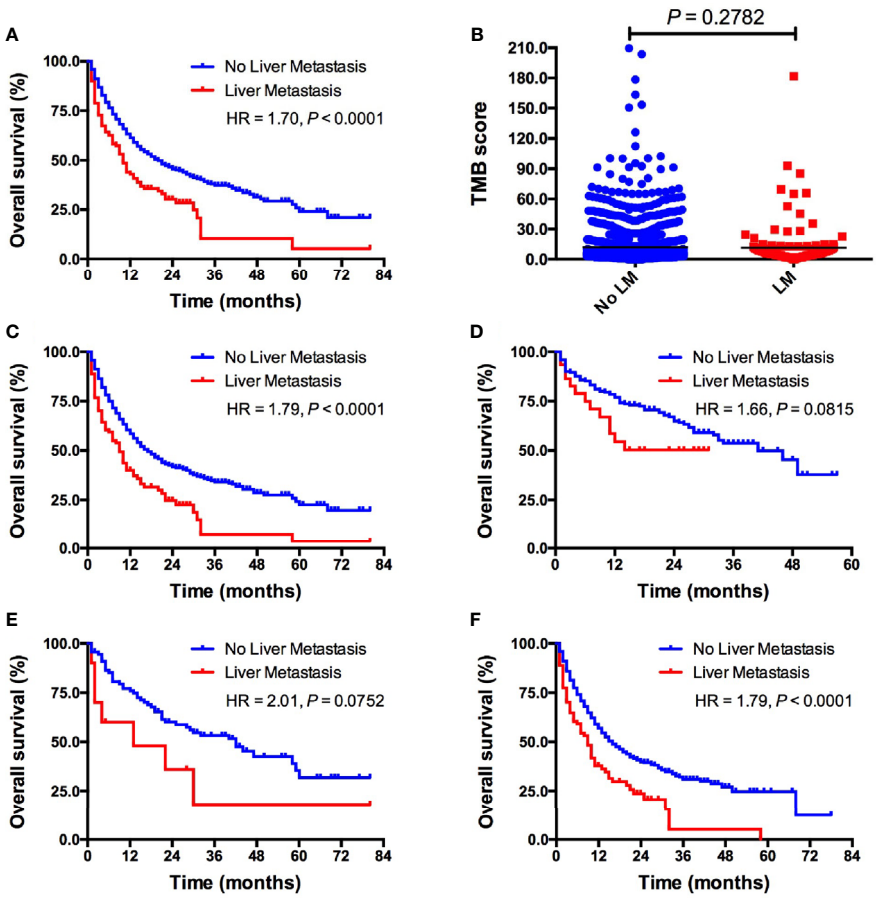

FIGURE 1 | Pan-cancer analysis of the predictive value of LM for ICIs treatment outcomes. **(A)** OS comparison between patients with *vs.* without LM in whole cohort; **(B)** TMB level comparison between patients with *vs.* without LM in whole cohort; **(C)** OS comparison between patients with *vs.* without LM in ICIs monotherapy group; **(D)** OS comparison between patients with *vs.* without LM in ICIs based combination therapy group; **(E)** OS comparison between patients with *vs.* without LM in PD-1/PD-L1 monotherapy group; **(F)** OS comparison between patients with *vs.* without LM inCTLA-4 monotherapy group. LM, liver metastasis; TMB, tumor mutational burden; ICI, immune checkpoint inhibitor.

TABLE 2 | Multivariate analyses of clinical parameters on OS.

Factor	HR (log rank)	95% CI	*P* value
Age (< 65/≥ 65)	1.003	0.873–1.152	0.971
Sex (Female/male)	1.116	0.971–1.284	0.122
Drug (monotherapy/combination)	1.797	1.450–2.227	<0.001
Liver metastasis (yes/no)	1.666	1.335–2.078	<0.001
Muation count (<median/≥median)	1.338	1.072–1.669	0.01
TMB score (<median/>median)	1.050	0.844–1.305	0.662
Tumor purity (<50/≥50)	1.153	1.005–1.332	0.042

HR, hazard ratio; CI, confidence interval; TMB, tumor mutational burden.

significantly lower in patients with LM than in patients without LM (4.3% *vs.* 28.9%, $P = 0.0118$; **Figure 2C**). The disease control rate (DCR) was similar between two groups (65.2% *vs.* 67.9%; **Figure 2C**). In multivariate analysis, LM was significantly associated with both shorter PFS (HR = 1.546, $P = 0.039$; **Supplemental Table S2**) and OS (HR = 1.543, $P = 0.046$; **Supplemental Table S1**). Additionally, PD-1/PD-L1 monotherapy as first-line treatment was significantly associated with longer PFS ($P = 0.020$; **Supplemental Table S1**) and OS ($P = 0.027$; **Supplemental Table S1**).

Features of Included Publication in the Meta-Analysis

Considering the negative predictive value of LM in NSCLC from both the online database and real-world cohort, we conducted a meta-analysis to compare the different treatment outcomes of anti-PD-1/PD-L1 based therapies in NSCLC with versus without LM. As shown in **Supplemental Figure S2**, 298 potentially relevant studies were screened. Most of the excluded publications were reviews, comments, duplications, or studies with incomplete data. The current study assessed 6,274 cases from 11 publications to investigate the distinct treatment outcomes of anti-PD-1/PD-L1 based therapies in NSCLC with versus without LM (22–32). The main features of the eligible studies are shown in **Supplemental Table S2**. Each included trial had the excellent methodologic quality (**Supplemental Table S3**).

TABLE 3 | Baseline characteristics of the population from real-world cohort.

Variables	All	Liver metastasis	No liver metastasis	*P* value
Total	182	23	159	
Age at diagnosis				
< 65 years	109	13	96	0.724
≥ 65 years	73	10	63	
Gender				
Male	146	17	129	0.417
Female	36	6	30	
Smoking history				
Never	75	11	64	0.490
Ever/current	107	12	95	
ECOG PS				
0	16	3	13	0.707
1–2	166	20	146	
Stage				
IIIB	12	2	10	0.988
IV	170	21	149	
Histological type				
Adenocarcinoma	107	10	97	0.110
Squamous cell carcinoma	51	8	43	
Others	24	5	19	
Treatment line				
First	53	4	49	0.281
Second or above	129	19	110	

ECOG PS, Eastern Cooperative Oncology Group performance status.

Treatment Outcomes in NSCLC With Versus Without LM

The pooled results showed that anti-PD-1/PD-L1 based therapies was correlated with better OS (HR = 0.73, 95% CI: 0.64–0.83; $P < 0.05$; **Figure 3A**) and PFS (HR = 0.77, 95% CI: 0.60–0.94; $P < 0.05$; **Figure 3C**) when compared with standard chemotherapy in patients with LM. Similarly, the pooled results indicated that anti-PD-1/PD-L1 based therapies was associated with longer OS (HR = 0.71, 95% CI: 0.66–0.77; $P < 0.05$; **Figure 3B**) and PFS (HR = 0.66, 95% CI: 0.57–0.75; $P < 0.05$; **Figure 3D**) in patients without LM. Both results of OS showed low heterogeneity ($I^2 = 0.0\%$, $P = 0.454$; $I^2 = 0.0\%$; $P = 0.622$; respectively), but results of PFS showed high heterogeneity ($I^2 = 64.9\%$, $P = 0.004$; $I^2 = 72.9\%$; $P < 0.001$; respectively). Subgroup analysis revealed that anti-PD-1/PD-L1 monotherapy could not prolong PFS than chemotherapy in patients with LM while anti-PD-1/PD-L1 based combination therapy could significantly prolong PFS (**Supplemental Figure S3**). In patients without LM, both anti-PD-1/PD-L1 based monotherapy and combination therapy could simultaneously prolong PFS and OS (**Supplemental Figure S3**).

DISCUSSION

The present study reported that the presence of LM was correlated with significantly inferior treatment outcomes in ICI based monotherapy. However, it was not associated with significantly inferior OS in ICI based combination treatment group. In one of the most common solid tumors, the presence of LM was associated with significantly inferior treatment outcomes in patients with advanced NSCLC from both the pan-cancer and real-world cohort. Interestingly, meta-analysis revealed that anti-PD-1/PD-L1 based monotherapy and combination therapy could simultaneously prolong PFS and OS in NSCLC patients without LM. However, anti-PD-1/PD-L1 based monotherapy could not prolong PFS than chemotherapy in NSCLC patients with LM while anti-PD-1/PD-L1 based combination therapy could dramatically prolong both PFS and OS. Collectively, these findings indicate that the presence of LM was the negative predictive factor in patients with advanced cancers received ICIs monotherapy. ICI based combination therapy might overcome the intrinsic resistance of LM to ICI monotherapy while the optimal combinatorial strategies need further investigation.

As one of the most common distant metastasis in solid tumors, LM has unique the tumor immune microenvironment (3, 4). When LM-competent cells entered the liver, they would encounter a variety of cells including liver sinusoidal endothelial cells, liver-associated lymphocytes, Kupffer cells, hepatic stellate cells, dendritic cells, and portal fibroblasts (3, 4). All of them would have an impact on the biology of LM formation and progression. Previously, several elegant studies have unraveled that liver could promote the specific immune tolerance under the circumstance of viral infections, organ transplantation and autoimmune diseases *via* eliminating effector T cell, inducing effector T cell anergy and regulatory T cells (Tregs) (33–35). Whether LM could impair the systemic antitumoral immunity and ICI treatment outcomes remains unknown. Recently, several

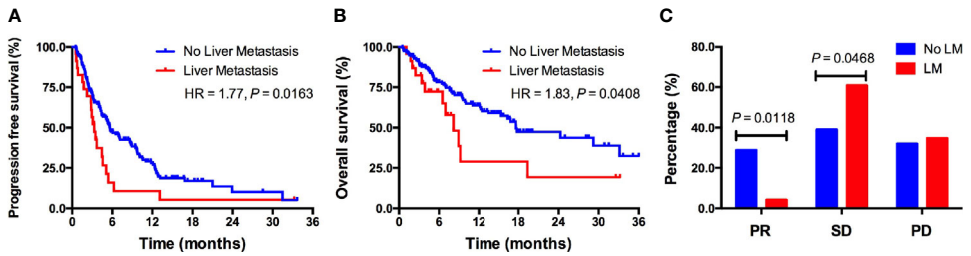

FIGURE 2 | The predictive value of LM for ICIs treatment outcomes in a real-world cohort. **(A)** Kaplan–Meier curve of PFS in patients with versus without LM; **(B)** Kaplan–Meier curve of OS in patients with versus without LM; **(C)** Response rate comparison between patients with versus without LM. LM, liver metastasis; PR, partial response; SD, stable disease; PD, disease progression.

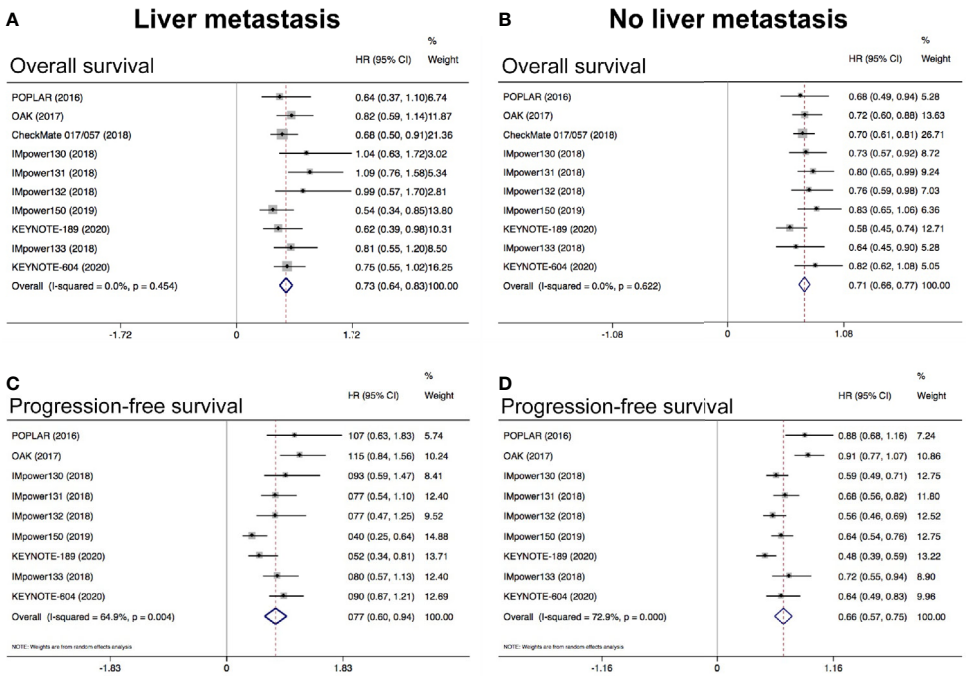

FIGURE 3 | Meta-analysis to evaluate the predictive value of LM in NSCLC treated with ICIs. **(A)** Pooled analysis of OS in patients with LM; **(B)** Pooled analysis of OS in patients without LM; **(C)** Pooled analysis of PFS in patients with LM; **(D)** Pooled analysis of PFS in patients without LM. LM, liver metastasis.

publications investigated the predictive value of LM for ICI efficacy. Paul et al. analyzed 336 patients with melanoma or NSCLC received pembrolizumab and reported that LM was associated with significantly reduced responses and PFS (13). Subsequently, a series of studies reported the negative predictive value of LM for ICI treatment in specific types of solid tumors (16, 36). Furthermore, our study indicated that the presence of LM was the pan-cancer negative predictive factor in patients received ICIs monotherapy. Interestingly, our data revealed that ICI based combination therapy could dramatically prolong both PFS and OS in patients with LM and the presence of LM did not significantly impair the efficacy of ICI based combination therapy. Taken together, these findings suggested that ICI monotherapy is insufficient to control the disease in patients with cancer and LM. Reasonable ICI based combination therapy need future investigation in this clinical scenario.

To unravel the mechanism of liver antitumoral immune tolerance in the context of cancer is the key to improve the clinical practice and prognosis of patients with LM. Several recent publications shed a light on this research area. Zhou et al. reported that LAG3 blockade could increase proliferation and effector cytokine production of intratumoral T-cells isolated from LM of colorectal cancer in response to both polyclonal and autologous tumor-specific stimulations, suggesting a new promising immunotherapeutic target for LM of colorectal cancer (37). James et al. observed that the presence of liver could suppress the systemic antitumor immunity in a dual-tumor immunocompetent mouse model (38). Mechanistically, coordinated activation of Tregs and modulation of intratumoral CD11b+ monocytes led to the antigen specific immune suppression. While Tregs were depleted or destabilized by using specific inhibitors, the antitumoral immune of PD-1 antibody could resuscitate within LM. More recently, Yu et al. found that LM could siphon activated CD8+ T cells from systemic circulation and induce antigen-specific Fas+CD8+ T cells undergo apoptosis following their interaction with FasL+CD11b+F4/80+ monocyte-derived macrophages (39). These immunosuppressive hepatic macrophages could be eliminated by liver-directed radiotherapy, which result in the increase of hepatic T cell survival and decrease of hepatic siphoning of T cells. These two elegant study together suggested the specific immune microenvironment of LM and ICI based combination therapy (e.g. plus CTLA-4 inhibitor, EZH2 inhibitors, radiotherapy, etc.) could rescue systemic antitumor immunity and improve the prognosis of cancer patients with LM.

These current findings had several significant limitations that should be acknowledged and treated with caution. First, relatively small number of eligible patients into the final real-world cohort analysis and the retrospective nature will inevitably have several biases such as selection bias. Meta-analysis is the archetypical observation and heterogeneous clinical trials were included without any technically correct information, making it not necessarily meaningful. Thus, the present findings must be cautiously interpreted and large-scale prospective study is eagerly warranted. Second, since PD-L1 expression results from online database was unavailable and real-world cohort did not record the PD-L1 expression, the impact of PD-L1 expression on the treatment outcomes could not be investigated. Third, details of patients with LM in published trials were not reported, making

further subgroup analysis difficult. Last but not least, the mechanisms of LM conferring poor prognosis in patients treated with ICI are not well stated. Since it is much difficult to obtain the paired primary and liver metastatic lesions in clinical practice, we cannot include any specific exome and/or transcriptomic features in the multivariate analysis. Therefore, currently, we cannot make a solid conclusion on the true predictive or prognostic significance of LM. In the future, we need comprehensively study the multi-omic features including genomic, transcriptomic, proteomic, metabolic and epigenomic features, especially single-cell transcriptome analysis and TCR sequencing of both primary lesions and LM to unravel the impact of specific immune clusters (e.g. macrophages, CD8+ T cells, Tregs, etc.) on tumor progression in the liver and ICI response, and then establish the true predictive or prognostic significance of LM in patients received ICIs therapy.

In conclusion, the current study indicated that the presence of LM was the negative predictive factor in cancer patients received ICIs monotherapy. ICI based combination therapy could dramatically prolong both PFS and OS in patients with LM and the presence of LM did not significantly impair the efficacy of ICI based combination therapy, suggesting it might overcome the intrinsic resistance of LM to ICIs monotherapy. However, due to the limited clinical and survival data from this study, the optimal combinatorial strategies in patients with LM are still unknown.

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 Wenzhou People's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors participated in the planning and execution of this study or analysis of the study data. TJ designed this study. All authors collected the data and conducted the relevant experiments. X-JC, LZ, E-DZ, and TJ collected the data and performed the statistical analyses. TJ and X-JC drafted the manuscript. LZ and E-DZ provided critical comments, suggestions 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/fimmu.2021.651086/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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A Multicenter Retrospective Study on the Prognosis of Stage III Unresectable Mutant Non-Small Cell Lung Cancer With Tyrosine Kinase Inhibitors Therapy

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Background: For unresectable stage III non-small cell lung cancer (NSCLC), concurrent chemoradiotherapy is nowadays the standard treatment. Patients with advanced NSCLC harboring driver-gene mutations benefit from Tyrosine Kinase Inhibitors (TKIs) Therapy. In a real-world setting, there is room for exploring the benefit of TKIs in stage III unresectable NSCLC patients with mutation.

Methods: A total of 81 patients from the Jinling Hospital and the Jiangsu Cancer Hospital with stage III unresectable mutant NSCLC applied targeted therapy were enrolled in this retrospective study. Patients with first-line application of TKIs were followed up to gain the situation of surgery qualifications, progression-free survival and overall survival, so as to evaluate the survival prognosis, then whether patients benefit and what kind of patients benefit most from TKI monotherapy treatment or its combination are explored.

Results: The median progression-free survival of involved 81 patients was 13.87 months (95% confidence interval (CI): 11.66–16.08), and the median survival was 41.47 months (95%CI: 20.11–62.83). The 5-year survival rates were 91.0, 80.3, 56.1, 45.5, and 32.5%, respectively. After first-line TKI therapy, seven patients (8.6%) were reevaluated as eligible for surgery and proceeded to surgery. Although no characteristics were found to be statistical prognostic, younger female non-smokers still tended to have a better prognosis with longer progression free survival and overall survival.

Conclusions: TKIs are a viable option for mutant stage III unresectable NSCLC patients who have achieved good clinical benefit from TKI. Patients who cannot tolerate chemoradiotherapy, especially those with driver gene mutations, can choose targeted therapy for first-line treatment.

Keywords: NSCLC, stage III unresectable, EGFR, TKI, surgery

INTRODUCTION

Lung cancer is currently one of the most malignant tumors, while five-year survival at all stages was about 19%. Once metastases are diagnosed, the rate directly declines to 5% (1). Non-small cell lung cancer accounts for 80–85% of lung carcinoma (2). The recognized mutant genes in NSCLC include EGFR, ALK, ROS, HER2, etcetera. As for EGFR mutation, the most typical types are exon 19 deletion and exon 21 mutation (3–5). Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are commonly used in advanced NSCLC with EGFR mutation (6). While the 5-year overall survival rate of standard concurrent chemoradiotherapy wandered around 20% (7), the long-term prognosis were 15–35% for unresectable or inoperable stage IIIA and 5–10% for stage IIIB (8). In unresectable ones, further studies are still needed that whether patients with driver gene mutation can benefit from first-line treatment with EGFR TKIs.

Previous studies have explored multiple treatments for stages III/IV advanced lung cancer. The Pacific study (9) showed that among patients with stage III unresectable NSCLC, the combination of standard concurrent chemoradiotherapy and durvalumab significantly prolonged overall survival compared with placebo (hazard ratio: 0.68; P = 0.0025) with similar safety as placebo. Meanwhile, previous studies have demonstrated no further benefit in survival prolongation with combination therapies based on CCRT or afatinib, like pembrolizumab (10), or cetuximab (11). Also, increasing the radiation dose to 74 Gy (high dose) paradoxically decreased survival compared with CCRT (12).

In this study, we collected eligible patients from two centers and included patients receiving first-line treatment with targeted drugs, then analyzed the clinical benefits of TKI therapy in these patients, especially the prognosis, and possible factors affecting survival outcomes.

We present the following article in accordance with the original article reporting checklist.

METHOD

Patients

In this study, the records of patients with NSCLC (including adenocarcinoma versus squamous) diagnosed by puncture and pathology admitted to the Jinling Hospital and the Jiangsu Cancer Hospital from January 1, 2005 to December 31, 2020 were retrieved. After sieve dereplication, stage III patients were selected according to TNM staging criteria of the UICC/AJCC 8th Edition (13). The driver gene detection results were then checked to exclude patients without mutations or untested, and to reserve those with positive driver genes (EGFR, ALK, ROS, HER2). Finally, we included patients with TKI therapy or TKI combination with chemotherapy as the first-line treatment. The study was approved by the Ethical Review Committee of the Affiliated Jinling Hospital (DBNJ20219).

Data Collection

We retrieved the medical records of the included 81 patients to obtain basic information such as age, gender, stage, driver gene, and mutation types. The specific medical records were then reviewed to obtain information on first-line treatment options, progression-free survival, second-line treatment, and whether or not surgery was performed, etc. Finally, patients were followed up for missing prognostic information such as overall survival, complication, and quality of life.

Statistical Analysis

Survival curves were plotted with Kaplan–Meier analysis, obtaining the median survival and the median progression-free survival, with 95% confidence intervals for both OS and PFS. Cox regression was used to explore the effect of factors such as gender, age, stage, genotype, smoking, chemotherapy combination and surgery on overall survival. All statistical analyses were calculated by SPSS statistics, and all statistical tests were all two-sided, and P values <0.05 would be considered statistically significant, with 95% confidence intervals.

RESULT

A total of 5,706 patients who had been admitted to our hospital were screened out from 19,872 admission records, and 81 were finally included. For patients who had already died, the specific overall survival was calculated, and for those who survived, the survival was calculated with January 31, 2021 as the end point of follow-up. As for patients who recently lost to follow-up, survival was defined as the time from the last follow-up. The flowchart of this study is shown in **Figure 1**.

Patient Characteristics

We collected the characteristics of 81 patients, as presented in **Table 1**. In this study, we included 42 (51.9%) elderly patients (≥65 years old) and 39 (48.1%) middle-aged patients. Among them, there were 20 cases of stage III A NSCLC (24, 7%), 45 cases of stage III B (55.6%) and nine cases of stage III C(11.1%); there were 35 males (43.2%) and 46 females (56.8%); Smoking, as an important factor, was also included in the study, with 11 smokers (13.6%), 57 non-smokers (70.4%) and 13 ex-smokers (16.0%).

Meanwhile, all 81 included patients had driver gene mutations, including 69 EGFR mutation (85.2%), 28 classical exon 19 deletions (34.6%), 17 exon 21 mutations (21%). In addition, there were nine ALK mutations (11.1%), one ROS (1.2%), and two HER2 mutation (2.5%).

In this study, a total of 63 patients (77.8%) chose TKI monotherapy as first-line treatment, while 18 patients were treated with TKI along with other therapies combined, including immunotherapy, chemotherapy or radiotherapy. Among them, nine (11.1%) patients received TKI along with chemotherapy, including pemetrexed, pemetrexed plus cisplatin. Six patients (7.4%) received radiotherapy on the basis of TKIs, and one patient underwent concurrent chemoradiotherapy. Immunotherapy involved only two patients, one in combination with bevacizumab, paclitaxel and the other in combination with bevacizumab alone.

Among the 81 patients, 75 (92.6%) chose first-generation TKIs as first-line treatment, with 51 (63.0%) receiving gefitinib,

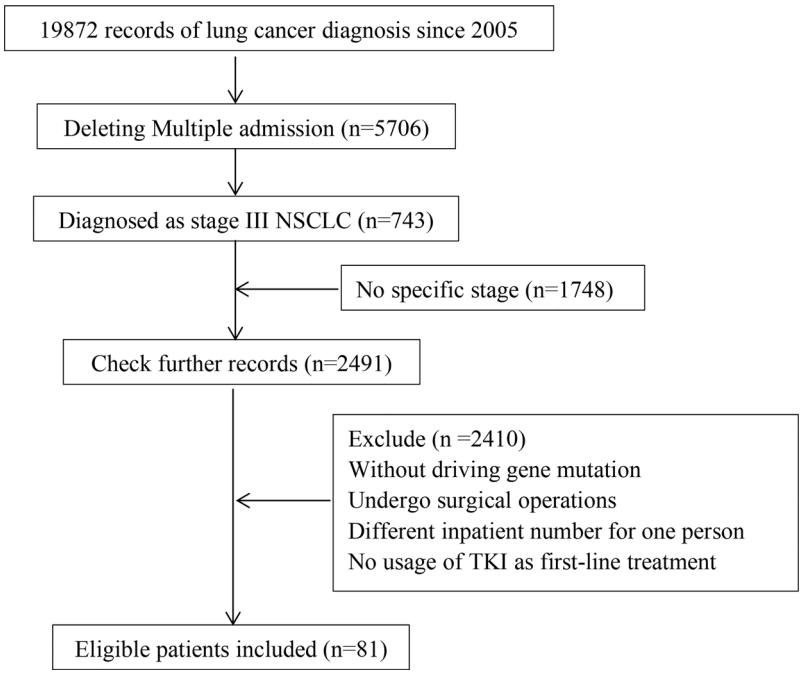

FIGURE 1 | The flowchart of this study. Including collection, screening, follow-up of in volved patients.

TABLE 1 | Characteristics of involved 81 patients.

Characteristic	Number (%)	Characteristic	Number (%)
Overall	81		
Gender		**Mutant gene**	
Male	35 (43.2)	**EGFR**	69 (85.2)
female	46 (56.8)	**ALK**	9 (11.1)
		ROS	1 (1.2)
		HER2	2 (2.5)
Age		**First-line treatment**	
Middle-age	39 (48.1)	**TKI**	63 (77.8)
Elderly	42 (51.9)	**TKI + chemotherapy**	9 (11.1)
		TKI + radiotherapy	6 (7.4)
		TKI + immunotherapy	2 (2.5)
		TKI+ chemoradiotherapy	1 (1.2)
Smoke	11 (13.6)	**Generation of TKI**	75 (92.6)
Smoker	57 (70.4)	**First-generation**	6 (7.4)
Non-smoker	13 (16.0)	**Second-generation**	
Ex-smoker			
Stage		**Surgery**	
Ⅲ A	20 (24,7)	**Surgery**	7 (8.6)
Ⅲ B	45 (55.6)	**No surgery & unknown**	68 (89.1)
Ⅲ C	9 (11.1)		
Missing	7 (8.6)		

Basic information of patients was obtained by retrieving medical records like gender, age, smoking, stage, mutation gene, first-line treatment, generations of TKIs, or surgery.

ten (12.3%) receiving erlotinib, and five (6.2%) receiving icotinib. Meanwhile, five patients (6.2%) received afatinib, one received ensartinib, two received second-generation TKIs, and no patients received third-generation in our study.

In second-line treatment, 17 patients continued with targeted therapy or its combination with other therapies, 20 switched to chemotherapy, and four patients were treated with combination of both. Among these 17 patients, three patients continued with first-generation TKIs, four with second-generation ones and ten with third-generation like osimertinib.

Survival and Prognosis

We followed up 81 patients and obtained their prognostic survival. Among them, 57 patients (70.4%) experienced disease progression, whereas 24 patients (29.6%) were lost to follow-up or did not yet progress. By the last follow-up, 31 patients (38.3%) had died and the remaining 50 patients (61.7%) had not yet reached the endpoint of death.

We calculated the overall survival on January 31, 2021 as the follow-up endpoint, and plotted progression free survival and overall survival curves, as shown in **Figure 2**. The median progression-free survival of all patients was 13.87 months (95% CI: 11.66–16.08) as well as the median overall survival was 41.47 months (95%CI: 20.11–62.83). The survival rates for 5 years were 91.0, 80.3, 56.1, 45.5 and 32.5%, respectively.

At the follow-up end point, we finally found that 23 patients (28.4%) had tumor progression and metastasis development to stage IV, with eight patients presenting with brain metastases, seven patients with bone metastases, five patients with pleural metastases or malignant pleural fluid, five patients with both lungs, one patient with liver, and one patient with abdominal cavity.

Influencing Factors of Survival

In this study, we included stage III unresectable NSCLC patients from two centers and obtained basic information and survival prognosis of these patients. Although patients treated with first-

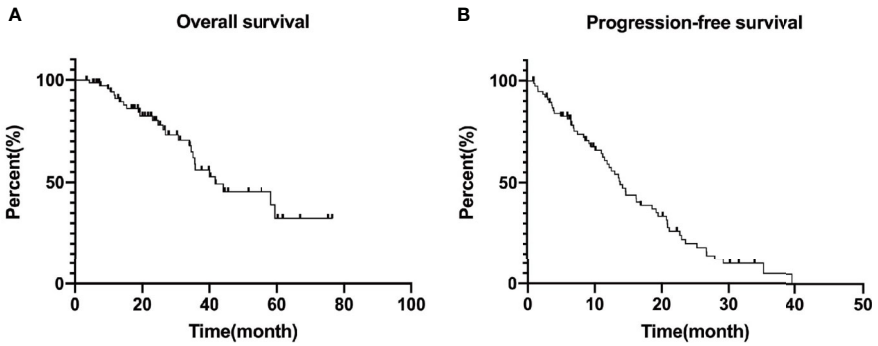

FIGURE 2 | Overall survival **(A)** and median progression free survival **(B)** curves of included patients. Progression free survival: 13.87 (95%CI: 11.66–16.08) months and Overall survival: 41.47 (95%CI: 20.11–62.83) months. The survival rates for 5 years are 91.0, 80.3, 56.1, 45.5, and 32.5%, respectively.

line TKI or TKI combination therapy achieved superior overall survival and outcomes in this study, some patients rapidly progressed in first-line therapy, thus affecting overall survival.

Previous studies have found that age, gender, TMN stage, smoking, surgery after first-line treatment and so on may have a statistically significant impact on survival. Subsequently, we explored possible factors which may influence survival and prognosis.

To conclude, Kaplan–Meier survival curve illustrated prolonged tendency in female patients compared with male patients in both OS and PFS, and statistical difference was found in PFS: 16.30 (11.35–21.25) *vs* 12.57 (8.90–16.24), P = 0.028. However, there is no statistical difference in OS: 59.60 (95% CI: 22.68–96.52) *vs* 35.40 (95% CI: 33.46–37.35, P = 0.065.

For elderly patients, the overall survival was 34.90 (11.68–58.12) months, and as for middle-aged patients, it was 43.87 (23.30–64.44) months, p = 0.327. Similar outcome was found in PFS: 16.27 (12.62–19.92) *vs* 12.57 (9.68–15.46) months, p = 0.220. Although P values were over 0.05, youth still probably influences prognosis.

Similarly, there is a trend of longer survival among non-smokers than that among smokers or ex-smokers. Median PFS was 11.07 (0.64–21.50) months for smokers, 16.97 (10.57–23.36) months for non-smokers, 12.23 (3.40–21.05) months for ex-smokers (p = 0.07), and median OS was 35.40 months for smokers, 58.33 (30.95–85.72) months for non-smokers; 34.90 (17.02–52.79) months for ex-smokers, p = 0.558.

In our study, 69 patients (85.2%) had EGFR mutations, and we explored the difference in survival prognosis of 28 patients (34.6%) with classic 19 deletion, compared with 17 patients (21%) with 21 mutations. The results showed that the PFS of the patients with 19 deletions was 16.27 (11.61–20.93) months, 11.93 (9.07–14.79) months in 21 mutations ones and 12.57 (5.61–19.53) months in other types of patients, p = 0.803. Overall survival for patients with 19 deletions was 58.33 (37.21–79.45) months, 21 mutations: 34.37 (12.49–56.25) months, p = 0.126. Patients with 19 deletions numerically survived longer and showed better outcome than patients with 21 mutations or other types.

In the Kaplan–Meier analysis, we found that gender and smoking might be prognostic factors for PFS and OS, so we conducted Cox regression analysis to further explore. We included gender, age, TMN stage, genetic subtype, smoking, first-line treatment, surgery, and also complications in the COX regression model, and calculated and analyzed hazard ratios and their 95% confidence intervals.

According to the results of the regression analysis, unfortunately, no influence was exerted on OS and PFS by all the factors: gender (p = 0.352/0.123, HR:0.682/0.354), age (p = 0.231/0.201, HR:1.495/2.130), TMN stage (p = 0.715/0.261), genotype: (p = 0.782/0.130), whether smoking (p = 0.462/0.507), first-line treatment (p = 0.923/0.646), whether surgery was performed (p = 0.967/0.977, HR:0.977/0.949), and complication (p = 0.112/0.212, HR:1.706/0.422).

To conclude, although we intended to find the TKI benefit population to guide the subsequent clinical choice, in our study, no independent factors were proven to have a prognostic impact statistically. Specific P values are listed in **Table 2**, and survival curves are plotted in **Figures 3** and **4**.

Prognosis of Operated Patients

In 81 patients of this study, after first-line TKI therapy, seven (8.6%) patients were reevaluated as eligible for surgery and proceeded to surgery who were three males and four females. By the end of the follow-up, two patients had reached the end point of follow-up and five patients were alive. The second patient had a survival of 34.37 months. Five patients had already progressed postoperatively, including abdominal cavity, brain, multiple bone metastases and recurrence in the postoperative stump, and the progression time was 29.20, 13.73, 7.5, 6.60, and 12.23 months successively. One patient was followed up for almost three years with no evidence of progression or metastases. The specific information of the seven patients is shown in **Table 3**.

Limited by the sample size and follow-up time, there was no statistically significant difference in overall survival between surgical and nonsurgical patients in this study. When considering TKIs as first-line therapy for patients with

TABLE 2 | The association between overall survival and progression free survival was calculated by Cox regression models.

Factors	P1 value	HR	95% CI	P2 value	HR	95% CI
Gender	0.352	0.682	0.305–1.525	0.123	0.354	0.095–1.323
Age	0.231	1.495	0.775–2.884	0.201	2.130	0.669–6.783
Stage	0.715			0.216		
Genotype	0.782			0.130		
19deletion	0.829	0.921	0.436–1.946	0.811	0.826	0.171–3.963
21mutation	0.655	1.225	0.504–2.977	0.170	2.991	14.304
Smoke	0.462			0.507		
Smoker	0.242	0.526	0.180–1.543	0.261	0.354	0.058–2.164
Ex-smoker	0.312	0.511	0.139–1.876	0.667	0.642	0.085–4.83
Firstline	0.923			0.646		
Surgery	0.967	0.977	0.334–2.856	0.963	0.949	0.105–8.580
Complication	0.112	1.706	0.882–3.299	0.212	0.422	0.109–1.636

P1 value, P value of overall survival; HR, hazard ratio; 95% CI, 95% confidence intervals; P2 value, P value of progression free survival. P values <0.05 were considered statistically significant, with 95% confidence intervals.

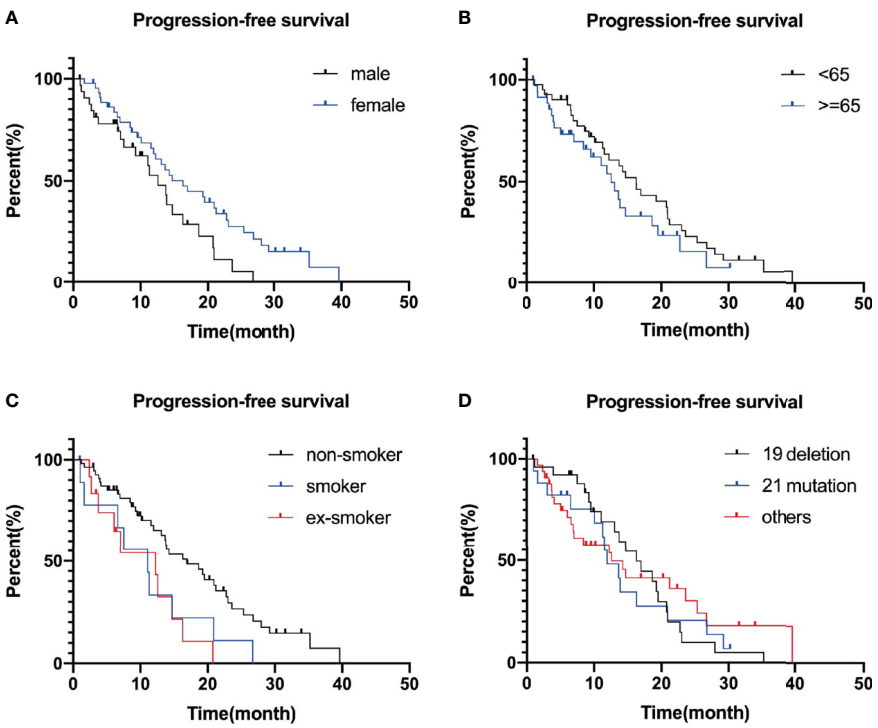

FIGURE 3 | Survival curves plotted separately by gender **(A)**, age **(B)**, smoking **(C)**, and genotype group **(D)**. P-values are 0.124, 0.168, 0.929, and 0.094, successively.

advanced NSCLC, the timing and effectiveness of surgery remain unclear.

Complications

For the 81 patients included, 25 (30.9%) developed complications in the first-line treatment. The major complications that occurred were hepatotoxicity and rash. Nine of these patients were detected abnormal liver enzyme indices, eight with TKI monotherapy and one with TKI combination chemotherapy. Three of them were finally forced to withdraw from first-line treatment due to continuing liver damage even with hepatoprotective treatment.

The second most serious complication was rash, which was observed in seven patients during follow-up. There were two cases of oral ulcer, one of gingival bleeding and one of thrombocytopenia and one of leukocytopenia. After apatinib administration, one patient had hypogeusia and hoarseness. Notably, among the six patients with TKI combined radiotherapy, two (33.3%) of them had inflammatory changes on chest X-rays, which were considered as radiation pneumonitis.

Due to the limited sample size, we failed to analyze and conclude the occurrence of complications may associated with the combination of chemotherapy, radiotherapy or immunotherapy.

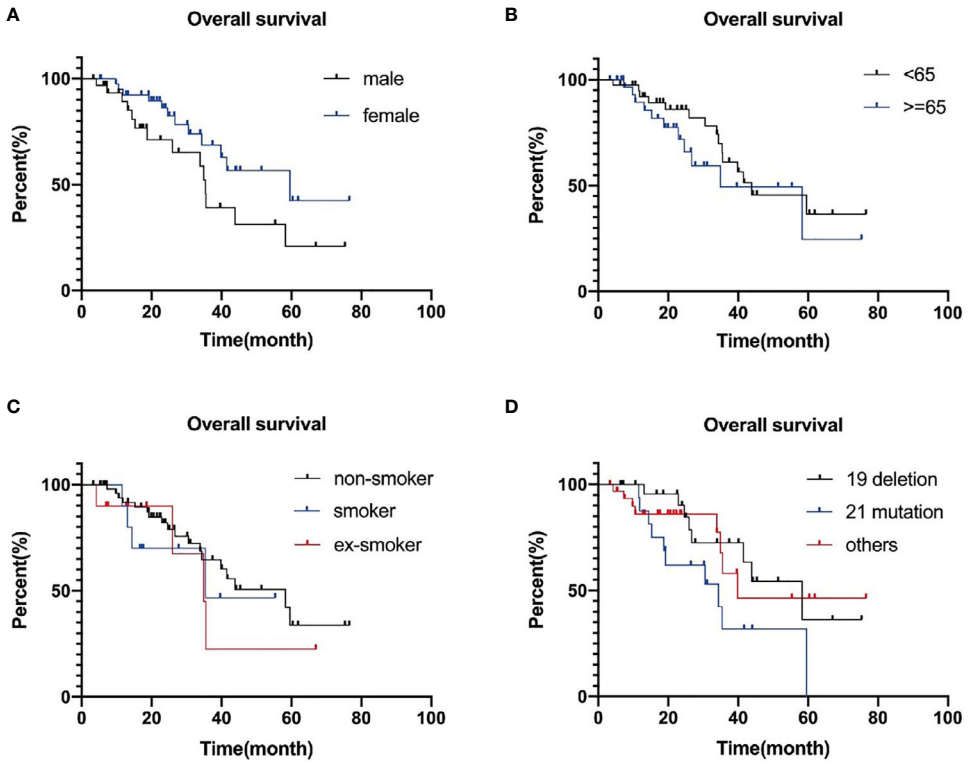

FIGURE 4 | Progression-free survival curves plotted separately by gender **(A)**, age **(B)**, smoking **(C)**, and genotype group **(D)**. P-values are 0.927, 0.299, 0.126, and 0.923, successively.

TABLE 3 | Prognosis of seven operated patients.

N	Gender	Age	Stage	TMN staging	Stage after surgery	TMN Staging after surgery	Gene	First-line treatment	metastasis	Living	PFS	PFS status	OS	OS status
1	Female	50	IIIA	T1bN2M0	IIB	T1aN2M0	EGFR	Icotinib	Unknown	Dead	8.67	0	/	1
2	Female	63	IIIB	T4N2M0	IIIB	T4N2M0	EGFR	Gefitinib	Abdominal	Dead	29.20	1	34.37	1
3	Male	46	IIIB	T3N2M0	IIIA	T1cN2M0	EGFR	Gefitinib + AP	Brain	Living	13.73	1	22.57	0
4	Male	36	IIIB	T1cN3M0	/	/	EGFR	Gefitinib	Progress	Living	7.50	1	16.67	0
5	Male	56	IIIB	T4N2M0	IIIA	T1bN2M0	EGFR&HER2	Gefitinib + AP	Bone	Living	6.60	1	17.60	0
6	Female	63	IIIA	T1N2M0	/	/	EGFR	Gefitinib	No metastasis	Living	/	0	44.23	0
7	Female	54	IIIA	T2N2M0	/	/	EGFR	Gefitinib	Recurrence	Living	12.23	1	12.77	0

AP, Pemetrexed + cisplatin; PFS, progression-free survival, definite from the start of first-line therapy to disease progression; OS, overall survival, definite from pathological diagnosis to patient death.

DISCUSSION

This study was a multicenter, real-world retrospective study involving stage III unresectable NSCLC patients carrying mutations in EGFR/ALK/ROS genes, recruited from the Jinling Hospital and the Jiangsu Cancer Hospital in Nanjing, Jiangsu Province. Our aim was to explore the prognosis of such patients after first-line applying first/second-generation TKIs or their combined therapy like chemotherapy, immunotherapy or radiotherapy, then explore which patients could clinically benefit most.

The treatment for unresectable stage III/IV advanced NSCLC is the focus of many investigators. Currently, stage III unresectable NSCLC are treated with concurrent chemoradiotherapy (CCRT) as the standard therapy (14). The investigators explored a combination of chemoradiotherapy that involved pembrolizumab, cetuximab and increased radiation dose. However, no other treatment is superior except durvalumab.

In 2018, Durm et al. (15) reported the results of a phase II study using pembrolizumab after CRT in 93 unresectable stage III NSCLC patients. The median OS was 22.4 months, the median PFS was 17 months, and the 2-year survival rate was 61.9%. Prolonging pembrolizumab treatment may be associated with prolonged PFS and OS, but unfortunately, not all patients benefit from consolidation immunotherapy (10). Studies also found that, combining with anti-EGFR antibody cetuximab did

not improve overall survival (12). When the radiotherapy dose was increased from the standard dose of 60 to 74 Gy, the median overall survival was 28.7 months and the 2-year survival rate was 58%, which was better than expected. But this therapy still did not improve overall survival and was potentially harmful. The phase III PROCLAIM study (16) also demonstrated that chemotherapy consolidation following pemetrexed cisplatin or etoposide cisplatin + radiotherapy was similarly not superior to standard chemoradiotherapy. Durvalumab in combination with a PD-L1 inhibitor after concurrent chemoradiotherapy significantly increase ORR and prolonged PFS and OS (HR: 0.68; P = 0.00251) (17–19). Other studies also demonstrated that, durvalumab was safe in patients and even similar to placebo in unresectable stage III NSCLC (20, 21).

Meanwhile, TKIs such as gefitinib and afatinib are also the standard first-line treatment options for advanced NSCLC patients with driver-gene mutations (22, 23). However, the clinical benefit of first-line administration of TKI therapy in such patients remains uncertain.

A retrospective study found that 56.6% of patients chose targeted agents as first-line therapy (4). Despite the average of acquired resistance after 9 to 14 months of EGFR-TKI therapy (24), previous studies have found that the application of targeted agents is still associated with a favorable survival benefit (25).

With the further development of targeted agents research, the emergence of second-generation and third-generation TKIs provided more options for patients and urged for clinical validation. Another real-world retrospective study (26) in 2020 analyzed 620 stage III/IV NSCLC patients with EGFR mutated. All patients had a PFS of 11.6 months and a median OS of 19.4 (17.5–21.7) months. The median PFS and 1 year survival rate for the three groups were: gefitinib 10.3 months, 69.1%; erlotinib 12.1 months, 71.6%; and afatinib 16.4 months, 78.2%. The median OS was 20.4 (17.5–27.8) months in the erlotinib arm and 17.5 (15.2–20.3) months in the gefitinib arm. Although the OS of afatinib arm was not reached in the study, it could still be concluded that afatinib had an advantage in prolonging PFS in patients. In LUX-Lung 7 (27), there was no significant difference in OS between afatinib and gefitinib, which was similar to the conclusion of our study. In our study, gefitinib was administered to 62.9% of patients. We explored the relationship between survival in patients treated with first or second generation TKIs, but found no statistical difference of PFS or OS (p = 0.903/0.799).

As for second-line TKIs, five patients were applied to afatinib, while no patient in the study received dacomitinib. Dacomitinib, a second-generation EGFR-TKI/HER2-TKI, was already approved in the United States, Japan and the European Commission for first-line monotherapy treatment in patients with EGFR mutation-positive inoperable or recurrent NSCLC, which may be a powerful new treatment option compared with gefitinib (28, 29). As a rare gene mutation, HER2 mutation was found in two patients included in our study, but both of them chose afatinib as first-line therapy. Although no patient received third-generation TKI monotherapy for first-line treatment in this study, the use of third-generation TKIs was clinically increasing. Unfortunately, a phase III clinical trial of a third-generation novel agent ASP8273 versus erlotinib or gefitinib in patients with advanced stage IIIB/IV NSCLC had to be discontinued due to excessive toxic side effects (30).

Meanwhile, other studies also found that in advanced NSCLC, different EGFR mutation types had different response rates to second-generation EGFR TKI like afatinib, in particular del 19 (31). But no difference of OS in first-line generation TKI was found between del 19 and L858R mutation (3) in this study, neither was found in our study.

There was no statistically significant difference in PFS or OS between del 19 and 21 mutation (p = 0.782/0.130).

A retrospective study compared the efficacy of TKIs monotherapy with CCRT in patients with stage IIIB lung adenocarcinoma with EFGR mutation. Although the 5-year OS rates in the TKI group compare with the CCRT group increased at a numerical level (30 and 26%), there was no statistically difference between the two groups (32). Considering that our study was just a single-arm, retrospective study and lacked a feasible chemotherapy control arm, we may not further conclude a survival advantage of EGFR TKIs monotherapy or combination therapy over CCRT. Since the poor response to monotherapy, many researchers turned to explore the possibility of combining TKI with other treatments. Whether the combination of chemotherapy and radiotherapy obtain more clinical benefits than single drug is a concern of many researchers. A multicenter phase II clinical trial compared the median OS of concurrent radiotherapy plus erlotinib with chemoradiotherapy. It was found that erlotinib group improved PFS, while patient tolerance of both groups was similar. Along with radiotherapy, TKI therapy showed better clinical benefits than chemotherapy in prolonging PFS. As for radiotherapy, radiation pneumonitis is one of the most serious complications affecting patient survival. Previous studies found that the incidence of radiation pneumonitis in patients with radiotherapy combined with first-generation TKI is as high as 40%, and the incidence of those over grade 3 is 20% (33). Among the six patients who were treated with radiotherapy along with TKI in our study, two patients showed inflammatory reactions on radiologic level, but none of them were severe but mild. RECEL study (34) found that erlotinib combined with thoracic radiotherapy significantly improved the median PFS compared with chemoradiotherapy, which suggested the value of EGFR-TKI with concurrent radiotherapy. In another retrospective study (35), among 45 unresectable stage III NSCLC patients receiving radiotherapy with EGFR-TKI, 17 patients (37.7%) suffered radiation pneumonitis, but achieved satisfactory PFS and OS: 27.9 (95% CI: 18.7–37.2) and 49.7 (95% CI: 27.7–71.8) months. A new phase II clinical trial (NCT0463659) is exploring the safety and efficacy of almonertinib and concurrent thoracic radiotherapy in patients with unresectable stage III EGFR-mutated NSCLC (36). If the patient is well tolerated, a combination of targeted drugs and radiotherapy may be chosen as first-line treatment. Further study may provide more objective basis for targeted therapy combined with radiotherapy in the treatment of advanced lung cancer.

Smoking status is a significant predictor of response and survival after underdoing EGFR TKIs treatment (37, 38). Previous studies have found that cigarette smoking dosage over 30 pack-years was an independent negative predictive factor and meanwhile smoking cessation combined with anti-EGFR treatment like erlotinib seems to be more effective in lung adenocarcinoma with EGFR mutation (39, 40). In two randomized trials comparing gefitinib (41) or erlotinib with placebo, non-smokers had a significant survival benefit. Our study showed similar outcomes to previous researches (42) with a prolongation of median non-smoking PFS with OS compared to those who smoked or quit, although they were not statistically different (p = 0.462/0.507), indicating that TKI therapy had better clinical benefits for non-smokers with positive driver gene. We also observed the prolongation of progression free survival and overall survival in female and middle-aged patients, but there was no statistical difference in the end.

Surgery has been widely used as radical treatment for tumor patients of resectable diseases. Preoperative and postoperative adjuvant chemotherapy seemed to have significant survival benefit (43, 44). Among stage III lung cancer patients, only some of them have access to surgery. Previous studies such as Yamamoto's (45) and Mazzoni's (46), reported that some patients regain the opportunity to undergo surgery after first-line treatment and finally excised. A multicenter study (47) showed that 14.8% of stage III patients underwent surgery. In previous studies, there was no significant difference in PFS between the surgery and non-surgery groups (HR = 0.91, 95% CI: 0.73–1.13) (48). In our study, seven (8.6%) patients ultimately underwent surgery. However, limited by the sample size, there was no benefit in PFS or OS between surgery patients and others (p = 0.967/0.977).

This study was a two-center retrospective study with a limited number of included patients, large interpatient heterogeneity and a proportion of patients lost to follow-up. Our study supported that the application of TKIs in stage III unresectable NSCLC patients with positive driver gene may achieve a good clinical benefit and is a considerable option. For patients who cannot bear chemoradiotherapy, especially those who have never smoked, if EGFR/ALK mutations occur, anti-EGFR/ALK therapy is considered as first-line treatment. However, further larger studies are still needed to validate this conclusion and explore the optimal treatment regimen for stage III unresectable mutant patients.

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 Ethical Review Committee of the Affiliated Jinling Hospital (DBNJ20219). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

(I) Conception and design: RW. (II) Administrative support: None. (III) Provision of study materials or patients: SY and JY. (IV) Collection and assembly of data: RW. (V) Data analysis and interpretation: None. All authors contributed to the article and approved the submitted version.

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Peripheral Blood Biomarkers for Early Diagnosis, Severity, and Prognosis of Checkpoint Inhibitor-Related Pneumonitis in Patients With Lung Cancer

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Background: Checkpoint inhibitor-related pneumonitis (CIP) is a potentially fatal immune-related adverse event that occurs during treatment with immune checkpoint inhibitors (ICIs). However, the roles played by peripheral blood parameters in CIP development remain unclear. Here, we aimed to identify which blood biomarkers correlated with the development and prognosis of CIP in patients with lung cancer.

Methods: We conducted a retrospective analysis of 87 patients with CIP (CIP group) and 87 patients without CIP (control group). Cytokines, blood routine, lactate dehydrogenase (LDH) and albumin (ALB) were collected at baseline (before ICIs), at onset of pneumonitis (in the CIP group), and before the last dose of ICI (in the control group). We compared the baseline values and changes over time in various blood parameters between the CIP and control groups. The CIP outcomes were collected and compared according to the median values of these parameters.

Results: Squamous carcinoma (odds ratio [OR]: 3.02; $p = 0.004$) and ICI monotherapy (OR: 6.56; $p = 0.004$) correlated with a high risk of CIP. In the CIP group, interleukin (IL)-6 and platelet-to-lymphocyte ratio (PLR) at CIP were significantly increased relative to baseline. By contrast, IL-6 and PLR reduced over time in the control group. Significant decrease in absolute lymphocyte count (ALC) and increases in IL-10, neutrophil to lymphocyte ratio (NLR), and LDH levels were observed from baseline to CIP. No significant change in these parameters was observed in the control group relative to baseline. ALB decreased in both groups, but the decrease in the CIP group was greater (9.21% *vs*. 2.44%; $p = 0.020$). High IL-6 levels (OR: 5.23, 95% confidence interval [CI]: 1.15–23.86; $p = 0.033$), and low levels of ALB (OR: 0.16, 95% CI: 0.04–0.64; $p = 0.009$) measured at the time of CIP symptom onset were associated with severe pneumonitis. Low concentration of IL-6 (hazard ratio [HR]: 0.17, 95% CI: 0.03–0.95; $p = 0.044$) and

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high ALB levels (HR: 0.28, 95% CI: 0.08–0.94; $p = 0.040$) were correlated with favorable overall survival in CIP.

Conclusions: Increase in IL-6, IL-10, NLR, PLR, and LDH levels or reduced ALC and ALB levels were associated with the occurrence of CIP in lung cancer patients. High IL-6 and low ALB levels at onset of CIP were related to severe grade and poor prognosis of CIP.

Keywords: checkpoint inhibitor-related pneumonitis, immune checkpoint inhibitor, interleukin-6, lymphocyte, albumin, lung cancer

INTRODUCTION

Immune checkpoint inhibitors (ICIs) provide enhanced survival benefits to patients with malignant tumors, including lung cancer (1, 2); however, ICIs sometimes cause a series of unique adverse events, known as immune-related adverse events (irAEs) (3). A review of 20 randomized controlled studies suggested that the incidence of fatal irAEs associated with programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) inhibitors was 0.43%, among which checkpoint inhibitor-related pneumonitis (CIP) was the most common (4). A meta-analysis showed that lung cancer was more likely than other cancers to result in all-grade or high-grade CIP (5). CIP lacks typical clinical symptoms, and 1/3 of patients with CIP are asymptomatic at the time of onset (6). The delayed treatment of CIP patients may lead to disease aggravation. The overall survival (OS) of patients with CIP who do not recover or whose symptoms worsen is significantly shortened compared with those who recovered from CIP (7). Therefore, determining the risk factors associated with CIP and early CIP identification is crucial. Previous studies showed that age, smoking status, pre-existing lung diseases, and chest radiotherapy history might be related to CIP occurrence (8–10). However, the sample sizes of CIP patients in these studies are small, and whether other risk factors may exist is also worthy of further study.

Blood-based biomarkers have the advantages of minimally invasive, easy to collect, and reproducible. Studies have shown that C-reactive protein (CRP), interleukin (IL)-6, blood cell counts, and cytokine levels are associated with irAEs (11). Recent data suggest that the neutrophil to lymphocyte ratio (NLR) may be related to irAE onset, severity, and subsequent prognosis (12). Similarly, increased NLR values may contribute to the diagnosis of ICI-associated myocarditis (13). A recent study indicated that elevated IL-6, IL-10, and eosinophil levels might be indicators of skin-related irAEs (14). However, a few reports have examined the association between peripheral blood biomarkers and CIP occurrence. Previous reports have shown that an increased anti-CD74 autoantibody was correlated with CIP occurrence (15). However, these biomarkers are not included in routine clinical tests, and their determination requires special equipment.

Previous studies have suggested that the OS of patients with irAEs was significantly longer than that of patients without irAEs. However, in the subgroup analysis, CIP was not significantly associated with ICI efficacy (16, 17). Conversely, a study by Fukihara et al. suggested that OS was significantly shorter among patients with CIP than among those without CIP (18). Another study showed that grades 1–2 CIP was associated with favorable OS, whereas grades 3–4 CIP was not (19). The survival time for CIP patients varies greatly. Therefore, determining whether peripheral blood markers can be used to predict OS in patients with CIP remains necessary.

This study was designed to identify the potential risk factors in baseline clinical characteristics associated with the occurrence of CIP and to investigate the association between clinically accessible biomarkers in peripheral blood and the development or prognosis of CIP.

MATERIALS AND METHODS

Patients

This retrospective, observational study was conducted at the First Affiliated Hospital of Guangzhou Medical University. Records for patients with unresectable stage III or IV primary lung cancer [according to the 2015 World Health Organization Classification of Lung Tumors (20)] treated with at least one dose of ICI between January 2016 and January 2021 were reviewed. Patients who developed CIP (CIP group) and randomly selected corresponding patients without CIP (control group) were included at a ratio of 1:1. Prior tuberculosis and bacterial and fungal infections in the lungs before immunotherapy were excluded. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the local Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University.

Diagnosis of CIP

CIP was diagnosed by two experienced pulmonologists and one chest radiologist, based on the guidelines of the National Comprehensive Cancer Network, the American Society for Clinical Oncology, and the European Society for Medical Oncology (21–23). We defined CIP as new-onset infiltrates on thoracic imaging and/or clinical symptoms of cough, shortness of breath, or wheezing that is likely to be caused by ICIs, and excluded other etiologies. For patients considering the diagnosis of CIP, several examinations were performed in order to exclude other lung diseases (e.g. pulmonary infections and tumor progression), such as bronchoalveolar lavage culture, sputum cultures and laboratory tests (routine blood test, procalcitonin,

tumor markers, arterial gas analysis, serous D-dimer and brain natriuretic peptide, etcetera). In addition, when patients with pulmonary infection had been a poor response to anti-infection treatment, CIP with pulmonary infection may be diagnosed. We compared pneumonitis extent and previous radiation field to exclude radiation-induced pneumonitis. If the diagnosis was not clear and the patient's physical condition allowed, a lung biopsy would be performed.

Data Collection and Outcome Assessment

The following information was retrospectively collected from each patient's medical records: patient demographics, pre-existing lung disease, tumor histology, Eastern Cooperative Oncology Group Performance Status (ECOG PS), radiation therapy administrations, treatment data, and driver gene status. The ECOG PS was evaluated prior to ICI treatment. Driver gene status was tested before any anti-tumor treatments were applied. In the CIP group, we also collected the time course of CIP, maximum CIP grade, and CIP outcomes. The severity of CIP was graded according to the Common Toxicity Criteria for Adverse Events (CTCAE version 4.0). In the CIP group, OS was calculated from the date of CIP diagnosis until death or the last follow-up date (April 1, 2021).

Among patients with CIP, we collected peripheral blood parameters at two time points: baseline (prior to ICI treatment), and at the time of CIP diagnosis. In the control group, we recorded these parameters at two time points: baseline before starting ICI treatment and before the last dose of ICI. Peripheral blood parameters included IL-2, IL-4, IL-6, IL-10, interferon-gamma (IFN-γ), tumor necrosis factor-α (TNF-α), absolute neutrophil count (ANC), absolute lymphocyte count (ALC), absolute eosinophil count (AEC), platelet count (PLT), lactate dehydrogenase (LDH), and albumin (ALB). The NLR was calculated as ANC divided by ALC. The platelet-to-lymphocyte ratio (PLR) was calculated by dividing PLT by ALC.

Statistical Analysis

Continuous variables were summarized as the median and interquartile range (IQR). Categorical data were summarized as the frequency (percentage). Differences in continuous variables at baseline were assessed using either an independent-samples t-test or the Mann–Whitney U test. Chi-square (χ^2) or Fisher's exact test was used to analyze categorical variables.

Logistic univariate analysis was used to determine which factors were associated with CIP. Multivariate logistic regression analysis was used to analyze those variables with p-value <0.1 in the univariate analysis to determine potential CIP risk factors. Changes in peripheral blood parameters over time were evaluated using a paired t-test or the Wilcoxon signed-rank test. The calculation of percentage change was performed as follows: (difference from baseline/baseline value) × 100. The Mann–Whitney U test was used to compare changes in blood parameters between the CIP and control groups. For those blood parameters with significant changes over time, the median value at the time of CIP diagnosis was used to perform logistic univariate and multivariate analyses to identify potential biomarkers associated with severe-grade CIP in the CIP group.

Finally, the Kaplan–Meier method was used to evaluate OS, with 95% confidence intervals (CIs), and the log-rank test was used to determine the significance of differences between two or more subgroups in CIP patients. A Cox proportional hazards model was used to identify prognostic factors associated with OS in the CIP group using multivariable survival analysis, including those variables with p-values <0.01 in the univariate analysis. Univariate and multivariate hazard ratios (HRs), with 95% CI values, were calculated.

Statistical analyses were conducted using IBM SPSS Statistics (Armonk, NY), version 25. All p-values were based on the two-sided hypothesis test, and a p-value <0.05 was considered significant.

RESULTS

Participants

A total of 848 patients with advanced lung cancer who were treated with ICIs at our institution were deemed eligible for potential study inclusion. Finally, 87 patients (10.3%) who developed CIP (CIP group) and 87 randomly selected patients without CIP (control group) were included in the analysis (**Figure 1**). All patients were treated with PD-1 or PD-L1 inhibitors, with PD-1 inhibitors being more commonly used. The demographic characteristics were similar between the CIP and control groups (**Table 1**). However, the distributions of tumor types and treatment data among cases and controls were significantly different. Squamous cell carcinoma was the most common histologic type among the CIP group (42.5%), whereas adenocarcinoma (62.1%) was the most common type in the control group. In addition, combination therapy (including ICI + chemotherapy and ICI + chemotherapy + antiangiogenic drugs) was the predominant treatment type for both groups, but ICI monotherapy comprised a larger proportion (25.3%) of treatment types in the CIP group (p <0.001). Compared with the control group, the CIP group had a higher frequency of prior radiation (10.3% *vs.* 20.7%; p = 0.006).

Among the 87 patients with CIP, the median age was 65 years (range: 18–85 years), and 83.9% were men. The median time from the initial administration of ICIs to the development of CIP was 3.8 months (range: 0.2–20.7 months). Among the CIP patients, 38 patients (43.7%) had severe (grades 3–5) CIP. The baseline TNF-α level of patients with CIP tended to be lower than that among those without CIP, but no significant difference was observed (p = 0.06; **Supplementary Table 1**).

In the univariate and multivariate analysis (**Table 2**), squamous carcinoma (odds ratio [OR]: 3.02, 95% CI: 1.41–6.43; p = 0.004) and ICI monotherapy (OR: 6.56, 95% CI: 1.79–23.98; p = 0.004) correlated independently and significantly with the occurrence of CIP.

Correlation of Biomarkers With CIP

IL-6 increased significantly from baseline to CIP [7.62 pg/ml (IQR: 5.42–17.46) to 11.81 pg/ml (IQR: 5.10–63.34); p = 0.001] in the CIP group. By contrast, a significant decrease in IL-6 levels was observed over time [6.66 pg/ml (IQR: 4.24–19.38) to 6.45 pg/ml

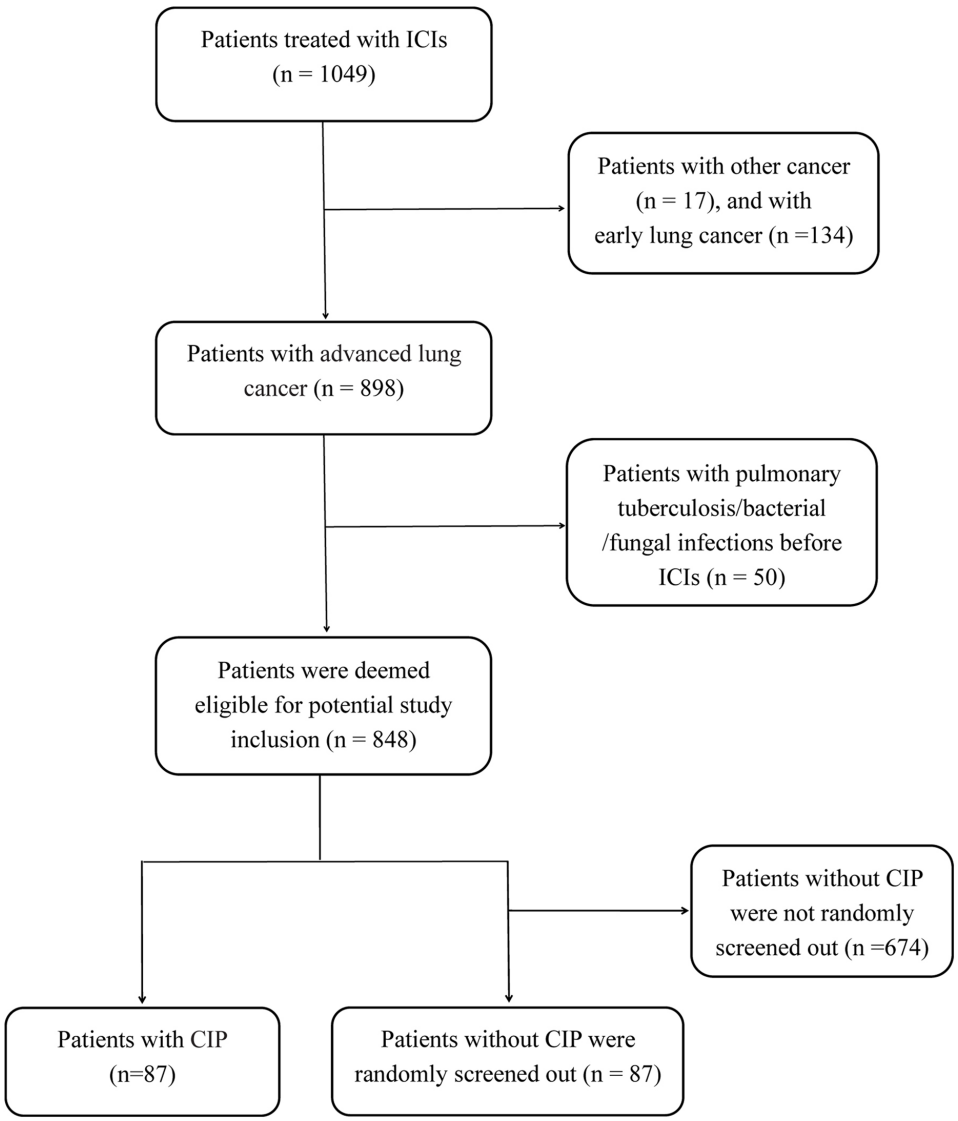

FIGURE 1 | The flow chart of study design and patients inclusion. ICIs, immune checkpoint inhibitors; CIP, checkpoint inhibitor-related pneumonitis.

(IQR: 3.92–12.79); p = 0.030] in the control group (**Figure 2A**). Similarly, the median levels of IL-10 at baseline and CIP were 2.41 and 3.79 pg/ml (p = 0.025), respectively in the CIP group, and no change in the IL-10 over time was observed among controls (p = 0.94; **Figure 2B**).

In the CIP group, ALC decreased significantly from baseline to CIP presentation [1.50 K/μl (IQR: 1.00–2.08) to 1.15 K/μl (IQR: 0.63–1.50); p <0.001]. However, ALC did not change over time in the control group [1.50 K/μl (IQR: 1.20–2.10) to 1.60 K/μl (IQR: 1.20–2.00); p = 0.52] (**Figure 2C**). Among CIP cases, a significant increase in NLR was observed from baseline to CIP presentation [3.58 (IQR: 2.44–6.79) to 5.38 (IQR: 3.07–10.32); p = 0.001]. However, no change in NLR over time was observed in the control group [2.82 (IQR: 1.97–4.58) to 2.31 (IQR: 1.55–4.29); p = 0.11] (**Figure 2D**). Similarly, an increase in the PLR was observed from baseline to CIP development [179.70 (IQR: 123.09–331.75) to 263.76 (IQR: 152.65–432.77); p = 0.008]. By contrast, the PLR decreased significantly from baseline to before the last ICI dose [161.11 (IQR: 121.05–231.58); p = 0.042] in the control group.

LDH of patients with CIP increased significantly from baseline to CIP [223.80 U/L (IQR, 177.03–398.93) to 257.85 U/L (IQR, 189.03–311.83); p = 0.049]. Nevertheless, there was no change in the LDH over time among patients without CIP (p = 0.37; **Figure 2E**). There was a significant decrease in the ALB from baseline to CIP [35.85 g/L (IQR, 33.45–39.25) to 33.80 g/L (IQR, 30.00–36.45); p <0.001]. Median ALB concentration was also comparable over time (36.95 *vs*. 36.70 g/L; p = 0.038) at baseline

TABLE 1 | Baseline characteristics in advanced lung cancer patients treated with ICIs.

Variables	CIP group (n = 87)	Control group (n = 87)	P-value
Age			
Median (range)	65 (18–85)	62 (31–83)	0.20
<65, n (%)	42 (48.3)	52 (59.8)	0.17
≥65, n (%)	45 (51.7)	35 (40.2)	
Gender, n (%)			0.26
Male	73 (83.9)	66 (75.9)	
Female	14 (16.1)	21 (24.1)	
Smoking status, n (%)			0.76
Current/former	45 (51.7)	42 (48.3)	
Never	42 (48.3)	45 (51.7)	
Preexisting lung diseases, n (%)	15 (17.2)	14 (16.1))	1
Histologic type, n (%)			**<0.001**
Squamous	37 (42.5)	13 (14.9)	
Adenocarcinoma	22 (25.3)	54 (62.1)	
NOS	3 (3.4)	3 (3.4)	
SCLC	10 (11.5)	9 (10.3)	
Others	15 (17.2)	8 (9.2)	
ECOG PS			0.08
0–1	82 (94.3)	74 (85.1)	
≥2	5 (5.7)	13 (14.9)	
Prior radiation, n (%)	18 (20.7)	9 (10.3)	0.06
EGFR/ALK mutation (initial biopsy/pre-TKI)	6 (6.9)	3 (3.4)	0.28
Treatment line, n (%)			
1	62 (71.3)	70 (80.5)	0.11
≥2	25 (28.7)	17 (19.5)	
ICI type, n (%)			1
PD-1	82 (94.3)	82 (94.3)	
PD-L1	5 (5.7)	5 (5.7)	
Treatment data, n (%)			**<0.001**
Monotherapy	22 (25.3)	3 (3.4)	
Combination therapy	65 (74.7)	84 (96.6)	

Bold values indicate p < 0.05; NOS, not otherwise specified; SCLC, small cell lung cancer; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor gene; ALK, anaplastic lymphoma kinase; TKI, tyrosine kinase inhibitors; ICI, immune checkpoint inhibitors; PD-1, programmed cell death protein-1; PD-L1, programmed death ligand-1; CIP, checkpoint inhibitor-related pneumonitis.

among cases. However, the decrease of ALB was higher in the CIP group than in the control group (9.21% *vs*. 2.44%; p = 0.020) (**Figure 2F**).

In the CIP group, IL-2, IL-4, IFN-γ, TNF-α, ANC, AEC, and PLT had no significant changes from baseline to presentation with CIP (**Supplementary Table 2**). No matter in the experimental group or the control group, in the subgroup analysis, changes in IL-6, IL-10, ALC, NLR, PLR, LDH and ALB over time were not statistically significant between the squamous carcinoma and non-squamous carcinoma groups, or between the combination therapy and monotherapy groups.

Correlation of Biomarkers and Severe CIP

During follow-up, severe CIP occurred in 38 cases (43.7%). In the logistic univariate analysis, high IL-6, NLR, and PLR levels were associated with severe pneumonitis (grade 3 or higher) in the CIP group. By contrast, high concentrations of ALC and ALB were negatively correlated with severe pneumonitis. Multivariate regression analysis showed that high levels of IL-6 (OR: 5.23, 95% CI: 1.15–23.86; p = 0.033) and low levels of ALB (OR: 0.16, 95% CI: 0.04–0.64; p = 0.009) were significantly associated with CIP (**Table 3**).

Correlation of Biomarkers and Overall Survival

Among all patients with CIP, the median OS was 11.1 months (95% CI: 4.4–17.8 months), and the one-year survival rate was 46.5%. We generated a univariate Cox proportional hazards regression model of variables measured at the time of pneumonitis diagnosis. The results showed that CIP grade, and IL-6, ALC, NLR, and ALB levels were significantly correlated with OS (**Table 4** and **Figure 3**). The median OS was significantly different according to treatment line (1st *vs* ≥2nd: 18.6 *vs* 5.5 months; HR: 0.37, 95% CI: 0.18–0.78; p = 0.009), CIP grade (1–2 *vs*. ≥3: 22.1 *vs* 3.7 months; HR: 0.11, 95.0% CI: 0.05–0.27; p <0.001), IL-6 (<11.81 *vs*. ≥11.81: 22.1 *vs* 6.1 months; HR: 0.07, 95.0% CI: 0.02–0.34; p = 0.001) (**Figure 4A**), ALC (≥1.15 *vs*. <1.15: 10.9 *vs*. 5.5 months; HR: 0.42, 95% CI: 0.20–0.92; p = 0.029), NLR (<5.38 *vs*. ≥5.38: 22.1 *vs*. 9.1 months; HR: 0.33, 95.0% CI: 0.15–0.74; p = 0.007), and ALB (≥33.80 *vs*. <33.80: 18.6 *vs*. 8.1 months; HR: 0.32, 95% CI: 0.14–0.73; p = 0.007) (**Figure 4B**).

In the multivariate Cox proportional hazards regression model, only IL-6 (<11.81 *vs*. ≥11.81: HR = 0.17, 95% CI: 0.03–0.95; p = 0.044) and ALB (≥33.80 *vs*. <33.80: HR = 0.28, 95% CI: 0.08–0.94; p = 0.040) were significantly and independently correlated with OS in patients with CIP (**Table 4**).

TABLE 2 | Univariate and multivariate logistic regression analysis for the risk factors of CIP.

Variables	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Age (≥65 *vs*. <65)	1.59 (0.87–2.9)	0.13	–	–
Gender (female *vs*. male)	1.67 (0.78–3.5)	0.19	–	–
Smoking (current or former *vs*. never)	1.15 (0.63–2.1)	0.65	–	–
ECOG PS (≥2 *vs*. <2)	0.35 (0.12–1.02)	0.054	0.38 (0.12–1.17)	0.09
Prior radiation	2.26 (0.95–5.36)	0.06	1.95 (0.75–5.02)	0.19
Histology (squamous *vs*. non-squamous)	3.86 (1.89–7.87)	**<0.001**	3.02 (1.41–6.43)	**0.004**
Treatment line (≥2nd *vs*. 1st)	1.66 (0.82–3.36)	0.16	–	–
Treatment (monotherapy *vs*. combination)	9.48 (2.72–33.04)	**<0.001**	6.56 (1.79–23.98)	**0.004**

Bold values indicate p < 0.05; CIP, checkpoint inhibitor-related pneumonitis; ECOG PS, Eastern Cooperative Oncology Group performance status; OR, odds ratio; CI, confidence interval.

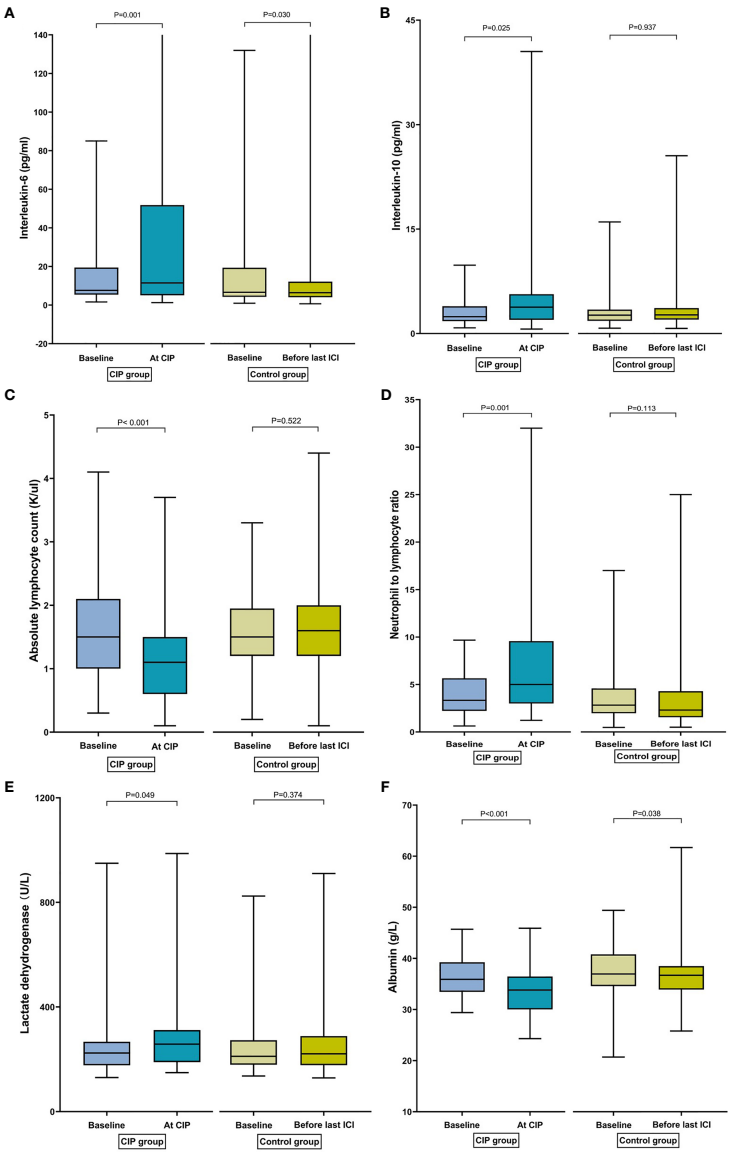

FIGURE 2 | Bar plots of peripheral blood parameters in patients with checkpoint inhibitor-related pneumonitis (CIP) and controls at different time points. **(A)** Interleukin-6. **(B)** Interleukin-10. **(C)** Absolute lymphocyte count. **(D)** Neutrophil-to-lymphocyte ratio. **(E)** Lactate dehydrogenase. **(F)** Albumin.

TABLE 3 | Univariate and multivariate logistic regression analysis for the risk factors of grades 3–4 CIP in the CIP group.

Variables	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	P-value	OR (95% CI)	P-value
IL-6 (≥11.81 *vs*. <11.81)	5.18 (1.73–15.49)	**0.003**	5.23 (1.15–23.86)	**0.033**
IL-10 (≥3.79 *vs*. <3.79)	2.62 (0.97–7.04)	0.057	1.85 (0.45–7.63)	0.39
ALC (≥1.15 *vs*. <1.15)	0.19 (0.07–0.52)	**0.001**	0.19 (0.03–1.08)	0.06
NLR (≥5.38 *vs*. <5.38)	7.23 (2.58–20.24)	**<0.001**	1.28 (0.25–6.70)	0.77
PLR (≥263.76 *vs*. <263.76)	2.94 (1.19–7.29)	**0.020**	1.76 (0.36–8.60)	0.48
LDH (≥257.85 *vs*. <257.85)	1.83 (0.73–4.58)	0.19	–	–
ALB (≥33.80 *vs*. <33.80)	0.18 (0.07–0.47)	**<0.001**	0.16 (0.04–0.64)	**0.009**

Bold values indicate p < 0.05; CIP, checkpoint inhibitor-related pneumonitis; IL-6, interleukin-6; IL-10, interleukin-10; the units for IL-6 and IL-10 are both pg/ml; ALC, absolute lymphocyte count, the unit for ALC is K/μl; NLR, neutrophil to lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; LDH, lactate dehydrogenase, the unit for LDH is U/L; ALB, albumin, the unit for ALB is g/L; OR, odds ratio; CI, confidence interval.

TABLE 4 | Cox proportional hazards regression analysis of clinical factors associated with overall survival of CIP patients.

Variables	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (≥65 *vs*. <65)	0.59 (0.28–1.28)	0.18	–	–
Gender (female *vs*. male)	0.84 (0.32–2.22)	0.72	–	–
Smoking (current or former *vs*. never)	1.01 (0.48–2.12)	0.99	–	–
Histology (squamous *vs*. non-squamous)	0.66 (0.30–1.49)	0.30	–	–
Treatment line (1st *vs*. ≥2nd)	0.37 (0.18–0.78)	**0.009**	0.61 (0.21–1.76)	0.36
Treatment (combination *vs*. monotherapy)	0.61 (0.27–1.41)	0.25	–	–
Grade of CIP (1–2 *vs*. ≥3)	0.11 (0.05–0.27)	**<0.001**	0.41 (0.11–1.56)	0.19
IL-6 (<11.81 *vs*. ≥11.81)	0.07 (0.02–0.34)	**0.001**	0.17 (0.03–0.95)	**0.044**
IL-10 (<3.79 *vs*. ≥3.79)	0.48 (0.20–1.14)	0.057	0.75 (0.27–2.04)	0.57
ALC (≥1.15 *vs*. <1.15)	0.42 (0.20–0.92)	**0.029**	0.29 (0.05–1.50)	0.14
NLR (<5.38 *vs*. ≥5.38)	0.33 (0.15–0.74)	**0.007**	1.11 (0.25–4.90)	0.89
PLR (<263.76 *vs*. ≥263.76)	0.52 (0.24–1.14)	0.09	1.35 (0.40–4.58)	0.63
LDH (<257.85 *vs*. ≥257.85)	0.54 (0.24–1.23)	0.14	–	–
ALB (≥33.80 *vs*. <33.80)	0.32 (0.14–0.73)	**0.007**	0.28 (0.08–0.94)	**0.040**

Bold values indicate p < 0.05; CIP, checkpoint inhibitor-related pneumonitis; IL-6, interleukin-6; IL-10, interleukin-10; the units for IL-6 and IL-10 are both pg/ml; ALC, absolute lymphocyte count, the unit for ALC is K/μl; NLR, neutrophil to lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; LDH, lactate dehydrogenase, the unit for LDH is U/L; ALB, albumin, the unit for ALB is g/L; HR, hazard ratio; CI, confidence interval.

DISCUSSION

This real-world, retrospective, observational study suggested that the histologic cancer type and ICI monotherapy may be risk factors of CIP occurrence. We found that IL-6, IL-10, ALC, NLR, PLR, LDH, and ALB levels changed significantly over time in patients with CIP. In addition, IL-6 and ALB levels at the time of CIP diagnosis were significantly correlated with severity and OS in CIP patients.

In our study, the overall CIP incidence was estimated at 10.3%, and 4.5% of patients developed grade 3 or higher CIP, which were larger proportions than those reported in previous clinical trials (24) but were consistent with a previous real-world study (10). The incidence of prior radiation was higher in CIP group than those in control group (20.7% *vs*. 10.3%; p = 0.06). In univariate logistic regression analysis, prior radiation tended to be associated with CIP (OR: 2.26, 95% CI: 0.95–5.36; p = 0.06). Multiple studies have shown that the history of prior radiotherapy could increase the risk of developing pneumonitis (8, 25). Our logistic regression analyses suggested that squamous carcinoma was associated with a high incidence of CIP. A previous study also reported that squamous carcinoma might be a risk factor for pneumonitis (26). One study showed that obstructive pneumonia may increase the risk of CIP (27). Most squamous cell carcinomas are central lung cancer, and obstructive pneumonia occurs more frequently, which may explain the increased incidence of pneumonitis in patients with squamous carcinoma. The association between pathological cancer types and CIP occurrence is worthy of further study. Our finding of a higher (OR: 6.56, 95% CI: 1.79–23.98; p = 0.004) CIP incidence among patients treated with ICI monotherapy was consistent with the findings of a recent meta-analysis (28), which showed that ICI monotherapy was associated with a higher risk of CIP (OR: 2.14, 95% CI: 1.12–4.80), compared with ICIs plus chemotherapy. This may be partly explained by cytotoxic chemotherapy drugs that can cause immunosuppression, and possibly the use of glucocorticoids as a pretreatment of chemotherapy, which may suppress the immune system as well as treat certain underlying lung diseases (e.g. asthma and chronic obstructive pulmonary disease) (28). In addition, antiangiogenic drugs (e.g. bevacizumab) could reduce vascular permeability and pulmonary exudation, which may contribute to the recovery of early pneumonitis (29). A case report showed that the addition of nintedanib to immunotherapy may prevent CIP (30).

With the development of irAEs, increased serum IL-6 and IL-10 levels have been demonstrated in case reports and retrospective studies with small samples (31–36). However, changes in the levels of these cytokines have only been reported in individual CIP cases. A case study showed a significant increase in IL-6 at the onset of CIP (37). Our study represents the first retrospective study to explore the relationship between cytokines and CIP development. We found that IL-6 and IL-10 levels increased significantly at CIP onset compared with those at baseline. However, the IL-10 levels remained unchanged, and the IL-6 levels decreased in patients without CIP over time. Elevated IL-6 was an independent biomarker for CIP severity and was an independent predictor for early death. In addition, high levels of IL-10 tended to be associated with severe CIP (p = 0.057). A study showed that the lymphocytes in the alveolar lavage fluid (BAL) of patients with CIP increased, predominantly CD4+ T helper (Th) lymphocytes (38). Th2 cells, an important subset of CD4 + cells, can produce cytokines (such as IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13), which in turn leads to excessive inflammation (39). These data supported the hypothesis that the excessive activation and proliferation of T cells cause an excessive cascade of cytokine release, which, in turn, causes an excessive immune response, leading to the occurrence of CIP. A previous case report showed that a patient developed severe cytokine release syndrome (CRS) after treatment with a PD-1 inhibitor (40). Thus, severe CIP may be related to CRS, which is a systemic inflammatory response caused by the release of inflammatory cytokines after the

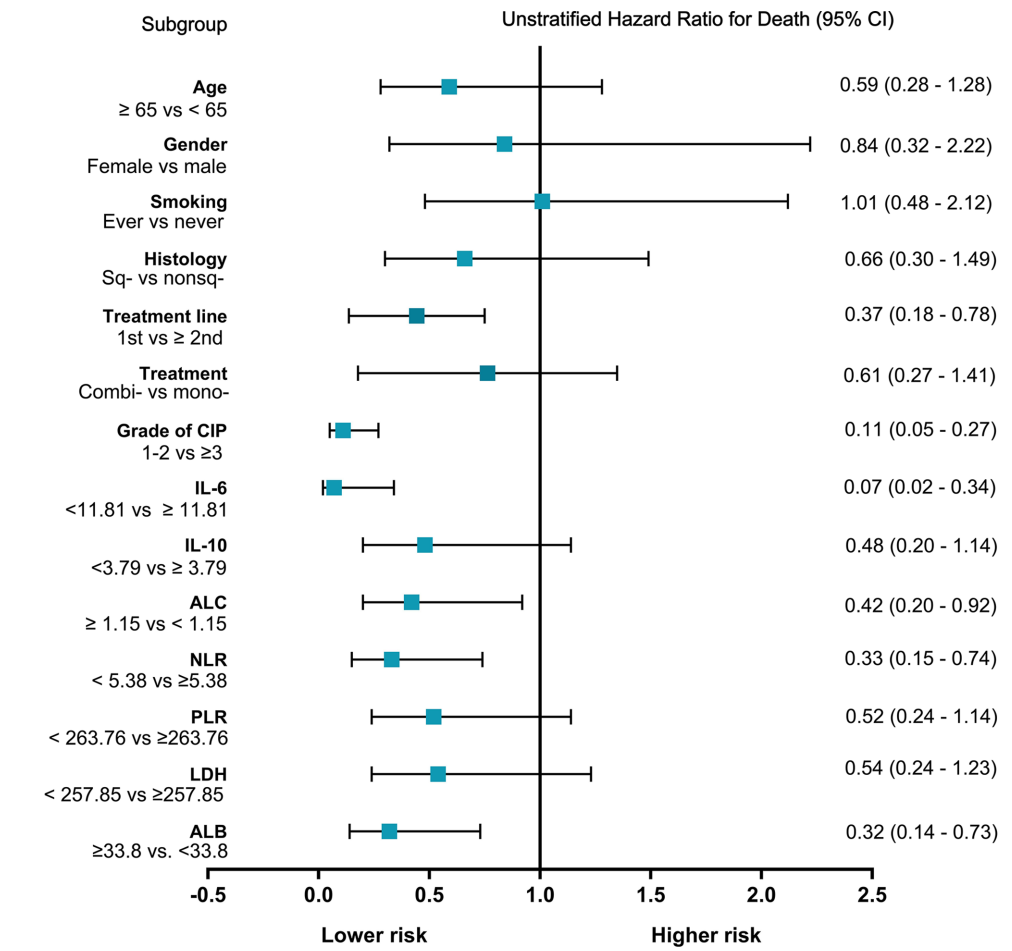

FIGURE 3 | Forest plot of subgroup analyses of prognostic factors for overall survival of checkpoint inhibitor pneumonitis (CIP). Sq-, squamous; nonsq-, nonsquamous; combi-, combination; mono-, monotherapy; IL-6, interleukin-6; IL-10, interleukin-10; the units for IL-6 and IL-10 are both pg/ml; ALC, absolute lymphocyte count, the unit for ALC is K/μl; NLR, neutrophil to lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; LDH, lactate dehydrogenase, the unit for LDH is U/L; ALB, albumin, the unit for ALB is g/L; CI, confidence interval.

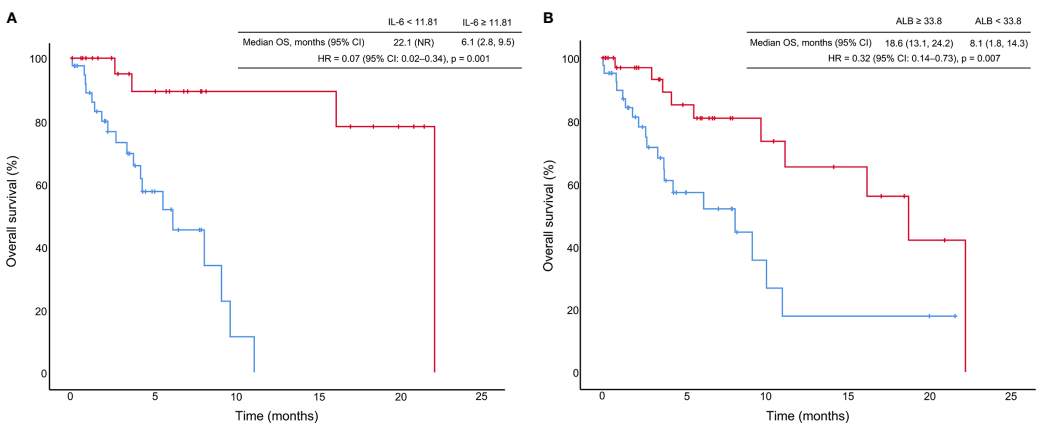

FIGURE 4 | Kaplan–Meier curves of overall survival (OS) stratified by interleukin-6 (IL-6) levels **(A)** and albumin (ALB) concentration **(B)**. HR, hazard ratio; CI, confidence interval; the unit for IL-6 is pg/ml; the unit for ALB is g/L.

activation of monocytes, macrophages, and other lymphocyte populations, and elevated IL-6 plays a key role in this process (41). Stroud et al. reported that 27/34 patients with irAEs had improved clinical symptoms after receiving tocilizumab (IL-6 inhibitors) (42). Thus, IL-6 inhibitors may be an option for individualized treatment of CIP patients.

We observed that peripheral blood ALC values decreased from baseline to CIP, whereas no change was observed in the control group. A previous study suggested that a higher baseline ALC level (>2000 cells/mL) was a risk factor for irAE (43). In univariate analysis, low ALC levels were correlated with severe pneumonitis. Fujisawa et al. reported that a decrease in ALC values was associated with the incidence of grades 3–4 CIP in melanoma patients treated with nivolumab (44). This phenomenon may be caused by the large number of lymphocytes transported from the blood that infiltrate the focus of pneumonitis, resulting in a reduction of ALCs in the circulating pool, especially in severe patients, which is manifested as reduced peripheral blood ALC values (45). CIP should be distinguished from pulmonary infections, especially bacterial pneumonia. Bacterial pneumonia is primarily characterized by increased neutrophils; however, in our study, CIP patients did not present with increased neutrophils, and changes in the NLR appeared to primarily be due to a decrease in lymphocytes. Therefore, decreased ALC values may represent an indicator that can be used to differentiate CIP from bacterial pneumonia.

In our study, NLR and PLR increased significantly in CIP compared with baseline values. In univariate analysis, the observed increases in these two biomarkers at the time of CIP symptom onset were associated with CIP severity. No previous data have examined the role of PLR in CIP detection. A recent report (12) by Matsukane et al. analyzed NLR fluctuations in solid tumors and found that increased NLR was significantly associated with the occurrence of irAEs, especially in pneumonitis. They also indicated that elevated NLR levels at the time of CIP diagnosis were correlated with the occurrence of high-grade CIP. Conversely, a study showed that NLR and PLR were not associated with irAEs but were associated with the response to ICI treatment (31). However, this study only included patients treated with cytotoxic T-lymphocyte-associated protein 4 (CTL-4) inhibitors and did not analyze specific organs.

Multiple studies have shown that NLR and PLR are associated with OS in lung cancer patients treated with ICIs (46–48). However, the relationship between these indicators and the OS of patients with CIP is rarely reported. The univariate analysis showed that elevated NLR and low ALC levels at the time of initial CIP symptom onset were associated with shorter OS in patients with CIP. In a previously published study, compared with patients with a rapid decrease in elevated NLR, those patients who maintain elevated NLR had a poorer OS (12).

Studies have reported that damaged lung tissue cells release LDH, leading to increased serum LDH levels and suggesting that elevated LDH may serve as an indicator of acute lung injury (49–52). However, whether LDH is elevated in CIP has not yet been reported. Our study found that LDH was significantly higher in CIP than at baseline. A previous study suggested that patients with LDH levels greater than twice the upper limit of the normal tended to have a reduced risk of severe irAEs than patients with normal LDH levels (53). However, our study found no correlation between baseline LDH and the occurrence of CIP. Additionally, no correlation was observed between LDH and the severity of CIP.

In the current study, decreased ALB levels were associated with CIP development. A previous study showed that low ALB level was a risk factor for CIP. CIP may result in the release of both proinflammatory and inflammatory cytokines, which increase capillary permeability and promote the entry of cell and plasma solutes (such as ALB) into lesion tissue, increasing the interstitial volume and changing the distribution of ALB, which manifest as a decrease in serum ALB (54). In the multivariate analysis, high ALB levels were negatively correlated with severe pneumonitis (OR: 0.16, 95% CI: 0.04–0.64). In addition, low ALB level was a predictor of poor OS. Consistent with a previous study, these results suggested that low serum ALB may serve as a biomarker of inflammation severity and was associated with reduced quality of life and longevity (54).

These data indicate that the measurement of these indicators could be performed when CIP is clinically suspected, particularly when other measurement methods, such as chest CT or chest X-ray, are not available or are inconclusive. In addition, these indicators may help identify patients who are at risk of severe CIP and may be used to predict CIP prognosis. However, this study has some limitations. First, this study is a real-world retrospective study. Second, we did not monitor all changes in these blood parameters from the beginning of ICI to the onset of CIP. Third, CIP was diagnosed by symptoms and radiology, and only 19.5% of patients were confirmed by histopathology.

In conclusion, squamous carcinoma and ICI monotherapy may represent risk factors for CIP development. Increases in IL-6, IL-10, NLR, PLR, and LDH levels or reductions in ALC and ALB levels during ICI treatment may also serve as biomarkers for early diagnosis of CIP. High levels of IL-6 and low concentrations of ALB at the time of initial onset of CIP symptoms were predictive of severe pneumonitis. Importantly, high IL-6 or low ALB levels could be applied to improve risk stratification in pneumonitis.

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 Institutional Review Board of the First Affiliated Hospital of Guangzhou Medical University (Guangzhou,

Guangdong, China). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XL, HD, and CZ designed the study. HD, YY, JW, GQ, and SL collected the patients' data. XL, HD, XX, ML, ZX, and YQ analyzed the data. XL, HD, YY, JW, and CZ drafted 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.2021.698832/full#supplementary-material

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Consideration of Surrogate Endpoints for Overall Survival Associated With First-Line Immunotherapy in Extensive-Stage Small Cell Lung Cancer

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Background: The combination of immune checkpoint inhibitors (ICIs) and chemotherapy is known to improve overall survival (OS) in patients with extensive-stage small cell lung cancer (ES-SCLC). ICIs have different response patterns and survival kinetics characteristics from those of the traditional chemotherapy. In first-line treatment for ES-SCLC, there is an urgent need for surrogate endpoints for the early and accurate prediction of OS. This study aimed to assess progression-free survival (PFS), milestone OS rate, milestone restricted mean survival time (RMST), overall response rate (ORR), and disease control rate (DCR) as proposed surrogate endpoints for OS in ES-SCLC for first-line immunotherapy trials.

Methods: Between January 1, 2013, and December 2020, published articles on randomized clinical trials of ICIs plus chemotherapy in patients with ES-SCLC as first-line therapy were searched in PubMed. Abstracts from the ESMO, ASCO, and WCLC, reported from 2018 onwards, were also searched. A weighted regression analysis based on the weighted least squares method was performed on log-transformed estimates of treatment effect, and the determination coefficient (R^2) was calculated to evaluate the association between treatment effect on the surrogate endpoint and OS.

Results: Seven trials, representing 3,009 patients, were included to make up a total of 16 analyzed arms. The ratio of the 12-month OS milestone rate (r = –0.790, P = 0.011, R^2 = 0.717) and 12-month OS milestone RMST (r = 0.798, P = 0.010, R^2 = 0.702) was strongly correlated with the hazard ratio (HR) for OS. The strongest association was observed between the ratio of the 24-month OS milestone RMST and the HR for OS (r = 0.922, P = 0.001, R^2 = 0.825). No associations were observed between the HR for OS and PFS and the RR for ORR and DCR.

Conclusions: The results suggested a strong correlation among the ratio of OS milestone rates at 12 months, ratios of OS milestone RMSTs at 12 and 24 months, and HR for OS. The results indicate that OS milestone rates and OS milestone RMSTs could be considered surrogate endpoints of OS in future first-line immunotherapy trials for ES-SCLC.

Keywords: small-cell lung cancer, surrogate endpoints, survival, immunotherapy, restricted mean survival time (RMST)

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BACKGROUND

Small cell lung cancer (SCLC) is a life-threatening cancer, and the median overall survival (OS) for patients with extensive-stage SCLC (ES-SCLC) is only 8–10 months. Recently, immunotherapy has attracted increasing attention as a favorable treatment for SCLC. The combination of immune checkpoint inhibitors (ICIs) and chemotherapy, as the first-line treatment for ES-SCLC, has been reported to significantly improve OS compared with chemotherapy alone (1, 2). To date, four phase III and several phase II studies have been published regarding first-line immunotherapy in ES-SCLC. However, the appropriate surrogate endpoints of OS in first-line immunotherapy treatment for ES-SCLC remain largely unknown.

Data from patient- and trial-level studies have shown that PFS is strongly correlated with OS in first-line treatment of ES-SCLC and is a potential surrogate endpoint of OS (3, 4). Disease control rate (DCR) and duration of response (DOR) are strong predictors of OS in relapsed SCLC and are surrogate endpoints of relapsed SCLC (5). Immunotherapy has a unique response pattern, and its survival kinetics is different from those of chemotherapy. In studies comparing immunotherapy with chemotherapy, for example IMPOWER133 study, two survival curves often overlap or intersect for the first 6 months; the survival curves do not diverge until approximately 6 months of the study. Long-term survival is achieved only in some patients (the platform appears at the tail of the curve). Under these circumstances, the suitability of PFS, ORR, or DCR as surrogate endpoints for OS in first-line immunotherapy for ES-SCLC should be evaluated. Since the survival curve of immunotherapy no longer follows the assumption of constant proportional hazards, the median OS cannot interpret all the characteristics of the survival curve; hence, immunotherapy trials face challenges in statistical design. Researchers are proactively exploring indicators that can promptly and accurately assess the effect of immunotherapy on OS of patients with ES-SCLC.

Recently, milestone survival and restricted mean survival time (RMST) have been explored as potential surrogate endpoints in immunotherapy trials. Milestone survival analysis is a cross-sectional assessment of OS at a clinically significant prespecified time point (6), which can capture the delayed clinical effect of immunotherapy. RMST has also been defined as the area under the survival curve for a specified time window (7, 8); it is a mean value. In studies with RMST as the endpoint the difference in RMST between the experimental and control groups represents the absolute benefit of OS. Several studies have used RMST as an endpoint. For example, KEYNOTE-604 study (9) used RMST as an endpoint of exploration; KEYNOTE-598 study (10) used RMST at 24 months as an indicator for the interim analysis; in addition, Bpharm et al. used RMST to reinterpret the study results of the CheckMate057 study (11). These studies suggest that surrogate endpoints are worth exploring in clinical studies of immunotherapy. However, the endpoints of the ES-SCLC study were set mainly from the experience of cytotoxic drug. The purpose of our study on the surrogate endpoints of immunotherapy in ES-SCLC is to provide a more suitable method for evaluating the efficacy. It provides a reference for the future clinical study design and a more comprehensive and pertinent interpretation of the current results of immunotherapy studies.

Here, we investigated the significance of 12-month OS milestone rate and 12- and 24-month OS milestone RMSTs as surrogate endpoints of OS in first-line immunotherapy for ES-SCLC. We analyzed the existing data on immunotherapy in treatment-naïve ES-SCLC patients to determine optimal surrogate endpoints that can predict OS early, reduce costs, and accelerate the development of ICIs in SCLC.

METHODS

Literature Search

The randomized controlled phase II and III clinical trials of first-line immunotherapy for ES-SCLC, published between January 2013 and December 2020, were identified based on a systematic electronic search in PubMed. Abstracts from the European Society for Medical Oncology (ESMO), American Society of Clinical Oncology (ASCO), and World Conference on Lung Cancer (WCLC) reported since 2018 were also searched. The authors were independently involved in the literature search. Search terms included "small cell lung cancer OR SCLC", "extensive disease", "first-line treatment", "PD-1/PD-L1", "CTLA-4", "pembrolizumab", "nivolumab", "atezolizumab", "durvalumab", "avelumab", "ipilimumab" and "chemotherapy". Relevant references of eligible clinical trials were also manually searched. A detailed flow diagram is presented in **Figure 1**.

Data Collection

Two researchers (LS and ZS) separately extracted and cross-checked the data. Where there was a difference in opinion on any of the extracted data, consensus was reached by involving a third researcher who evaluated the same data and made the final decision. We extracted the following information from the included literature: name and phase of study, number of patients, experimental arm(s) regimen, control arm regimen, primary endpoint, and system for classification. The milestone rates were calculated using the Kaplan–Meier estimates. The model-independent values of RMST data were extracted from Kaplan–Meier curves using DigitizeIt Version 2.2 (www.digitizeit.xyz), and the area under the calculated curve was described according to a previously described method (12, 13).

Statistical Analysis

Hazard ratios (HRs) were used to quantify the treatment effects on PFS and OS while RRs were used to quantify the treatment effects on ORR and DCR. The ratios of milestone RMST and OS milestone rates were used to quantify effects of the 12- or 24-month OS milestone RMST and 12-month OS milestone rates. Spearman rank correlation coefficients (rs) were calculated to evaluate the correlation between effects of treatment on surrogate endpoints and the HRs of OS. The correlation coefficient, r, ranged from −1 to 1 (an r value closer to 1 indicates a stronger correlation).

A weighted regression analysis based on the weighted least squares (WLS) method was performed on estimates of log-

transformed treatment effect weighted by sample size of arms, and the determination coefficient R^2 was calculated to reflect the strength of the association between treatment effects on the surrogate endpoints and HRs of OS. Data were analyzed using the R software (version 3.4.3; https://cran.r-project.org/bin/windows/base/old/3.4.3/). All tests were two-sided, and P-values <0.05 were considered statistically significant.

RESULTS

Trials Included in the Analysis

Table 1 lists the basic information of the included studies. Seven trials (three randomized phase II and four randomized phase III trials), representing 3,009 patients, for a total of 16 analysis arms were included (**Figure 1**). Two trials used the three-arm design, while five used the two-arm parallel control design. The primary endpoints of three trials were PFS, while two focused on OS and two focused on OS and PFS. Five trials investigated programmed cell death ligand 1(PD-L1)/programmed cell death 1(PD-1) inhibitors, while two assessed cytotoxic T-cell lymphocyte antigen-4 (CTLA-4) inhibitors. PFS and OS were reported in seven, ORR in six, and DCR in five studies. The 12-month milestone OS rate and 12-month OS milestone RMST could be extracted from seven trials, and the 24-month OS milestone RMST from six trials.

Analysis

A significantly strong positive correlation was identified between the 12-month OS milestone rate and HR for OS (r = −0.790, P = 0.011, n = 9). The weighted regression model was as follows: Log (HRos) = −0.099 − 0.567 × log (ratio of the 12-month OS milestone rate). The R^2 value of the weighted regression line was 0.717 (P = 0.004), indicating that 71.7% of variability among the effects on OS could be explained by the ratio of the 12-month OS milestone rate (**Figure 2**).

Since KEYNOTE-604 (9) reported 12- and 24-month RMSTs of PFS and OS, we conducted sensitivity verification between the recalculated RMST and the reported data. The results showed that recalculated RMSTs were identical to data reported in the original articles (**Table S1**). Meanwhile, 12-month OS milestone rate, 12/24-month OS milestone RMSTs, HR of PFS, HR of OS for all included trials were shown in **Table S2**; estimated median OS and HR of OS and that compared with original reported data were listed in **Table S3**.

The ratio of the 12-month OS milestone RMST was strongly correlated with the OS HR (r = 0.798, P = 0.010, n = 9). The following regression formula was used: Log (HRos) = −0.160 +

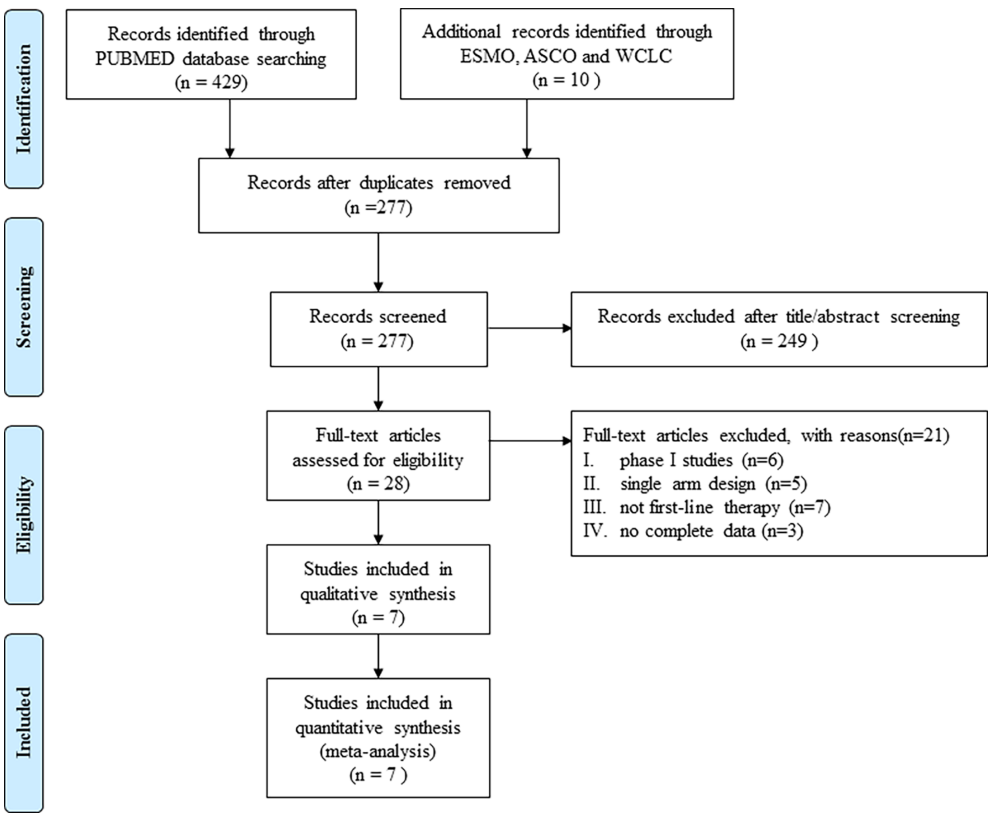

FIGURE 1 | Flowchart of study inclusions and exclusions.

TABLE 1 | Basic information of the included studies.

Study	Phase	Experimental Arm(s)	Control Arm	Primary endpoints	No. of patients	System for classifying response	Study arms
KEYNOTE-604 (9)	III	Pembrolizumab + EP/EC	Placebo + EP/EC	PFS and OS	453	RECIST 1.1	2
IMpower133 (1, 14)	III	Atezolizumab + EC	Placebo + EC	PFS and OS	403	RECIST 1.1	2
EA5161 (15)	II	Nivolumab + EP/EC	EP/EC	PFS	145	RECIST 1.1	2
CASPIAN (2, 16)	III	A:Durvalumab + EP/EC B:Durvalumab + tremelimumab + EP/EC	EP/EC	OS	805	RECIST 1.1	3
Reck2012 (17)	II	A: phased-ipilimumab + paclitaxel + carboplatin B: concurrent-ipilimumab + paclitaxel + carboplatin	Placebo + paclitaxel + carboplatin	irPFS	130	mWHO& irRC	3
Reck2016 (18)	III	Ipilimumab + EP/EC	Placebo + EP/EC	OS	954	mWHO	2
EORTC (19)	II	Pembrolizumab + EP/EC	Placebo + EP/EC	PFS	119	NR	2

2.337 × Log (ratio of the 12-month OS milestone RMST). R^2 was 0.702 (P = 0.0048), suggesting that the ratio of the 12-month OS milestone RMST could explain 70.2% of HRos outcomes (**Figure 3**).

Additionally, we observed the strongest correlation between the 24-month OS milestone RMST and OS HR (r = 0.922, P = 0.001, n = 8). The equation for the resulting line was as follows: Log (HRos) = − 0.063 + 1.794 × Log (ratio of the 24-month OS milestone RMST). R^2 was 0.825 (P = 0.002), suggesting that the ratio of the 24-month OS milestone RMST could explain 82.5% of HRos outcomes (**Figure 4**).

No correlation was found between the HR for PFS and HR for OS (r = 0.449, P = 0.225, n = 9). The weighted regression model was as follows: Log (HRos) = −0.033 + 0.758× Log (HR_{PFS}); R^2 was 0.315 (P = 0.116) (**Figure 5A**).

The RR for ORR tended to be correlated with the HR for OS, but the correlation was not statistically significant (r = −0.675, P = 0.066, n = 8). The weighted regression model was obtained using the following formula: Log (HRos) = −0.175 − 0.426 × Log (RR_{ORR}); R^2 was 0.233 (P = 0.226) (**Figure 5B**).

Similarly, no correlation was found between the RR for DCR and HR for OS. The correlation coefficient between RR for DCR and HR for OS was −0.232 (P = 0.658, n = 6). The weighted regression model was obtained using the following formula: Log (HRos) = −0.185 − 0.229 × log (RR_{DCR}); R^2 was 0.018 (P = 0.798) (**Figure 5C**).

DISCUSSION

To our best knowledge, this is a specific study to evaluate the trial-level surrogacy endpoints for OS, focusing on first-line immunotherapy for ES-SCLC. We found a strong correlation between OS HR and the ratio of OS milestone rates at 12 months

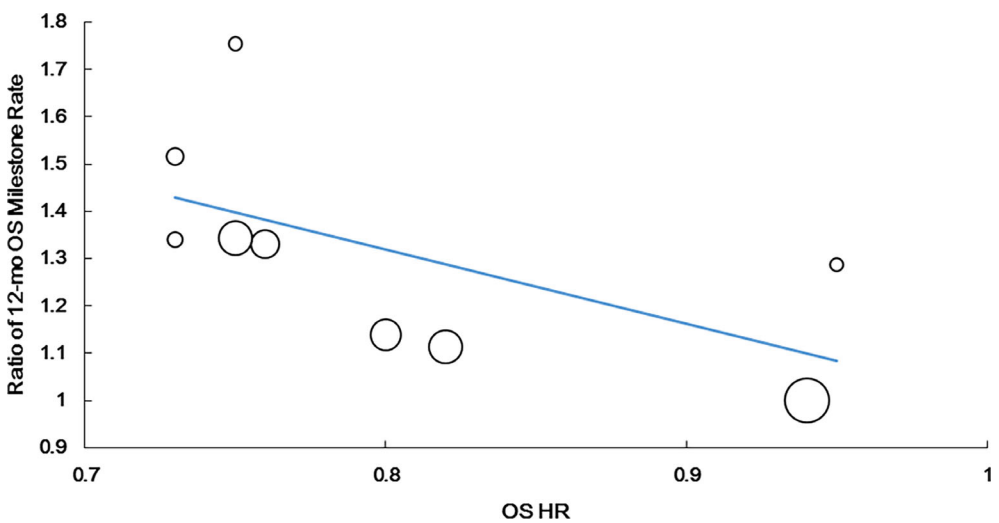

FIGURE 2 | Correlation of treatment effects on the overall survival (OS) hazard ratio (HR) with the ratios of 12-month OS milestone rate.

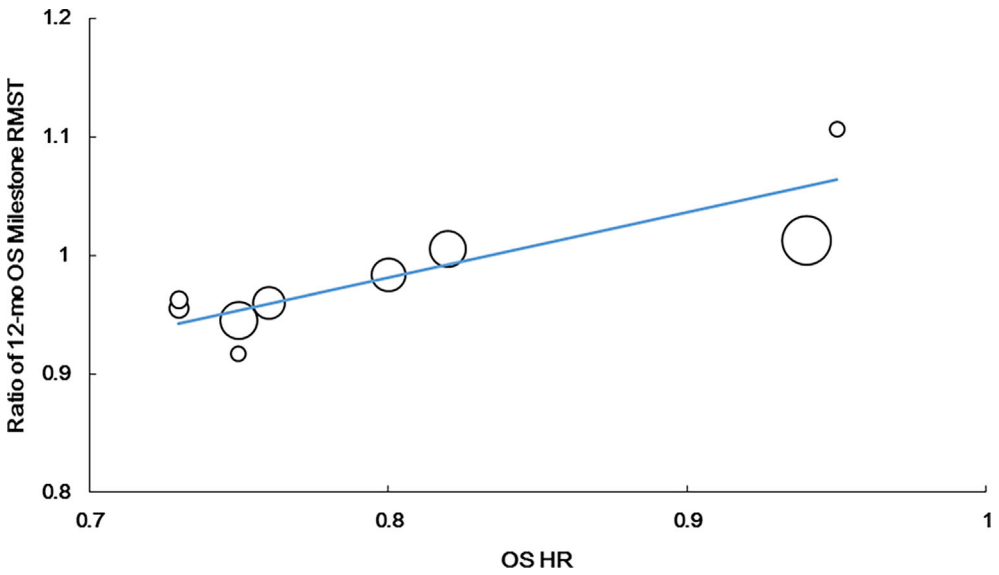

FIGURE 3 | Correlation of treatment effects on the overall survival (OS) hazard ratio (HR) with the ratios of 12-month OS milestone RMST.

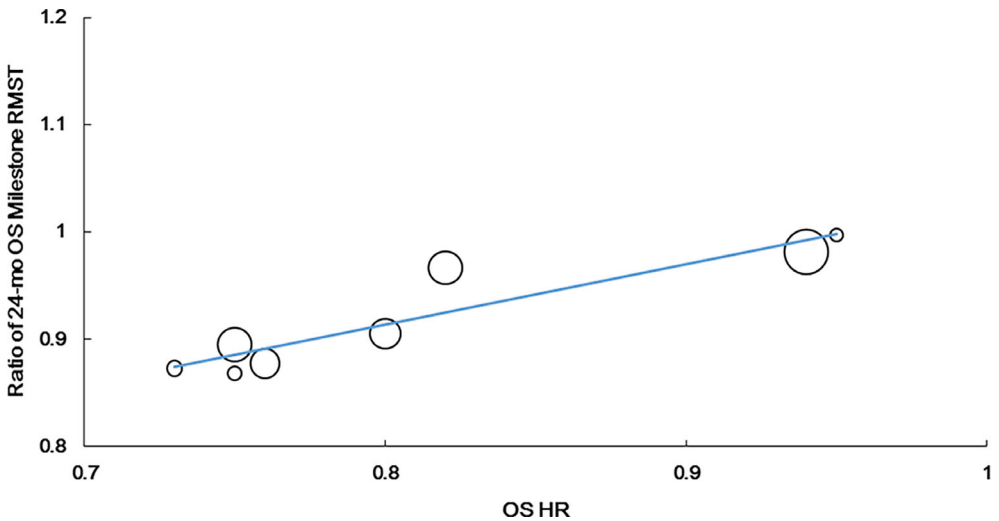

FIGURE 4 | Correlation of treatment effects on the overall survival (OS) hazard ratio (HR) with the ratios of 24-month OS milestone RMST.

or the ratios of RMST at 12 and 24 months. However, no correlation was observed between the HR of OS and PFS and the RR of ORR and DCR, which were unreliable surrogate endpoints of first-line immunotherapy for ES-SCLC.

A study by Chen et al., using 42 trials, evaluated the roles of PFS, ORR, and DCR as surrogate endpoints for OS in first-line therapy for ES-SCLC (20). Although the HR of PFS could explain 72% of the HR outcomes of OS, only three immunotherapy trials were included in this study. In addition, it was found that all three immunotherapy trials were below the weighted regression line. Consistent to the results of our study, the analysis of the correlation between the HR of OS and RR of ORR and DCR, suggested that the OS of immunotherapy cannot be accurately evaluated using ORR and DCR.

Although PFS was a potential surrogate endpoint for OS in ES-SCLC patients treated with chemotherapy, PFS was frequently inconsistent with OS in patients from the trials of first-line immunotherapy for ES-SCLC. In the CASPIAN study (2), compared to chemotherapy alone, durvalumab plus chemotherapy did not prolong PFS; however, it led to a statistically significant improvement in OS. In our study, no correlation was found between the HR for PFS and OS, respectively. This finding indicates that in the

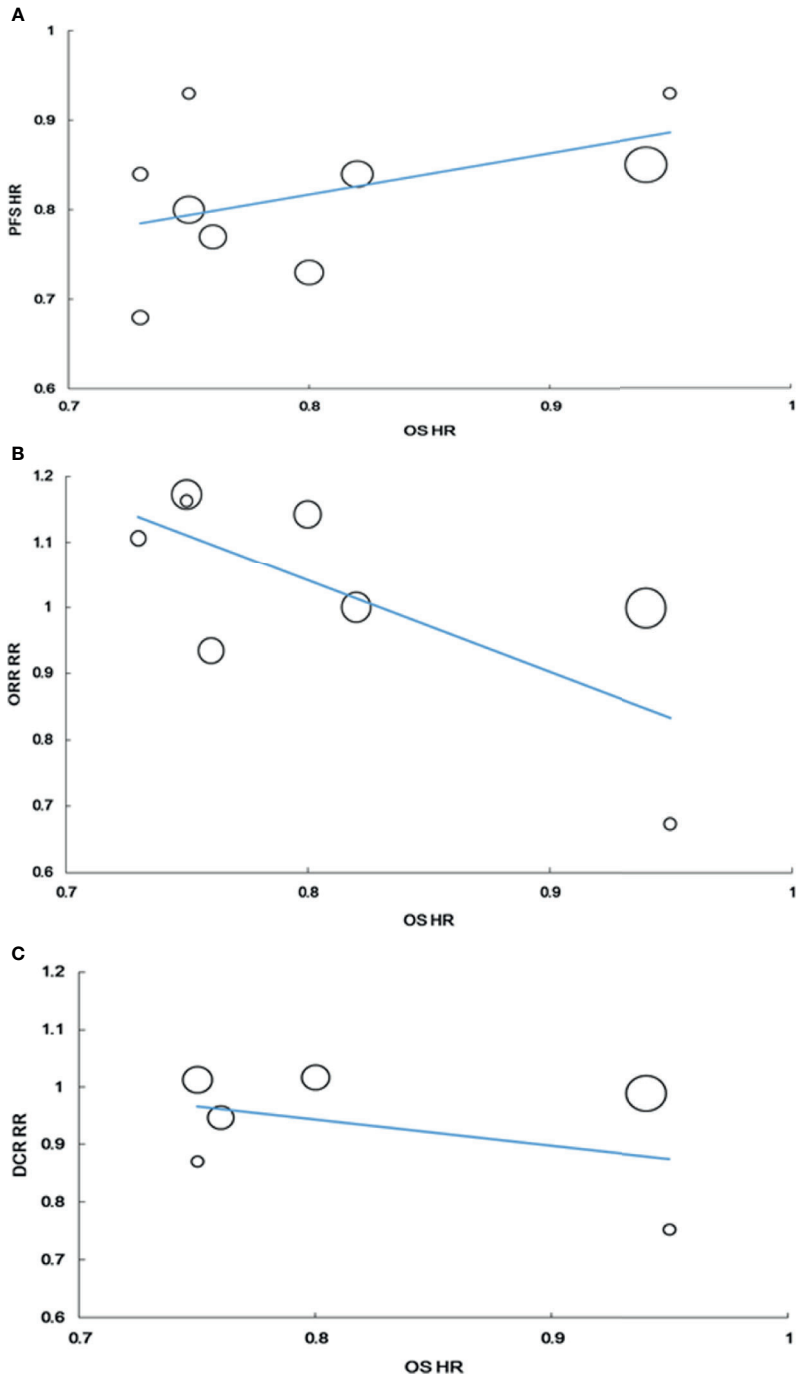

FIGURE 5 | Correlation of treatment effects on the overall survival (OS) hazard ratio (HR) with the PFS HR **(A)**, with the RR of ORR **(B)**, with the RR of DCR **(C)**.

era of immunotherapy, PFS is no longer an ideal surrogate endpoint for OS as a first-line immunotherapy for ES-SCLC.

In a milestone survival analysis, Blumenthal et al. (21) observed a strong correlation between the 12-month OS milestone rate and OS HR in NSCLC immunotherapy studies. In our study, there was a very strong correlation between the HR for OS and the ratio of 12-month OS milestone rate of first-line immunotherapy for ES-SCLC. The OS milestone rate can be used as a potential surrogate endpoint for OS. Both the IMpower133 study and CASPIAN study considered the estimated number of OS events as the interim analysis time point; interim analyses of the two studies were performed at a median follow-up of 13.9 and 14.2 months,

respectively, at approximately 60% maturity of OS (1, 2). However, the 12-month OS milestone rate analysis could predict the OS of first-line immunotherapy for ES-SCLC approximately 2 months in advance. Further studies are needed to determine whether 12 months is the ideal time point to perform the OS milestone rate analysis. Besides, the OS milestone rate is a cross-sectional analysis at a predetermined time point (22), which makes it difficult to summarize the survival curve in its entirety. RMST represents the distribution of any time event at a presetting and clinically meaningful time point (8), which can explain all survival information before the presetting time point. It is an absolute measure of survival time and can robustly interpret therapeutic efficacy. In our study, the ratios of OS milestone RMSTs at both 12 and 24 months were strongly correlated with HR for OS, particularly that of the OS milestone RMST at 24 months. In the KEYNOTE-604 study (9), although PFS of the interim analysis was inconsistent with OS in the final analysis, the 12-month PFS and 24-month OS RMSTs were favorable for combined treatment with pembrolizumab and chemotherapy. This can explain the divergent results and suggests that the OS milestone RMST could more accurately predict OS. Further investigations of the OS milestone RMST as a surrogate endpoint of first-line immunotherapy trials for ES-SCLC in the future are needed.

Limitations

Our study has several limitations. First, we did not acquire detailed individual patient data; we only evaluated data at the trial-level. Patient-level data may provide more reliable data support for the issue of surrogate endpoints as first-line immunotherapy for SCLC. Second, although our study included all first-line immunotherapy trials for ES-SCLC, the included studies were heterogeneous, comprising phase III and II studies, evaluation criteria of Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, modified World Health Organization (WHO) criteria, and immune-related response criteria (irRC). Third, our study found that OS milestone rate and OS milestone RMST were better associated with OS, both of which require a presetting time point for analysis. However, determining the ideal time point is challenging. If the effect of immunotherapy is assessed too early, it may not be sufficiently significant. Moreover, the curve of OS still overlaps at 6 months, as seen from several phase III studies of first-line immunotherapy for ES-SCLC. So, we calculated RMSTs at 12 months and 24 months. We found that RMSTs at 12 months and 24 months had a strong correlation with OS, respectively (R^2 = 0.702 and R^2 = 0.825). The correlation was statistically stronger at 24 months. In addition, the curves of OS of the three phase III studies approached the plateau about 24 months. Since only three phase 3 studies have been published, more data are needed to confirm whether RMST at 24 months is the most appropriate. Fourth, these indicators for predicting OS are statistically calculated which is not intuitive and convenient for clinicians to use. We suggest that there will be more intuitive and objective evaluation indicators in the future with the presentation of more clinical research data of SCLC immunotherapy and deeper exploration of the survival dynamics of immunotherapy. Finally, data included in our study were limited, as immunotherapy in ES-SCLC is still in its infancy. Moreover, up to now the studies published have just about one year follow-up.

CONCLUSIONS

In this study, the ratios of 12-month OS milestone rate and 12- and 24-month milestone RMSTs were found to be strongly correlated with the HR for OS. OS milestone rate and OS milestone RMST are promising surrogate endpoints of OS in first-line immunotherapy trials for ES-SCLC. OS milestone survival rate and OS milestone RMST could predict OS earlier and more accurately and are worth considering as intermediate endpoints of first-line immunotherapy trials of ES-SCLC in the 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.

AUTHOR CONTRIBUTIONS

Conception and design: SZ, SL, and YiC. Administrative support: YiC. Provision of study materials or patients: SZ, SL and YaC. Collection and assembly of data: SZ, SL and PZ. Data analysis and interpretation: SL and SX. Manuscript writing: All authors. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.696010/full#supplementary-material

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CASE REPORT
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Case Report: Abscopal Effect of Microwave Ablation in a Patient With Advanced Squamous NSCLC and Resistance to Immunotherapy

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Currently, immunotherapy has been a backbone in the treatment of advanced non-small cell lung cancer (NSCLC) without driver gene mutations. However, only a small proportion of NSCLC patients respond to immune checkpoint inhibitors, and majority of patients with initial response will develop acquired resistance at 5 years, which usually manifests as oligo-progression or oligo-metastases. Evidence from multiple clinical trials indicates that local consolidative therapies could improve the prognosis of oligometastatic NSCLC patients. Herein, we reported a case of advanced squamous lung cancer which showed a durable abscopal effect from microwave ablation after acquired resistance of immunotherapy.

Keywords: ablation, immunotherapy, lung cancer, abscopal effect, oligo-progression

INTRODUCTION

Lung cancer remains a malignant disease with high incidence and mortality (1). With the advent of immune checkpoint inhibitors, programmed cell-death protein 1 (PD-1) or programmed cell death 1 ligand 1 (PD-L1) blockade has become a backbone as first-line treatment, and a standard of care as second-line therapy for patients with advanced NSCLC and EGFR/ALK wildtype (2). However, the clinical outcomes of immunotherapies are not always satisfying. Schoenfeld AJ et al. found that 74% of patients with initial response to immunotherapy will develop acquired resistance at 5 years, and 56% of them experienced oligo-progression (3). In recent years, locoregional therapies have been widely used to treat patients with oligo-progression or oligo-metastases and showed impressive outcomes in multiple solid tumors (4, 5). Among these local therapies, microwave ablation (MWA) is increasingly used in clinical practice due to its advantages of producing larger ablation zones over shorter periods of time. Herein, we report an abscopal effect of MWA in a 69-year-old patient with metastatic squamous lung cancer. We performed a right lower lung lesion ablation after resistance of immunotherapy and observed tumor shrinkage in 4R/7 lymph node metastatic lesions. We posit that the present case report will provide novel insight into the treatment of advanced NSCLC in clinical practice.

CASE PRESENTATION

In June 2018, a 69-year-old male with a 30 pack–year smoking history was referred to Shanghai Pulmonary Hospital for a right lower lobe mass and 4R/7 lymphadenopathy (**Figure 1**), along with severe chronic obstructive pulmonary disease (COPD). Ultrasound-guided bronchial biopsy revealed squamous cell carcinoma, and genetic testing showed negativity for driver gene mutations. The patient was initially treated with vinorelbine 40 mg/m^2 d1, 8 and cisplatin 60 mg/m^2 d1–2. However, after four cycles, his symptoms worsened, and chest computed tomography (CT) scan confirmed a progressive disease (PD). Hence, the chemotherapy regimen was shifted to albumin-bound paclitaxel 200 mg/m^2 d1, 8. The maximum diameter of primary lesion shrank 28% during the treatment of albumin-bound paclitaxel. However, after four cycles the primary lung lesion was still not effectively controlled, and disease progression in the chest was confirmed by imaging (**Figure 2A**), and ECT bone scan revealed a new lesion in the right tibia, indicating the occurrence of bone metastases. In addition, the PD-L1 expression of this patient showed negative results.

In August 2019, the patient participated in a single-arm phase II clinical study of camrelizumab plus apatinib for advanced NSCLC. After four cycles, a partial response (PR) was observed in December 2019 (**Figure 2B**), with a PFS of 12.8 months. Oligo-progression in the chest was found in August 2020, with enlarged primary lung lesion and mediastinal 4R/7 lymphadenopathy (**Figure 2C**). Given that this patient had severe COPD and could not tolerate radiotherapy in the lung and mediastinum simultaneously, CT-guided microwave ablation was utilized to eliminate the primary tumor in September 2020. One month later, chest CT scan showed the right lower lobe mass was gradually absorbed. Surprisingly, the enlarged 4R/7 lymph nodes shrank significantly at the same time and continued to decrease by subsequent follow-up scans, indicating an abscopal effect of local ablation (**Figure 2D**). Thus, we cancelled the original plan of radiotherapy for him and decided to continue anti-PD-1 immunotherapy as before. Until the last follow-up in March 2021, the patient had not shown any signs of disease progression and obtained a durable response. The timeline treatment administration from the episode of care was presented in **Figure 3**.

DISCUSSION

In this report, we presented a successful case of a patient with advanced squamous cell lung cancer who showed an abscopal effect of local ablation. This patient showed initial response to PD-1 blockade and VEGFR-TKI after the failure of traditional chemotherapy. However, he developed acquired resistance thereafter with an oligo-progression. Emerging evidence demonstrated that locoregional therapies improved overall survival in oligo-progressive NSCLC patients and became a standard therapeutic strategy after resistance to molecular targeted therapy (6, 7). In the present case, we applied

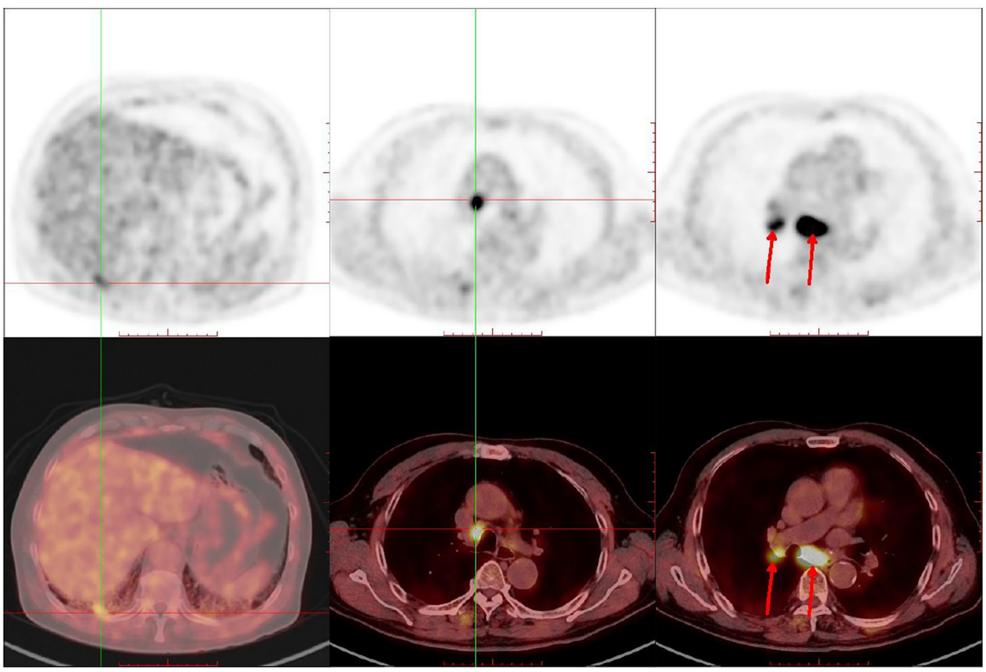

FIGURE 1 | PET/CT revealed a right lower lobe mass and 4R/7 lymphadenopathy before treatment.

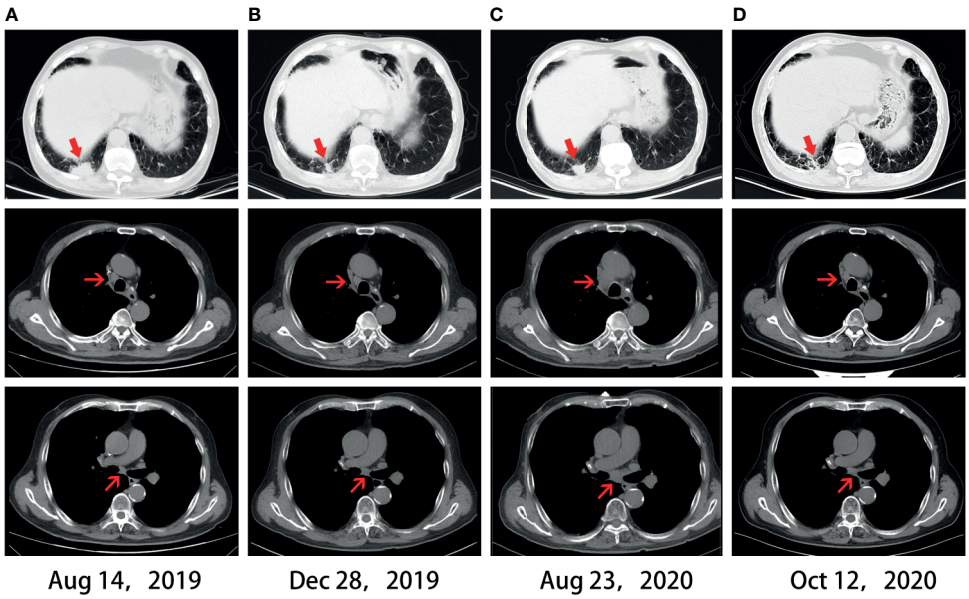

FIGURE 2 | Chest CT scans. **(A)** CT before immunotherapy. **(B)** CT revealed a partial response after four months of immunotherapy. **(C)** CT revealed disease progression after acquired resistance of immunotherapy. **(D)** CT revealed an abscopal effect after one month of local ablation.

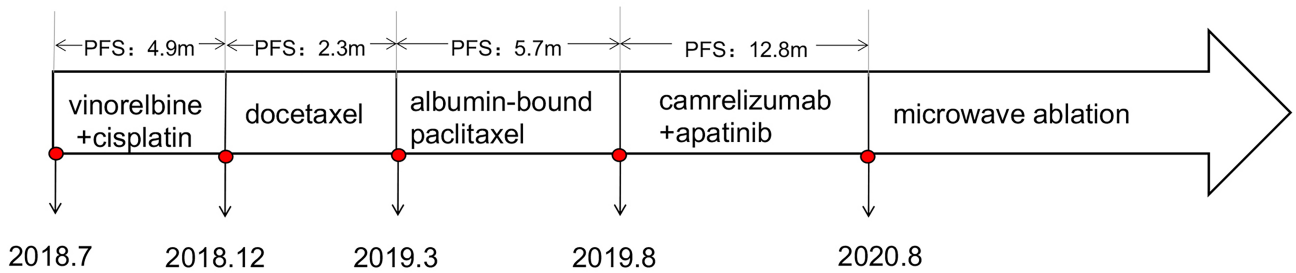

FIGURE 3 | Timeline of treatment administration from the episode of care.

local ablative therapy in oligo-progressive NSCLC after immunotherapy resistance and observed a durable abscopal effect, which highlighted the importance of local ablation in cancer immunotherapy.

Although thermal ablative techniques are becoming more frequent in lung cancer, the mechanism of their systemic immunomodulatory effects remains controversial. Thermal ablation mainly includes radiofrequency ablation (RFA), microwave ablation (MWA), and argon–helium knife cryotherapy (8). Among them, MWA showed favored features of shorter ablation times and potentially larger ablation zones (9, 10). Typically, MWA provides high thermal energy to cause tumor necrosis as an *in situ* antigen and thereby initiating systemic immune response, which is similar to radiation-induced abscopal effect (11). In addition, thermal ablation could change tumor microenvironment by promoting the infiltration of tumor-specific T cells. Previously, Zerbini et al. demonstrated that circulating tumor-specific T cells and natural killer (NK) cells were activated and enhanced by RFA that was applied to hepatocellular carcinoma (12). The increase of T cells, NK cells, or macrophages within the tumor microenvironment after thermal ablation needs to be validated by more experimental studies in the future.

To the best of our knowledge, this is the first case that showed a durable abscopal effect of MWA in squamous NSCLC after acquired resistance of immunotherapy. Local ablation eliminated the primary lesion and exerted an abscopal effect on the distant lesions by boosting the immune system; local ablation might provide a novel strategy for patients who developed acquired resistance to immunotherapy. NSCLC patients with multiple

metastases might also benefit from local ablation therapy due to the appearance of abscopal effect. Therefore, the application of local ablative therapies showed a superior potency in the area of immunotherapy than targeted therapy. Additionally, the abscopal effects of radiotherapy have been observed in previous reports (13, 14), and local ablative therapies might be an alternative surrogate.

Nevertheless, there exist several limitations in our report. The application of MWA is still not widely used in clinical practice for lung cancer. Therefore, clinical trials that compare the efficacy of local ablation with other therapies, such as radiotherapy, are urgently needed. In addition, the mechanism of how local ablation stimulates abscopal effect after resistance to immunotherapy needs to be clarified further. Our case sheds light foroptimal approach in patients with lung cancer who developed acquired resistance to immunotherapy.

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

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

CS and MY drafted the manuscript. YP, DX, and BC collected materials and prepared figures. SR and CZ critically revised the final manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Tryptophan and Its Metabolites in Lung Cancer: Basic Functions and Clinical Significance

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Lung cancer is the most lethal malignancy worldwide. Recently, it has been recognized that metabolic reprogramming is a complex and multifaceted factor, contributing to the process of lung cancer. Tryptophan (Try) is an essential amino acid, and Try and its metabolites can regulate the progression of lung cancer. Here, we review the pleiotropic functions of the Try metabolic pathway, its metabolites, and key enzymes in the pathogenic process of lung cancer, including modulating the tumor environment, promoting immune suppression, and drug resistance. We summarize the recent advance in therapeutic drugs targeting the Try metabolism and kynurenine pathway and their clinical trials.

Keywords: tryptophan, lung cancer, kynurenine pathway, IDO, TDO

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INTRODUCTION

Lung cancer (LC) is one of the most common malignancies worldwide and has a high mortality rate (1). Previous studies have shown that lung carcinogenesis is attributed to the gain-functional mutation of several cancer-associated genes, including the epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene homolog (KRAS), and v-raf murine sarcoma viral oncogene homolog B1 (BRAF) (2–4). Actually, therapeutic drugs targeting these molecules have been demonstrated to prolong the survival of LC patients, particularly for non-small cell lung cancer (NSCLC) patients. However, therapeutic efficacy of these drugs is limited due to rapid development of drug resistance in LC patients (4–6). Therefore, other effective treatments are urgently needed. Currently, cancer has been thought not to be a genetic disease, rather than a metabolic disease, which is associated with tumor immune escape (7, 8). It is well known that tumor cells usually undergo aerobic glycolysis for their glucose metabolism, known as the Warburg effect (9). Moreover, extensive studies have revealed that alternations in metabolisms are not only for glucose, but also for amino acid, lipid, nucleotide, and others in cancer (10). Notably, tryptophan (Try) metabolism is a particularly compelling physiological context in LC because of its complex and multifaceted effect on LC cells and cancer-associated cells in immune escape (11).

Try cannot be synthesized directly by the human body and has the lowest levels in the human body among 20 essential amino acids such that it depends on food protein. Similar to other essential amino acids, Try is essential for biosynthesizing cellular protein and formatting cytoskeleton (12). In the circulation, most Try binds to albumin for transportation and only 10%–20% of it remain free amino acid (13, 14). The free Try is mainly degraded through the kynurenine (KYN) pathway and is

metabolized to form serotonin or other metabolites (15). Try plays a significantly physiological role in synthesizing proteins. However, the metabolic formation of serotonin and the KYN pathway-mediated metabolism, together with the lack of its endogenous production, may make Try shortage that can impair the protein synthesis. In the KYN pathway, Try is firstly converted to formyl-kynurenine, which is rapidly degraded to KYN by key enzymes of indoleamine-pyrrole 2,3-dioxygenase (IDO)1, IDO2, and tryptophan 2,3-dioxygenase (TDO), particularly by IDO1 (14, 16). Next, KYN is catalyzed into a series of metabolites, including anthranilic acid (AA), kynurenic acid (KA), xanthurenic acid (XA), 3-hydroxyanthranilic (3-HAA), quinolinic acid (QA), and NAD+ (17). In the lung, Try degradation is mainly catalyzed by IDO1 because IDO1 is constitutively expressed in many organs while TDO is predominantly expressed in the liver (18). Previous studies have shown that most Try metabolites in the KYN pathway are associated with the development of many diseases, including cancer. Actually, the IDO1-related Try metabolites are associated with lung cancer development (19, 20). This review aims to summarize the research advance in how Try and its metabolites contribute to the development and progression of LC.

THE EXPRESSION AND BIOLOGICAL FUNCTIONS OF Try METABOLITES IN LC

Try and Its Metabolites in LC

A previous study has indicated that circulating Try levels decrease in patients with lung, gastric, colorectal, breast, and prostate cancer (21). Recent studies using liquid chromatography mass spectrometry (LC-MS) have found that plasma Try and XA levels decrease and 3-HAA increases in 19 NSCLC patients, relative to 10 non-tumor healthy controls (22, 23). Similarly, high-performance liquid chromatography-fluorescence detection (HPLC-FD) or gas chromatography mass spectrometry (GC-MS) analyses reveal that the concentrations of serum Try in LC patients are significantly lower than that in the controls (24, 25). Moreover, patients with lung adenocarcinoma (LADC) tend to have lower serum Try concentrations than those with lung squamous carcinoma (LSCC), which may be related to its regulatory function in the proliferation and metastasis of different types of cancers (24). However, there is no significant difference in the levels of serum Try during the progression of lung cancer. Accordingly, the levels of serum or plasma Try may be useful for the diagnosis of LC with a specificity of >92% (24). Interestingly, a study reveals that cisplatin-resistant LC cells consume more Try than non-resistant cells (26), suggesting that Try levels may be associated with the development of drug resistance in LC cells. However, how the levels of circulating Try are associated with levels of Try in the tumor microenvironment remains to be investigated.

The decrease in circulating Try may be attributed to several reasons. First, the enhanced expression and activity of Try-metabolizing enzymes in LC patients can promote Try metabolism, decreasing the levels of Try in the circulation and tumor (27). Second, LC patients may have malnutrition and poor digestion/absorption so that they may intake less Try from foods (24). Last, over-consumption of Try-contained foods may disorder Try metabolism, especially in advanced stage of lung cancer (24, 28) because Try is an essential component for cytoskeleton and protein synthesis in LC.

Decreased circulating Try is a crucial metabolic feature in LC patients. Accordingly, Try levels may combine with other metabolic molecules for diagnosis of LC. Actually, the levels of serum Try, alanine, valine, isoleucine, histidine, and ornithine have a diagnostic value for NSCLC with an area under the receiver-operator characteristic (ROC) of >0.80, and effectively discriminate neoplastic patients from healthy subjects (29, 30). LC patients display decreased levels of serum Try, threonine, citrulline, and histidine and increased proline, isoleucine, phenylalanine, and ornithine, leading to an area under curve (AUC) of 0.80, but the Try metabolite profile does not distinguish different pathological types of LC (29, 31). Consistently, HPLC-FD analysis indicates that a combination of six metabolites [L-tryptophan, hypoxanthine, inosine, indoleacrylic acid, acylcarnitine C10:1, and lysoPC (18:2)] effectively separates NSCLC patients from non-tumor subjects with an AUC of 0.99 (32).

IDO1, IDO2, and TDO in LC

IDO1

The IDO1 is a key enzyme in the KYN pathway, particularly in the lung. Previous studies have detected IDO expression in tumor cells, blood vessels, stromal cells of NSCLC patients, as well as in dendritic cells (DCs) in the tumor environment and tumor-related lymph nodes in patients with LC (33). However, the function of IDO1 in endothelial cells has yet been understood (34). The expression of IDO promotes KYN accumulation, which may dilate blood vessels (35). Accordingly, it is possible that IDO1 deficiency may reduce vascular-related adverse reaction of some therapeutic drugs pharmacologically (35). Besides, IDO1 mRNA transcripts are upregulated in lung tissues (36) and the serum KYN : Try ratio (KTR), an indicative of IDO activity, is greater in LC patients than healthy subjects (37), supporting the notion that higher KTR is associated with increased risk for LC (38), especially for LSCC in heavy smokers, because AhR (aryl hydrocarbon receptor) activates the carcinogenesis pathway of benzo(a)pyrene (BaP), a strong lung carcinogen derived from tobacco smoking (39). High levels of IDO1 expression can enhance LC cell invasion *in vitro* and distant metastasis into the brain, liver, and bone *in vivo*, while IDO1 inhibition attenuates their invasion and distant metastasis in rodents (40). Similarly, IDO1 inhibition also inhibits the lung metastasis of breast cancer and improves the survival of tumor-bearing animals (41, 42). Furthermore, IDO1-deficient mice are partially resistant to cancer growth in a Lewis rat model of lung carcinoma (43).

The activity and expression of IDO1 are associated with diagnosis, prognosis, and therapeutic responses in LC (44–48). IDO1 activity may be a valuable biomarker for evaluating the response to immunotherapy, and its levels may help in choosing therapy for LC patients, who are sensitive to immunotherapy (49).

Similarly, increased IDO1 activity is detected in LC patients, who initially respond to immune checkpoint inhibitors (ICIs) and later exhibit cancer progression, leading to a worse prognosis (44). Furthermore, increased IDO1 activity is closely associated with worse survival of NSCLC patients receiving explicit radiotherapy (48, 50). However, these studies were performed in small groups of patients. Therefore, further prospective studies with a larger population are necessary to validate the prognostic value of IDO1 activity in LC patients following radiotherapy. Interestingly, elevated IDO1 expression is associated with better outcome in lung adenosquamous carcinoma patients, especially for those after surgical resection of the tumor (51). The discrepancy may stem from different studying populations. While previous studies mainly focus on patients with unresectable LC and patients receiving chemotherapy or chemoradiation, this study centers on LC patients after radical surgery (51). It is possible that IDO1 activity may have different values in prognosis of different stages of LC following varying therapeutic strategies.

IDO2 and TDO

IDO2 and TDO are other key enzymes for Try degradation (52–55). Although the IDO1 and IDO2 genes are highly homologous at human chromosome 8 and tightly connected (56, 57), the IDO2 catalytic activity is much weaker than that of IDO1 *in vitro* and *in vivo* (58). Actually, there is no significant difference in the concentrations of plasma Try and KYN between wild-type and IDO2-deficient mice (59). Human TDO gene sequence has a low homology (16%) with the IDO1, but their protein catalytic domains have a high similarity (60) and TDO is predominantly expressed in the liver (61). Similar to IDO1, upregulated IDO2 and TDO may be associated with immune escape in some types of tumors (53, 55, 62, 63). Previous studies have reported that TDO enhances the migration and invasion of glioblastoma and breast cancer cells *in vitro* and treatment with a TDO inhibitor significantly inhibits distant metastasis in mice (64–66). Furthermore, $IDO2^{-/-}$ mice display a decreased tumor size compared with wild-type mice (67). Pharmacological inhibition of TDO reduces the number of lung tumor nodules in mice (68). Apparently, enhanced IDO2 and TDO expression and activity may promote the progression and metastasis of LC and their activity is indistinguishable (49). Similar to the function of IDO1, upregulated IDO2 expression is linked to worse prognosis in NSCLC (53). Therefore, IDO2 inhibitors may be valuable for targeting LC and IDO2 may be a biomarker for immunotherapy (69). Moreover, there is little information on whether IDO2 expression is associated with resistance to cisplatin in LC patients and what the value of IDO2 is in diagnosis and prognosis of LC (26, 63). Therefore, further studies are warranted to address these questions.

Try METABOLITES AND IMMUNE ESCAPE IN LC

The immune escape is a "hallmark of cancer" (70, 71). Tumor immune escape refers to the phenomenon, in which tumor cells can grow and metastasize by avoiding recognition and attack by the immune system through various mechanisms (72). Currently, IDO1 has been suggested to be important for immune escape of LC. First, upregulated IDO1 expression promotes the degradation of Try and the accumulation of its metabolites (such as KYN, 3-HAA and others) in LC. These metabolites act on various immune cells, including T cells (naive CD4+ T cells, Th17, and Treg), antigen-presenting cells (APC, DCs, and macrophages), and NK cells, and lead to immune escape. The promising mechanisms by which Try metabolites induce cancer immune tolerance and immunosuppression are summarized in **Figure 1**.

First, Try is an essential amino acid for immune cell proliferation, and Try depletion results in T-cell apoptosis, which is one major reason for cancer immunosuppression (73). The decreased Try levels can inhibit T-cell proliferation by activating general control over nonderepressible 2 (GCN2) kinase and suppressing the mTOR signaling, a target of rapamycin (74–76). The GCN2 is a serine/threonine kinase and can phosphorylate eukaryotic initiation factor 2a kinase (eIF2a) in the presence of low concentration of Try, inhibiting protein synthesis and T cell proliferation (74). Activated GCN2 can also promote the differentiation of naïve CD4+ T cells into Tregs (16, 74, 77). Furthermore, GCN2 can alter the phenotype of DCs and macrophages (75, 76, 78), making them prone to immunosuppression to promote tumorigenesis. In contrast, other studies argue that GCN2 is a sensor of amino acid starvation and its activation is not dependent on a low Try level, rather than deficiency in many amino acids (79, 80). Actually, T cells with GCN2 deficiency have similar activity to wild-type T cells in B16 melanoma-bearing mice (79), which contradicts the tumor promotion of GCN2. Apparently, there may be another mechanism that senses Try-deprived condition to regulate T cell immunity against tumor. The mTOR signaling appears to be a possible candidate (81, 82) because inhibition of mTOR complex 1 (mTORC1) can induce T-cell autophagy and anergy in the tumor microenvironment (83). Moreover, mTORC1 inhibition can also induce Treg cells to suppress anti-tumor immune responses (82).

Second, increased KYN can lead to immune tolerance by inhibiting T cell proliferation and inducing T cell apoptosis to promote tumor growth (38). The KYN is a ligand of AhR, and its activation promotes Treg cell differentiation that can directly inhibit anti-tumor immune responses, contributing to cancer immune escape (77, 84). The AhR activation can also direct DCs and macrophages toward an immune-suppressive phenotype (85–87). The AhR activation enhances IL-10 synthesis and secretion, and inhibits the IFN-β signaling in DCs, but induces IL-10 and IFN-α production in NK cells, respectively. Consistently, higher frequency of Tregs is detected in mice bearing cisplatin-resistant tumors than those bearing cisplatin-sensitive tumors (26).

Third, the downstream metabolites (such as 3-HAA and QA) of KYN can also induce T-cell apoptosis (88), contributing to immune tolerance. Recent studies have shown that QA can inhibit the proliferation of cancer-specific CD8+ cytotoxic T

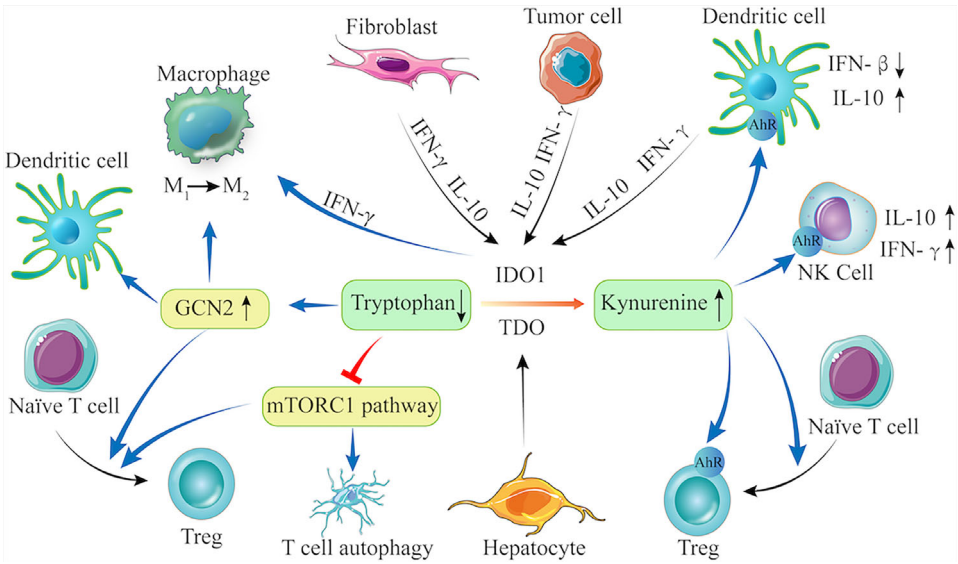

FIGURE 1 | The Try-IDO1/TDO-KYN pathway and immune escape. IDO1 is constitutively expressed in fibroblast, tumor cells, and DCs, and can be upregulated by IL-10 and IFN-γ, whereas TDO is only expressed in hepatocytes. When IDO1 and TDO are activated, they promote Try degradation and KYN accumulation. Try depletion can activate GCN2 and inhibit the mTORC1 signaling. The KYN can bind to AhR in NK cells, Tregs, and DCs. Therefore, the Try-IDO1/TDO-KYN pathway cooperatively modulates immune cells (e.g., DCs, macrophage, Treg, and T cells) to regulate anti-inflammatory cytokine production, leading to enhanced immunosuppression in the tumor microenvironment.

and NK cells to promote tumor growth (89). Furthermore, LC patients with lower plasma 3-HAA, the precursor of QA, benefit more from ICI treatment, suggesting that plasma 3-HAA levels may be a biomarker for predicting the response of LC patients to ICIs (23). The lower plasma 3-HAA may reflect less immunosuppression in patients because 3-HAA can promote Treg responses to produce high levels of TGF-β that decrease effector T-cell function, leading to immunosuppression (90). However, its precise mechanism in tumor immunity is not clear.

Next, IDO1 expression can be regulated by cytokines, such as IL-10 and IFN-γ (91, 92) while IDO1 inhibition can enhance T-cell proliferation and infiltration in the tumor environment and IL-2 production (93). Furthermore, IDO1 or IDO2 deficiency can modulate the tumor microenvironment by reducing KTR, enhancing immune cell infiltration and IFN-γ production (67). TDO and IDO2 act as the Try-metabolizing enzymes and can also promote Try degradation, resulting in immune regulation similar to IDO1. However, there are few reports and further studies are needed.

Last, IDO1 and TDO catalyze the production of several downstream Try metabolites, such as KYN (64, 84), KA (94), and XA (66), which can activate the AhR and may contribute to the immune modulation of IDO1 and TDO. Interestingly, KYN can directly bind and activate the AhR, with a high affinity at low picomolar levels (95). However, whether similar mechanisms also apply to other polar metabolites that activate the AhR, such as KA, remains to be investigated. In addition, AhR can regulate IDO-related regulatory phenotype in DCs (96). Here, an autocrine IDO–KYN/AhR–IDO feedback loop may contribute to the immune modulation (97, 98).

THE CLINICAL APPLICATIONS OF Try METABOLITES IN LC

Enhanced IDO1 expression and activity can evade immunosurveillance and are associated with poor prognosis of LC. Therefore, inhibition of IDO1 may be an ideal strategy for intervention of LC. There are several direct IDO1 inhibitors available, including epacadostat and navoximod that neither directly kill tumor cells, nor spontaneously initiate an immune response (99). Unlike epacadostat, the Try mimetic indoximod (D-1MT, 1-methyl-D-tryptophan) is the first non-enzyme inhibitory drug that targets the IDO1 pathway and can inhibit lung tumor growth *in vivo* (100–102). Indoximod can directly act on immune cells by creating an artificially Try-related signal, relieving the IDO1-mediated immunosuppression (99). There are ongoing clinical trials that investigate anti-IDO1 agents as monotherapy or adjuvant therapies with other drugs for various solid tumors. The clinical trials of anti-IDO1 agents for different combination strategies, such as combination with ICIs, other immunomodulators, and chemotherapy, are summarized in **Table 1**.

Epacadostat, a small-molecule IDO1 inhibitor, was developed by Incyte and is being tested for its therapeutic efficacy and safety in an advanced stage of clinical trial (103). The phase I/II KeyNote 037/ECHO 301 trial to test the safety and efficacy of different doses of epacadostat combined with 200 mg pembrolizumab (i.e., an anti-PD1 agent) every 3 weeks (Q3W) in 62 patients with different types of cancers has achieved promising results (104). There were 24% of patients experiencing high-grade toxicities but no treatment-related

TABLE 1 | Clinical trials for the potential drugs targeting the IDO1/TDO-KYN pathway.

Indication	Tumor type	Combination	Status	ClinicalTrials.gov	Phase
IDO inhibitor: Epacadostat	Metastatic NSCLC	Pembrolizumab	Complete	NCT03322540	II
	Metastatic NSCLC	Pembrolizumab and Platinum-based Chemotherapy	Completed	NCT03322566	II
	NSCLC	Nivolumab	Terminated	NCT03348904	III
	Extensive Stage Small Cell Lung Cancer	Pembrolizumab	Withdrawn	NCT03402880	II
	NSCLC, UC	Atezolizumab	Terminated	NCT02298153	I
	Advanced Solid Tumor, NSCLC	Sirolimus	Recruiting	NCT03217669	I
	Solid Tumor, Advanced Malignancies, Metastatic Cancer	Azacitidine and Pembrolizumab	Completed	NCT02959437	I/II
	Solid Tumor, Head and Neck Cancer, Lung Cancer, UC	Durvalumab (MEDI4736)	Completed	NCT02318277	I/II
	B-cell Malignancies, CRC, Head and Neck Cancer, LC, Lymphoma, Melanoma, Ovarian Cancer, Glioblastoma	Nivolumab	Completed	NCT02327078	I/II
	Microsatellite-instability High CRC, Endometrial Cancer, Head and Neck Cancer, HCC, GC, Lung Cancer, Lymphoma, RCC, Ovarian Cancer, Solid Tumor, UC, Melanoma, Bladder Cancer, TNBC	Pembrolizumab	Active, not recruiting	NCT02178722	I/II
	NSCLC	Pembrolizumab and chemotherapy	Completed	NCT02862457	I
	Solid Tumor	INCB001158 and Pembrolizumab	Terminated	NCT03361228	I/II
	Advanced Malignancies, Metastatic Cancer	INCAGN01876 and Immune Therapies	Completed	NCT03277352	I/II
	Solid Tumor	Pembrolizumab and Chemotherapy	Completed	NCT03085914	I/II
	Solid Tumor	Nivolumab and Immune Therapies	Active, not recruiting	NCT03347123	I/II
IDO inhibitor: Navoximod	Advanced solid tumor	–	Completed	NCT02048709	I
IDO inhibitor: BMS-986205	NSCLC	Nivolumab and Chemotherapy	Withdrawn	NCT03417037	III
	Advanced Cancer, Melanoma, NSCLC	Nivolumab and Ipilimumab	Recruiting	NCT02658890	I/II
IDO inhibitor: MK-7162	Advanced solid tumor	Pembrolizumab	Recruiting	NCT03364049	I
IDO inhibitor: LY3381916	LY3381916 alone or in combination with LY3300054 in solid tumors	LY3300054	Recruiting	NCT03343613	I
IDO inhibitor: KHK2455	Locally advanced or metastatic solid tumors	Mogamulizumab	Recruiting	NCT02867007	I
IDO pathway modulator: Indoximod (D-1-MT)	NSCLC, Progression of NSCLC, NSCLC Recurrent	Tergenpumatucel-L and docetaxel	Active, not recruiting	NCT02460367	I
	Metastatic or refractory solid tumors	N/A	Completed	NCT00567931	I
	Relapsed or Refractory Solid Tumors	–	Terminated	NCT00739609	I
IDO pathway modulator: NLG-802	Advanced solid tumors	N/A	Recruiting	NCT03164603	I
Dual IDO1/TDO inhibitor: HTI1090/ SHR9146	Advanced solid tumors	SHR-1210 and apatinib	Not yet recruiting	NCT03491631	I
	Advanced solid tumors	N/A	Recruiting	NCT03208959	I
IDO Peptide Vaccination	NSCLC	–	Completed	NCT01219348	I

Data accessed from https://www.clinicaltrials.gov/ on January 15, 2021. UC, urothelial cancer; CRC, colorectal cancer; HCC, hepatocellular carcinoma; RCC, renal carcinoma; N/A, not applicable.

death and 12 out of 22 patients obtained objective responses. Unfortunately, a further phase III clinical trial with epacadostat 100 mg twice a day (BID) and pembrolizumab 200 mg (Q3W) failed to improve progression-free survival (PFS) in patients with metastatic melanoma (105). Because of the limitations of this trial, further clinical trials are necessary to test its therapeutic efficacy and safety.

The phase I ECHO-110 study was designed to test epacadostat at different doses combined with atezolizumab (i.e., an anti-PD-L1 agent) 1,200 mg Q3W in 29 patients with

stage IIIB/IV NSCLC, who had previously been treated with ≥1 prior line of platinum-based chemotherapy (≥2 cycles), but not with checkpoint/IDO inhibitors. Similarly, 7 out of 29 patients displayed high-grade toxicities but no treatment-related death. Epacadostat at a dose up to 300 mg BID combined with atezolizumab 1,200 mg Q3W was well-tolerated in patients with previously treated NSCLC (103). However, only one patient achieved objective response. The low therapeutic efficacy may stem from the fact of almost all patients with negative PD-L1 expression. Similarly, the single-arm combination of the ECHO-301 trial also failed, lining with the results from other Phase II and III trials conducted in different settings (17) and was converted into the randomized phase II trials of epacadostat combined with pembrolizumab in LC patients. In addition, the KEYNOTE-715-06/ECHO-306-06 trials with the combination of epacadostat, pembrolizumab, and platinum-based chemotherapy did not obtain promising benefit in overall response rate in NSCLC patients (Clinicaltrial.gov.). These observations suggest that the combination of Epacadostat and a PD-1/PD-L1 blockade may not be valuable for patients with PD-L1 negative LC. However, whether this treatment strategy can achieve positive responses in PD-L1 expressing NSCLC or whether combination with platinum-based chemotherapy can achieve a better outcome in NSCLC patients has not been clarified. The ongoing, randomized, phase 2 ECHO-305 (NCT03322540) and ECHO-306 (NCT03322566) trials may give promising results.

New IDO inhibitors, such as navoximod (NLG-919/GDC-0919) and BMS-986205, are also being tested in clinical trials (106). In a phase I study of the IDO1 inhibitor, combination of navoximod and atezolizumab displayed acceptable safety, tolerability, and pharmacokinetics, but not clear beneficial evidence of navoximod in patients with advanced solid tumors (107).

There are questions on whether epacadostat doses used in the ECHO-301 trial could effectively inhibit IDO1 activity in the tumor microenvironment and whether targeting multiple enzymes in the KYN pathway to control Try metabolism would benefit to these patients (58).

There are also ongoing trials testing IDO1 and TDO dual inhibitors such as HTI-1090 (SHR9146) as a monotherapy for solid tumors (NCT03208959). The dual inhibitor of DN1406131 is being tested for its safety in healthy subjects (NCT03641794) while RG70099 from Curadev/Roche is still in preclinical development (108).

In a word, most researchers have focused on IDO/TDO inhibitors for the treatment of LC, and some of them have already been tested in clinical trials. However, the current therapeutic efficacy appears limited. Thus, further studies are necessary to understand the biological functions of Try and its metabolites in the development and progression of LC. Given that the KYN downstream metabolites have profound functions in regulating T cell immunity against LC, these metabolites and their catalyzing enzymes may be explored for development of therapies for LC. Similarly, combination of IDO/TDO inhibitors and other therapies (chemotherapy, radiotherapy, targeted therapy, and immunotherapy) should be pursued to determine the safety and therapeutic efficacy in LC. Previous studies have demonstrated that patient's metabolism (BMI variation and hypercholesterolemia) has a significant impact on the outcome of PD1 inhibitor treatment in LC patients (109, 110). Some drugs can regulate body metabolism and are significantly related to clinical outcomes of ICI treatment in LC patients (111, 112). Metformin, an effective agent for the management of type 2 diabetes mellitus, in combination with ICI treatment can improve the anticancer effects of ICIs (113, 114). Statins can inhibit cholesterol production (115) and is associated with better clinical outcome of anti-PD1 treatment in advanced NSCLC patients in an intensity-dependent manner (111). However, IDO1 as an immune checkpoint is not as well studied as PD1, and the role of patient metabolism and drugs involved in its regulation on the outcome of patients treated with IDO/TDO inhibitors needs to be further confirmed. If demonstrated, IDO/TDO inhibitors may benefit many patients with LC.

CONCLUSION

Currently, modulation of Try metabolism has been used for diagnosis, prognosis, and therapies for LC. The levels of circulating IDO activity and downstream metabolites (3-HAA, QA, KA, etc.) can be used to predict the efficacy of different treatments in LC (116, 117). However, the results are inconsistent, which may be caused by limitations, such as small sample size, inconsistent measurement methods, influence of the gender, tumor stage, and tumor heterogeneity. Hence, further studies are needed in multi-centers with a larger population, standardized measurement methods, paired samples, and detailed analysis for different stages and pathological types of LC. Currently, some metabolites, enzyme inhibitors targeting immune checkpoints, and modulators have been developed for the diagnosis and treatment of LC. Because the change in metabolomics is one of the factors for the development of cancer, it will be wise to integrate the role of metabolomic changes in the pathogenesis of LC and consider other factors together for the development of therapeutic strategies for LC. Therefore, further studies are necessary to understand the process of complicated Try metabolism and its regulation in different types and stages of LC.

AUTHOR CONTRIBUTIONS

CL performed the literature search and drafted the manuscript and figures. HZ edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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ARID1A, ARID1B, and *ARID2* Mutations Serve as Potential Biomarkers for Immune Checkpoint Blockade in Patients With Non-Small Cell Lung Cancer

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Worldwide, non-small cell lung cancer (NSCLC) has the highest morbidity and mortality of all malignancies. The lack of responsiveness to checkpoint inhibitors is a central problem in the modern era of cancer immunotherapy, with the rapid development of immune checkpoint inhibitors (ICIs) in recent years. The human switch/sucrose nonfermentable (SWI/SNF) chromatin-remodeling complex has been reported to be recurrently mutated in patients with cancer, and those with SWI/SNF mutations have been reported to be sensitive to ICIs. Six reported cohorts, a total of 3416 patients, were used to analyze the mutation status of ARID1A, ARID1B, ARID2 and SMARCA4 in patients with NSCLC and the effect of mutations on prognosis after ICIs. Finally, a nomogram was established to guide the clinical use of ICIs. The results show that patients with NSCLC who have ARID1A, ARID1B, and ARID2 mutations of the SWI/SNF complex were more likely to benefit from ICI therapy.

Keywords: non-small cell lung cancer, immunotherapy, SWI/SNF complex, PD-1/PD-L1 inhibitors, anti-PD1/PD-L1

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INTRODUCTION

Lung cancer has the highest morbidity and mortality of all malignancies worldwide, with 80% - 85% of histological types diagnosed as non-small cell lung cancer (NSCLC). According to cancer statistics, worldwide, 9.6 million cancer deaths occurred in 2018, of which lung cancer showed the highest incidence and mortality (1). Recently, advances in understanding the complex relationship between tumor cells and the immune response have resulted in a paradigm shift in cancer immunology, and new and more effective approaches to cancer immunotherapy. Immune checkpoint inhibitors (ICIs), such as programmed cell death 1/programmed death ligand 1 (PD-1/PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) blockade, enable the adaptive immune response to recognize and kill tumor cells, revolutionizing the standard of care for several

cancers, including NSCLC. Several clinical trials have shown that ICI therapy is effective for first- and second-line treatments of advanced NSCLC, consolidated treatment of locally advanced NSCLC, and neoadjuvant treatment of early NSCLC. However, despite the promising efficacy of immunotherapy in NSCLC, the success of ICIs is currently limited to a small subset of patients, with the overall response rate to anti–PD-1 or PD-L1 therapy only 20%- 30% (2, 3). Thus, strategies are needed to identify the most suitable candidates for ICIs. To date, several clinical predictors of the ICI response in NSCLC have been identified (e.g., mutational and neoantigen loads, and PDL-1 expression), with PD-L1 expression being used in clinical practice to select patients for therapy. However, the quantitative detection of PD-L1 as a prediction index requires antibodies and staining platforms, which contribute to differences in the accuracy of PD-L1 levels, which may affect the predictive value. Moreover, clinical trials have shown that second-line treatment with anti–PD-1 or anti-PD-L1 antibodies may even be effective in patients with no PD-L1 expression on their tumor or immune cells (4), whereas patients with high PD-L1 expression sometimes fail to respond to anti–PD-1/PD-L1 therapy (5). The tumor mutation burden (TMB) (6), the total number of mutations per megabase in the coding regions of tumor cells, and neoantigen load, which indicate the neoantigens produced by tumor cells to active T cells, are other predictors of therapeutic efficacy. Some researchers have found that a high TMB and neoantigen load are associated with an improved response to ICI treatment (6–8), whereas others found no significant difference (9–11). Therefore, the establishment of new predictors to identify suitable candidates for immunotherapy is a central challenge in the modern era of cancer immunotherapy.

The human switch/sucrose nonfermentable (SWI/SNF) chromatin-remodeling complex is encoded by multi-gene families recurrently mutated in cancer. Previous studies have shown that tumors, such as renal clear cell carcinoma, harboring SWI/SNF mutations are sensitive to ICIs. Meanwhile, mutations in SWI/SNF complex genes, such as *SMARCA4*, *ARID1A*, *ARID1B*, and *ARID2*, affect the clinical outcomes of ICI treatments in patients with NSCLC. However, studies on the role of mutations of the SWI/SNF complex in ICI therapy for patients with NSCLC are lacking

In this study, publicly available profiles were collected and integrated, and a comprehensive analysis was performed to investigate the role of SWI/SNF complex gene mutations in the prognosis of patients with NSCLC treated with anti-PD-1/PD-L1 ICIs.

METHODS

Data Sources

Whole-exome sequencing (WES) data of 1144 NSCLC cases from The Cancer Genome Atlas (TCGA) cohort (12) was obtained through cBioPortal (http://www.cbioportal.org/). The RNA-seq data of 515 LUAD and 501 LUSC were downloaded from the TCGA (https://portal.gdc.cancer.gov/). Five available clinical cohorts with 2272 patients who underwent ICI therapy at the Memorial Sloan Kettering Cancer Center (MSKCC) (9, 13–16) were included in this study. Detailed information for each cohort is shown in **Table 1**. Neoantigen data were obtained using a tumor immunograph network (https://tcia.at/home) (17). Tumor-infiltrating lymphocytes based on RNA-sequencing (seq) data were obtained from TIMER (http://timer.comp-genomics.org/) (18).

Assessment of the TMB

Mutation profiles were assessed by WES in Hellmann (14), Naiyer (16), and TCGA cohorts and by next-generation sequencing in Zehir (13), Rizvi (15), and Samstein (9) cohorts. The TMB is the number of gene synonymous variants per million base-pairs detected in tumor tissue. The TMB was defined as the number of non-silent somatic mutation counts

TABLE 1 | Baseline data of 3416 patients with non-small cell lung cancer.

Characteristic		TCGA Cohort n=1144	Zehir Cohort n=1567	Samstein Cohort n=355	Hellmann Cohort n=75	Rizvi Cohort n=240	Naiyer Cohort n=35
Gender	Male	673 (59%)	681 (43%)	166 (48%)	37 (49%)	118 (49%)	16 (46%)
	Female	468 (41%)	886 (57%)	178 (52%)	38 (51%)	122 (51%)	19 (54%)
Age	>60	695 (71%)	NA	246 (72%)	47 (63%)	156 (65%)	19 (54%)
	<=60	253 (26%)	NA	98 (28%)	28 (37%)	86 (35%)	16 (46%)
Smoking status	Ever	976 (85%)	972 (62%)	NA	60 (80%)	197 (80%)	30 (86%)
	Never	111 (10%)	334 (21%)	NA	15 (20%)	47 (20%)	5 (14%)
	Unknown	57 (5%)	261 (17%)	NA	0	0	0
Histology	AD	660 (58%)	1268 (81%)	268 (78%)	Non-SCC:59 (79%)	186 (78%)	30 (86%)
	SCC	484 (42%)	163 (10%)	44 (13%)	16 (21%)	34 (14%)	4 (11%)
	Others	0	136 (9%)	30 (9%)	0	20 (8%)	1 (3%)
Treatment type	PD1/ PDL1	NA	NA	324 (91%)	0	206 (86%)	35 (100%)
	CTLA4	NA	NA	0	0	0	0
	Combo	NA	NA	20 (6%)	75 (100%)	34 (14%)	0
PDL1	PDL1	0	NA	NA	70 (93%)	86 (36%)	30 (86%)
	CD274	1015 (89%)	NA	NA	0	0	0
Neoantigen		1053 (92%)	NA	NA	75 (100%)	NA	NA

NA, Not Available.

in coding regions. A TMB-low population was defined as patients with <10 mut/MB (9).

Messenger RNA Expression Profiling Analysis of Immune-Related Signatures

Tumor immune microenvironment-related signatures, including chemokines, chemokine receptors, immunostimulators, and immunoinhibitors, were compared. Associations between SWI/SNF complex gene mutations and relevant immune-related genes were analyzed in 1016 patients from the TCGA cohort, for whom both RNA-seq and DNA-seq data were available. The list of immune genes was mainly based on published articles that summarized genes related to immunotherapy. The list of 63 immune genes is provided in **Supplementary Table 1**.

Construction of an Integrated Prognostic Classifier Model

As shown in **Supplementary Table 2**, univariate Cox regression analysis was used to screen for factors significantly associated with progression-free survival (PFS). Smoking history, treatment type (anti–PD-1/PD-L1 or anti-CTLA4), PD-L1 immunohistochemistry (IHC) score, TMB, SWI/SNF mutation status, and epidermal growth factor receptor (EGFR) mutation status were included for further analysis of the Rizvi cohort. A multivariate Cox regression analysis model was constructed using elected factors and "rms", "foreign", "survival", "tidyverse", and "survivalROC" packages of R. A calibration curve of the nomogram was made for internal verification. The risk score was calculated according to its regression coefficient, and patients were divided into low- and high-risk score groups according to the cutoff value. The Naiyer cohort was used as an external validation cohort to validate the model.

Statistical Analyses

Statistical analyses were performed using R version 3.6.2. The packages: "ggplot2", "rms", "foreign", "survivalROC", and "survival" were used for statistical and graphics analyses, and the packages "survival" and "survminer" were used for survival analysis. Pearson's correlation coefficient was used to analyze the correlation between two continuous variables. An independent sample *t*-test was used to compare two groups of samples. The Wilcoxon test was used to compare multiple groups of samples, and the log-rank test was used to compare two or more survival curves. $P < 0.05$ was considered statistically significant. The Benjamin & Hochberg method was used to adjust the *P* value.

RESULTS

Demographic and Clinical Characteristics of the Study Cohorts

Basic information of the six cohorts is shown in **Table 1**. A total of 3416 patients, 1711 females and 1691 males, were included in this study. The study participants comprised 2412 patients with adenocarcinoma and 745 patients with squamous carcinoma; 2235 patients were smokers and 512 were non-smokers, and the median age was 61 years. **Table 1** shows the demographic and clinical characteristics of the study cohorts.

SWI/SNF Complex Genes Were Frequently Mutated in Patients With NSCLC

Of the 3416 NSCLC patients, approximately 25% had at least one SWI/SNF complex gene mutation; of these, 9% harbored *SMARCA4* mutations, 8% harbored *ARID1A* mutations, 5% harbored *ARID2* mutations, and 4% harbored *ARID1B* mutations. **Figure 1** shows detailed mutations for each gene.

Additionally, SWI/SNF complex gene mutations rarely occurred simultaneously with V-Ki-ras2 Kirsten ratsarcoma viral oncogene homolog (KRAS) and EGFR mutations (**Supplementary Figure 1**).

The association between mutations of SWI/SNF complex genes with demographic and clinical factors, such as sex, age, smoking status, histology, and distant metastasis, was analyzed. Mutations in SWI/SNF complex genes were found to be significantly frequent in smokers, indicating that tobacco exposure may significantly impact mutations in the SWI/SNF complex. Additionally, *ARID1A* and *ARID2* mutations were more frequently found in males, *SMARCA4* was more frequently mutated in patients with

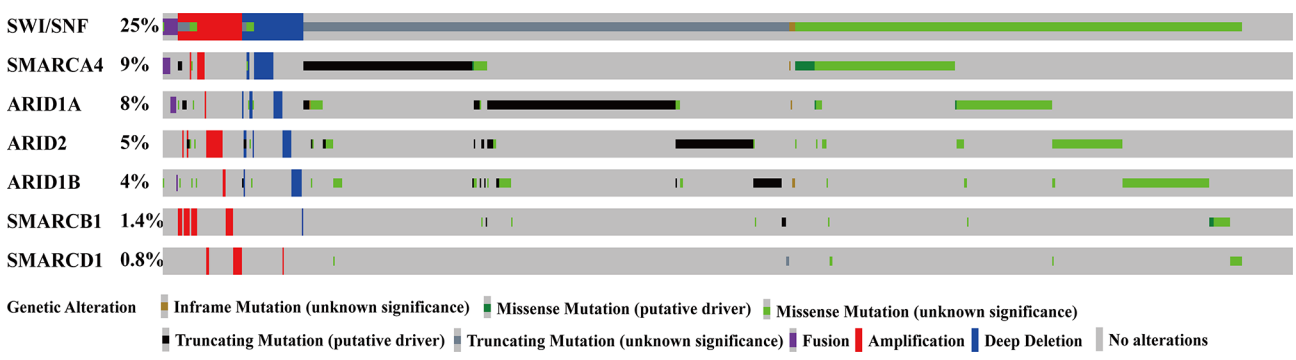

FIGURE 1 | SWI/SNF complex genes were frequently mutated in patients with NSCLC. SWI/SNF, human switch/sucrose nonfermentable; NSCLC, non-small cell lung cancer.

TABLE 2 | Correlation analysis of SMARCA4,ARID1A,ARID1B,ARID2,SMARCB1,SMARCD1 gene mutations with gender, pathological status, age, smoking status, whether distal metastasis.

Characteristic		Gender		Histology			Age		Smoking Status		Metastasis	
		Man	Woman	AD	SCC	Other	>60	<=60	Yes	No	Yes	No
SMARCA4	Wild	1386	1439	1944	683	157	714	267	2066	483	224	683
	Mutation	140	120	202	24	20	49	27	212	21	13	44
	P		0.140		<0.001		0.119		<0.001		0.871	
ARID1A	Wild	1399	1454	1994	651	150	723	273	2085	482	223	685
	Mutation	133	105	152	56	27	40	21	193	22	14	42
	P		0.039		0.457		0.235		0.002		1	
ARID2	Wild	1430	1495	2024	677	171	724	277	2149	483	221	691
	Mutation	96	64	122	30	6	39	17	129	21	16	36
	P		0.006		0.139		0.663		0.178		0.369	
ARID1B	Wild	1470	1488	2076	664	170	721	272	2172	495	225	687
	Mutation	56	71	70	43	7	42	22	106	9	12	40
	P		0.216		0.001		0.227		0.003		0.925	
SMARCB1	Wild	1506	1549	2130	703	173	754	290	2254	500	233	724
	Mutation	20	10	16	4	4	9	4	24	4	4	3
	p		0.058		0.619		0.811		0.597		0.117	
SMARCD1	Wild	1516	1550	2133	703	175	737	278	2261	503	236	722
	Mutation	10	9	13	4	2	5	3	17	1	1	5
	P		0.782		0.905		0.539		0.165		1	

adenocarcinoma, and the *ARID1B* mutation was more frequently found in patients with squamous carcinoma, which were all statistically significant (**Table 2**).

ARID1A, *ARID1B*, and *ARID2* Mutations Are Associated With Better Outcomes for Patients With NSCLC Treated With ICIs

No significant difference in PFS and overall survival (OS) was observed between wild-type (WT) and *SMARCA4* mutation groups in either Samstein or Hellmann, Rizvi, and Naiyer (HRN) cohorts. However, patients with *ARID1B* mutations had a better median PFS [mPFS; 22.4 *vs.* 4; hazard ratio (HR) = 0.442; 95% confidence interval (95% CI) = 0.235–0.833; P = 0.0092; **Supplementary Figure 2A**]. Patients with *ARID1A*, *ARID1B*, and *ARID2* mutations had better OS, although the difference was not significant; this may be due to the limited number of cases with mutations (**Supplementary Figure 1B**). Patients with an *ARID1A* or *ARID1B* mutation treated with ICIs had a median OS (mOS) of 21 months compared to 11 months for the WT group. Patients with an *ARID2* mutation treated with ICIs had an mOS of 36 months compared to 11 months for the WT group (**Supplementary Figure 2B**).

ARID1A, *ARID1B*, and *ARID2* analyses were combined. Patients with at least one mutation in one of the three genes were defined as the SWI/SNF complex mutation group, and the remaining patients were defined as the WT group. In a survival analysis of the two cohorts, for the HRN cohort, the mPFS of the mutant and WT groups was 6.2 *vs.* 3.8 months, respectively (P = 0.0069; HR = 0.638; 95% CI = 0.459–0.887; **Figure 2A**), whereas the mOS of the mutant and WT groups was 22 *vs.* 10 months, respectively (P = 0.0089; HR = 0.604; 95% CI = 0.408–0.894) in the Samstein cohort (**Figure 2B**).

We suspected that the increase in the number of cumulative mutations in SWI/SNF complex genes would improve immunotherapy efficacy. Therefore, patients without SWI/SNF complex gene mutations were defined as the WT group, patients with one mutation were defined as the one-mutation group, and patients with two or more mutations were defined as the co-mutation group. Survival analysis of the HRN and Samstein cohorts demonstrated that the mPFS values in the HRN cohort of the one-mutation and WT groups were 6.2 and 3.8 months, respectively (P = 0.025; HR = 1.907; 95% CI = 0.474–7.675; **Figure 2C**), whereas mOS in the Samstein cohort of the one-mutation and WT groups was 22 vs. 10 months, respectively (P = 0.032; HR = 1.883; 95% CI = 0.467–7.594) in the Samstein cohort (**Figure 2D**). However, due to the small number of patients, the co-mutation group only showed a better mPFS or mOS than the mutation group in the HRN cohort (**Figures 2C, D**).

Tendency of Patients With SWI/SNF Mutations to Have High TMB and Neoantigen Loads

In the Rizvi and Hellmann groups, higher PD-L1 IHC scores were observed in the any *SWI/SNF* and *ARID1B* mutation groups, and lower PD-L1 IHC scores were observed in the *SMARCA4* mutation group (**Supplementary Figure 3A**).

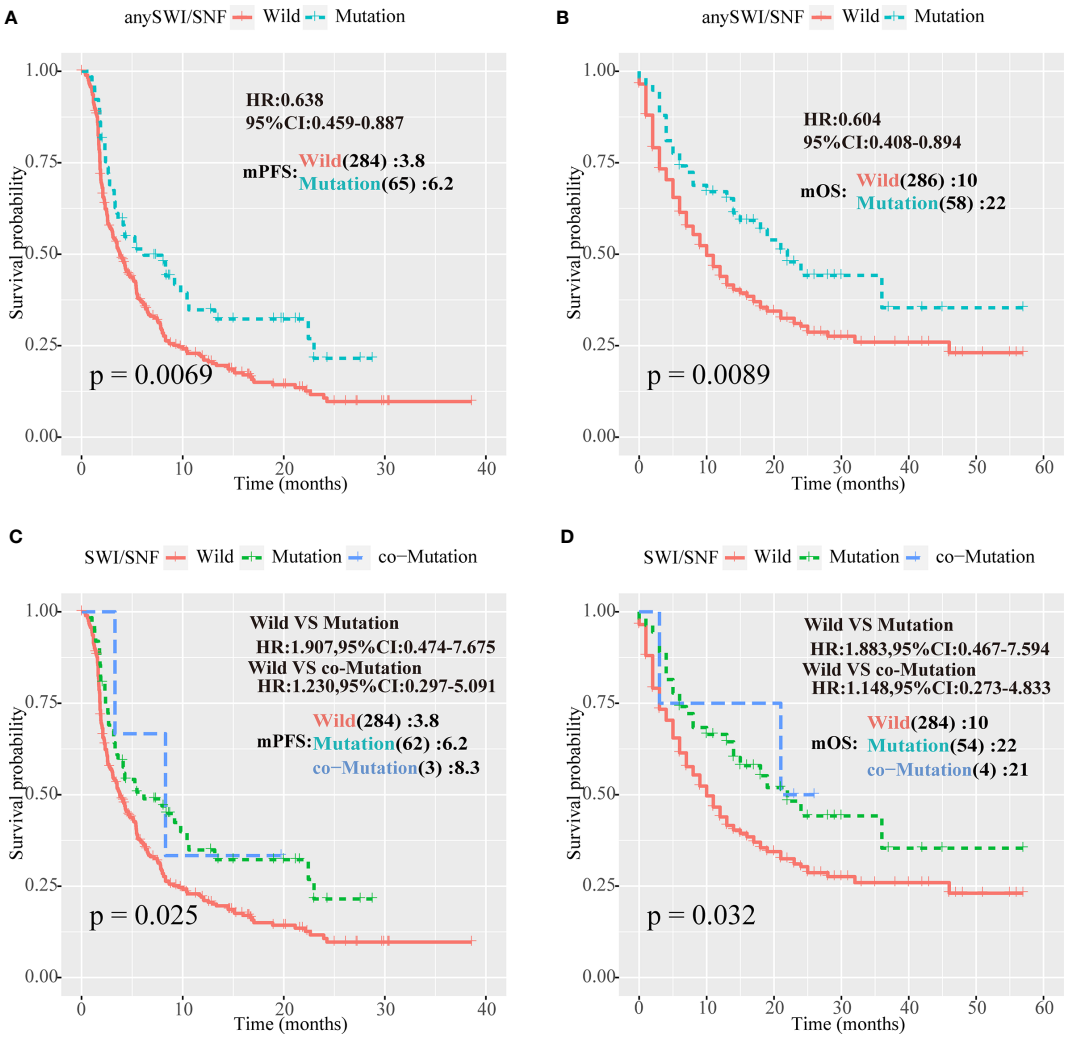

FIGURE 2 | Human switch/sucrose nonfermentable (SWI/SNF) complex mutations were associated with better outcomes for patients with NSCLC treated with PD-1/PD-L1 inhibitors. **(A, B)** Survival curves of progression-free survival (PFS) for the Hellmann, Rizvi, and Naiyer (HRN) cohort, and of overall survival (OS) for the Samstein cohort according to *ARID1A*, *ARID1B*, and *ARID2* mutations in patients with non-small cell lung cancer (NSCLC). Patients with at least one mutation in one of the three genes were part of the human switch/sucrose nonfermentable (SWI/SNF) complex mutation group, and the remaining patients were part of the wild-type (WT) group. **(C, D)** PFS curve for the Rizvi cohort and OS for the Samstein cohort according to *ARID1A*, *ARID1B*, and *ARID2* mutations in NSCLC patients. Patients with no mutations in any of the three genes formed the WT group, patients with one mutation were part of the one-mutation group, and patients with two or more mutations formed the co-mutation group.

In patients with NSCLC and low PD-L1 scores (<50), the mPFS of patients with any SWI/SNF complex mutation was superior to that of WT patients treated with ICIs (8.3 *vs.* 3.7 months; P = 0.001; HR = 0.420; 95% CI = 0.246–0.717; **Supplementary Figure 3B**).

In the Zehir, Samstein, Rizvi, and Hellmann cohorts, the TMB of patients with *ARID1A*, *ARID1B*, and *ARID2* mutations of the SWI/SNF complex mutation group was significantly higher than that of the WT group (P < 0.001; **Figure 3A**, left). Similarly, in the TCGA cohort, the TMB of patients with *ARID1A*, *ARID1B*, and *ARID2* gene mutations was significantly higher than that of the WT group (P < 0.001; **Figure 3A**, right).

In TMB-high (>10) patients with NSCLC, the mPFS of patients with any SWI/SNF complex mutation was superior to that of WT patients (8.3 *vs.* 3.8 months; P = 0.058; HR = 0.618; 95% CI = 0.374–1.022; **Figure 3C**). In TMB-high patients with NSCLC in the Samstein cohort, the mOS of patients with any SWI/SNF complex mutation was significantly superior to that of WT patients (36 *vs.* 12 months; P = 0.028; HR = 0.536; 95% CI = 0.302–0.954; **Figure 3D**). There was no significant difference between any SWI/SNF mutation and WT subgroups in the mPFS or mOS of TMB-low patients with NSCLC in the two cohorts (**Supplementary Figures 3C, D**). Moreover, in the non-ICIs treated NSCLC population, the mutations of the SWI/SNF

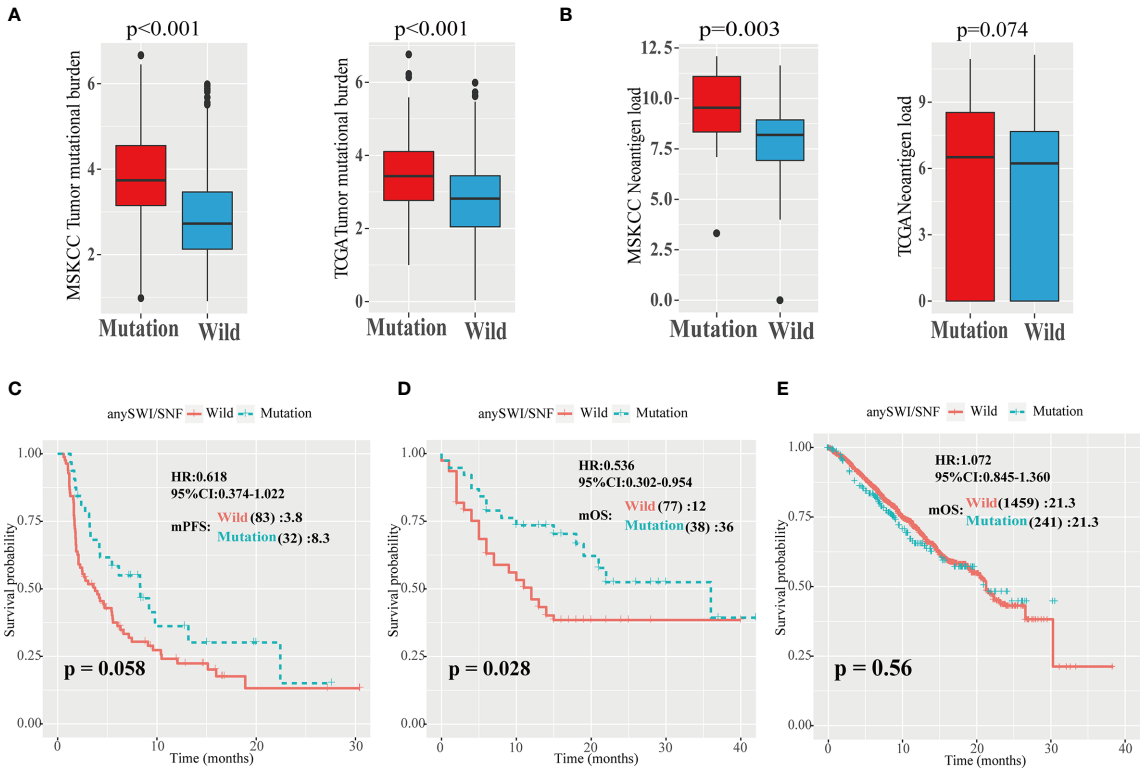

FIGURE 3 | High TMB and neoantigen load of patients with SWI/SNF mutations. **(A)** Analysis of tumor mutation burden (TMB) values in five independent Memorial Sloan Kettering Cancer Center (MSKCC) cohorts, including the Zehir, Samstein, Rizvi, Naiyer, and Hellmann cohorts (left), and The Cancer Genome Atlas (TCGA) cohort (right). **(B)** Analysis of neoantigen load in Hellmann (left) and TCGA (right) cohorts. **(C, D)** Progression-free survival (PFS) curves of patients with non-small cell lung cancer (NSCLC) in the TMB-high group of the Hellmann, Rizvi, and Naiyer (HRN) **(C)** and Samstein **(D)** cohorts based on *ARID1A*, *ARID1B*, and *ARID2* mutations. SWI/SNF, human switch/sucrose nonfermentable. **(E)** Overall survival (OS) curves of patients with non-small cell lung cancer (NSCLC) in the Zehir cohort based on the human switch/sucrose nonfermentable (SWI/SNF) mutation status.

complex did not have a better survival benefit (**Figure 3E**). Therefore, SWI/SNF is a prognostic indicator and a true predictor independent on PD-L1 and TMB.

The relationship between neoantigen load and SWI/SNF complex mutations was also explored. It was found that patients with any SWI/SNF complex gene mutation had elevated neoantigen loads (P = 0.003; **Figure 3B**).

Decreased Activated Dendritic Cells and Monocyte Infiltration, and Altered Immune Microenvironment, in NSCLC Patients With *ARID1A*, *ARID1B*, or *ARID2* Mutation

To investigate correlations between the infiltration of immune cells and SWI/SNF complex gene mutations, 22 immune cell types were analyzed using expression data from the TCGA dataset. The immune infiltration levels of monocytes, myeloid dendritic cell activated, and T-cell CD4$^+$ memory resting cells were decreased in patients with an *ARID1A*, *ARID1B*, or *ARID2* mutation. However, macrophage M1 and T-cell follicular helper cell levels were increased in patients with an *ARID1A*, *ARID1B*, or *ARID2* mutation (**Figures 4A, B**).

The expression levels of chemokines, chemokine receptors, immunoinhibitors, and immunostimulators were also analyzed to further explore whether SWI/SNF complex mutations affect the expression of immune-related cytokines (**Supplementary Table 1**). Patients with SWI/SNF complex gene mutations were found to have lower expression levels of the following gene clusters: chemokines (CCL17, CXCL17, and CXCL16; **Figure 4C**), chemokine receptors (CXCR2, CXCR1, and CCR2; **Figure 4C**), immunoinhibitors (BTLA, CD244, HAVCR2, and LGALS9; **Figure 4D**), and immunostimulators (NT5E and TMIGD2; **Figure 4D**).

Construction of an Integrated Prognostic Classifier Model for Predicting the Efficacy of ICI Therapy

Univariate analysis showed that PD-L1 score, TMB, SWI/SNF mutation status, smoking history, EGFR mutation status and treatment type, were statistically significant in predicting PFS in the Rizvi cohort. A nomogram was then developed to predict 6- and 12-month PFS using the above six factors in the Rizvi cohort (**Figure 5A**). Receiver operating characteristic (ROC) analysis

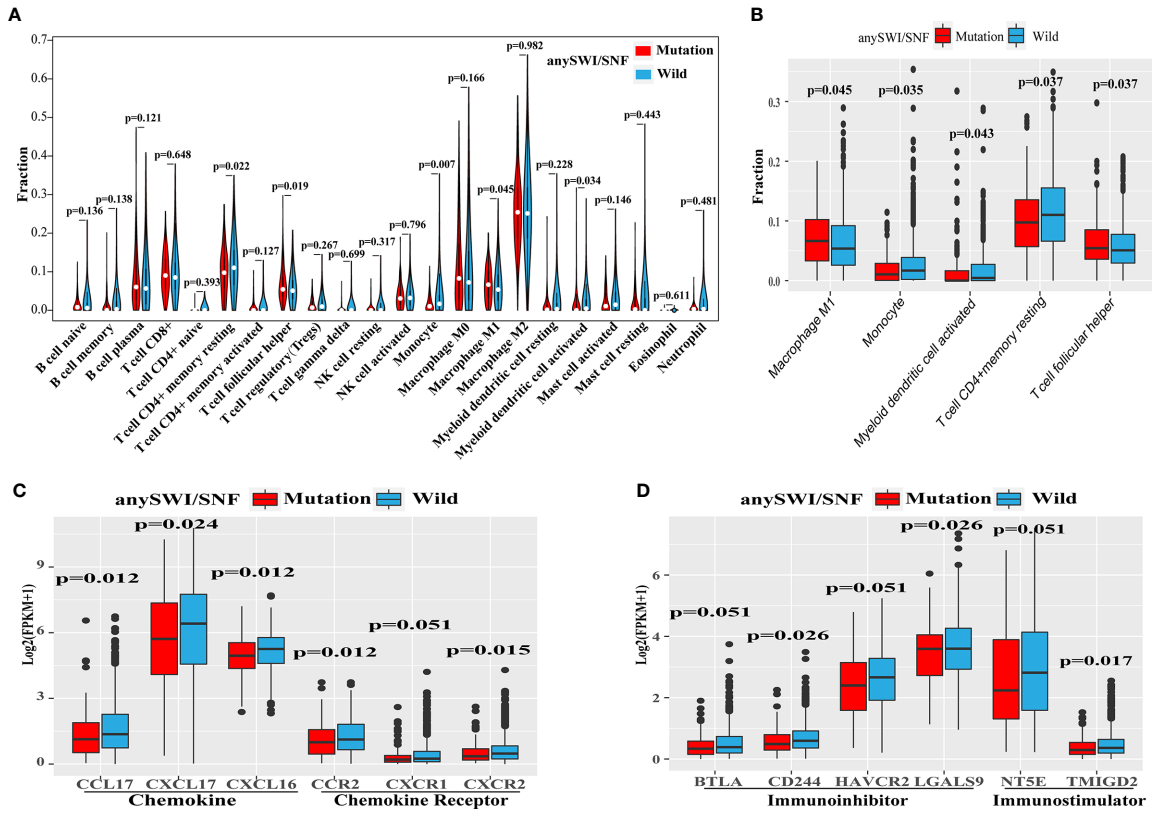

FIGURE 4 | Altered immune microenvironment in SWI/SNF mutation patients. **(A)** Violin plot of the relative infiltration of 22 immune cell types in The Cancer Genome Atlas (TCGA) cohort. **(B)** Immune infiltration of monocytes or dendritic cells according to the human switch/sucrose nonfermentable (SWI/SNF) complex mutation status of the TCGA cohort. **(C, D)** Expression of chemokines or chemokine receptors **(C)** and immunoinhibitors or immunostimulators **(D)** according to the SWI/SNF complex mutation status of the TCGA cohort.

indicated good accuracy of this model (area under the curve [AUC] of 6-month survival, 0.779; AUC of 12-month survival, 0.854; **Figure 5B**); the calibration curve also suggested an acceptable accuracy (**Figure 5D**). The PFS survival curve showed that the low-risk group had a better mPFS than the high-risk group (6.6 *vs.* 2.5; $P < 0.001$; HR = 2.847; 95% CI = 1.761–4.691; **Figure 5C**). Furthermore, the Naiyer cohort was used as an external validation cohort to verify the prognostic value of this immune signature. The ROC curve suggested that this immune signature was highly consistent with the ideal model (AUC of 6-month survival, 0.824; AUC of 12-month survival, 0.901; **Figure 5E**). The PFS survival curve showed that the low-risk group also had a better mPFS than the high-risk group (14.5 *vs.* 3.3; $P = 0.0015$; HR = 3.442; 95% CI = 1.288–9.197; **Figure 5F**).

DISCUSSION

The lack of responsiveness to checkpoint inhibitors is a central problem in the modern era of cancer immunotherapy. At present, a PD-L1 score measured by IHC is the standard predictive biomarker for anti-PD-1/PD-L1 ICI therapy. However, clinical trials have shown the deficiency of this biomarker as a predictor of such therapy (2, 3).

In this study, SWI/SNF complex genes were frequently mutated in patients with NSCLC. Furthermore, patients with NSCLC treated with PD-1/PD-L1/CTLA-4 inhibitors and having *ARID1A*, *ARID1B*, or *ARID2* mutations of the SWI/SNF complex showed better outcomes in comparison to those without such mutations. The mOS of patients with at least one of these mutations was 22 months compared to 10 months for the WT group ($P = 0.0089$; HR = 0.604; 95% CI = 0.408–0.894; **Figure 2B**), whereas the mPFS of patients with at least one of these mutations was 6.2 *vs* 3.8 months for the WT group ($P = 0.0069$; HR = 0.638; 95% CI = 0.459–0.887; **Figure 2A**). Additionally, cumulative mutations of the SWI/SNF complex were beneficial to the efficacy of ICI therapy. The mPFS for the co-mutation group was 8.3 months compared to 3.8 months for the WT group (**Figure 2C**). Moreover, in the non-ICIs-treated NSCLC population, the mutations of the SWI/SNF complex did not have a better survival benefit (**Figure 3E**). This indicates that the SWI/SNF complex mutation has a survival benefit for NSCLC patients treated with ICIs. Furthermore, a comprehensive predictive classifier model was built to evaluate the efficacy of ICI therapy according to SWI/SNF mutation status and clinical factors, such as smoking history, treatment type, PD-L1 score, and TMB.

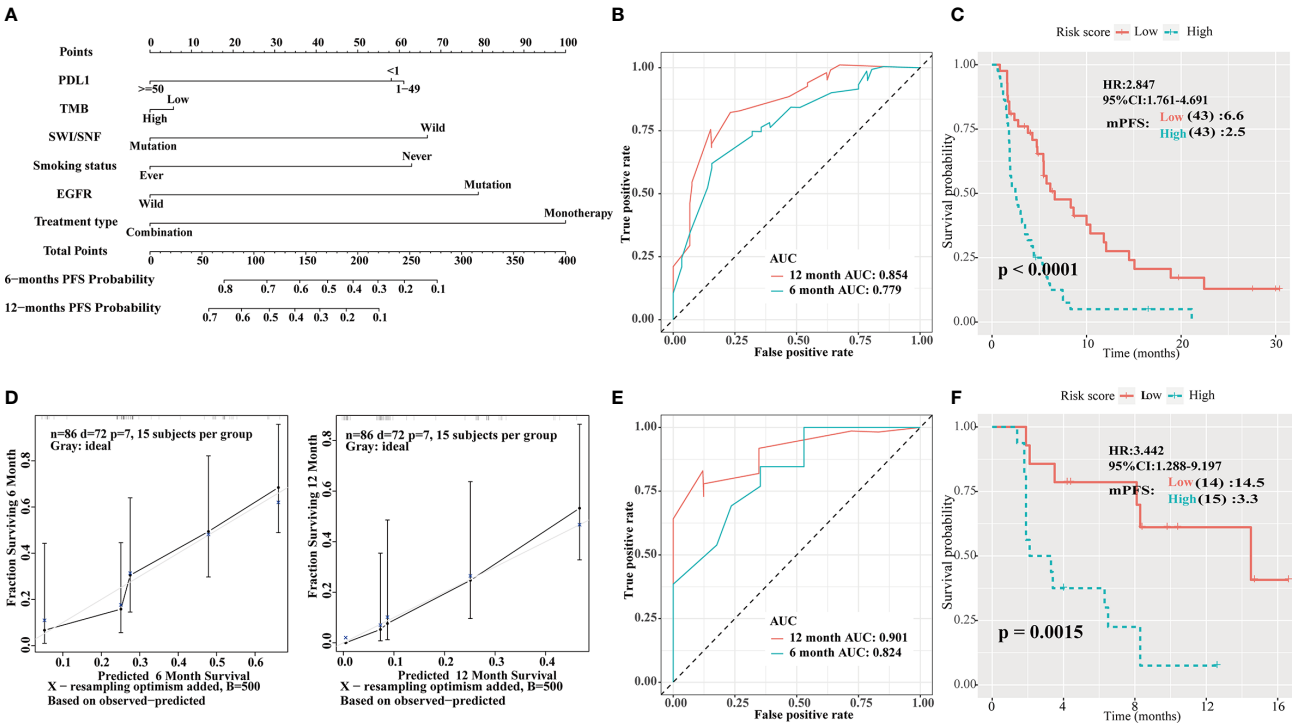

FIGURE 5 | Construction of an integrated prognostic classifier model. **(A)** Nomogram based on programmed death ligand 1 (PD-L1) score, tumor mutation burden (TMB), human switch/sucrose nonfermentable (SWI/SNF) mutation status, smoking history, epidermal growth factor receptor (EGFR) mutation status, and treatment type of the Rizvi cohort. **(B)** Receiver operating characteristic (ROC) curves for predicting progression-free survival (PFS) of the nomogram in the Rizvi cohort. **(C)** Calibration plot of the nomogram for the probability of PFS at 6 (left) and 12 (right) months in the Rizvi cohort. **(D)** Survival curve of PFS with the nomogram in the Rizvi cohort. The risk score was calculated according to the regression coefficient. The cohort was divided into low- and high-risk score groups for Kaplan-Meier curve analysis. **(E)** ROC curves for predicting PFS of the nomogram in the Naiyer cohort. **(F)** Survival curve of PFS with the nomogram according to the risk score in the Naiyer cohort.

ROC curves for 6 and 12 months were drawn. AUCs were calculated as 0.779 and 0.854 for the test cohort, and 0.824 and 0.901 for the validation cohort, respectively. The risk score was calculated according to the regression coefficient. The low-risk group showed better mPFS (2.5 *vs.* 6.6; $P < 0.001$; HR = 2.847; 95% CI = 1.761–4.691 for the Rizvi cohort; **Figure 5D** and 3.3 *vs.* 14.5; $P = 0.0015$; HR = 3.442; 95% CI = 1.288–9.197 for the Naiyer cohort; **Figure 5F**). These results revealed the roles of *ARID1A*, *ARID1B*, and *ARID2* mutations in predicting the outcome for patients with NSCLC treated with ICIs. These findings indicated that a comprehensive model, including SWI/SNF complex mutation status and other clinical factors, will guide the use of immunotherapy and provide a reference for individualized immunotherapy against NSCLC.

The central function of the SWI/SNF complex is the coordinated regulation of gene expression programs by remodeling chromatin structure and regulating transcription by remodeling nucleosome occupancy at critical DNA elements. To investigate whether mutations of the SWI/SNF complex can influence the expression of PD-L1, scores for PD-L1 were compared between datasets from Rizvi and Hellmann cohorts, in which PD-L1 scores were available from IHC assays. PD-L1 mRNA expression levels were also compared to the TCGA dataset, in which PD-L1 RNA-sequencing data were available. Higher PD-L1 scores were observed in the *ARIDA1B* mutation group and lower PD-L1 scores were observed in the *SMARCA4* mutation group, with no significant difference in mRNA expression in the TCGA cohort. Further investigation will help reveal whether the SWI/SNF complex is involved in the regulation of PD-L1 and thus whether it plays a role in mediating immune escape in the context of lung cancer.

In this study, *ARID1A*, *ARID1B*, and *ARID2* gene mutations of the SWI/SNF complex were associated with increased TMB and neoantigen load. TMB, the total number of mutations per megabase in the coding regions of tumor cells, reflects the instability of tumor cells (8, 19). Because the activation of adaptive immunity requires antigen recognition, increased antigen recognition indicates a greater immune response. A high TMB may indicate that more neoantigens can be produced by tumor cells to activate T cells suppressed by immune checkpoint molecules. As increased TMB is associated with increased neoantigen load, this is usually associated with greater immunogenicity and a stronger immune response (19). Furthermore, our study also revealed that although a difference between any SWI/SNF mutation and WT subgroups was not apparent in terms of mPFS or mOS in TMB-low patients with NSCLC, in TMB-high patients, the mPFS or mOS of patients with *ARID1A*, *ARID1B*, or *ARID2* mutations was superior to those of WT patients (8.3 *vs.* 3.8 months; $P = 0.058$; HR = 0.618; 95% CI =

0.374–1.022 for PFS; and 36 *vs.* 12 months; $P = 0.028$; HR = 0.536; 95% CI = 0.302–0.954 for OS). These results indicated that, in TMB-high patients, *ARID1A*, *ARID1B*, and *ARID2* mutations indeed enhanced the immune response to PD-1/PD-L1 blockade. Additionally, the mPFS of PD-L1-low patients with at least one of these mutations was 8.3 months compared to 3.7 months for the WT group ($P = 0.001$; HR = 0.420; 95% CI = 0.246–0.7170; **Supplementary Figure 3C**). The mOS of TMB-high patients with at least one of these mutations was 36 months compared to 12 months for the WT group ($P = 0.028$; HR = 0.536; 95% CI = 0.302–0.954; **Figure 3D**).

These results also indicated that the immune microenvironment was altered in NSCLC patients who had *ARID1A*, *ARID1B*, or *ARID2* mutations. Compared with patients of the WT group, patients with mutations showed decreased the percentage of M1 macrophages, T helper cells, resting memory CD4+ T cells, monocytes and activated dendritic cells. In previous reports, the increased infiltration of M1 macrophages and follicular T helper cells is related to the better prognosis of lung cancer (20, 21). Meanwhile, the activation of resting memory CD4+ T cells has been reported to contribute to the progression and development of lung adenocarcinoma (22). Monocytes have also been reported as immunosuppressive cells in small cell lung cancer (23). Presently, there is still a lack of research on the relationship between the above cell infiltration and SWI/SNF complex. Moreover, the expression of chemokines (CCL17, CXCL17, and CXCL16), chemokine receptors (CXCR2, CXCR1, and CCR2), immunoinhibitors (BTLA, CD244, HAVCR2, and LGALS9), and immunostimulators (NT5E and TMIGD2) was reduced (**Figure 4C, D**). Cytokines play an important role in the differentiation, maturation, and migration of various immune cells (24, 25). CCL17, CXCR2, LGALS9 and NT5E recruits regulatory T cells into tumors as a mechanism of anti-tumor immune impairment (26–29). CXCL17 induces immature myeloid dendritic cells to infiltrate human pancreatic cancer, thereby promoting the immune response (30, 31). CXCL16 also plays an important role in enhancing the immune function of breast cancer by attracting T cell infiltration (32). Meanwhile, monocytes recruited by CCR2 will increase the number of lung metastases in breast cancer (33). BTLA and HAVCR2 mediate the inhibition of human tumor specific CD8 + T cells (28, 34), and CD244 mediates the dysfunction of natural killer cells (35). The relationship between these genes and the SWI/SNF complex is still unknown. Activated T-cell recruitment to tumor sites is necessary to mediate tumor cell killing (33). The efficacy of anti-PD-1 immunotherapy can be predicted according to the degree of immune cell infiltration, as determined by chemokines and entry through tumor blood vessels (33, 36). Therefore, further investigation of the roles of the SWI/SNF complex and the immune microenvironment will help us understand the mechanism of PD-1/PD-L1 blockade.

CONCLUSION

The results of this study demonstrated that patients with *ARID1A*, *ARID1B*, or *ARID2* mutations were more likely to benefit from ICIs. A clinical prognosis prediction model will help guide the use of immunotherapy in patients with NSCLC and provide a reference for individualized immunotherapy of NSCLC in the 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

GZ, YL and RS wrote the main manuscript text. ZZ, SX, and CC prepared Figures. PC, HZ, ML, and ZP contributed to data analysis. HL and JC contributed to data acquisition. 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/fimmu.2021.670040/full#supplementary-material

Supplementary Figure 1 | Human switch/sucrose nonfermentable (SWI/SNF) complex gene mutations rarely occurred simultaneously with KRAS and EGFR mutations.

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TNF-Alpha Pathway Alternation Predicts Survival of Immune Checkpoint Inhibitors in Non-Small Cell Lung Cancer

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Translational research on immune checkpoint inhibitors (ICIs) has been underway. However, in the unselected population, only a few patients benefit from ICIs. Therefore, screening predictive markers of ICI efficacy has become the current focus of attention. We collected mutation and clinical data from an ICI-treated non-small cell lung cancer (NSCLC) cohort. Then, a univariate Cox regression model was used to analyze the relationship between tumor necrosis factor α signaling mutated (TNFα-MT) and the prognosis of immunotherapy for NSCLC. We retrospectively collected 36 NSCLC patients (local-cohort) from the Zhujiang Hospital of Southern Medical University and performed whole-exome sequencing (WES). The expression and mutation data of The Cancer Genome Atlas (TCGA)-NSCLC cohort were used to explore the association between TNFα-MT and the immune microenvironment. A local cohort was used to validate the association between TNFα-MT and immunogenicity. TNFα-MT was associated with significantly prolonged overall survival (OS) in NSCLC patients after receiving immunotherapy. Additionally, TNFα-MT is related to high immunogenicity (tumor mutational burden, neoantigen load, and DNA damage response signaling mutations) and enrichment of infiltrating immune cells. These results suggest that TNFα-MT may serve as a potential clinical biomarker for NSCLC patients receiving ICIs.

Keywords: TNFα, NSCLC, ICIs, biomarker, tumor microenvironment

INTRODUCTION

Lung cancer is a disease with very high morbidity and mortality among all malignant tumors in the world (1–3). In the past decades, the 5-year overall survival (OS) rate of patients with advanced lung cancer has been only 5% (4). Histologically, lung cancer is mainly divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for more than 85% of all lung cancer cases and is the most common histological subtype (5, 6). The current main treatment plan for NSCLC is a comprehensive treatment based on surgery, radiotherapy, chemotherapy and molecular targeted therapy.

With the advent of the era of precision medicine, targeting programmed cell death protein 1/programmed cell death ligand-1 (PD-1/PD-L1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) has revolutionized cancer treatment and improved the long-term survival rate of patients with advanced NSCLC (7–10). However, growing evidence have shown that anti-PD-1/PD-L1 monotherapy produces long-lasting (>6 months) clinical benefits for only a small number of patients (15% to 19.4% in phase I/II clinical trials) (7–9, 11); thus, biomarkers with high specificity and detection rates are needed to predict the efficacy of PD-1/PD-L1 immune checkpoint inhibitors (ICIs).

Currently, PD-L1 expression is approved as a biomarker for immunotherapy (12, 13); PD-L1 is an inducible and dynamic biomarker for ICI treatment for multiple cancer types. Additionally, PD-L1 is expressed not only on the surface of tumor cells but also on immune cells in tumor tissues, and its expression can be affected by cell growth mediator such as IFNγ. Therefore, the expression is still an imperfect biomarker for predicting the efficacy of anti-PD-1/PD-L1 therapy in NSCLC (14–16). Tumor mutational burden (TMB) can also be used as a marker for determining the efficacy of immunotherapy. However, these markers also have some limitations (17–19). For example, it is difficult to standardize the "high" and "low" cut-off of TMB, the consistency of using different platforms to detect TMB, and the DNA quality assessment methods of biopsy specimens. Thus, screening predictictive biomarkers of ICI efficacy has become the current focus of clinical practice.

Growing evidence shows that specific pathway mutations or specific gene mutations are related to the prognosis of immunotherapy (20, 21). The ZFHX3 mutation is associated with a favorable prognosis for NSCLC receiving ICIs. Studies have shown that the damaged DNA repair mechanism, which results in enhanced immunogenicity and a high mutation load (22). The damaged DNA repair mechanism in patients with NSCLC indicated a sensitive response to PD-1/PD-L1 inhibitors (23). Teo et al. showed that DNA damage response (DDR) pathway mutations may be related to a satisfactory clinical response and significantly prolonged progression-free survival (PFS) and OS in patients with urothelial carcinoma after receiving immunotherapy (21). In addition, Wang et al. showed that comutations in the DDR pathway can be a potential marker for immunotherapy in multiple tumor types (20).

Recently, the immune microenvironment has been discovered to play a vital role in the efficacy of immunotherapy. Studies have shown that tumor-infiltrating lymphocytes (TILs), cytotoxic signatures, and pro-inflammatory mediators are related to favorable immunotherapy efficacy and clinical outcomes (24–26). The past decade has witnessed the importance of a thorough understanding of the cell-intrinsic mechanisms that determine a tumor's susceptibility to T cell antitumor activity, which was beginning to provide key mechanistic insights into the clinical benefit of potentiating tumor-intrinsic signaling for boosting responses to ICIs (27). The activation of tumor-intrinsic signaling regulates and promotes the immunosuppressive tumor microenvironment, which includes exclusion and dysfunction of effective immunocytes and recruitment and differentiation of immunosuppressive cells (28). TNFα, as a weighted marker of Th1 cells, further mediates antitumor immunity and promotes tumor senescence (29). TNFα promotes the transformation and antitumor functions of TILs and increases the efficacy of ICIs (30). Vredevoogd et al. found that selective reduction of the TNF cytotoxicity threshold increases the susceptibility of tumors to immunotherapy (31). TNF-related apoptosis-inducing ligand (TRAIL) contribute to the antitumor activity of cytotoxic T cells by inducing proliferative arrest and/or apoptosis (32). However, the impact of TNFα-MT on the clinical prognosis of NSCLC patients undergoing immunotherapy is still unclear and needs further exploration. In this study, we mainly analyzed how the mutation status of the TNFα pathway affects the prognosis of ICIs in NSCLC patients from the aspects of tumor immunogenicity and the immune microenvironment.

METHODS

Clinical Samples

We used the cBioPortal to download mutation data and clinical data from an NSCLC cohort receiving ICIs (33). This cohort, with 344 patients with NSCLC, was defined as the ICI-treated cohort for subsequent analysis. Additionally, we retrospectively collected 36 NSCLC patients (defined as local cohort) from the Zhujiang Hospital of Southern Medical University and performed whole-exome sequencing (WES). Sample preparation, sequencing and raw data processing methods are detailed in the supplementary methods. This study was approved by the Ethics Committee of the Zhujiang Hospital of Southern Medical University, and the patients signed informed consent forms. We used the "TCGAbiolinks" package (34) to download the clinical data, transcription data and mutation data of the TCGA-LUAD and TCGA-LUSC cohorts. We combined the TCGA-LUAD and TCGA-LUSC cohorts into one cohort (TCGA-NSCLC cohort) and used this cohort for downstream analysis. The clinical characteristics of ICI-treated NSCLC, local-NSCLC and TCGA-NSCLC cohort were shown in the **Tables S1–S3**.

Mutation Data Preprocessing and Immunogenicity Data

First, the mutation data were screened with the maftools package (35) according to the nonsynonymous mutation types. Then, we collected the TNFα pathway gene set from the Molecular Signatures Database (MSigDB) (**Table S4**). If the number of mutations in the pathway was 0, then the sample was considered wild type (TNFα-WT); otherwise, it was considered mutant (TNFα-MT). The definitions of TNFα-WT and TNFα-MT were applied to all cohorts in this study. Regarding TMB, TMB score in the ICI-treated cohort was directly obtained from the public data set; in the local cohort and the TCGA-NSCLC cohort, TMB was calculated according to published study. Additionally, the neoantigen load (NAL) and MANTIS scores in the TCGA-NSCLC cohort were reported by previous researchers (36, 37).

The DNA damage response (DDR) pathway gene set was obtained from the MSigDB (38). We used the number of nonsynonymous mutations to estimate the number of DDR pathway mutations.

Immune Microenvironment Analysis and Gene Set Enrichment Analysis

The expression data from the NSCLC cohort and the CIBERSORT algorithm (39) (1000 iterations; parameters: default) were used to evaluate the proportions of twenty-two immune cell types. Additionally, immune-related genes, immune checkpoint-related genes and immune cell fractions were obtained from previous studies. The limma package was used to analyze differences in the expression data of NSCLC patients. After the difference analysis, the data were used as input in the clusterProfiler package (40), and the enrichment scores (ESs) of Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Reactome pathways were calculated.

Statistical Analysis

A univariate Cox regression model was used to evaluate the effect of the TNFα pathway and clinical characteristics on the prognosis of patients in the ICI-treated cohort, and hazard ratios (HRs) and 95% confidence intervals (CIs) were used to evaluate their influence. The Wilcoxon rank-sum test was used to compare the differences in continuous variables between the two groups. Fisher's exact test was used to compare the differences in categorical variables between the two groups. Kaplan-Meier (KM) analysis was used to evaluate the relationship between TNFα-MT and OS, and the log-rank P value was used to reflect significant differences. $P < 0.05$ was considered statistically significant, and all statistical tests were two-sided. R software (version 3.6) was used for statistical analysis.

RESULTS

TNFα-MT Is a Predictor of Prolonged Survival for Patients Receiving Immunotherapy

To explore whether the mutation status of the TNFα pathway can predict the prognosis of patients receiving ICIs for NSCLC, we downloaded the mutation data and survival data of an ICI-treated NSCLC cohort from the cBioPortal website (39). The detailed analysis process is shown in **Figure 1A**. Next, we divided all patients into two groups based on the nonsynonymous mutation status of the TNFα pathway, namely, the TNFα-MT group and the TNFα-WT group. Clinical data, such as age (old *vs*. young), sex (male *vs*. female), histological type (non-LUAD *vs* LUAD), and sample type (metastasis *vs* primary), other pathways (WNT signaling and INFγ singnaling) were not related to the survival of patients in the ICI-treated cohort, but the mutation status of the TNFα pathway was closely associated with the clinical prognosis of patients receiving ICIs ($P < 0.05$; **Figure 1B**). Compared with the TNFα-WT group, the TNFα-MT group had a significantly longer OS (log-rank $P = 0.02$; HR = 0.72; 95% CI: 0.55-0.95; **Figure 1C**).

A Panoramic View of Gene Mutations in Different TNFα-MT States

To explore the differences in the frequencies of somatic mutations between TNFα-MT and TNFα-WT, we analyzed the top 20 somatic mutations in the ICI-treated cohort and the TCGA-NSCLC cohort. First, in the ICI-treated cohort, we found that among the top 20 mutated genes, the TNFα-MT group had higher mutation rate of TP53 (71% *vs* 58%; $P < 0.05$), FAT1 (15% *vs* 7%; $P < 0.05$) and ARID1A (13% *vs* 6%; $P < 0.05$). Concerning other clinical information, including age, sex, histological type and sample type, there was no significant difference between the TNFα-MT and TNFα-WT groups (**Figure 2A**). Next, we conducted a similar analysis on the TCGA-NSCLC cohort (**Figure 2B**), and the results showed that the TNFα-MT group had a significantly higher frequency of somatic mutations, including all 20 with the highest mutation frequencies ($P < 0.05$), but the only tumor suppressor gene included in these mutations was TP53. Compared with the TNFα-WT group, the TNFα-MT group had a higher proportion of men ($P < 0.01$). The results of the mutual exclusivity analysis of the top 20 mutated genes in the ICI-treated and TCGA-NSCLC cohorts are shown in **Figure S1**.

The TNFα-MT Group Has Higher Immunogenicity Than the TNFα-WT Group

To explore the difference between the immunogenicity of the TNFα-MT and TNFα-WT groups, we further elaborated on the number of mutations in the DDR pathway, TMB and NAL. First, we downloaded the gene sets of 8 DDR pathways from the MSigDB and merged all genes related to the DDR pathway into a merged DDR pathway. In the ICI-treated cohort, we found that the TNFα-MT group had a significantly higher number of mutations in the double-strand break (DSB), Fanconi anemia (FA), homologous recombination (HR), nucleotide excision repair (NER), nonhomologous end joining (NHEJ), single-strand break (SSB), and merged DDR pathways than the TNFα-WT group (all $P < 0.05$; **Figure 3A**). In the TCGA-NSCLC cohort, the TNFα-MT group had more mutations in all DDR-related pathways (all $P < 0.05$; **Figure 3B**). Then, we used the local cohort from the Zhujiang Hospital of Southern Medical University for further verification. In the local cohort, we also found that TNFα-MT patients had a higher number of mutations in the DDR pathway (all $P < 0.05$; **Figure 3C**). Additionally, there was a significant difference in DDR signaling mutations according to the mutation status of different TNFα pathways. Regardless of the cohort examined (i.e., the ICI-treated cohort, the TCGA-NSCLC cohort or the local cohort), the TNFα-MT group had a higher TMB than the TNFα-WT group (all $P < 0.05$; **Figures 3D–F**). The TCGA-NSCLC cohort has a significantly high NAL (**Figure 3G**). The MANTIS score can be used to evaluate the microsatellite instability (MSI) status; the higher the score is, the closer its status is to microsatellite instability-high (MSI-H). The MANTIS score of the TNFα-MT group was significantly higher than that

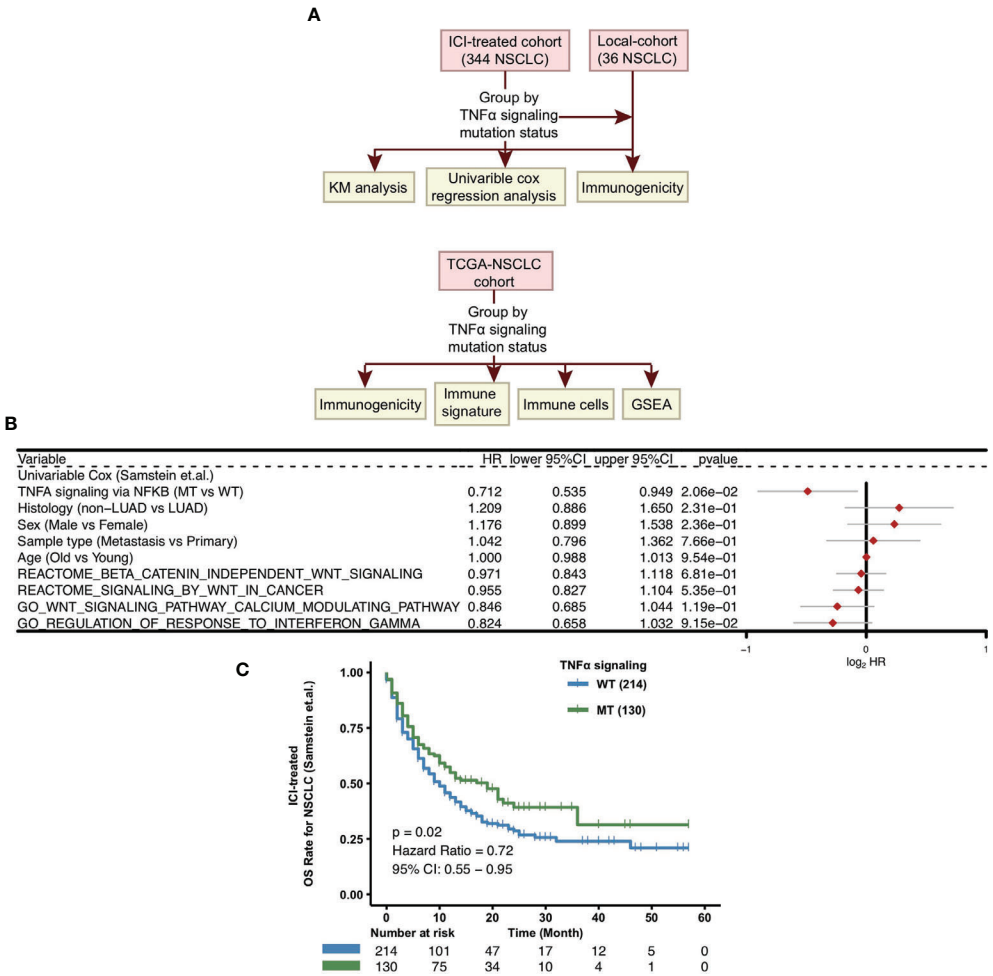

FIGURE 1 | Predictive values of clinical characteristics and the TNFα signaling mutation status on ICI outcomes. **(A)** Flow chart of the establishment of the clinical cohorts and subsequent analyses. **(B)** Forest plot of the results of the univariate Cox regression analyses. **(C)** KM survival curves for OS in NSCLC patients from the ICI-treated cohort. NSCLC, non small-cell lung cancer; TCGA, The Cancer Genome Atlas; ICI, immune checkpoint inhibitors; OS, overall survival; KM, Kaplan Meier.

of the TNFα-WT group (**Figure 3H**). In addition, the patients in the TNFα-MT group smoked more pack years than those in the TNFα-WT group (P <0.05; **Figure 3I**).

Differences in Immune Microenvironment Between the TNFα-MT and TNFα-WT Groups

To explore the differences in the immune microenvironment between the TNFα-MT and TNFα-WT groups, we compared immune-related genes, immune cell signatures and immune cell types. As the target of ICIs, immune checkpoints are very important in the course of immunotherapy. In the TCGA-NSCLC cohort, we found that the expression levels of PD-L1 (CD274), LAG3 and CD276 were significantly higher in the TNFα-MT group than in the TNFα-WT group (all P <0.05; **Figure 4A**). **Figure 4B** shows typical cases for each TPS level (3 TNFα-MT *vs* 3 TNFα-WT cases). Additionally, some immune-related genes, such as cytotoxicity markers (GZMB), chemokine markers (CXCL9 and CXCL10) and cytokine-related genes (IFNG), were significantly increased in the TNFα-MT group (all P <0.05); **Figure 4C**). At the level of immune cell infiltration, the TNFα-MT group showed a significant enrichment in M1 macrophages, activated memory CD4+ T cells, CD8+ T cells and follicular helper T cells (all P <0.05; **Figure 4D**). Correlation analysis showed that a high number of mutations in TNFα signaling were associated with a high infiltration level of activated immune cells (such as activated memory CD4+ T cells, CD8+ T cells and follicular helper T cells) (R > 0; P <0.05; **Figure 4E**). In contrast, the number of mutations in TNFα signaling was negatively associated with the proportion of Tregs (R <0; P <0.05; **Figure 4E**). The difference analysis of some immune-related signatures showed that the TNFα-MT group had significantly more BCR richness and higher proportions of Th2 cells and TILs than the TNFα-WT group (all P <0.05; **Figure 4F**).

GSEA can be used to examine differences of the enrichment degree of signaling activity between two groups. Therefore, we

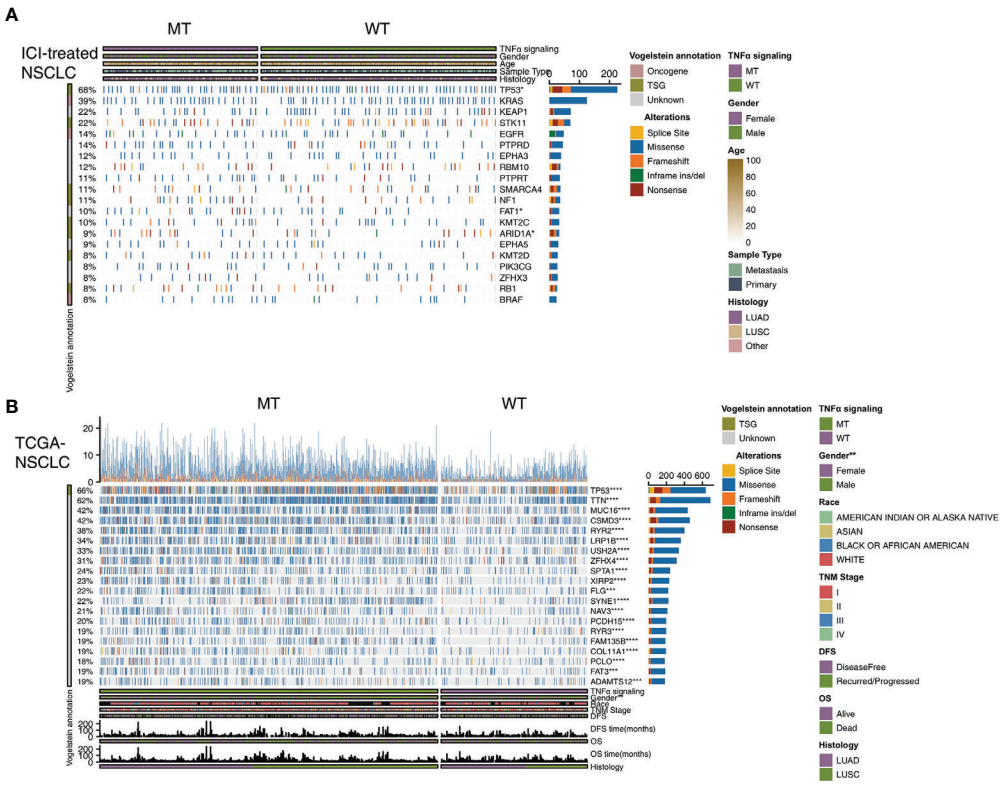

FIGURE 2 | Genomic profiles of NSCLC patients in the ICI-treated **(A)** and TCGA-NSCLC **(B)** cohorts. The top 20 genes with the highest mutation frequencies and the corresponding clinical information are shown in the figure. (*P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001; Fisher's exact test). NSCLC, non small-cell lung cancer; TCGA, The Cancer Genome Atlas; ICI, immune checkpoint inhibitors.

used GSEA to compare the ESs between the TNFα-MT and TNFα-WT groups. GSEA showed that the activities of immune-related pathways, such as lymphocyte migration activities involved in the inflammatory response, negative regulation of B cell apoptosis, BCR downstream activity, antigen processing and presentation, were significantly increased in the TNFα-MT group (all P <0.05, ES >0; **Figure 5**).

DISCUSSION

Although ICIs have changed the treatment strategies of NSCLC patients with the development of immunotherapy in recent years, only a small number of patients fully or partially respond to and benefit from ICIs (24, 25, 41). Therefore, for NSCLC patients to better produce an antitumor immune response from ICI treatment and obtain better prognostic outcomes, it is necessary to identify clinically predictive markers. As a stimulatory cytokine, TNFα contributes to the antitumor activity of cytotoxic T cells by inducing proliferative arrest and/or apoptosis, and further enhances tumor cytotoxicity threshold to T cell-derived TNF (31). In this study, we explored the association between TNFα-MT and the prognosis of NSCLC patients receiving ICIs. First, through a univariate Cox regression model and KM analysis, it was found that only TNFα-MT was associated with a favorable prognosis of patients receiving ICIs. Next, we aimed to explain why TNFα-MT was associated with improved clinical benefits in patients from the perspective of the immune microenvironment (**Figure 6**). Patients with TNFα-MT have significantly higher immunogenicity, proportion levels of infiltrating activated immune cells, expression levels of chemokines and cytotoxic markers and MANTIS scores than patients with TNFα-WT. Additionally, we retrospectively collected 36 NSCLC samples from the Zhujiang Hospital of Southern Medical University to further verify the results described above.

ICIs exert an antitumor effect by restoring T cell-mediated antitumor immune function and have become the new clinical treatment approaches for NSCLC. The tumor microenvironment (TME) consists of blood vessels, cancer-associated fibroblasts (CAFs), the extracellular matrix (ECM) and TILs (42). Studies have shown that a local immune imbalance in tumor tissues or tissues surrounding tumors; the systemic immune status, including the number and activity of T cell subsets; antigen recognition, capture, and presentation capabilities; and other host immune stress capabilities also affect immune checkpoints, important aspects that affect the clinical efficacy of inhibitors (19, 24, 25, 41, 43–46).

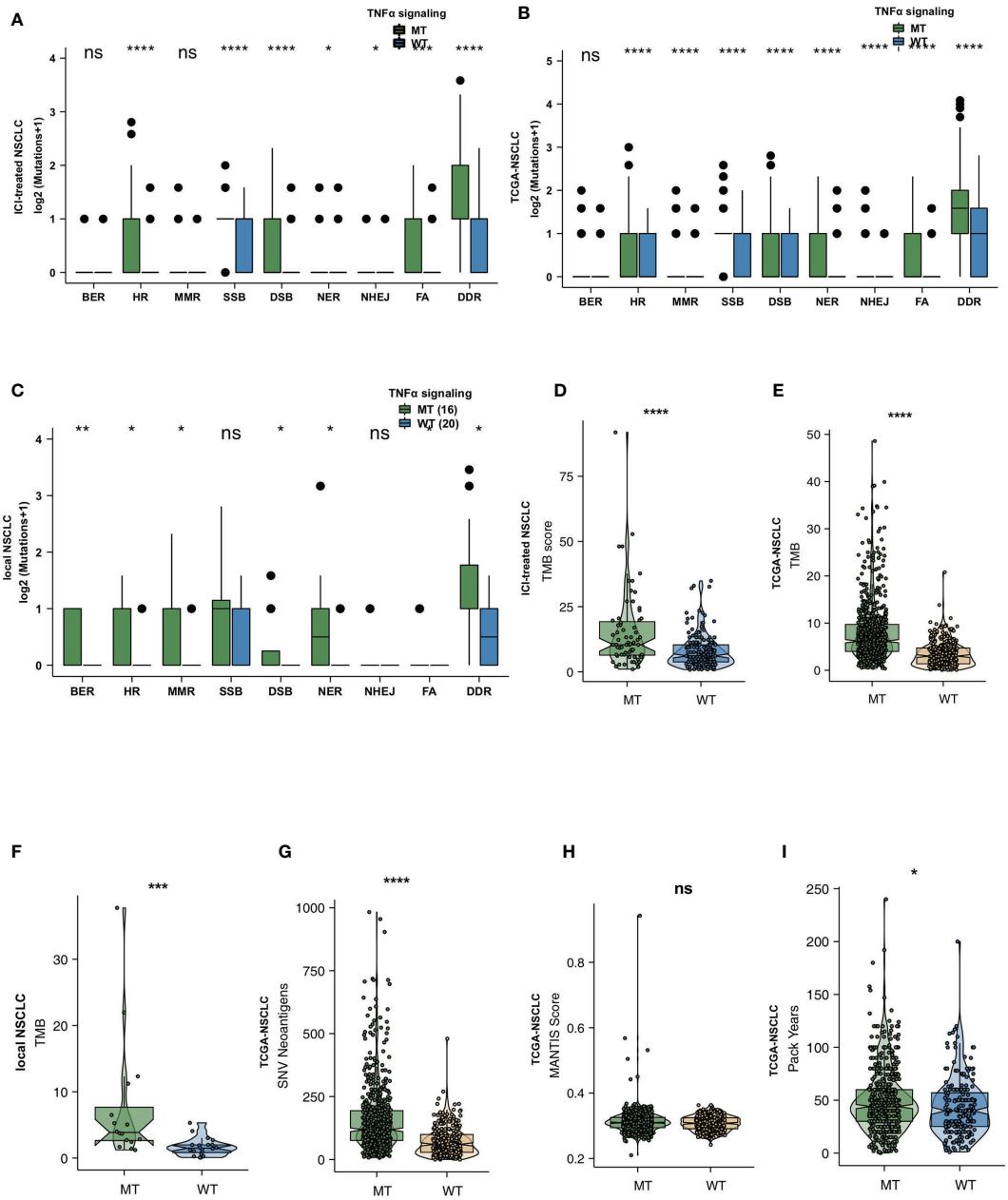

FIGURE 3 | TNFα-MT NSCLC was associated with enhanced tumor immunogenicity. Comparison of DNA damage-related gene set alterations between TNFα-MT and TNFα-WT tumors in the ICI-treated NSCLC **(A)**, TCGA-NSCLC **(B)** and local NSCLC **(C)** cohorts. Comparison of TMB between TNFα-MT and TNFα-WT tumors in the ICI-treated NSCLC **(D)**, TCGA-NSCLC **(E)** and local NSCLC **(F)** cohorts. Comparison of NAL between TNFα-MT and TNFα-WT tumors in the TCGA-NSCLC cohort **(G)**. Comparison of the MANTIS score between TNFα-MT and TNFα-WT tumors in the TCGA-NSCLC cohort **(H)**. Comparison of pack years between TNFα-MT and TNFα-WT tumors in the TCGA-NSCLC cohort **(I)**. (*P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001; Wilcoxon rank-sum test). NSCLC, non small-cell lung cancer; TCGA, The Cancer Genome Atlas; ICI, immune checkpoint inhibitors. ns, not significant.

TILs, especially CD4+ and CD8+ T cells and their immunoregulatory cytokines, play a key role in adaptive immunity. CD8+ T cells produce IFNγ, TNF and granzyme B by binding to T cell receptors and tumor cells, leading to tumor cell clearance (47). However, a variety of such factors have been associated with extrinsic resistance to PD-L1/PD-1 blockade immunotherapy (48). For example, irreversible T cell exhaustion was associated with response or resistance to ICI therapy. Treg cells can directly inhibit the antitumor effect of CD8+ T cells (49). In addition, continuous antigen exposure can

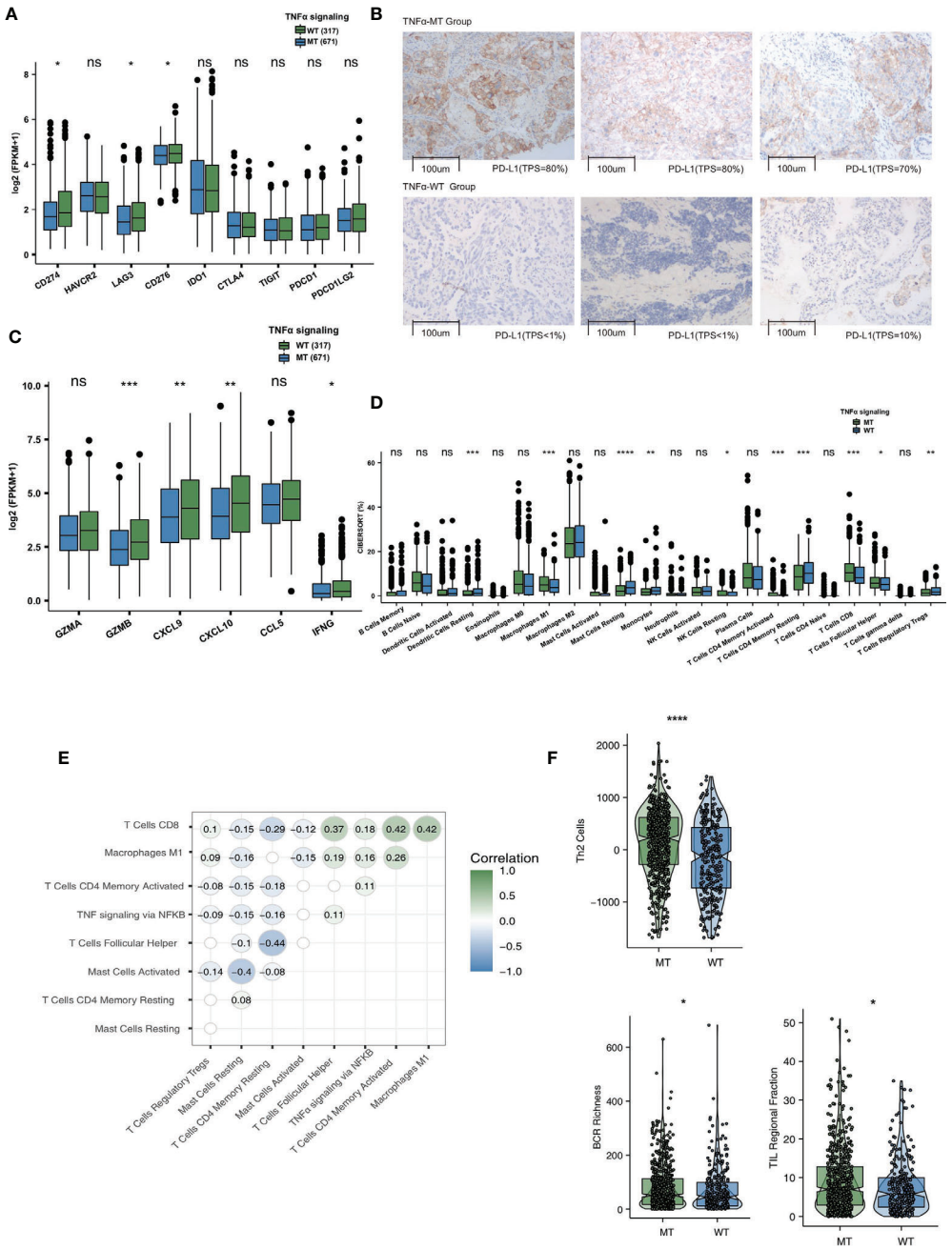

FIGURE 4 | TNFα-MT NSCLC was associated with a significant enrichment of immune cells and enhanced immune scores. **(A)** Comparison of the expression of immune checkpoints between TNFα-MT and TNFα-WT tumors in the TCGA-NSCLC cohort. **(B)** The typical cases for each TPS level between the TNFα-MT (3 samples) and TNFα-WT (3 samples) groups in the Local-NSCLC. **(C)** Comparison of the expression of immune-related genes between TNFα-MT and TNFα-WT tumors in the TCGA-NSCLC cohort. **(D)** Comparison of immune cells between TNFα-MT and TNFα-WT tumors in the TCGA-NSCLC cohort. **(E)** Correlation analysis between the proportions of several immune cell types and number of TNFα signaling mutations. **(F)** Comparison of immune scores between TNFα-MT and TNFα-WT tumors in the TCGA-NSCLC cohort. (*P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001; Wilcoxon rank-sum test). NSCLC, non small-cell lung cancer; TCGA, The Cancer Genome Atlas; TPS, Tumor Proportion Score. ns, not significant.

cause T cell dysfunction or exhaustion, which is characterized by the loss of effector and memory functions (50). PD-(L)1 inhibitors exert an antitumor effect by reactivating the immune response of T cells to tumors (51). Additionally, studies have found that the baseline status of TILs can also be used as a predictive biomarker for ICI therapy. In a retrospective study of a series of patients (52–56), such as those with colorectal cancer (CRC), melanoma and NSCLC, TILs in tumor biopsy samples

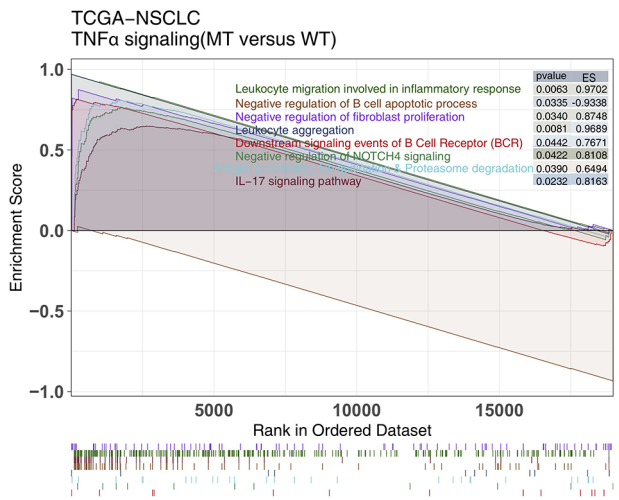

FIGURE 5 | The results of GSEA. The color of the curve corresponds to the font color of the pathway. GSEA of hallmark gene sets downloaded from the MSigDB. Each run was performed with 1000 permutations. Enrichment results with significant differences between TNFα-MT and TNFα-WT tumors are shown. MSigDB, The Molecular Signatures Database; GSEA, Gene Set Enrichment Analysis.

were related to favorable OS. Patients with stage III NSCLC receiving immunotherapy have a higher CD8+ TIL density had longer PFS and OS than NSCLC patients a lower CD8+ TILs (57).

In this study, the immune microenvironment of patients with TNFα-MT was significantly enriched in activated memory CD4+ T cells and CD8+ T cells. Tumor-associated macrophages (TAMs) are an important component of immune infiltration in NSCLC. They are highly plastic and exhibit a variety of phenotypes, including the M1 type (classical activation, antitumor activity and proinflammatory response) and the M2 type (nonclassical activation, proangiogenesis and the immunosuppression of original tumor activity) (58). Also, TNF plays a key role in the polarization of macrophages, such as the transformation of myeloid-derived suppressor cells (MDSCs) into M1-like macrophages, which exert antitumor functions (59).

In addition to T cell exhaustion, the release of immunosuppressive cytokines, another extrinsic factor, linked to resistance to ICI therapy (60, 61). However, inflammatory cytokines enriched in the immune microenvironment also play a vital role in the antitumor immune response. For example, chemokines such as CXCL10 and CXCL9 can enhance immune infiltration and antitumor immunity by recruiting CD8+ T cells, dendritic cells (DCs) and natural killer (NK) cells (62). IFNγ can support the proliferation and differentiation of CD8+ T cells (63, 64). Dong et al. demonstrated that IFNγ pretreatment could help CAR-T achieve better therapeutic effects on solid tumors (63). Defects in IFN signal transduction within cancer cells contributed to intrinsic resistance to PD-1 blockade immunotherapy. Gao et al. found that genomic defects in IFNγ pathway genes as primary resistance factor impaired melanoma rejection upon anti-CTLA-4 therapy (60). Additionally, Evgin et al. indicated that type I IFN has negative consequences for CAR T cell viability, and rendering CAR T cells insensitive to type I IFN facilitates combination therapy (62).

The specific immune signature (cytotoxic T lymphocytes signature) is also associated with the prognosis of patients after receiving ICIs (65). Highly expressed cytotoxic markers, such as CD8A, CD8B, GZMA, GZMB and PRF1, are associated with an improved prognosis of immunotherapy (65–67). Recently, CTLA-4, PD-1, TIM-3, TIGIT and other cooperative inhibitory molecules have been shown to be expressed on the surface of immune cells to downregulate immunity, which was another extrinsic resistance factor to ICIs (68). These cells function to protect the host from excessive immune damage. The success of CTLA-4 or PD-1/PD-L1 blockade catalyzed the enthusiasm for a new class of antibody that block negative immune checkpoint regulators for cancer therapy (69). In this study, patients with TNFα-MT had higher expression levels of immune checkpoints, such as PD-L1 (CD274), LAG3 and CD276, than patients with TNFα-WT.

Tumor immunogenicity has also been shown to be related to the efficacy of immunotherapy, which can be assessed *via* TMB, NAL, MSI-H, DDR pathway mutations and antigen processing and presentation signatures (70–73). Insufficient tumor antigenicity was another intrinsic factor contributing to immunoresistance (74). Alterations in the DDR pathway may lead to the accumulation of uncorrected DNA damage and ultimately increase tumor immunogenicity (18, 44, 75, 76). In this study, we found that patients with TNFα-MT had higher immunogenicity, which was manifested as an upregulated TMB and NAL. The MANTIS score can also be used to evaluate the MSI score. The higher the score is, the closer its status is to MSI-H. The MANTIS score of the TNFα-MT group was significantly higher than that of the TNFα-WT group. Based on the results described above, we believe that the upregulated immunogenicity in the TNFα-MT group may represent one of the potential factors that results in these patients having a satisfactory clinical prognosis after receiving immunotherapy (77).

Although this study, from the perspective of the immune microenvironment (i.e., immune cells, immune-related signatures, immunogenicity, and cytokines) explored the impact of TNFα signaling mutations on the prognosis of NSCLC patients receiving ICIs, there are still some limitations. First, we only analyzed a cohort of patients receiving immunotherapy; therefore, we hope to recruit more NSCLC patients receiving immunotherapy for follow-up verification. Second, in the ICI-treated cohort, only the targeted sequencing data were analyzed; this mutation data were far less than those of WES, and transcriptome, proteomics and other genomic data were lacking. Third, we used only the TCGA-NSCLC cohort and a local cohort containing 36 NSCLC patients from the Zhujiang Hospital of Southern Medical University for verification. Fourth, we did not perform related cell experiments or animal experiments to directly prove our hypothesis; corresponding cell experiments and animal experiments will be done in the future. Fifth, TNF-MT signature may indeed be a mirror of a T-

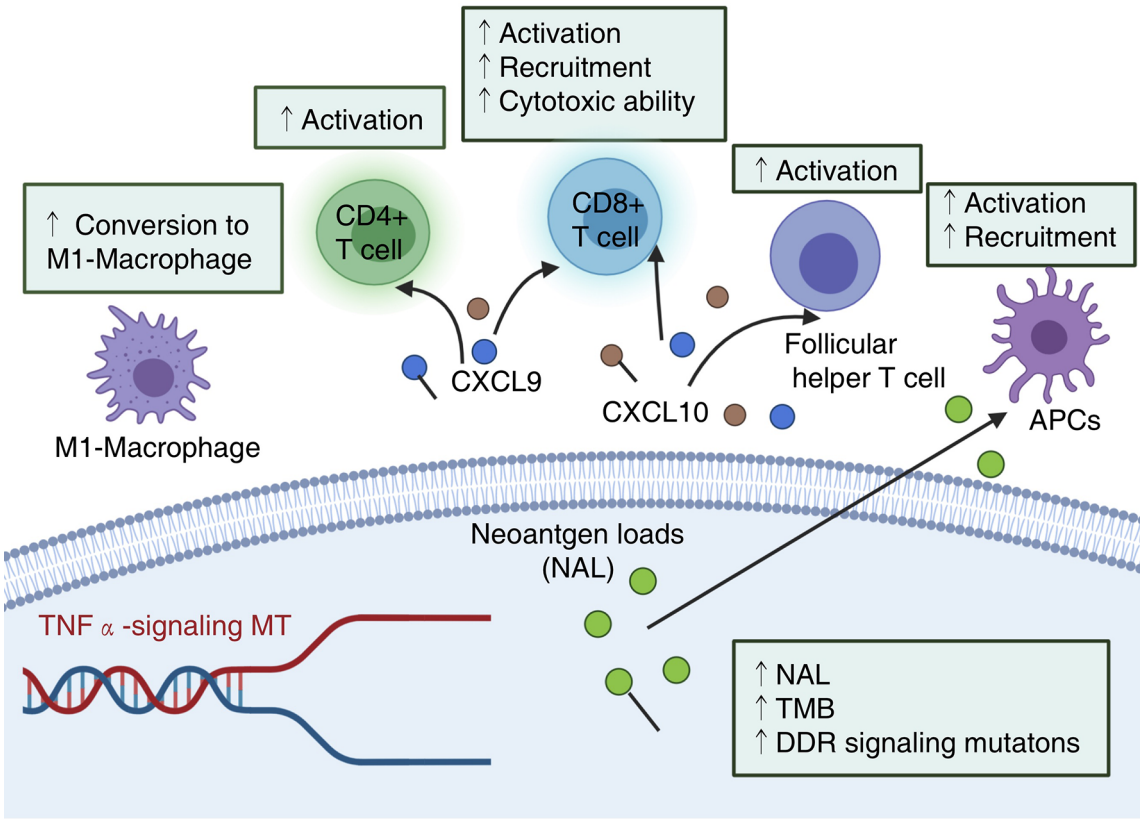

FIGURE 6 | Potential mechanism underlying the prognostic value of TNFα-MT. NAL, neoantigen loads; TMB, Tumor mutational burden; DDR, DNA damage response.

cell cytotoxicity signature, but this is only a hypothesis, because we are more to elaborate the correlation between TNF-MT signature and TIME. We hope that we can further explore association between the T-cell cytotoxicity signature and prognosis of immunotherapy. Finally, we hope that we can collect more cancer types to validate the role of TNFα signaling on the prognosis related to immunotherapy.

CONCLUSIONS

In this study, compared with TNFα-WT NSCLC, TNFα-MT NSCLC had a better prognosis for immunotherapy. Additionally, we found that TNFα-MT showed a significant enrichment in activated immune cells, upregulated immunogenicity and increased immune-related signatures. Therefore, TNFα-MT may serve as potential biomarkers for clinically guiding NSCLC patients to receive immunotherapy.

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 patients/participants provided their written informed consent to participate in this study and the research presented here has been performed in accordance with the Declaration of Helsinki and has been approved by the ethics committee of the Zhujiang Hospital of Southern Medical University.

AUTHOR CONTRIBUTIONS

Writing-original draft, AL. Conceptualization, PL and JZ. Investigation, AL. Writing-review and editing, AL, HZ, HM, PL and JZ. Formal analysis, AL, HZ and HM. Visualization, AL, HZ, HM, ZD and TG. 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/fimmu.2021.667875/full#supplementary-material

Supplementary Figure 1 | Results of the cooccurrence/mutual exclusivity analysis of the top 20 mutated genes in the ICI-treated NSCLC **(A)** and TCGA-NSCLC **(B)** cohorts.

Supplementary Table 1 | Baseline characteristics of NSCLC patients (ICI-treated NSCLC cohort).

Supplementary Table 2 | Baseline characteristics of NSCLC patients (TCGA-NSCLC cohort).

Supplementary Table 3 | Baseline characteristics of NSCLC patients (Local-NSCLC cohrot).

Supplementary Table 4 | The TNFα pathway gene set from the Molecular Signatures Database (MSigDB).

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The Prognostic Significance of the Continuous Administration of Anti-PD-1 Antibody *via* Continuation or Rechallenge After the Occurrence of Immune-Related Adverse Events

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Objectives: Although immune checkpoint inhibitors (ICIs) have been shown to improve overall survival (OS) in advanced non-small-cell lung cancer (NSCLC) patients, ICIs sometimes cause various types of immune-related adverse events (irAEs), which lead to the interruption of ICI treatment. This study aims to evaluate the clinical significance of the continuation of ICIs in NSCLC patients with irAEs and to assess the safety and efficacy of the readministration of ICIs after their discontinuation due to irAEs.

Methods: We retrospectively identified patients with advanced NSCLC who were treated with first- to third-line anti-programmed cell death-1 (PD-1) therapy from January 2016 through October 2017 at multiple institutions belonging to the Niigata Lung Cancer Treatment Group. Progression-free survival (PFS) and OS from the initiation of ICI treatment were analyzed in patients with and without irAEs, with and without ICI interruption, and with and without ICI readministration. A 6-week landmark analysis of PFS and OS was performed to minimize the lead-time bias associated with time-dependent factors.

Results: Of 231 patients who received anti-PD-1 antibodies, 93 patients (40%) developed irAEs. Of 84 eligible patients with irAEs, 32 patients (14%) continued ICIs, and OS was significantly longer in patients who continued ICIs than that in patients who discontinued ICIs [not reached (95% CI: NE-NE) *vs*. not reached (95% CI: 22.4–NE);

p = 0.025]. Of 52 patients who discontinued ICIs, 14 patients (6.1%) readministered ICIs, and OS in patients with ICI readministration was significantly longer than that in patients without ICI readministration [not reached (95% CI: NE-NE) *vs*. not reached (95% CI: 8.4–NE); p = 0.031].

Conclusion: The current study demonstrated that both the continuation and readministration of ICIs after irAE occurrence improved OS compared to the permanent interruption of ICIs in NSCLC patients with ICI-related irAEs.

Keywords: drug therapy, immune-related adverse event, immunology, NSCLC, PD-1

INTRODUCTION

Immune checkpoint inhibitors (ICIs), such as anti-programmed cell death-1 (PD-1) and anti-programmed cell death ligand-1 (PD-L1) antibodies, have achieved durable responses in some patients with non-small-cell lung cancer (NSCLC) (1–6). Anti-PD-1/PD-L1 therapy has become the standard of care for advanced NSCLC patients.

Treatment with ICIs is often accompanied by immune-related adverse events (irAEs), and irAEs can be lethal or the main reason for the discontinuation of ICIs. The decision of whether to continue or discontinue ICIs after the occurrence of irAEs is generally based on the type of irAE and its severity (7). We previously reported that ICI-related interstitial lung disease (ILD), whose appearance was ground-glass opacities (GGOs), was a significant predictor of poor survival outcomes (8). However, recent evidence has demonstrated that the occurrence of irAEs is associated with better survival outcomes in patients with NSCLC (9–13). Several retrospective studies have also shown that there were some cases in which the effects of ICIs were sustained even after the discontinuation of treatment due to ICI-related irAEs (14, 15). In contrast, a retrospective study reported that the interruption of ICIs due to irAEs was associated with a lower overall survival (OS) than continuous ICI treatment (16). Furthermore, the clinical outcomes of the rechallenge of ICIs in patients who recovered from irAEs remain unclear.

This study aims to assess the significance of the continuation of ICIs in NSCLC patients who developed irAEs and to evaluate the safety and efficacy of the readministration of ICIs in patients who discontinued ICI treatment due to ICI-related irAEs.

MATERIALS AND METHODS

Study Design and Patients

We retrospectively analyzed the medical records of patients with advanced NSCLC who were treated with single-agent anti-PD-1 as first- to third-line therapy at multiple institutions belonging to the Niigata Lung Cancer Treatment Group from January 2016 to October 2017. To prevent selection bias, all consecutive patients who met eligibility were enrolled. This study was approved by the institutional review board of each participating institution.

Study Assessment

The following data were collected retrospectively for all patients: demographics, phenotypes of cancers, types of anti-PD-1 therapies, and irAEs. Treatment responses were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria version 1.1. Each irAE was graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. Progression-free survival (PFS) was measured as the time from the start of anti-PD-1 therapy to progressive disease (PD) or death due to any cause. OS was measured as the time from the first administration of immunotherapy to death due to any cause. The objective response rate (ORR) was defined as the percentage of patients assessed as having complete response (CR) or partial response (PR) of all patients treated with anti-PD-1 therapy. The disease control rate (DCR) was defined as the percentage of patients assessed as having CR, PR, or stable disease (SD) of all patients treated with anti-PD-1 therapy. Treatment interruption was defined as either the delay or cessation of ICI treatment due to irAEs. Patients with ICI readministration were defined as those who were readministered a PD-1 inhibitor at least one time after the interruption of ICI treatment due to irAEs. Patients without ICI readministration were defined as those whose ICI treatment was permanently stopped due to irAEs.

Statistical Analysis

Kaplan–Meier survival curves were constructed for PFS and OS, and differences between groups were identified using the log-rank test. The univariate Cox proportional hazards model was used to assess the effects of the presence of irAEs, the continuation of ICIs, and the readministration of ICIs on PFS and OS. Continuous variables are presented as the median (range) and were compared by two-sided *t*-tests. Comparisons between groups were performed by Fisher's exact test or the chi-square test. To minimize the lead-time bias associated with time-dependent factors, we performed a 6-week landmark analysis including only patients who were alive or whose disease was

Abbreviations: ICI, immune checkpoint inhibitor; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1; NSCLC, non-small-cell lung cancer; ILD, interstitial lung disease; GGO, ground-glass opacity; PFS, progression-free survival; OS, overall survival; irAE, immune-related adverse event; NOS, not otherwise specified; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ORR, objective response rate; DCR, disease control rate.

under control at 43 days after the initiation of anti-PD-1 therapy, which is the median time of onset of irAEs, for PFS and OS. For this 6-week landmark analysis, we excluded 66 patients for PFS and 17 patients for OS in the analysis of **Figure 2**, 13 patients for PFS and three patients for OS in the analysis of **Figure 3**, and 12 patients for PFS and two patients for OS in the analysis of **Figure 4** because these patients had PD or died for any cause within 6 weeks of initiation of anti-PD-1 therapy. Additionally, we excluded seven patients who experienced irAEs after the discontinuation of anti-PD-1 treatment due to PD and two patients who died suddenly for unknown reasons after developing irAEs (**Figures 1**, **3** and **Table 4**). All the reported p-values were two-sided, and $p < 0.05$ was considered significant. Statistical analysis was performed using JMP 14.2.0 statistical software (SAS Institute, Cary, NC, USA).

RESULTS

Patient Characteristics

In total, 231 patients were enrolled in this study. Among these patients, 93 patients (40%) developed irAEs (**Figure 1**). The baseline characteristics at the initiation of anti-PD-1 therapy of patients with and without irAEs are presented in **Table 1**. The percentages of males, current or former smokers, squamous cell carcinoma, and pembrolizumab use were significantly higher in patients with irAEs than those in patients without irAEs. On the other hand, the percentage of epidermal growth factor receptor (*EGFR)* mutations was lower in patients with irAEs than that in patients without irAEs. Other clinical features, including age, Eastern Cooperative Oncology Group performance status, treatment line, and PD-L1 expression, were not significantly different.

Association of Immune-Related Adverse Events With Clinical Outcomes

The distribution of irAEs is shown in **Table 2**. The Kaplan–Meier curves of the 6-week landmark analysis for PFS and OS in patients with and without irAEs are shown in **Figure 2**. The median PFS was significantly longer in patients with irAEs than that in patients without irAEs [14.3 (95% CI: 9.0–16.5) *vs*. 4.8 (95% CI: 3.2–7.6); $p < 0.001$]. The median OS was also significantly longer in patients with irAEs than that in patients without irAEs [not achieved (95% CI: NE–NE) *vs*. 21.0 (95% CI: 15.1–NE); $p = 0.005$]. The hazard ratios estimated by the Cox proportional hazards model were as follows: the PFS hazard ratio was 0.51 (95% CI: 0.34–0.75; $p < 0.001$), and the OS hazard ratio was 0.49 (95% CI: 0.30–0.81; $p = 0.005$). Furthermore, the ORR and DCR were significantly higher in patients with irAEs than those in patients without irAEs (**Table 3**).

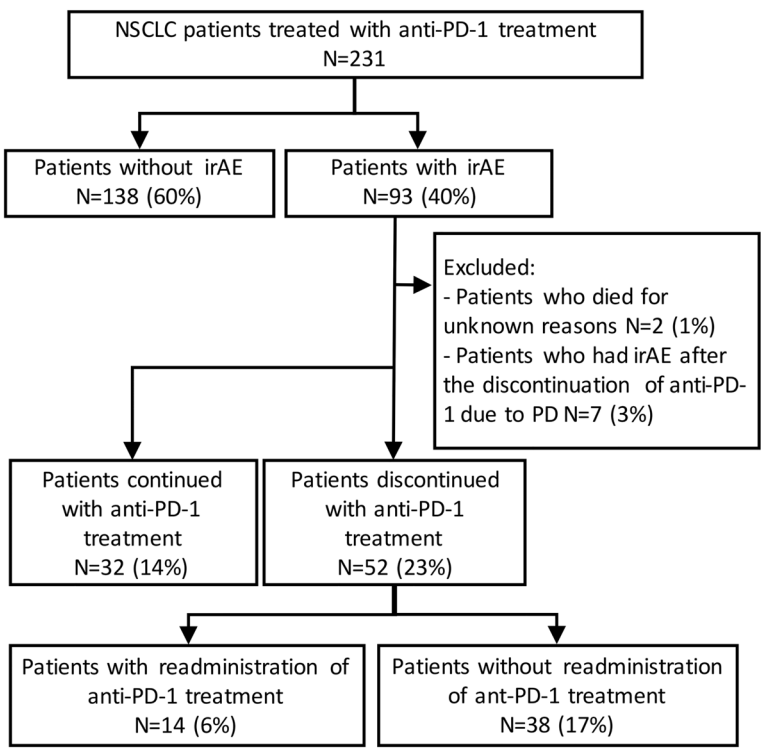

FIGURE 1 | Patient flow diagram. NSCLC, non-small-cell lung cancer; PD-1, programmed cell death-1; irAE, immune-related adverse event.

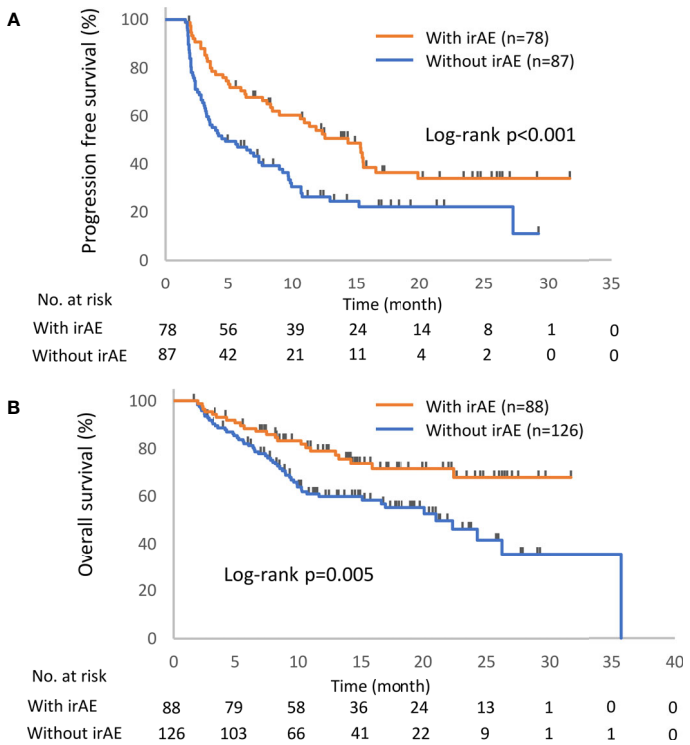

FIGURE 2 | Kaplan–Meier curves for the 6-week landmark analysis of the progression-free survival **(A)** and overall survival **(B)** of patients with or without irAEs. IrAE, immune-related adverse event.

TABLE 1 | Patients' characteristics at anti-PD-1 therapy.

Clinical feature		With irAEs (total, n = 93)	Without irAEs (total, n = 138)	p-value
Median age, years (range)		67 (41–84)	68 (38–82)	0.4[a]
Sex, n (%)	Female/male	14 (15)/79 (85)	41 (30)/97 (70)	0.016[b]
Smoking status, n (%)	Current or former	84 (90)	102 (74)	0.004[b]
	Never	9 (9.7)	36 (26)	
PS, n (%)	0–1	80 (86)	108 (78)	0.19[b]
	≥2	13 (14)	30 (22)	
Stage, n (%)	III	12 (13)	9 (7)	0.23[b]
	IV	45 (48)	76 (55)	
	Recurrent	36 (39)	53 (38)	
Histology, n (%)	Adenocarcinoma	36 (39)	96 (69)	<0.001[b]
	Squamous cell carcinoma	46 (49)	34 (25)	
	Others	11 (12)	8 (6)	
Driver mutation, n (%)	*EGFR*	1 (1)	12 (9)	0.017[c]
Treatment line of anti-PD-1	first line	21 (23)	17 (12)	0.06[b]
therapy, n (%)	second, third line	72 (77)	121 (88)	
PD-L1 expression, n (%)	≥50%	28 (30)	26 (19)	0.16[c]
	1%–49%	5 (5)	8 (6)	
	<1%	7 (8)	7 (5)	
	Unknown	53 (57)	97 (70)	
Anti-PD-1 therapy, n (%)	Nivolumab	63 (68)	113 (82)	0.02[b]
	Pembrolizumab	30 (32)	25 (18)	
Median duration between initial anti-PD-1 treatment to the first irAE onset, days (range)		43 (0–522)		

Differences between groups were identified using [a]Student's t-test, [b]chi-square test, or [c]Fisher's exact test. PS, performance status; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand 1; irAE, immune-related adverse event; EGFR, epidermal growth factor receptor.

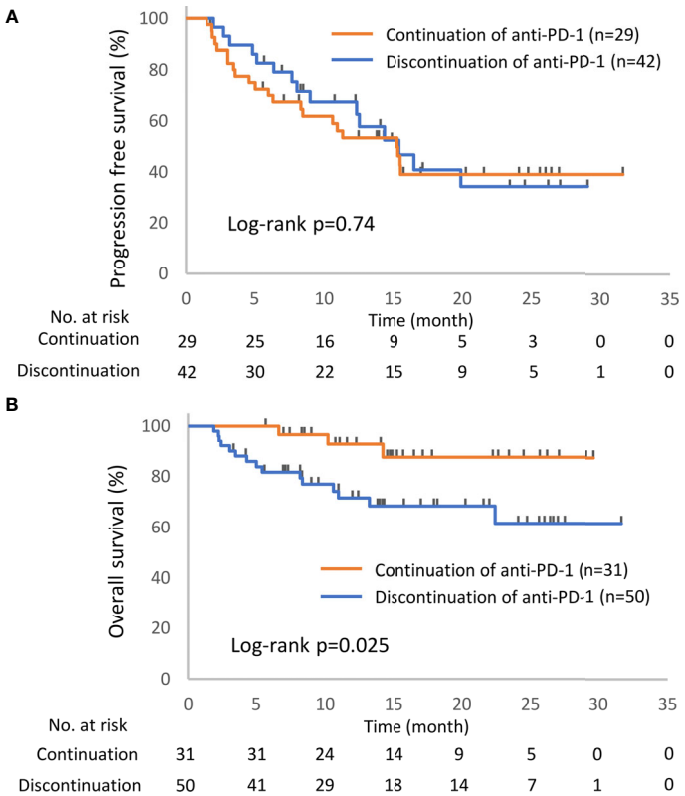

FIGURE 3 | Kaplan–Meier curves for the 6-week landmark analysis of the progression-free survival **(A)** and overall survival **(B)** of patients continuing or stopping PD-1 treatment after irAE occurrence. PD-1, programmed cell death-1; irAE, immune-related adverse event.

TABLE 2 | Distribution of irAEs.

Phenotypes of irAEs	Total (n = 231)	CTCAE G3–4	CTCAE G5	Therapy continued	Systemic steroid	IrAEs improved
Pneumonitis, n (%)	33 (14)	11 (5)	1 (0.4)	1 (0.4)	24 (10)	31 (13)
Thyroid dysfunction, n (%)	26 (11)	2 (1)	0 (0)	17 (7.4)	0 (0)	23 (10)
Rash, n (%)	14 (6)	2 (1)	0 (0)	8 (3.5)	3 (1)	13 (6)
Pyrexia, n (%)	10 (4.3)	1 (0.4)	0 (0)	6 (3)	1 (0.4)	10 (4.3)
Diarrhea/colitis, n (%)	9 (4)	0 (0)	0 (0)	5 (2)	3 (1)	6 (3)
Adrenal insufficiency, n (%)	7 (3)	2 (1)	0 (0)	3 (1)	7 (3)	7 (3)
Infusion reaction, n (%)	6 (3)	0 (0)	0 (0)	3 (1)	3 (1)	6 (3)
Pruritus, n (%)	3 (1)	0 (0)	0 (0)	2 (1)	0 (0)	2 (1)
Liver dysfunction, n (%)	3 (1)	1 (0.4)	0 (0)	0 (0)	1 (0.4)	3 (1)
Anorexia, n (%)	2 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.4)
Neuropathy, n (%)	2 (1)	1 (0.4)	0 (0)	0 (0)	2 (1)	1 (0.4)
Fatigue, n (%)	2 (1)	0 (0)	0 (0)	1 (0.4)	0 (0)	1 (0.4)
Nausea, n (%)	1 (0.4)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.4)
Proteinuria, n (%)	1 (0.4)	0 (0)	0 (0)	1 (0.4)	0 (0)	1 (0.4)
Uveitis, n (%)	1 (0.4)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.4)
Thrombocytopenia, n (%)	1 (0.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Autoimmune myositis, n (%)	1 (0.4)	1 (0.4)	0 (0)	0 (0)	1 (0.4)	0 (0)
Fulminant type 1 diabetes, n (%)	1 (0.4)	1 (0.4)	0 (0)	0 (0)	0 (0)	1 (0.4)
Depression, n (%)	1 (0.4)	1 (0.4)	0 (0)	1 (0.4)	0 (0)	1 (0.4)
Death NOS, n (%)	1 (0.4)	0 (0)	1 (0.4)	0 (0)	0 (0)	0 (0)

IrAE, immune-related adverse event; CTCAE, Common Terminology Criteria for Adverse Events; Death NOS, death that cannot be attributed to a CTCAE term associated with Grade 5.

TABLE 3 | Association between irAEs and treatment responses.

	All (n = 231)	With irAE (n = 93)	Without irAE (n = 138)	p-value
CR, n (%)	5 (2)	4 (4)	1 (1)	
PR, n (%)	69 (30)	41 (44)	28 (20)	
SD, n (%)	51 (22)	22 (24)	29 (21)	
PD, n (%)	96 (42)	19 (20)	77 (56)	
NE, n (%)	10 (4)	7 (8)	3 (2)	
ORR, n (%)	74 (32)	45 (48)	29 (21)	<0.001[a]
DCR, n (%)	125 (54)	67 (72)	58 (42)	<0.001[a]

Differences between groups were identified using [a]chi-square test. IrAE, immune-related adverse event; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; ORR, objective response rate; DCR, disease control rate

Association of Immune Checkpoint Inhibitor Interruption Due to Immune-Related Adverse Events With Clinical Outcomes

Of the 93 patients with irAEs, 32 patients continued anti-PD-1 treatment, and 52 patients discontinued anti-PD-1 treatment (**Supplementary Table S1**). Patients who died for unknown reasons (n = 2) and those who had irAEs after the discontinuation of anti-PD-1 treatment due to PD (n = 7) were excluded from the following analysis (**Figure 1**). The Kaplan–Meier curves of the 6-week landmark analysis for patients who continued and discontinued anti-PD-1 therapy are shown in **Figure 3**. The median PFS was not different between patients who continued and discontinued anti-PD-1 treatment [15.4 (95% CI: 9.0–NE) *vs.* 15.3 (95% CI: 8.3–NE); p = 0.76]. However, the median OS was significantly longer in patients who continued ICIs than that in patients who discontinued ICIs [not reached (95% CI: NE–NE) *vs.* not reached (95% CI: 22.4–NE); p = 0.025]. The hazard ratios estimated by the Cox proportional hazards model were as follows: the PFS hazard ratio was 0.9 (95% CI: 0.47–1.74; p = 0.76), and the OS hazard ratio was 0.27 (95% CI: 0.077–0.92; p = 0.036). In terms of irAE phenotypes, the percentage of patients who experienced immune-related pneumonitis was higher in the anti-PD-1 treatment interruption group than that in the anti-PD-1 continuation group (**Table 4**). On the other hand, the percentage of patients who experienced immune-related thyroid dysfunction was higher in the anti-PD-1 continuation group

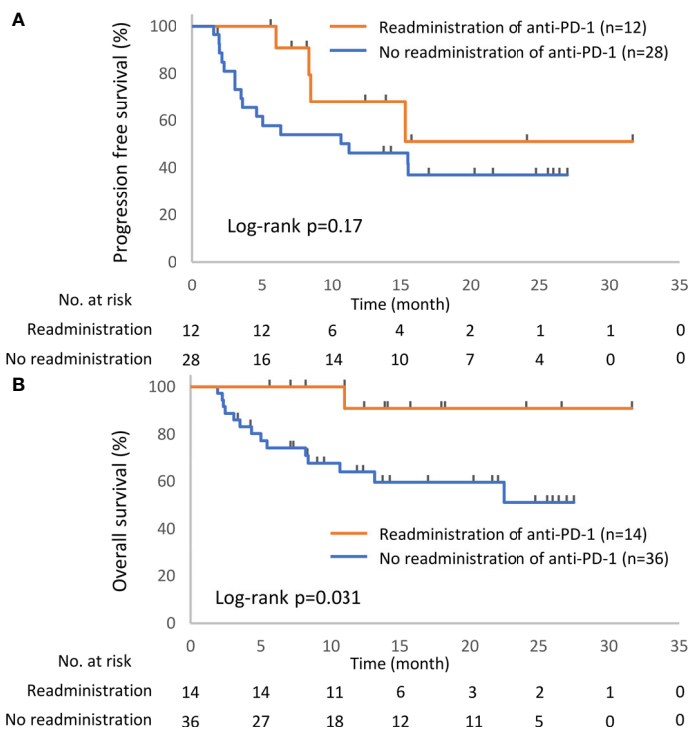

FIGURE 4 | Kaplan–Meier curves for the 6-week landmark analysis of the progression-free survival **(A)** and overall survival **(B)** of patients with or without the readministration of anti-PD-1 treatment after the discontinuation of anti-PD-1 treatment due to irAEs. PD-1, programmed cell death-1; irAE, immune-related adverse event.

TABLE 4 | Characteristics of the initial irAEs and clinical courses, related treatment interruption.

		Anti-PD-1 treatment interruption (n = 52)	Anti-PD-1 treatment continuation (n = 32)	p-value
Phenotypes of irAE	Pneumonitis, n (%)	29 (54)	1 (3)	<0.001[a]
	Diarrhea, n (%)	3 (6)	5 (16)	0.25[a]
	Adrenal insufficiency, n (%)	3 (6)	3 (9)	0.67[a]
	Infusion reaction, n (%)	3 (6)	3 (9)	0.67[a]
	Thyroid dysfunction, n (%)	9 (17)	14 (44)	0.017[b]
	Pyrexia, n (%)	5 (10)	5 (15)	0.50[a]
	Rash, n (%)	7 (13)	6 (19)	0.73[b]
CTCAE grade ≥3, n (%)		20 (38)	0 (0)	<0.001[a]
Median duration between initial anti-PD-1 treatment to the first irAE onset, days (range)		40 (0–522)	60 (0–384)	1[c]
ORR, n (%)		24 (46)	21 (66)	0.13[b]
DCR, n (%)		36 (69)	27 (84)	0.19[b]

Differences between groups were identified using [a]Fisher's exact test, [b]chi-square test, or [c]Student's t-test. IrAE, immune-related adverse event; CTCAE, Common Terminology Criteria for Adverse Events; PD-1, programmed cell death-1; ORR, overall response rate; DCR, disease control rate.

than that in the anti-PD-1 interruption group. As expected, the percentage of patients who experienced grade 3 or higher irAEs was higher in the anti-PD-1 interruption group than that in the anti-PD-1 continuation group. Other phenotypes of irAEs, the timing of the first irAE, ORR, and DCR were not different among patients with anti-PD-1 interruption and those with anti-PD-1 continuation.

Association of Immune Checkpoint Inhibitor Readministration With Clinical Outcomes

In total, 52 patients discontinued anti-PD-1 treatment, and 14 patients were readministered ICIs (**Supplementary Table S2**). All patients had received the same type of anti-PD-1 inhibitor prior to discontinuation. The Kaplan–Meier curves of the 6-week landmark analysis for patients readministered and not readministered anti-PD-1 therapy are shown in **Figure 4**. Two patients who were readministered ICIs after PD were excluded from the PFS analysis. The median PFS was not significantly different between patients with and without the readministration of anti-PD-1 treatment [15.3 (95% CI: 8.3–NE) *vs.* 11.3 (95% CI: 3.5–NE); p = 0.17]. On the other hand, the median OS was significantly longer in patients with ICI readministration than that in patients without ICI readministration [not reached (95% CI: NE–NE) *vs.* not reached (95% CI: 8.4–NE); p = 0.031]. The hazard ratios estimated by the Cox proportional hazards model were as follows: the PFS hazard ratio was 0.46 (95% CI: 0.16–1.41; p = 0.18), and the OS hazard ratio was 0.15 (95% CI: 0.019–1.1; p = 0.063). The characteristics of the initial irAEs stratified by readministration are shown in **Table 5**. The percentage of patients who experienced immune-related pneumonitis at initial ICI treatment was significantly higher in patients who did not receive ICI readministration than that in patients who did receive ICI readministration. The percentage of patients who experienced grade 3 or higher irAEs was not

TABLE 5 | Characteristics of the initial irAEs and clinical courses, related readministration of anti-PD-1 treatment.

		With readministration of anti-PD-1 treatment (n = 14)	Without readministration of anti-PD-1 treatment (n = 38)	p-value
Phenotypes of IrAEs, n (%)	Pyrexia, n (%)	3 (21)	2 (5)	0.11[a]
	Diarrhea/Colitis, n (%)	2 (14)	1 (3)	0.17[a]
	Adrenal insufficiency, n (%)	2 (14)	1 (3)	0.17[a]
	Liver dysfunction, n (%)	2 (14)	1 (3)	0.17[a]
	Pneumonitis, n (%)	3 (21)	26 (68)	0.004[a]
	Thyroid dysfunction, n (%)	3 (21)	6 (16)	0.69[a]
	Rash, n (%)	2 (14)	5 (13)	1[a]
	Fulminant type 1 diabetes, n (%)	1 (7)	0 (0)	0.27[a]
	Infusion reaction, n (%)	0 (0)	3 (8)	0.56[a]
	Neuropathy, n (%)	0 (0)	2 (5)	1[a]
CTCAE grade ≥3, n (%)		4 (29)	15 (39)	0.53[a]
ORR to the initial anti-PD-1 therapy, n (%)		10 (71)	14 (37)	0.057[b]
Median time from the last administration of the initial anti-PD-1 to the readministration of anti-PD-1, days (range)		70 (22–414)	NA	
Subsequent systemic therapy after anti-PD-1 therapies, n (%)		3 (21)	14 (37)	0.34[a]

Differences between groups were identified using [a]Fisher's exact test or [b]chi-square test. IrAE, immune-related adverse event; CTCAE, Common Terminology Criteria for Adverse Events; ORR, overall response rate; PD-1, programmed cell death-1; NA, not applicable.

TABLE 6 | Details of recurrent and new irAEs out of 14 patients with readministered ICI after irAE occurrence.

Case	IrAEs that caused first interruption of ICI	CTCAE grade	IrAEs with readministration	CTCAE grade	ICI continuation	ICI after readministration
1	Pneumonitis	1	Pneumonitis	1	Continued	NA
2	Thyroid dysfunction	3	Thyroid dysfunction	3	Discontinued	Permanently interrupted
3	Liver dysfunction	1	Thyroid dysfunction	1	Continued	NA
4	Diarrhea	2	Anorexia	1	Discontinued	Permanently interrupted

IrAE, immune-related adverse event; CTCAE, Common Terminology Criteria for Adverse Events; ICI, immune checkpoint inhibitor; NA, not applicable.

significantly different between patients with ICI readministration and those without ICI readministration. There were no differences between the anti-PD-1 readministration and permanent interruption groups regarding other phenotypes of irAEs or subsequent systemic therapy after anti-PD-1 treatment (**Table 5**). The recurrent and new irAEs that developed after the readministration of anti-PD-1 treatment are detailed in **Table 6**. In 14 patients who were readministered ICIs, two had recurrent irAEs (14%), and two developed new irAEs (14%). Only one patient developed a severe recurrent irAE that was CTCAE grade 3. We have summarized initial irAEs, tumor responses to first anti-PD-1 therapy, and clinical outcomes in patients with continuation and those with or without readministration of anti-PD-1 therapy in **Supplementary Table S3**.

DISCUSSION

This study demonstrated the prognostic significance of the occurrence of irAEs, ICI continuation after the development of irAEs, and ICI rechallenge after the interruption of ICIs due to irAEs. Patients with irAEs had a better prognosis than those without irAEs (**Figure 2**), and the continuation or rechallenge of ICIs was associated with better survival times than the permanent interruption of ICIs due to irAEs (**Figures 3**, **4** and **Supplementary Figure S1**). Although some patients with ICI readministration experienced the recurrence of the same or new irAEs, most of these recurrent irAEs were controllable (**Table 6**).

There have been no reports that simultaneously evaluated the significance of ICI continuation and readministration after the occurrence of irAEs in NSCLC patients. In a retrospective study verifying the impact of ICI interruption due to irAEs, the median OS was worse in patients with ICI interruption than that in others with continuous ICI administration (16). Several retrospective studies have examined the safety of ICI rechallenge after initial irAE occurrence in cancer patients and indicated that the safety of ICI rechallenge was acceptable (17–19). A retrospective study of NSCLC demonstrated that among patients with ICI interruption at the time of initial irAE occurrence, ICI rechallenge prolonged OS in patients who had no treatment response before irAE onset (17). While these previous studies focused on only the initial irAEs, our study included all irAEs that occurred during the whole clinical course. The current study showed that among patients with irAEs, although permanent ICI interruption was associated with poor prognosis, ICI readministration and ICI continuation improved prognostic outcomes (**Supplementary Figure S1**). The better prognosis of patients with ICI continuation or readministration than that in patients with permanent ICI interruption may be biologically plausible. The blocking effect of PD-1/PD-L1 is generally expected to diminish with the interruption of ICIs because the binding of the anti-PD-1 antibody to PD-1-positive tumor-infiltrating $CD8^+$ T cells is transient (20). Although not related to irAEs, a randomized phase 3b/4 study of NSCLC that compared patients who continued nivolumab for more than 1 year with those who discontinued nivolumab after 1 year of treatment demonstrated that continuous ICI therapy had better clinical outcomes (21). Collectively, these findings suggest the significance of continuously administering ICIs as much as possible after irAE occurrence *via* continuation or readministration.

In the current study, there was no PFS advantage from the continuation or readministration of ICIs in patients with ICI-related irAEs (**Figures 3**, **4**). As a prospective study of patients with nonsquamous NSCLC showed that there was no difference in PFS between nivolumab and docetaxel (1), the efficacy of single-agent ICI therapy might not be able to be evaluated properly by PFS.

Unexpectedly, our study demonstrated no difference in the frequency of irAEs whose CTCAE grade was over 3 between patients with ICI readministration and those with permanent ICI interruption (**Tables 4**, **5**). This result might suggest that clinicians aggressively readministered ICIs to patients whose irAEs had been severe but improved. Indeed, better survival outcomes were observed in patients who had experienced grade 3–4 irAEs and received the readministration of anti-PD-1 therapy (**Supplementary Figure S2**). Although Johnson et al. (22) suggested that severe or life-threatening toxicity is one of the factors that argues against ICI rechallenge, the readministration of ICIs might be considered in patients whose irAEs had been severe but recovered. However, it is noteworthy in the current study that the frequency of pneumonitis as an irAE was significantly higher in patients who discontinued ICIs and in those who permanently interrupted ICIs (**Tables 4**, **5**). In addition, our study suggests that the readministration of anti-PD-1 therapy had no survival benefit in patients with pneumonitis (**Supplementary Figure S2**). Several meta-analyses have reported that pneumonitis is one of the most common fatal irAEs in patients treated with ICIs (23, 24). Therefore, although there has been no evidence that the continuous administration or readministration of ICIs tends to lead to fatal irAEs, it should be noted that the continuation or readministration of ICIs to patients who experienced irAEs such as pneumonitis, which could be fatal if exacerbated, should be carefully determined on a patient-by-patient basis.

The limitations of the current study include the relatively small number of patients with ICI readministration and the retrospective nature of the study. Clinicians might have tended to

avoid continuing and readministering ICIs to patients with irAEs such as pneumonitis, which could be fatal if exacerbated. There is no detailed analysis for each irAE in this study. In addition, response rate to initial anti-PD-1 therapies in patients with readministration tended to be higher than that in patients without readministration (ORR 71% *vs*. 37%, p = 0.057; **Table 5**). There is a possibility that clinicians might have tended to readminister anti-PD-1 therapy to patients with good tumor response to initial ICI even with irAEs.

In summary, we retrospectively investigated the clinical significance of the continuation and readministration of single-agent anti-PD-1 therapy in NSCLC patients with ICI-related irAEs. The continuation and readministration of ICIs significantly prolonged OS, and their safety was acceptable. A future prospective study is needed to establish optimal treatment strategies for patients with irAEs.

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 Niigata University Graduate School of Medical and Dental Sciences, Niigata Prefectural Shibata Hospital, Niigata City General Hospital, Niigata Cancer Center Hospital, Saiseikai Niigata Hospital, Nishi-Niigata Chuo National Hospital, Niigata Prefectural Central Hospital, Nagaoka Chuo General Hospital, Nagaoka Red Cross Hospital, Niigata Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TO devised this study. TO and SW designed the protocol. SW, TO, SS, KN, KI, RK, TM, TA, SM, HT, MO, MTe, NM, TI, AI, KS, HY, MH enrolled patients into the study. TF and SW co-wrote this manuscript, conducted data analysis, and prepared figures and tables. 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.2021.704475/full#supplementary-material

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Prognostic Value of the Pretreatment Lung Immune Prognostic Index in Advanced Small Cell Lung Cancer Patients Treated With First-Line PD-1/PD-L1 Inhibitors Plus Chemotherapy

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Background: Lung immune prognostic index (LIPI) refers to a biomarker combining derived neutrophil-to-lymphocyte ratio (dNLR) and lactate dehydrogenase (LDH). Its prognostic effect on advanced small cell lung cancer (SCLC) patients receiving programmed cell death 1/programmed cell death ligand-1 (PD-1/PD-L1) inhibitors plus chemotherapy as first-line treatment remains unclear. Our research investigated the relationship between pretreatment LIPI and the prognosis of patients receiving first-line PD-1/PD-L1 inhibitors plus chemotherapy.

Methods: Advanced SCLC patients receiving PD-1/PD-L1 inhibitors plus chemotherapy as first-line treatment from Jan 2015 to Oct 2020 were included. Based on the values of dNLR and LDH, the study population was divided into two groups: LIPI good and LIPI intermediate/poor. The Kaplan-Meier method was used to compute the median survival time and the log-rank test was used to compare the two groups. Univariate and multivariate analyses were used to examine the correlation between the pretreatment LIPI and clinical outcomes.

Results: One hundred patients were included in this study, of which, 64% were LIPI good (dNLR < 4.0 and LDH < 283 U/L), 11% were LIPI poor (dNLR ≥ 4.0 and LDH ≥ 283 U/L), and the remaining 25% were LIPI intermediate. The LIPI good group had better progression-free survival (PFS) (median: 8.4 *vs* 4.7 months, $p = 0.02$) and overall survival (OS) (median: 23.8 *vs* 13.3 months, $p = 0.0006$) than the LIPI intermediate/poor group. Multivariate analysis showed that pretreatment LIPI intermediate/poor was an independent risk factor for OS (HR: 2.34; 95%CI, 1.13, 4.86; $p = 0.02$). Subgroup analysis

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showed that pretreatment LIPI good was associated with better PFS and OS in males, extensive disease (ED), PD-1 inhibitor treatment, smokers, and liver metastasis ($p < 0.05$).

Conclusions: Pretreatment LIPI could serve as a prognostic biomarker for advanced SCLC patients receiving first-line PD-1/PD-L1 inhibitors plus chemotherapy.

Keywords: small cell lung cancer, immune checkpoint inhibitor, first-line, lung immune prognostic index, prognosis

INTRODUCTION

Small-cell lung cancer (SCLC) constitutes 13 - 15% of total lung cancer cases, and is characterized by rapid progression and early distant metastasis (1, 2). Over 90% of SCLC patients are elders or past heavy smokers (3). One-third of SCLC patients are classified as having limited disease (LD), and the others as having extensive disease (ED) according to the Veteran's Administration Lung Cancer Study Group Staging System (4, 5). Despite sensitivity to first-line chemotherapy, most SCLC cases recur in one year and are insensitive to second-line treatment (6). The median overall survival (OS) is 15–20 months for patients with LD, and 8–13 months for those with ED (7).

Immune checkpoint inhibitors (ICIs), especially programmed cell death 1/programmed cell death ligand-1 (PD-1/PD-L1) inhibitors, have revolutionized the treatment landscape of various cancers. Recently, the IMpower 133 and CASPIAN studies have demonstrated that a combination of atezolizumab or durvalumab and chemotherapy could improve clinical outcomes of SCLC patients as compared to those using chemotherapy alone (8, 9). The phase II EA5161 study has demonstrated the addition of nivolumab at first-line treatment significantly improved the progression-free survival (PFS) and OS of ES-SCLC patients (median PFS: 5.5 *vs* 4.6 months, $p = 0.012$; median OS: 11.3 *vs* 8.5 months, $p = 0.038$) (10). The phase III KEYNOTE-604 study showed that advanced SCLC patients receiving first-line pembrolizumab plus chemotherapy had better OS compared with those receiving chemotherapy alone, but the difference did not meet the predefined statistical threshold (11). A meta analysis study found that both PD-L1 inhibitors and PD-1 inhibitors plus chemotherapy as first-line treatment could provide a significant improvement of survival time compared with chemotherapy alone for advanced SCLC patients (12). FDA has approved PD-1 inhibitors as third-line treatment in 2018 and PD-L1 inhibitors as first-line treatment in 2020 for patients with ED or relapsed SCLC, which is an important advancement for SCLC patients.

SCLC patients have a relatively high tumor mutation burden (13), but it has not been proven to serve as a clear predictor in patients receiving ICI treatment (8, 14). PD-L1 expression is low or absent in SCLC patients, but it is still not used as a predictive biomarker in SCLC patients receiving ICI treatment (15). Currently, no prognostic biomarkers can definitely guide the application of ICIs in patients with SCLC. Therefore, identifying biomarkers to select patients who are likely to respond to immunotherapy is crucial. Systemic inflammation plays a critical role in the occurrence and development of cancer (16). Previous studies have reported the prognostic role of systemic inflammation indicators in non-small cell lung cancer (NSCLC) patients receiving immunotherapy, including neutrophil-to-lymphocyte ratio (NLR) and lactate dehydrogenase (LDH) (17–22). Several studies showed that the lung immune prognostic index (LIPI), combining derived NLR (dNLR, absolute neutrophil count/[white blood cell concentration−absolute neutrophil count]) and LDH, could predict survival in advanced NSCLC patients receiving immunotherapy (23, 24). However, there is a lack of studies describing the prognostic value of pretreatment LIPI in advanced SCLC patients receiving PD-1/PD-L1 inhibitor treatment. Therefore, we aim to investigate whether pretreatment LIPI was related to the prognosis of advanced SCLC patients treated with first-line PD-1/PD-L1 inhibitors plus chemotherapy.

METHODS

Study Design and Patients

The study was carried out at the Chinese PLA general hospital (Beijing, China). Advanced SCLC patients receiving PD-1/PD-L1 inhibitors plus chemotherapy as first-line treatment from Jan 2015 to Oct 2020 were included. The inclusion criteria were as follows (1): patients who were diagnosed with SCLC (2); patients receiving first-line PD-1/PD-L1 inhibitors plus chemotherapy; and (3) patients who were treated with at least two cycles of PD-1/PD-L1 inhibitors. The exclusion criteria were (1): absence of efficacy assessment; and (2) absence of pretreatment blood test results. Clinical characteristics as well as pretreatment blood laboratory test results were recorded. Clinical characteristics included age, sex, stage, smoking history, ICI drugs, Eastern Cooperative Oncology Group Performance Status (ECOG PS), sites of metastasis and efficacy, and pretreatment blood test results included total white blood cell count, absolute neutrophil count, absolute lymphocyte count, and LDH levels. This research was authorized by the Ethics Committee of Chinese PLA General Hospital and performed according to the principles of the Declaration of Helsinki.

LD is defined as a disease limited to one hemithorax, local mediastinal lymph nodes, and ipsilateral supraclavicular lymph nodes, which can be included in a tolerable radiation field; ED includes the cases not classified as LD (25). Blood tests were conducted within 5 days before the first cycle of immunotherapy. LIPI was calculated by dNLR (absolute neutrophil count/[white blood cell concentration−absolute neutrophil count]) and LDH, and cutoff values of dNLR and LDH were calculated using X-tile software based on data (26), which were 4.0 U/L and 283 U/L, respectively. Patients were stratified into LIPI good (dNLR < 4.0 and LDH < 283 U/L) and

LIPI intermediate/poor groups (intermediate: dNLR < 4.0 and LDH ≥ 283 U/L, or dNLR ≥ 4.0 and LDH < 283 U/L; poor: dNLR ≥ 4.0 and LDH ≥ 283 U/L) groups.

Treatment responses were assessed every two cycles of ICI treatment by two independent investigators (ZZ and LL) according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, including complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). PFS was defined as the period from the first ICI treatment to disease progression or death (whichever occurred first). OS was defined as the period from the first ICI treatment to death. All patients were followed up through telephone counseling and searching electronic medical records with a cutoff date of March 16, 2021.

Statistical Analysis

Statistical analyses were conducted using IBM SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (La Jolla, CA, USA). X-tile 3.6.1 software (Yale University, New Haven, CT, USA) was used to identify the optimal cut-off values for dNLR and LDH. Kaplan-Meier curves were used to analyze OS and PFS, and the differences were evaluated by log-rank test. Chi-square or Fisher's exact test was used to compare categorical variables. Hazard ratio (HR) with its 95% confidence interval (CI) was estimated by Cox proportional hazards models. Univariate and multivariate analyses were conducted to determine the independent prognostic value of pretreatment LIPI. The variables with $p < 0.05$ in the univariate analysis were eligible to be included in the multivariate analysis. Phi correlation coefficients were calculated to determine the association between each pair of the dichotomous variables. All statistical tests were two-sided with a statistical significance of $p < 0.05$.

RESULTS

Patient Clinical Characteristics

A total of 110 SCLC patients receiving first-line PD-1/PD-L1 inhibitors were identified, of which, four patients received only one dose of PD-1/PD-L1 inhibitors, and six patients had no pretreatment blood test results (**Figure 1**). Finally, 100 SCLC patients were included for data analysis. Most of those patients (87%) received platinum-etoposide chemotherapy (45% carboplatin and 42% cis-platinum), and the other patients (13%) received nab-paclitaxel and etoposide. Moreover, 65% of

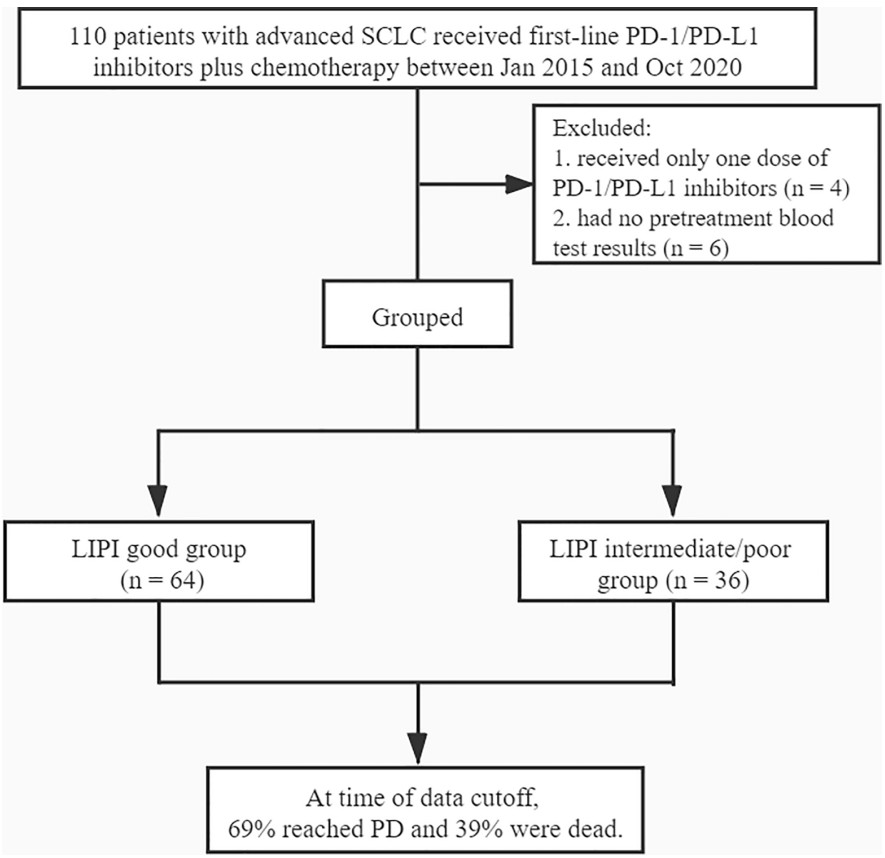

FIGURE 1 | Diagram of the study.

the patients received PD-1 inhibitors (nivolumab, pembrolizumab or sintilimab), and 35% received PD-L1 inhibitors (atezolizumab or durvalumab). Patients had a maximum of 4-6 cycles of chemotherapy as first-line treatment. The median follow-up time was 19.2 months. Detailed clinical data of the patients are summarized in **Table 1**. The median age was 60 years (range: 32–82). Among the 100 patients, 88% were males, 74% had an ED, 94% had an ECOG PS of 0–1, 79% had a smoking history, 22% had brain metastasis, 24% had liver metastasis, and 29% had bone metastasis. Of the patients, 60%, 31%, and 9% had PR, SD, and PD, respectively; 78% had dNLR < 4.0, and 75% had LDH < 283 U/L. Patients in the LIPI good, LIPI intermediate, and LIPI poor groups were 64%, 25%, and 11%, respectively.

TABLE 1 | Characteristics of patients with advanced SCLC.

Characteristics	No. of patients (n = 100)	Percentage (%)
Age (year), median (range)	60 (32–82)	
<60	48	48
≥60	52	52
Sex		
Male	88	88
Female	12	12
Stage		
LD	26	26
ED	74	74
Smoking history		
Never smoke	21	21
Smoke	79	79
ICI Drugs		
PD-1 inhibitor	65	65
PD-L1 inhibitor	35	35
Chemotherapy		
Platinum plus etoposide	87	87
Nab-paclitaxel plus etoposide	13	13
ECOG PS		
0–1	94	94
≥2	6	6
Brain metastasis		
Yes	22	22
No	78	78
Liver metastasis		
Yes	24	24
No	76	76
Bone metastasis		
Yes	29	29
No	71	71
Treatment efficacy		
PR	60	60
SD	31	31
PD	9	9
dNLR		
<4.0	78	78
≥4.0	22	22
LDH (U/L)		
<283	75	75
≥283	25	25
Pretreatment LIPI		
Good	64	64
Intermediate	25	25
Poor	11	11

LD, limited disease; ED, extensive disease; ECOG PS, Eastern Cooperative Oncology Group Performance Status; ICI, immune checkpoint inhibitor; PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; dNLR, derived neutrophil-to-lymphocyte ratio; LDH, lactate dehydrogenase; LIPI, Lung immune prognostic index; PR, partial response; SD, steady disease; PD, progressive disease.

Univariate and Multivariate Analysis for PFS and OS

At time of data cutoff, 69% of the patients reached PD and 39% died. LIPI good was associated with better PFS than LIPI intermediate/poor (median: 8.4 *vs* 4.7 months, $p = 0.02$) (**Figure 2**). Univariate analysis demonstrated that ECOG PS 0–1, no bone metastasis, and pretreatment LIPI good were related to better PFS in SCLC patients receiving first-line ICI treatment ($p < 0.05$). Before multivariate analysis, the pairwise correlation coefficients of ECOG PS, bone metastasis, and pretreatment LIPI were calculated to determine the potential correlation between each pair of these variables. All the correlation coefficients were below 0.5, indicating that there was a low correlation between each pair of these variables (**Table 2**). After multivariate analysis, the results indicated that ECOG PS ≥ 2 (HR: 2.58; 95%CI, 1.10, 6.04; $p = 0.03$) and bone metastasis (HR: 2.53; 95%CI, 1.47, 4.37; $p = 0.001$) were independent risk factors for PFS. In contrast, pretreatment LIPI intermediate/poor (HR: 1.42; 95%CI, 0.84, 2.39; $p = 0.19$) was not an independent risk factor for PFS in multivariate analysis (**Table 3**).

As shown in **Figure 2**, patients with LIPI good had better OS than those with LIPI intermediate/poor (median: 23.8 *vs* 13.3 months, $p = 0.0006$). Univariate analysis showed that PD-1 inhibitor treatment, LD, ECOG PS 0–1, no liver metastasis, no bone metastasis, and pretreatment LIPI good were related to better OS ($p < 0.05$). All the pairwise correlation coefficients of ICIs drugs, stage, ECOG PS, liver metastasis, bone metastasis, and pretreatment LIPI were below 0.5 (**Table 2**). After multivariate analysis, the results showed that PD-L1 inhibitors (HR: 2.37; 95%CI, 1.10, 5.11; $p = 0.03$), ECOG PS ≥ 2 (HR: 6.96; 95%CI, 2.25, 21.55; $p = 0.001$), liver metastasis (HR: 2.66; 95%CI, 1.19, 5.93; $p = 0.02$), bone metastasis (HR: 4.61; 95%CI, 2.01, 10.59; $p < 0.001$), and LIPI intermediate/poor (HR: 2.34; 95%CI, 1.13, 4.86; $p = 0.02$) were independent risk factors for OS (**Table 4**).

Subgroup Analysis of Relationship Between LIPI and Survival Outcomes

We evaluated the differences in patients' characteristics between the LIPI good and LIPI intermediate/poor groups. The results indicated that age, liver metastasis, and bone metastasis were not balanced between the two groups ($p < 0.05$) (**Table 5**). Subgroup analysis stratified by these characteristics was further conducted. As shown in **Figures 3**, **4**, LIPI good was associated with better PFS and OS compared with LIPI intermediate/poor in males, smokers, those with ED, those receiving PD-1 inhibitors, and those with liver metastasis ($p < 0.05$).

DISCUSSION

Although ICIs have been established as an important option for treating patients with SCLC, these drugs are not beneficial for all

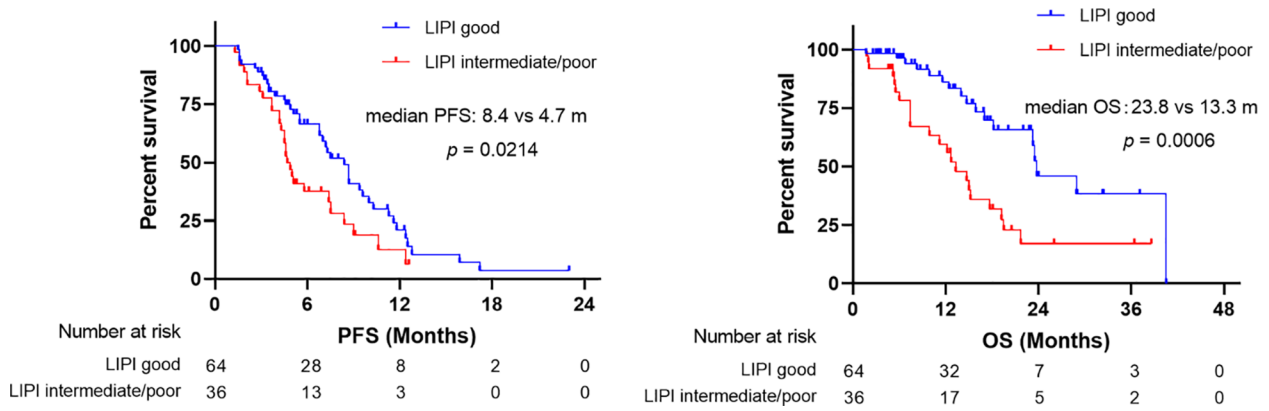

FIGURE 2 | Association between pretreatment LIPI with PFS and OS.

TABLE 2 | Correlation coefficient between each pair of the variables selected by univariate analysis.

Correlation coefficients	ECOG PS	Bone metastasis	Pretreatment LIPI	ICI drugs	Stage	Liver metastasis
ECOG PS	–	0.117	0.161	-0.185	0.150	0.351
Bone metastasis	0.117	–	0.347	0.085	0.379	0.415
Pretreatment LIPI	0.161	0.347	–	-0.026	0.160	0.310
ICI drugs	-0.185	0.085	-0.026	–	-0.043	0.128
Stage	0.150	0.379	0.160	-0.043	–	0.333
Liver metastasis	0.351	0.415	0.310	0.128	0.333	–

ECOG PS, Eastern Cooperative Oncology Group Performance Status; LIPI, lung immune prognostic index; ICI, immune checkpoint inhibitor.

TABLE 3 | Univariate and multivariate analysis for PFS in SCLC patients treated with ICIs.

Variable	Category	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p-value*	HR (95%CI)	*p-value*
Age (year)	≥60 *vs* <60	1.14 (0.71, 1.84)	0.59	—	—
Sex	Female *vs* Male	1.28 (0.63, 2.58)	0.50	—	—
Smoking history	Yes *vs* No	0.58 (0.33, 1.02)	0.06	—	—
ICI drugs	PD-L1 inhibitors *vs* PD-1 inhibitors	1.35 (0.82, 2.22)	0.24	—	—
Stage	ED *vs* LD	0.99 (0.57, 1.72)	0.98	—	—
ECOG PS	≥2 *vs* 0–1	2.71 (1.16, 6.35)	0.02	2.58 (1.10, 6.04)	0.03
Brain metastasis	Yes *vs* No	1.13 (0.64, 1.98)	0.68	—	—
Liver metastasis	Yes *vs* No	1.57 (0.91, 2.69)	0.10	—	—
Bone metastasis	Yes *vs* No	2.81 (1.67, 4.73)	<0.001	2.53 (1.47, 4.37)	0.001
Pretreatment LIPI	Intermediate/Poor *vs* Good	1.76 (1.08, 2.89)	0.03	1.42 (0.84, 2.39)	0.19

ICI, immune checkpoint inhibitor; PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; LD, limited disease; ED, extensive disease; ECOG PS, Eastern Cooperative Oncology Group Performance Status; LIPI, lung immune prognostic index; HR, hazard ratio; CI, confidence interval.

patients. The method of selecting SCLC patients who could respond to immunotherapy remains unclear. Inflammatory markers have been found to be correlated with the survival of patients with lung cancer (27–34). The LIPI, calculated by dNLR and LDH, has been investigated as a prognostic factor for lung cancer. Mezquita et al. (23) reported that pretreatment LIPI was related to clinical outcomes of advanced NSCLC with ICI treatment, but not chemotherapy. However, Kazandjian et al. (35) demonstrated that LIPI was an important prognostic biomarker irrespective of treatment modality in NSCLC. Sonehara et al. (36) first revealed that LIPI could be used as a prognostic biomarker for SCLC patients, but the sample size was

TABLE 4 | Univariate and multivariate analysis for OS in SCLC patients treated with ICIs.

Variable	Category	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p-value*	HR (95%CI)	*p-value*
Age (year)	≥60 *vs* <60	1.22 (0.65, 2.32)	0.54	—	—
Sex	Female *vs* Male	0.34 (0.08, 1.40)	0.13	—	—
Smoking history	Yes *vs* No	1.74 (0.68, 4.46)	0.25	—	—
ICI drugs	PD-L1 inhibitors *vs* PD-1 inhibitors	2.20 (1.11, 4.35)	0.02	2.37 (1.10, 5.11)	0.03
Stage	ED *vs* LD	3.20 (1.13, 9.03)	0.03	0.98 (0.29, 3.28)	0.97
ECOG PS	≥2 *vs* 0–1	6.30 (2.58, 15.36)	<0.001	6.96 (2.25, 21.55)	0.001
Brain metastasis	Yes *vs* No	1.83 (0.90, 3.71)	0.09	—	—
Liver metastasis	Yes *vs* No	4.58 (2.39, 8.78)	<0.001	2.66 (1.19, 5.93)	0.02
Bone metastasis	Yes *vs* No	5.61 (2.86, 10.97)	<0.001	4.61 (2.01, 10.59)	<0.001
Pretreatment LIPI	Intermediate/Poor *vs* Good	2.93 (1.54, 5.60)	0.001	2.34 (1.13, 4.86)	0.02

ICI, immune checkpoint inhibitor; PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; LD, limited disease; ED, extensive disease; ECOG PS, Eastern Cooperative Oncology Group Performance Status; LIPI, lung immune prognostic index; HR, hazard ratio; CI, confidence interval.

small, and the study involved patients without ICIs as first-line treatment. Other previous studies showed that pretreatment LIPI was a prognostic biomarker in ED-SCLC patients receiving chemotherapy or LD-SCLC patients (37, 38). In a recent retrospective study with data from a randomized clinical trial, inflammatory markers, including LIPI, were evaluated in ED-SCLC patients receiving atezolizumab and chemotherapy, and the results showed that LIPI was not an independent prognostic factor (39). However, their study had a small sample size and the patient population in the prospective clinical trial could not represent the entire SCLC population receiving first-line PD-1/PD-L1 inhibitor treatment.

TABLE 5 | Differences of patients' characteristics between the two groups.

Characteristics	Pretreatment LIPI		*p-value*
	Good	Intermediate/poor	
Age (year)			
<60	36	12	0.037
≥60	28	24	
Sex			
Male	53	35	0.051
Female	11	1	
Stage			
LD	20	6	0.154
ED	44	30	
Smoking history			
Never smoke	17	4	0.079
Smoke	47	32	
ICI drugs			
PD-1 inhibitors	41	24	0.83
PD-L1 inhibitors	23	12	
ECOG PS			
0–1	62	32	0.184
≥2	2	4	
Brain metastasis			
Yes	13	9	0.621
No	51	27	
Liver metastasis			
Yes	9	15	0.003
No	55	21	
Bone metastasis			
Yes	11	18	0.001
No	53	18	

ICI, immune checkpoint inhibitor; PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; LD, limited disease; ED, extensive disease; ECOG PS, Eastern Cooperative Oncology Group Performance Status; LIPI, lung immune prognostic index; HR, hazard ratio; CI, confidence interval.

To the best of our knowledge, this is the first study to demonstrate the relationship between pretreatment LIPI and the survival outcomes of SCLC patients receiving first-line ICI treatment. In previous studies, the included cohorts were divided into three groups (LIPI good, LIPI intermediate, and LIPI poor) (37–39). However, no obvious differences were reported between the LIPI intermediate and LIPI poor groups in terms of OS (37). In addition, few untreated patients had a poor LIPI score (11% patients in our study). Therefore, it might be more appropriate if the cohort was separated into two groups (LIPI good and LIPI intermediate/poor). In a previous study on the association of pretreatment LIPI with survival time in advanced hepatocellular carcinoma patients, the population was also divided into two groups (LIPI good and LIPI intermediate/poor) (40). Our findings showed that pretreatment LIPI was associated with PFS and OS in SCLC patients with first-line ICI treatment in univariate analysis. Multivariate analysis showed that pretreatment LIPI was an independent prognostic factor for OS, but not for PFS. However, the negative results of PFS should be interpreted with caution owing to the retrospective nature of this study. PFS was influenced by multiple factors, such as the frequency of evaluation of tumors. Conversely, the difference in OS between the LIPI good group and LIPI intermediate/poor group is more convincing. In addition, although multivariate analysis took many factors into consideration, other factors not included in the analysis, such as PD-L1, TMB and antibiotic therapy (41), may also affect the final results. We further conducted a subgroup analysis by patients' characteristics, and the results indicated that the LIPI good group had better PFS and OS than the LIPI intermediate/poor group, especially in subgroups of males, smokers, those

PFS subgroup	No. of patients	Intermediate/Poor events (%)	Good events (%)	HR (95 % CI)	*p-value*
Overall	100	36 (36)	64 (64)	1.77 (1.08–2.91)	0.023
Age (year)					
< 60	48	12 (12)	36 (36)	1.96 (0.93–4.16)	0.078
≥ 60	52	24 (24)	28 (28)	1.64 (0.81–3.33)	0.168
Sex					
Male	88	35 (35)	53 (53)	1.87 (1.11–3.17)	0.020
Female	12	1 (1)	11 (11)	2.89 (0.30–28.11)	0.360
ECOG PS					
0–1	94	32 (32)	62 (62)	1.61 (0.95–2.71)	0.076
≥ 2	6	4 (4)	2 (2)	2.33 (0.26–21.39)	0.020
Stage					
LD	26	6 (6)	20 (20)	1.36 (0.41–4.54)	0.621
ED	74	30 (30)	44 (44)	1.91 (1.09–3.35)	0.023
ICI drugs					
PD-1 inhibitors	65	24 (24)	41 (41)	2.39 (1.26–4.52)	0.007
PD-L1 inhibitors	35	12 (12)	23 (23)	1.20 (0.52–2.77)	0.665
Smoking history					
Never smoker	21	4 (4)	17 (17)	1.52 (0.40–5.77)	0.539
Smoker	79	32 (32)	47 (47)	2.12 (1.21–3.72)	0.009
Brain metastasis					
Yes	22	9 (9)	13 (13)	3.00 (1.06–8.52)	0.037
No	78	27 (27)	51 (51)	1.45 (0.82–2.59)	0.205
Liver metastasis					
Yes	24	15 (15)	9 (9)	5.62 (1.46–21.68)	0.012
No	76	21 (21)	55 (55)	1.35 (0.73–2.47)	0.337
Bone metastasis					
Yes	29	18 (18)	11 (11)	1.79 (0.78–4.13)	0.173
No	71	18 (18)	53 (53)	1.23 (0.61–2.47)	0.568

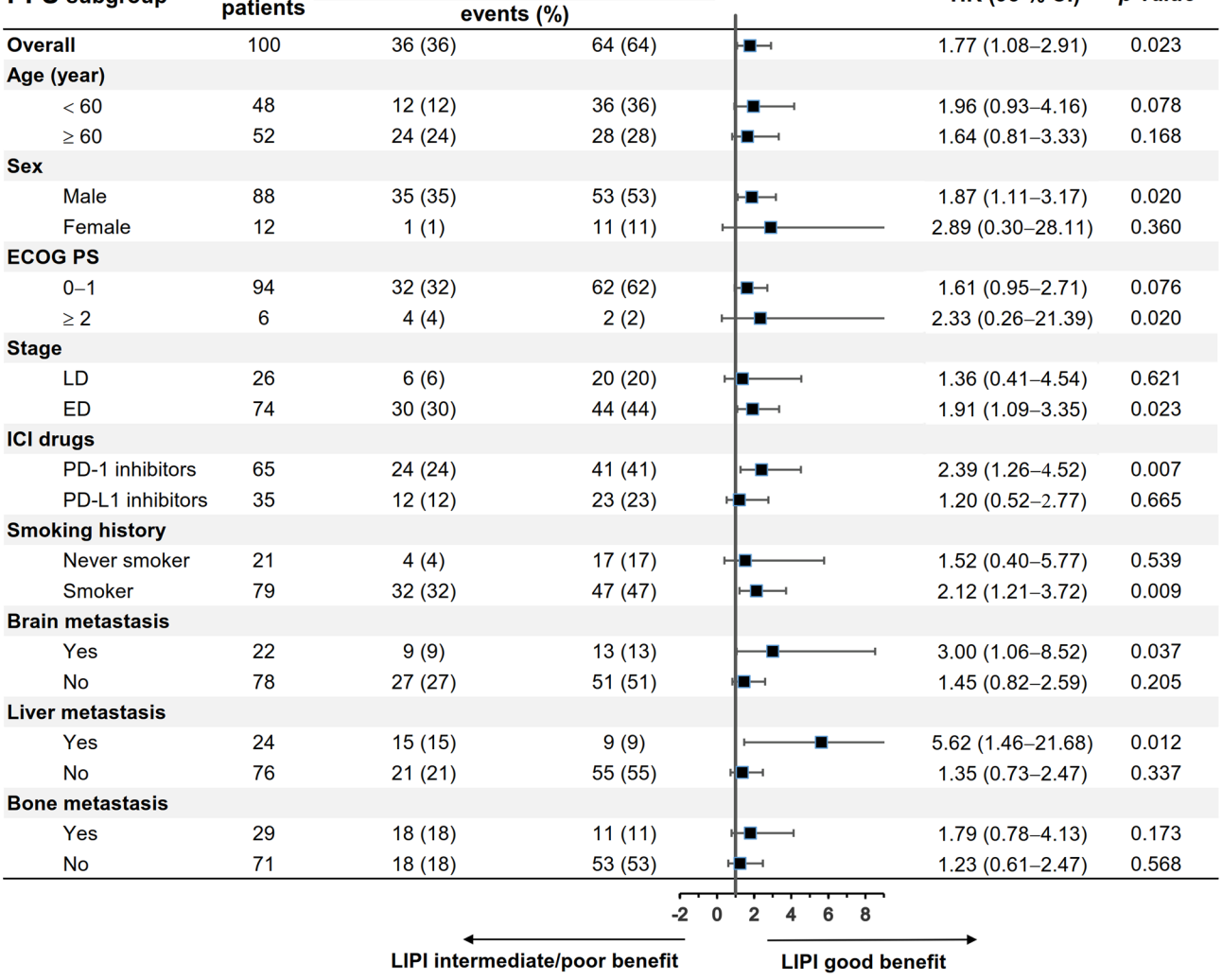

FIGURE 3 | Subgroup analysis of the association between pretreatment LIPI and PFS.

with ED, those receiving PD-1 inhibitor treatment, and those with liver metastasis, which revealed that the pretreatment LIPI might be prognostic only for specific subgroups of SCLC patients. However, these results need further investigation.

There were several limitations to this study. Firstly, it was a single-center retrospective study with a small sample size; therefore, some confounding factors and selective bias could not be avoided. Because the sample size of the LIPI poor group was too small, we divided the cohort into two groups (LIPI good and LIPI intermediate/poor) rather than three groups (LIPI good, LIPI intermediate, and LIPI poor) to conduct analyses. Secondly, considering the promising results of nivolumab plus chemotherapy as first-line treatment in SCLC patients in the EA5161 study and the accessibility and affordability of PD-L1 inhibitors in Chinese patients, 65% of the patients in this study were treated with PD-1 inhibitors, though only PD-L1 inhibitors have been approved as first-line treatment in SCLC patients by FDA. Thus, the interpretation of our results should be cautious due to drug selecting bias. Lastly, the cutoff values of dNLR and LDH were data-based and calculated using X-tile software, which may not have been optimal. Nevertheless, our study offered a simple and non-invasive method to help identify advanced SCLC patients who could benefit from first-line ICI plus chemotherapy treatment in clinical practice.

Our findings showed the prognostic value of pretreatment LIPI in advanced SCLC patients receiving first-line ICI treatment combined with chemotherapy, especially in males, those with ED, those receiving PD-1 inhibitor treatment, smokers, and those with

OS subgroup	No. of patients	Intermediate/Poor events (%)	Good events (%)	HR (95 % CI)	*p-value*
Overall	100	36 (36)	64 (64)	2.94 (1.54–5.60)	0.001
Age (year)					
< 60	48	12 (12)	36 (36)	5.83 (2.07–16.48)	0.001
≥ 60	52	24 (24)	28 (28)	2.07 (0.79–5.42)	0.140
Sex					
Male	88	35 (35)	53 (53)	2.61 (1.34–5.07)	0.005
Female	12	1 (1)	11 (11)	5.75 (0.31–106.44)	0.241
ECOG PS					
0–1	94	32 (32)	62 (62)	2.70 (1.34–5.43)	0.006
≥ 2	6	4 (4)	2 (2)	1.97 (0.21–18.35)	0.553
Stage					
LD	26	6 (6)	20 (20)	6.98 (0.72–67.57)	0.093
ED	74	30 (30)	44 (44)	2.50 (1.27–4.94)	0.008
ICI drugs					
PD-1 inhibitors	65	24 (24)	41 (41)	3.52 (1.52–8.17)	0.003
PD-L1 inhibitors	35	12 (12)	23 (23)	1.95 (0.67–5.71)	0.221
Smoking history					
Never smoker	21	4 (4)	17 (17)	12.91 (1.34–124.33)	0.027
Smoker	79	32 (32)	47 (47)	2.33 (1.16–4.65)	0.017
Brain metastasis					
Yes	22	9 (9)	13 (13)	3.64 (0.94–14.18)	0.062
No	78	27 (27)	51 (51)	2.69 (1.26–5.75)	0.011
Liver metastasis					
Yes	24	15 (15)	9 (9)	5.24 (1.37–19.94)	0.015
No	76	21 (21)	55 (55)	2.20 (0.93–5.19)	0.072
Bone metastasis					
Yes	29	18 (18)	11 (11)	2.12 (0.81–5.51)	0.125
No	71	18 (18)	53 (53)	2.17 (0.81–5.85)	0.124

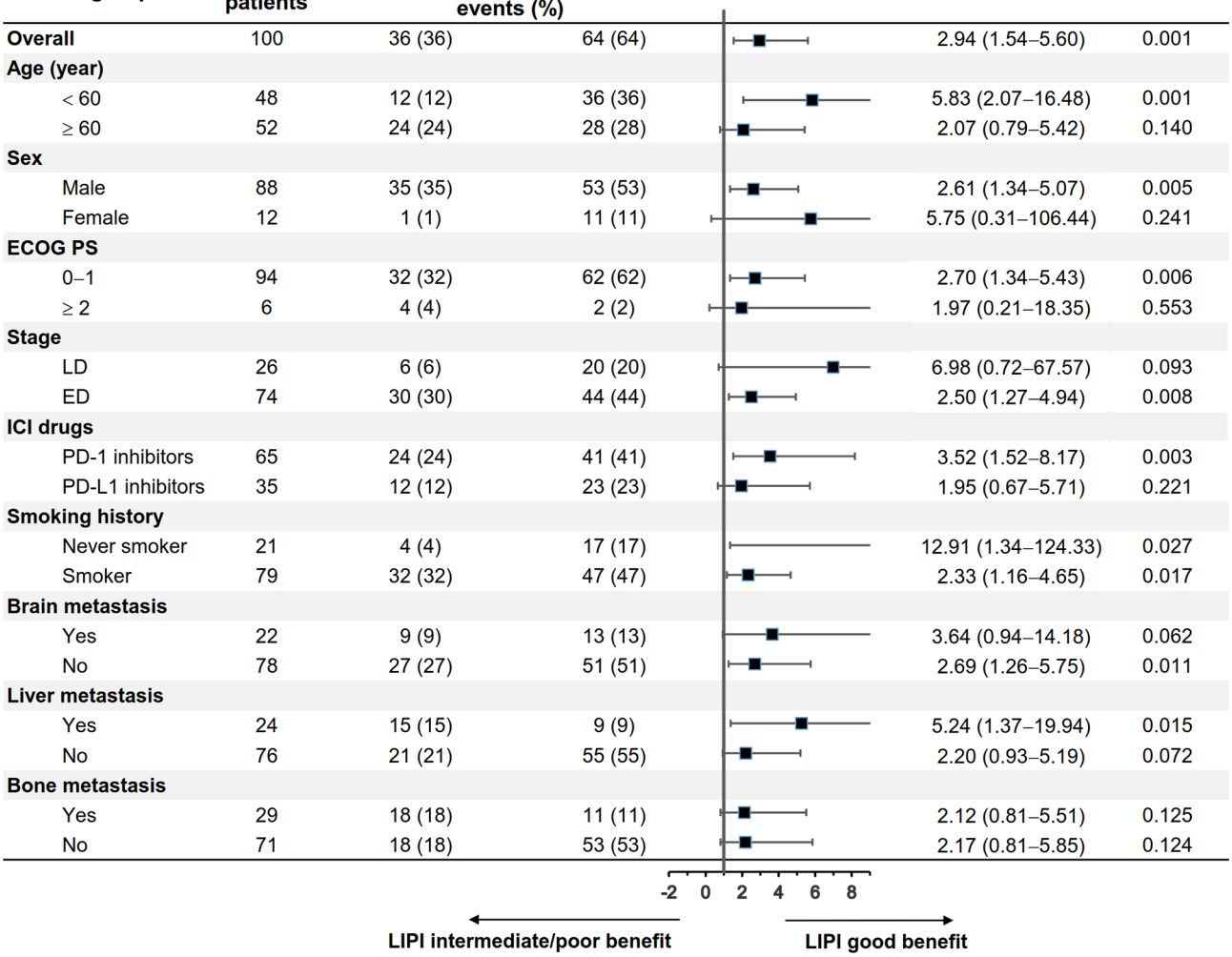

FIGURE 4 | Subgroup analysis of the association between pretreatment LIPI and OS.

liver metastasis. Pretreatment LIPI might serve as a useful tool to identify patients who may benefit from this treatment regimen.

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 protocol was approved by the Ethics Committee of Chinese PLA general hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YH conceived the idea of this article. LL completed the work of acquisition of data. ZZ and LL shared the task of analysis, interpretation of data, and manuscript writing. All authors participated in discussing and revising the manuscript. All authors contributed to the article and approved the submitted version.

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