IMMUNOLOGY AND IMMUNOTHERAPY OF HEAD AND NECK CANCER

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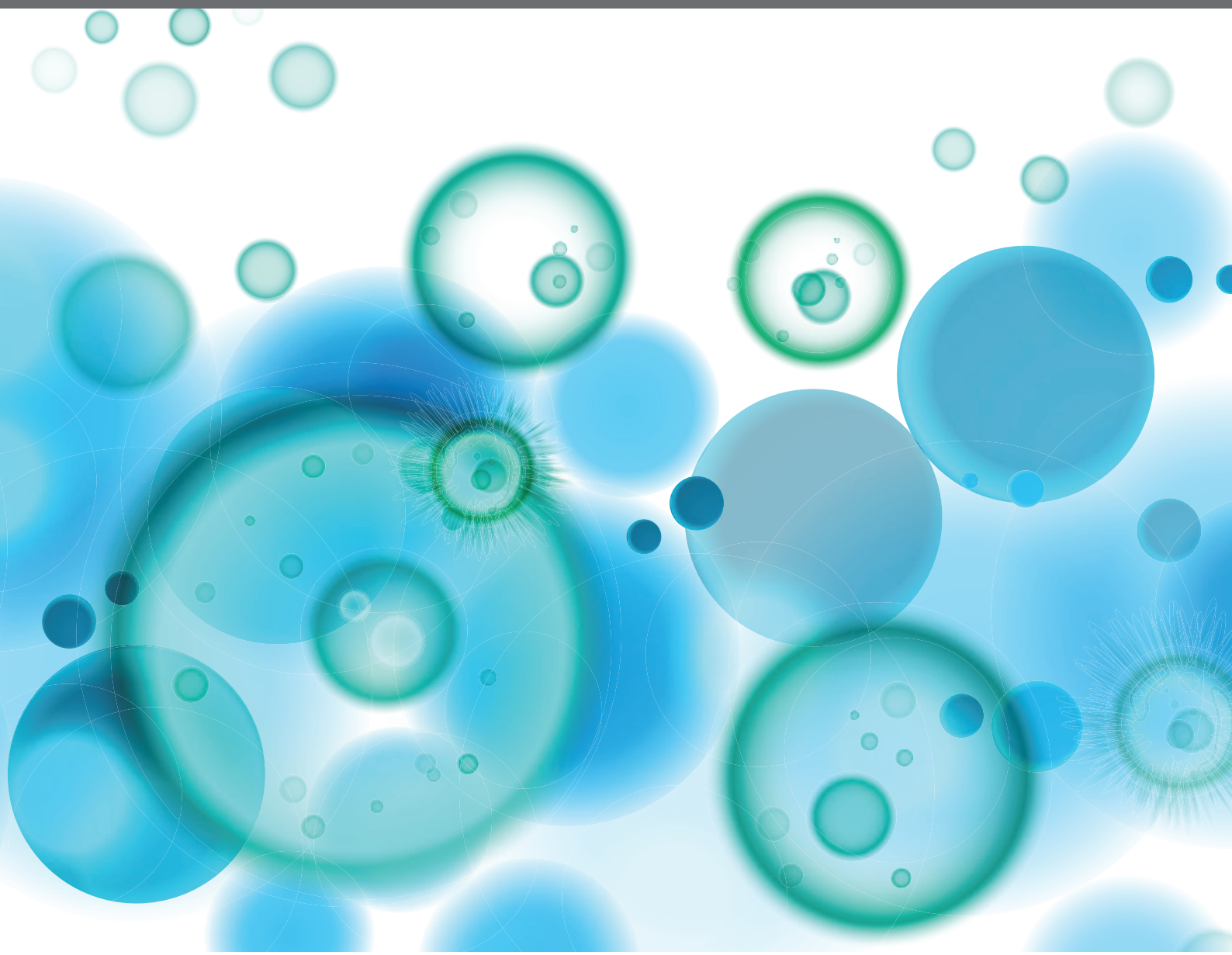

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IMMUNOLOGY AND IMMUNOTHERAPY OF HEAD AND NECK CANCER

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EDITORIAL
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Editorial: Immunology and Immunotherapy of Head and Neck Cancer

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Keywords: head and neck cancer, immunotherapy, biomarkers, PD-1/PD-L1, HPV

Editorial on the Research Topic

Immunology and Immunotherapy of Head and Neck Cancer

Immunotherapy targeting programmed death-1/programmed death ligand-1 (PD-1/PD-L1) has been established as the standard of care in the first line of treatment for recurrent metastatic (R/M) HNC, either in combination with chemotherapy or as monotherapy for PD-L1 positive tumors, as was meticulously reviewed in the current topic by Hsieh et al. Nonetheless, results from several clinical studies have shown limited efficacy of immune checkpoint inhibition in earlier disease stages. For locally advanced disease immunotherapy has been explored in two distinct frameworks; 1. in combination with chemoradiotherapy for treatment intensification in high risk cases, or 2. as an alternative resource, aiming at the de-intensification of high-toxicity chemoradiotherapy regimens, in low-risk cases. Immunotherapy in the neoadjuvant setting is also investigated in several clinical trials, primarily targeting high risk/HPV negative cases, either as single agent or in chemotherapy combinations. Although induction immunotherapy exhibited an acceptable safety profile in most clinical trials, it has not been studied in randomized studies and thus has not received approval in the neoadjuvant setting. Additionally, for locally advanced disease, Saddawi-Konefka et al. also provide a comprehensive summary on the current immunotherapy-related preclinical and clinical data. As the addition of PD-L1 inhibitor to cisplatin chemoradiotherapy has failed to demonstrate advantage over cisplatin chemoradiotherapy alone in randomized III trials, the authors highlight the importance of optimizing therapeutic sequence as a potential way to maximize benefit from immune checkpoint inhibition. Moreover, in an interesting case report included in the present Research Topic, Nie et al. propose an alternative treatment approach for locally recurrent disease after surgery, which involves the combination of an PD-1/PD-L1 inhibitor with an EGFR targeted agent, followed by radiotherapy. The combined regimen led to nearly complete tumor regression and consequent improvement in a PD-L1 positive case, where prognosis with conventional therapeutic options would be otherwise dismal. However, it should be noted that the observed positive outcome was accompanied by a grade IV autoimmune adverse event, indicating the need to perform a cost-benefit evaluation of the proposed schema in larger patient cohorts.

Biomarker analysis performed as part of clinical trials, which led to the introduction of immunotherapy in R/M HNC management, have shown a direct correlation of drug efficacy with PD-L1 protein expression, which resulted in PD-L1 becoming the only clinically approved predictive biomarker of immunotherapy response in this type of cancer. However, as a significant

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proportion of PD-L1 positive cases experience no clinical benefit, a large body of research has focused on the tumor microenvironment (TME) in search of diverse predictive biomarkers with potential clinical applicability. In their article on the present Research Topic, Zhao et al. identified the immune-inhibitory functions of IL-21 in HNC TME and described its negative prognostic effect. Using patient and healthy-donor tissue samples, they found association of increased IL21+CD4+ T helper cells' presence in stroma with advanced disease stage and poor survival outcomes. Additionally, IL-21 prompted cell polarization towards the regulatory (Treg) phenotype and induced Treg generation and expansion utilizing a PD-1/PD-L1 interaction-dependent mechanism. Interestingly, IL21- stimulated Tregs specifically, appeared to suppress immunity against tumor associated antigens, an effect that was reversed by dual IL-21 and PD-1 inhibition. The above finding provides insight on a possible mechanism of resistance to immunotherapy in HNC and at the same time sets the groundwork for further validation of IL-21 as a potential predictive biomarker for response to PD1 checkpoint inhibitors.

A different approach in biomarker development involves gene expression analysis and molecular characterization of head and neck tumors. Accordingly, RNA analysis on PD-L1 differential expression among HNC patients by Wu et al., revealed that increased baseline PD-L1 expression correlated with poor prognosis and the "PD-L1 high" subgroup exhibited upregulated tumor-associated macrophage gene expression, as well as increased expression of epithelial mesenchymal transition-related genes. The authors also conclude that the same patient category demonstrated improved response both to immunotherapy and chemotherapy agents. Additionally, Wang et al. proposed a 21 long-noncoding-RNA-pair immune related signature, derived from TCGA data, as both a prognostic tool and an immunotherapy predictive biomarker. It should be noted that although gene signatures often exhibit promising predictive value, they are hard to validate in large real-world cohorts and as their identification requires costly assays, they represent controversial candidates for clinical implementation as companion diagnostic tests.

Moreover, HPV positivity is known to characterize a biologically distinct sub-category of head and neck tumors, with favorable prognosis and abundant immune infiltration. As findings from previous studies have failed to draw definite conclusions on the potential implication of HPV status in immunotherapy outcomes, Xu et al. attempt to shed further light on the Research Topic by analyzing combined study data. In their meta-analysis of 814 patients with known HPV status, they observed a clear association of HPV positivity with increased objective response rate and overall survival in anti-PD-1/PD-L1 treated cases and the same favorable trend was observed for disease control rate and progression- free survival. The above findings suggest that HPV- related pre-existing tumor immunogenicity enhances the therapeutic effect of immune checkpoint inhibition.

In conclusion, the present Research Topic has accumulated impactful articles aiming at the compilation of current knowledge on immunotherapy for HNC, as well as the illustration of novel findings on immunotherapy biomarkers.

AUTHOR CONTRIBUTIONS

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

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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SYSTEMATIC REVIEW
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Programmed Death-1/Programmed Death-Ligand 1-Axis Blockade in Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma Stratified by Human Papillomavirus Status: A Systematic Review and Meta-Analysis

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Background: Programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) inhibitors have provided clinical benefit to head and neck squamous cell carcinoma (HNSCC) patients in recent clinical trials. However, it remains unclear as to whether human papillomavirus (HPV) status is associated with improved clinical outcome of anti-PD-1 or anti-PD-L1 immunotherapy in HNSCC.

Methods: PubMed, EMBASE, Cochrane Library, and Web of Science were systematically searched up to February 28, 2021. Published clinical trials of HNSCC patients treated with only PD-1 or PD-L1 inhibitors were selected. The primary or secondary outcome of these studies included objective response rate (ORR) stratified by HPV status. The pooled odds ratio (OR) and hazard ratio (HR) were estimated using a fixed-effect model.

Results: A total of seven eligible studies comprising 814 patients were included. The ORR of HPV positive HNSCC patients was significantly higher than that of HPV negative HNSCC patients (OR = 1.77; 95%CI = 1.14-2.74; P = 0.01), and this favorable effect occurred in pooled anti-PD-L1 trials (OR = 2.66; 95%CI = 1.16-6.11; P = 0.02). In comparison, the pooled OR was 1.51 in anti-PD-1 trials (95%CI = 0.90-2.54; P = 0.12). Survival analysis indicated that HPV positive HNSCC patients had a lower risk of overall

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death as compared to HPV negative HNSCC patients (HR = 0.77; 95%CI = 0.60–0.99; P = 0.04).

Conclusions: HPV positive HNSCC patients display improved outcomes with PD-1/PD-L1 axis blockade as compared to HPV negative HNSCC patients. These improved outcomes are likely driven to a greater extent by anti-PD-L1 inhibitors. However, randomized controlled trials with greater numbers of patients are needed for validation of these early findings.

Keywords: human papilloma virus, immune checkpoint blockade, head and neck squamous cell carcinoma, anti-PD-1, anti-PD-L1

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer globally, with 600,000 cases diagnosed annually and mortality rates as high as 40%–50% (1). The vast majority of head and neck cancers are squamous cell carcinomas, which arise within different anatomical subsites. Therapeutic strategies for HNSCC include surgery, chemotherapy, radiotherapy, and targeted agents, including small molecular inhibitors or antibodies (2). Despite advances in treatment, the estimated 5-year overall survival rate of HNSCC has not significantly improved (3). Recently, there have been several studies that show immune checkpoint blockade appears to provide a promising new avenue for treatment in HNSCC (4, 5).

Programmed death-1 (PD-1), a member of the immunoglobulin superfamily associated with CD28 and CTLA-4, may be expressed on the surface of activated T cells, B cells, and monocytes (6). Programmed death-ligand 1 (PD-L1) binding to PD-1 on T cells results in suppression of the T cell immune response (7). Cancer cells may develop several mechanisms of escaping immune-mediated surveillance and death, including surface expression of PD-L1 (8). The interruption of PD-1 engagement by its ligand reinvigorates the immune system, allowing immune-mediated anti-cancer responses to resume, leading to marked clinical responses in some cancers (9). Anti-PD-1 and anti-PD-L1 antibodies such as nivolumab, pembrolizumab, cemiplimab, and atezolizumab, durvalumab, avelumab have shown promising results in several cancer types (10, 11). Nivolumab and pembrolizumab have been approved as first-line agents in recurrent/metastatic HNSCC patients by the United States Food and Drug Administration (FDA) (7, 12).

Despite a declining trend in smoking and drinking rates in the United States, the incidence of a proportion of HNSCC related to human papillomavirus (HPV) infection has been increasing (13). HPV positive and negative HNSCC are considered two entirely different types of cancer, in part due to their unique molecular landscapes (14). HPV associated oncogenes E6 and E7 drive oncogenesis in HNSCC by inactivating tumor suppressors TP53 and Rb and activating oncogenic signaling pathways including EGFR and PI3K etc (15, 16). Nevertheless, numerous clinical studies have demonstrated that HPV positivity in HNSCC confers a clear survival benefit as compared to HPV negative HNSCC patients after surgery with or without chemoradiotherapy (17, 18). One possible factor contributing to this survival difference is that HPV may elicit inherent local or systemic immunity against tumor cells in HNSCC patients, even in the absence of therapy (19), leading to the hypothesis that HPV positive HNSCC patients may show increased benefit from immune checkpoint blockade.

There have been conflicting results from published HNSCC clinical trials involving either anti-PD-1 or anti-PD-L1 therapy. The HAWK (20) study concluded that HPV positive patients had a higher objective response rate and survival rate than HPV negative patients. However, Keynote012 (21), NCT01375842 (22), and Keynote055 (23) trials reported that HNSCC patients' tumor response did not correlate with HPV status. Data from two recent meta-analyses (24, 25) suggest there is a trend towards significance favoring higher response rates in HPV positive vs. HPV negative tumors in patients receiving anti-PD-1/PD-L1 therapy. One study by Wang et al. (25) used odds ratio (OR) in the analysis of overall survival, however this calculation does not take into account the effect of time. Furthermore, key limitations in these studies include a lack of stratification by anti-PD-1 or anti-PD-L1 therapy separately and inadequate selection of trials based on what is publicly available. Based on these inconsistent findings, we posited that there might be a difference in outcomes in HPV positive patients treated with immunotherapy depending on the use of either PD-1 or PD-L1 agents disrupting the PD-1/PD-L1 axis.

To further understand the importance of HPV status in HNSCC patients treated with anti-PD-1 or anti-PD-L1 agents, we systematically pooled the results from available trials together and conducted the present meta-analysis, which ultimately may help inform further investigation and ultimately clinical decision making.

MATERIALS AND METHODS

Search Strategy and Eligibility Criteria

This systematic review and meta-analysis was conducted according to the Cochrane Handbook for Systematic Reviews of Interventions (26) and reported by adhering to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses: the PRISMA Statement (27). Our protocol has been registered in the PROSPERO platform (ID: CRD42020175779).

Two independent authors systematically searched PubMed, EMBASE, Cochrane Library, and Web of Science for relevant articles published in English until February 28, 2021. Our search strategies included the following terms: "HPV or Human papillomavirus", "Immunotherapy or Cemiplimab or Atezolizumab or Nivolumab or Pembrolizumab or Durvalumab or Avelumab or PD-1 or PD-L1 or PD1 or PDL1 or checkpoint" and "head and neck or head and neck cancer or head and neck neoplasm or head and neck tumor or head and neck carcinoma or HNC or HNSCC or SCCHN". The complete search strategies used are found in **Supplementary Data**. We also manually checked the reference lists of identified studies and reviews to include more eligible trials. The search results were imported into Endnote (version 9.2).

Studies were included if they satisfied the following criteria: clinical trials of HNSCC patients treated with only a PD-1 or PD-L1 inhibitor agent, regardless of region, race, age, and gender; studies with a primary or secondary outcome that included objective response rate (ORR); reporting of ORR stratified by HPV status; studies reported in English. Clinical trials allowing participants with prior exposure to any immune-checkpoint blockade were excluded. If the same clinical study was reported in more than one publication, only the one with the most recent or complete data was analyzed. The methodological quality of randomized controlled trials (RCT) was evaluated by the recommendations in the Cochrane Collaboration handbook (26) to assess the risk of bias. The quality of non-RCTs was judged by the Newcastle-Ottawa Scale (28) by two of the authors independently.

Data Extraction

We included the following data extracted from the eligible studies: trial name, publication year, study design, drug and dose, number of participants, age, gender, HPV status, anatomical subsite, ORR, overall survival (OS), progression-free survival (PFS), median time of follow-up, median OS, median PFS, median duration of response and the median time to response. The disease control rate (DCR) was extracted as the percentage of patients with complete response, partial response, or stable disease in the trial according to the guideline of response evaluation criteria in solid tumors (RECIST version 1.1) (29).

Statistical Analysis

Continuous variables were reported as mean ± standard deviation (or median and range). Categorical variables were expressed as count and percentage. Measures of ORR and DCR stratified by HPV status were assessed by odds ratio (OR) and 95% confidence interval (CI). Subgroup analysis of ORR was performed according to the treatment agent used. OS and PFS data were evaluated by hazard ratio (HR) and 95% CI, and Tierney methodology was used for calculation if the data were not directly available in the original report (30). Statistical heterogeneity was detected using the Cochran Q chi-square test and inconsistency index (I^2). If the studies were low heterogeneity (P>0.1, I^2 < 50%), a fixed-effects model was used. Otherwise, a random-effects model was applied. We did not assess publication bias because only a small number of studies were included in the meta-analysis (n_{max} = 7). All statistical analyses were conducted with Review Manager version 5.3 and STATA version 16.

RESULTS

Study Search, Selection, and Characteristics

A literature search identified 829 records after removing duplicates, and seven studies (20–23, 31–33) met the inclusion criteria after screening by title, abstract, and full text (**Figure 1**). The seven clinical trials included patients treated with anti-PD-1/PD-L1 agents and standard treatment and were comprised of two randomized controlled trials (RCT) and five single-arm trials.

The summary of the risk of bias for the two RCTs was shown in **Supplementary Figure 1**. The Newcastle-Ottowa Scale (28) score of the five single-arm studies was 5 (**Supplementary Table 1**).

Patients Characteristics

A total of 814 patients were included, 671 (82.4%) of which had HPV status reported (**Table 1**). Most of the patients were male (80.5%); the mean of the median age was 60.2 years (range 20-90) across the included trials. There were 217 (32.3%) HPV positive patients and 454 (67.7%) HPV negative patients. A summary of the anatomical subsites included was reported in **Supplementary Table 2**. The most common subsite in included trials was the oropharynx (n = 259, 31.8%). As shown in **Table 2**, the median OS and duration of response across the included studies were longer than the standard therapy arm in the Checkmate141 study (6.0-13.0 months vs. 5.1 months, 7.4-12.4 months vs. 4.0 months) (31).

Higher Objective Response Rate in HPV Positive HNSCC Patients

We conducted a pooled analysis to assess the clinical efficacy of anti-PD-1/PD-L1 agents in HNSCC patients grouped by agents and HPV status.

A total of 665 patients from seven studies with a reported ORR were included in this analysis. As shown in **Figure 2A**, the results revealed that HPV positive patients had a higher ORR than HPV negative patients, regardless of anti-PD-1 or anti-PD-L1 treatment (ORR: 21.5% vs 13.7%, odds ratio (OR) = 1.77, 95% confidence interval (95%CI) = 1.14-2.74; P = 0.01). Subgroup analysis demonstrated that the pooled OR with use of anti-PD-1 agents was 1.51 (95%CI = 0.90–2.54; P = 0.12). In comparison, the pooled OR with use of anti-PD-L1 agents was 2.66 (95%CI = 1.16–6.11; P = 0.02) (**Figure 2A**).

Favorable Overall Survival in HPV Positive HNSCC Patients

447 patients available from four studies showed that HPV positive patients had significantly better overall survival than

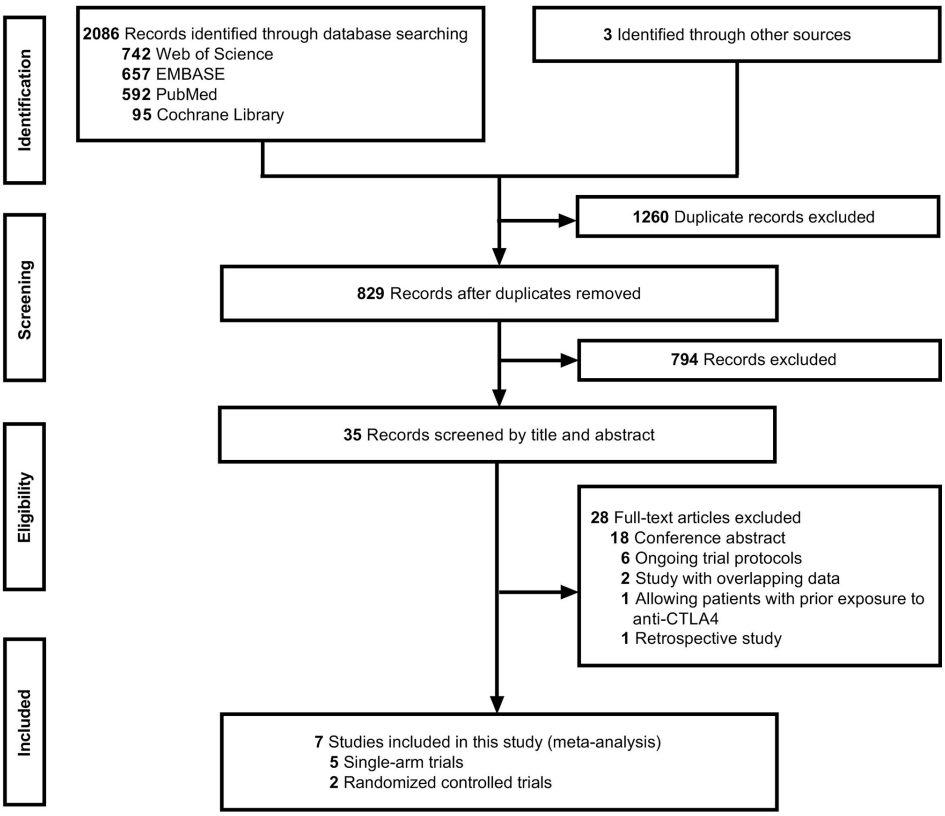

FIGURE 1 | Study selection followed by PRISMA diagram.

TABLE 1 | The characteristics of the included studies.

Study	Year	Study design (Open-label)	Drug and dose	N	Age (median, range)	Male (%)	HPV status			
							Method	+ (%)	- (%)	unknown (%)
Anti-PD-1										
Checkmate141 2y update	2018	Randomized, phase III	Nivolumab 3mg/kg, iv, every 2weeks	240	59 (29-83)	197 (82)	OPC used p16 IHC test, >70% is +	64 (27)	56 (23)	120 (50)
Keynote012	2016	Non-randomized, multicenter, multi-cohort, phase I b	Pembrolizumab 10mg/kg, iv, every 2 weeks	60	63 (20-83)	49 (82)	p16 IHC test, >70% is +	23 (38)	37 (62)	0 (0)
Keynote012 expansion	2016	Non-randomized, multicenter, multi-cohort, phase I b	Pembrolizumab 200mg, iv, every 3 weeks	132	60 (25-84)	110 (83)	the site investigator	28 (21)	104 (79)	0 (0)
Keynote055	2017	Multicenter, single-arm, phase II	Pembrolizumab 200 mg, iv, every 3 weeks	171	60 (33-90)	138 (81)	Local institution (most use p16 IHC test)	37 (22)	131 (77)	3 (1)
Anti-PD-L1										
HAWK	2019	Single-arm, phase II	Durvalumab 10 mg/kg, iv, every 2 weeks	112	60 (24-84)	80 (71)	p16 IHC test, FISH or PCR	34 (30)	65 (58)	13 (12)
CONDOR	2018	Randomized, multicenter, phase II	Durvalumab 10 mg/kg, iv, every 2 weeks	67	62 (23-82)	54 (81)	Medical records, local or central testing	18 (27)	49 (73)	0 (0)
NCT01375842	2018	Phase I a	Atezolizumab 15mg/kg, 20mg/kg, or a 1200-mg fixed dose, iv, every 3weeks	32[a]	62 (32-78)	27 (84)	PCR	13 (41)	12 (38)	3 (9)

N, number of patients; PD-1, programmed death 1; PD-L1, programmed death-ligand 1; HPV, human papillomavirus; +, positive; -, negative; iv, intravenous; OPC, oropharyngeal cancer; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; PCR, polymerase chain reaction.

[a]*In NCT01375842, four patients with nasopharyngeal cancer were excluded from the HPV analysis population.*

TABLE 2 | The response and survival time of included studies.

Study	Drug	ORR (%)	DCR (%)	Median follow-up (months)	Median OS (months)	Median PFS (months)	Median duration of response (months)	Median time to response (months)
Anti-PD-1								
Checkmate141 2y update, 2018	Nivolumab	Niv: 13.3 IC[a]: 5.8	36.3 41.3	NA[b]	7.7 5.1	2.0 2.3	9.7 4.0	2.1 2.0
Keynote012, 2016	Pembrolizumab	21.4	48.2	14 (IQR, 4-14)	13.0	2.0	12.4	1.9
Keynote012 expansion, 2016	Pembrolizumab	17.7	34.9	9 (IQR, 3-11)	8.0	2.0	not reached	2.0
Keynote055, 2017	Pembrolizumab	16.4	35.7	7 (range, 0-17)	8.0	2.1	8.0	2.0
Anti-PD-L1								
HAWK, 2019	Durvalumab	16.2	22.5	6.1 (range, 0.2-24.3)	7.1	2.1	10.3	2.0
CONDOR, 2019	Durvalumab	9.0	14.9	6.0 (range, 0.3-18.0)	6.0	1.9	not reached	4.1
NCT01375842, 2018	Atezolizumab	21.9	40.6	NA[b]	6.0	2.6	7.4	NA

PD-1, Programmed death 1; PD-L1, Programmed death-ligand 1; Niv, Nivolumab group; IC, investigator's choice group; NA, not available; ORR, objective response rate; DCR, disease control rate; OS, overall survival; PFS, progression-free survival; IQR, interquartile range.

[a]*Patients in this group are treated with standard single agent of investigator's choice, such as methotrexate, docetaxel or cetuximab.*

[b]*The follow-up time of 2-year update of Checkmate141 and NCT01375842 is of a minimum of 24.2 and 14 months, respectively.*

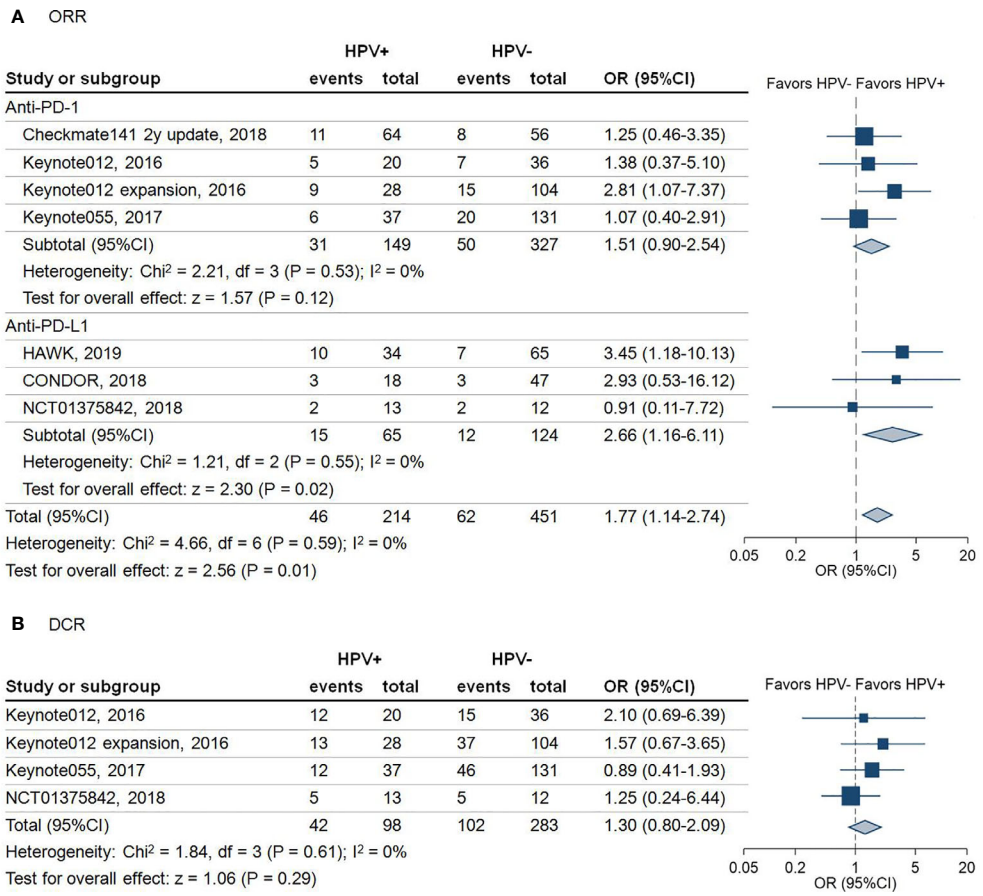

FIGURE 2 | Forest plot for the efficacy of anti-programmed death-1/programmed death-ligand 1 agents in patients with different human papillomavirus status. **(A)** Objective response rate (ORR) of the included studies stratified by the type of immune checkpoint blockade. Squares indicate adjusted effect size (odds ratio [OR]). Horizontal lines represent 95% confidence interval (95%CI). Diamonds represent the pooled ORs; **(B)** Disease control rate (DCR) of the included studies. Squares indicate adjusted effect size (odds ratio [OR]). Horizontal lines represent 95%CI. Diamonds represent the pooled ORs. Pooled ORs were calculated using the fixed-effect model. PD-1, Programmed death 1; PD-L1, Programmed death-ligand 1; HPV, human papillomavirus; +, positive; -, negative.

HPV negative patients (hazard ratio (HR) = 0.77; 95%CI = 0.60–0.99; P = 0.04) (**Figure 3A**). Because the overall survival (OS) data stratified by HPV status was not provided in the original trials, we were not capable of performing subgroup analysis of OS by anti-PD-1 and anti-PD-L1 separately.

Due to not all the trials reporting disease control rate (DCR) and progression-free survival (PFS), there were only 381 patients from four trials involved in the analysis of DCR. The pooled analysis demonstrated that HPV positive patients are 1.3 times more likely to achieve DCR compared with HPV negative patients (DCR: 42.9% vs 36.0%, OR = 1.30, 95%CI = 0.80–2.09; P = 0.29) (**Figure 2B**). However, this outcome did not achieve statistical significance. A similar trend of PFS in HPV positive over HPV negative status was noticed in 224 HNSCC patients (HR = 0.88; 95%CI = 0.63–1.22; P = 0.45) (**Figure 3B**).

DISCUSSION

The impact of immunotherapy in the treatment of head and neck squamous cell carcinoma (HNSCC) has been rapidly progressing, with an associated survival benefit in approximately 20-30% of patients (31, 34, 35). We hypothesized that the distinct immunological tumor landscapes of HPV positive and negative HNSCC patients might confer a difference in survival rates and tumor response after anti-PD-1/PD-L1 therapy (36). Previous evidence suggests that HPV may promote the expression of PD-L1 and PD-1 mediated through an IFN-γ related response (37, 38). Consequently, this may support the hypothesis that HPV is a favorable factor in both anti-PD-1 and anti-PD-L1 treated cancer patients. Through analysis of seven studies, including 814 patients, we demonstrated that HPV positive HNSCC patients treated with anti-PD-1 or anti-PD-L1 agents displayed significantly longer OS than HPV negative HNSCC patients, which is in concordance with a similar association observed in HPV positive vs. negative patients undergoing surgery or chemoradiotherapy (17, 18). While the difference in PFS and DCR was not significant between HPV positive and negative patients, the limited number of studies here may be a potential factor influencing this result. Specifically, there were only four studies that reported DCR and two studies that reported PFS. As more trial data emerge with longer follow-up time, the true association of HPV status with DCR and PFS will be more definitively ascertained.

To our knowledge, this is the first study to report an association with improved outcomes using anti-PD-L1 agents in HPV positive HNSCC patients. The explanation for this observed association is likely complicated and multifactorial. In addition to binding to PD-1, PD-L1 may inhibit T cell proliferation and induce immune tolerance *in vivo* and *in vitro via* the interaction with other receptors such as CD80 (39). Blocking PD-L1 on dendritic cells (DC) relieves cis sequestration of CD80, which allows CD80/CD28 interaction to enhance T cell priming (40). We have previously shown that HPV positive HNSCC patients might have a higher proportion of DCs than HPV negative patients (41). Furthermore, it has been shown that the HPV16 E7 oncoprotein may promote increased CD80 expression on DCs (42). Therefore, combined blockade of PD-1/PD-L1 and CD80/PD-L1 interactions by anti-PD-L1 agents in HPV positive HNSCC patients may represent a possible mechanism for increased benefit in these patients. Additionally, HPV16 E6/E7 oncoprotein may promote Akt

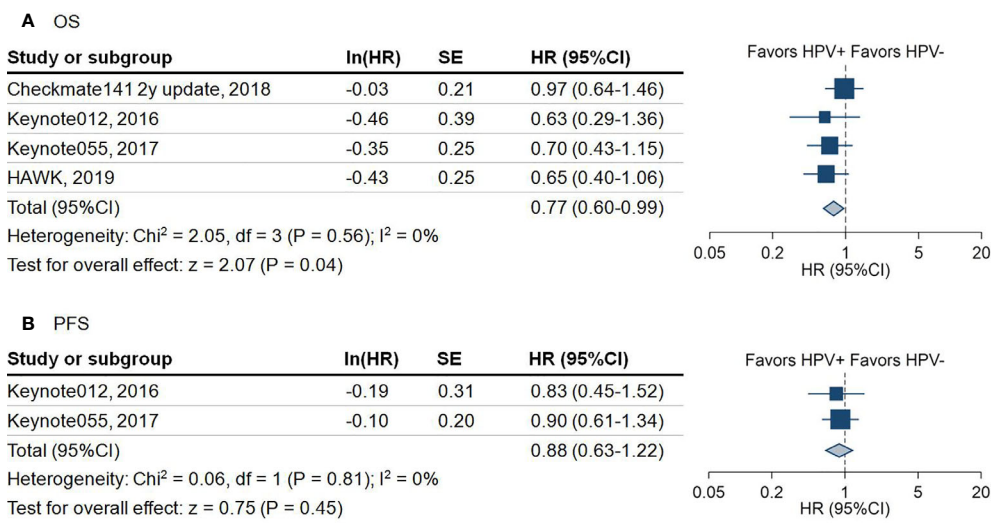

FIGURE 3 | Forest plot for the survival outcome of anti-programmed death-1/programmed death-ligand 1 agents in patients with different human papillomavirus status. **(A)** Overall survival (OS) of included studies. Squares indicate adjusted effect size (hazard ratio [HR]). Horizontal lines represent 95% confidence interval (95% CI). Diamonds represent the pooled HRs. **(B)** Progression-free survival (PFS) of included studies. Squares indicate adjusted effect size (hazard ratio [HR]). Horizontal lines represent 95%CI. Diamonds represent the pooled HRs. Pooled HRs were calculated using the fixed-effect model. PD-1, Programmed death 1; PD-L1, Programmed death-ligand 1; HPV, human papillomavirus; +, positive; -, negative; SE, standard error.

activity (43) and glucose consumption (44). *In vitro* culture of tumor cell lines with anti-PD-L1 directly might decrease AKT phosphorylation and glucose uptake in the absence of PD-1-expressing T cells (45), which may directly restrain tumor cell growth in turn. This could be another potential mechanism of increased benefit from anti-PD-L1 agents in HPV positive HNSCC patients. While these pathways provide a rational hypothesis towards explaining the difference in outcomes seen in our analysis, this does not imply that the mode of action of anti-PD-1/anti-PD-L1 inhibitors is different in HPV positive HNSCC patients compared with HPV negative. Rather, this demonstration in outcome difference suggests a need for translational research to better elucidate the underlying mechanism.

We noticed that there was a higher ORR in HPV positive over HPV negative HNSCC patients undergoing anti-PD-1 therapy in our analysis; however, this difference failed to reach significance ($P = 0.12$). As more and higher-quality trial data emerges, this difference may also trend toward significance. Importantly, the number of patients in our anti-PD-1 treated studies (476 patients) outnumber those of the anti-PD-L1 studies (189 patients) in our analysis, suggesting that the preferential effect of anti-PD-L1 therapy cannot merely be explained secondary to low numbers in the anti-PD-1 treated studies. Furthermore, the proportion of HPV positive patients in the two subgroups of anti-PD-1 and anti-PD-L1 studies is similar (31.3% vs. 34.4%, **Figure 2A**), underlining the evenness of HPV positive patients across the two different treatment-subgroups. From an immune perspective, it has been theorized in previous studies that anti-PD-1 agents would have a more extensive effect beyond the PD-L1 pathway (46), with the notion that blocking the PD-1 receptor would interfere with interactions with multiple ligands, including PD-L1 and PD-L2 (47). Here, it is important to note that PD-L2 expression is variable, and it is not as highly expressed as PD-L1 on tumor and immune cells, making its role in anti-cancer immune suppression unclear (48, 49). Differences in the expression of PD-L2 between HPV-positive and HPV-negative tumors may offer a potential explanation for the differences observed in our study.

Previous work has been published that investigates the relationship between HPV status and response to anti-PD-1/PD-L1 therapy (24, 25, 50). Patel et al. (24) and Wang et al. (25) both showed a trend towards significance for higher response rates in HPV positive vs. HPV negative tumors in patients receiving anti-PD-1/PD-L1 therapy. Additionally, a recent report indicated that immune checkpoint blockade immunotherapy enhances ORR in HPV positive HNSCC patients compared with HPV negative patients (50). However, a major limitation of this report is that it only included four clinical trials, which does not encompass the full scope of the present literature on the topic. Furthermore, the key limitation in these previously published studies is the lack of stratification by anti-PD-1 or anti-PD-L1 therapy separately. Our study is the first to do so, and this has demonstrated the possibility of a meaningful difference in outcome for HPV positive HNSCC patients treated with either anti-PD-1 or anti-PD-L1 blockers. In our study, we used restricted inclusion criteria to ensure the quality of all the eligible studies (Keynote-040 (34) and Keynote-048 (35) were excluded due to no published ORR for HPV positive patients, and other data from trials like EAGLE (NCT02369874), NCT02684253 (51) were not available yet), but were still able to include a larger number of patients with HPV information.

Limitations

Our study has several limitations. Firstly, some information was not available in all included studies, which prevented us from performing other informative sub-analyses such as stratification by anatomic subsite, the combination of PD-L1 expression and HPV subgroups, and drug dosage. In further studies, anatomic subsite in the head and neck is an essential factor to consider as it is known that the prognostic value of HPV status, based on available data, is thus far limited to the oropharynx (52). Particularly, as PFS is really the gold standard for the effect of therapy, the limited data of PFS restrict the value of our conclusion. Additionally, as there are no HPV diagnostic tests with FDA regulatory approval for head and neck cancers, the methodology to determine HPV status across the included trials differed based on the local institution or licensed lab. The most common method in the included trials was p16 immunohistological staining applied to oropharyngeal cancer as well as non-oropharyngeal cancer, which may be imperfect and would therefore bring bias, albeit minor, to the analysis (**Table 1**). Furthermore, a vast majority of HPV positive tumors are located within the oropharynx. The overall PD-L1 expression information is summarized in **Supplementary Table 3**. Nevertheless, the interaction between PD-L1 and HPV is difficult to estimate due to the currently available data. It is possible that elements of our analysis are confounded by differences in response and survival related to the subsite and PD-L1 expression itself. These considerations should be addressed carefully as more randomized controlled trial data matures in order to confirm our findings and to explore other possible factors related to response to immunotherapy in HNSCC.

CONCLUSION

HPV positive HNSCC patients display improved outcomes with PD-1/PD-L1 axis blockade as compared to HPV negative HNSCC patients. These improved outcomes are likely driven to a greater extent by anti-PD-L1 inhibitors. However, randomized controlled trials with greater numbers of patients are needed for validation of these early findings.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

GZ, YL, RM, and XZ conceived and designed the study. YX, DH, and IW extracted, analyzed, and interpreted the data. YX and GZ drafted the article. TS, CM, YL, RM, and XZ did the critical revision of the article for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Identification of Immune-Related LncRNA Pairs for Predicting Prognosis and Immunotherapeutic Response in Head and Neck Squamous Cell Carcinoma

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Long noncoding RNAs (lncRNAs) have multiple functions with regard to the cancer immunity response and the tumor microenvironment. The prognosis of head and neck squamous cell carcinoma (HNSCC) is still poor currently, and it may be effective to predict the clinical outcome and immunotherapeutic response of HNSCC by immunogenic analysis. Therefore, by using univariate COX analysis and Lasso Cox regression, we identified a signature consisting of 21 immune-related lncRNA pairs (IRLPs) that predicted clinical outcome and Immunotherapeutic response in HNSCC. Specifically, it was associated with immune cell infiltration (i.e., T cells CD4 memory resting, CD8 T cells, macrophages M0, M2, and NK cells), and more importantly this signature was strongly related with immune checkpoint inhibitors (ICIs) [such as PDCD1 ($r = -0.35$, $P < 0.001$), CTLA4 ($r = -0.26$, $P < 0.001$), LAG3 ($r = -0.22$, $P < 0.001$) and HAVCR2 ($r = -0.2$, $P < 0.001$)] and immunotherapy-related biomarkers (MMR and HLA). The present study highlighted the value of the 21 IRLPs signature as a predictor of prognosis and immunotherapeutic response in HNSCC.

Keywords: immune-related lncRNA pairs (IRLPs), head and neck squamous cell carcinoma (HNSCC), immune checkpoint inhibitors (ICIs), prognosis, immunotherapies

Abbreviations: HNSCC, Head and neck squamous cell carcinoma; OS, Overall survival; IRLPs, Immune related lncRNA pairs; TCGA, The Cancer Genome Atlas; LASSO, Least absolute shrinkage and selection operator; ICIs, Immune checkpoint inhibitors; FPKM, Fragments per Kilobase Million; DElncRNAs, Differentially expressed lncRNAs; CIBERSOFT, Celltype Identification by Estimating Relative Subsets of RNA Transcripts; GSEA, Gene set enrichment analysis; MMR, Mismatch repair; TMB, Tumor mutation burden; ROC, Receiver operating character; AUC, Area under curve; LncRNAs, Long non-coding RNAs; FDR, False discovery rate; MCP-counter, microenvironment cell population count.

INTRODUCTION

HNSCC is the sixth most common malignant tumor. About 600,000 people worldwide suffer from this disease, and about 300,000 patients die from the disease every year (1). Long-term repeated inflammation is considered to be one of the main causes of the disease, including smoking, drinking, repeated trauma, and human papillomavirus infection (2). HNSCC is characterized by local invasion, regional lymph nodes, and poor prognosis (3). Particularly, patients with advanced HNSCC may require multiple modes of combined treatment, but the quality of the prognosis is not optimistic. Therefore, early detection and in-depth understanding of the characteristics of cancer cells, and accurate diagnosis are the keys to successful treatment. There is an urgent need to study new and sensitive HNSCC tumor prognostic markers to reduce the number of HNSCC patients who are not diagnosed before the onset of the invasive disease.

Cancer immunotherapy aims to enhance the activity of the immune system against cancer and has been the main driving force for personalized treatment (4, 5). In recent decades, immunotherapy has developed rapidly and has become a treatment method for many cancers (6). Several immunotherapies, including immune checkpoint inhibitors, have been developed. In some studies, the expression of PD-L1 in HNSCC is usually higher, with a positive rate of 46% to 100% (7). The reversal of immune rejection mediated by tadalafil and antitumor vaccines also resulted in the up-regulation of PD-L1 in recurrent HNSCC, indicating that immune checkpoint therapy may be equally effective in patients with recurrent HNSCC (8). Relevant research on HNSCC patients is also in full swing, which is expected to improve the survival of HNSCC patients (9, 10). Although these findings support the importance of HNSCC immunology, its molecular mechanism is still unclear, especially for immune-related gnomic effects.

LncRNA is a type of noncoding RNA with 200 nucleotides that does not code for protein (11). lncRNAs are ubiquitous in the genome. They regulate 70% of human gene expression and cannot function in a universal way because they can interact with DNA, RNA, and proteins and exhibit either enhancement or inhibition. Its expression disorder is closely related to the occurrence and development of HNSCC (12, 13). Recent evidence shows that lncRNAs change not only the genome or transcriptome topology but also the immune microenvironment, which contributes to the main phenotype of cancer (14). LncRNA is involved in directing the expression of genes related to immune cell activation, which leads to the tumor's immune cell infiltration (15). With the development of high-throughput gene sequencing technology and the establishment of large-scale gene expression data sets, cancer researchers are able to accurately identify tumor-related prognostic biomarkers (16). However, there was a batch effect on the detected gene expression levels due to the different platforms and time of testing for gene expression, which may lead to the inaccuracy of the analysis results and bring some difficulties to the comprehensive utilization of data (17). Recently, researchers have provided a new way to solve this difficulty, which can overcome the batch effect of different platforms. The way is to normalize and scale the expression matrix based on the relative ranking of gene expression levels (18, 19). Specifically, we used these immune-related lncRNA expression levels in each sample to compare pairwise and construct IRLPs. In a specific sample, if the expression value of the first irlncRNA is greater than the second irlncRNA, the score of this IRLP in the sample is 1; otherwise, it is 0. The score of each IRLP in all samples was calculated, and IRLPs with low variation were removed (IRLP with a score of 1 or 0 in more than 80% of the sample in any data set) (20). Finally, IRLPs with higher variability were identified for further analysis. This method has produced reliable results in multiple studies. Li et al. validated individualized prognostic markers for pancreatic cancer by integrating IRGPs, presenting a conceivable method for deciding on a preoperative treatment (21). Li et al. constructed IRLPs to predict overall survival in patients with osteosarcoma and to provide potential guidance for patients who might benefit from immunotherapy (22). These studies about IRGPs have important clinical significance for the personalized treatment and prognosis of cancer patients. This method has produced reliable results in multiple studies. Li et al. validated individualized prognostic markers for pancreatic cancer by integrating IRGPs, presenting a conceivable method for deciding on a preoperative treatment (21). Li et al. constructed IRLPs to predict overall survival in patients with osteosarcoma and to provide potential guidance for patients who might benefit from immunotherapy (22). These studies about IRGPs have important clinical significance for the personalized treatment and prognosis of cancer patients.

However, there have been no studies on the clinical relevance and prognostic significance of IRLPs in HNSCC.

In conclusion, in terms of the accuracy of cancer prediction models, the combination of two biomarkers is better than simple genes (19). We integrated the sequencing samples of 546 HNSCC patients based on the TCGA data set. Univariate COX analysis and Lasso Cox regression are used to determine reliable IRLPs. These IRLPs signatures can predict the clinical outcome of HNSCC and establish a prognostic model of risk associated with immune gene pairs. We found that IRLPs are powerful prognostic biomarkers and predictors of HNSCC.

MATERIALS AND METHODS

Clinical Sample and Data Collection

Gene expression quantification data (FPKM and counts format) for HNSCC were downloaded from TCGA (https://portal.gdc.cancer.gov/). Then 44 normal samples and 502 HNSCC samples were obtained. The RNA expression matrix was extracted separately by annotations using the Gencode (GENCODE v 26) GTF file and normalized. Genes whose expression was "0" in 90% of HNSCC patients were removed. Clinical data were downloaded from the UCSC Xena website (https://xena.ucsc.edu/). To analyze the correlation of lncRNA expression signatures with the prognosis of HNSCC patients, we filtered out samples without survival

information. Then, we selected a total of 499 patients. Significant lncRNA-pathway pairs across 33 cancer types with each lncRNA having an activity in immune pathways (lncRES) score> 0.995 and a false discovery rate (FDR) < 0.05 were downloaded from Immlnc (http://biobigdata.hrbmu.edu.cn/ImmLnc/index.jsp) (23). The list of immune-related lncRNAs in HNSCC was extracted separately. Stromal scores and immune scores of HNSCC were calculated by applying the ESTIMATE algorithm and downloaded from the website (https://bioinformatics.mdanderson.org/estimate/index.html) (24).

Analysis of Differentially Expressed lncRNAs

We obtained DElncRNAs between normal and tumor tissues, where P value < 0.05 and log2-fold change (FC) > 1.5 were used as the cutoffs by using the R package 'edgeR' (25). Then, we filtered DEirlncRNAs by matching the list of immune-related lncRNA in HNSCC. The R package 'heatmap' was used to display the eight selected irlncRNAs.

Identification of Prognostic-Related IRLPs in Patients With HNSCC

We then used the lncRNA expression levels of these lncRNAs in each sample for pairwise comparison to construct irlncRNAs. In a specific sample, if the expression value of the first irlncRNAs is greater than that of the second irlncRNAs, the score of this IRLPs in the sample is 1; otherwise, it is 0. The score of each IRLP in all samples was calculated, and IRLPs with low variation were removed (IRLPs with a score of 1 or 0 in less than 20% of the sample in any data set). Finally, IRLPs with higher variability were identified for further analysis. Univariate Cox regression analysis was performed on these IRLPs in the TCGA cohort and IRLPs with $p < 0.0001$ were considered prognostic-related IRLPs and used for subsequent analysis.

Construction and Evaluation of Signatures Based on IRLPs

Lasso Cox regression analysis was performed on the above-mentioned prognostic-related IRLPs, and finally an optimal model composed of 21 IRLPs was determined. Subsequently, the optimal model based IRLPs signature of each patient was calculated. In the 3-year overall survival TCGA cohort, time-dependent ROC curve analysis was used to determine the optimal cutoff value for IRLPs signature (22, 26). According to the cutoff value of the IRLPs signature, patients were divided into high-risk group and low-risk group. The log-rank test was used to evaluate the overall survival difference between the low-risk group and the high-risk group, and the KM survival curve was drawn. ROC curve analysis was used to evaluate the sensitivity and specificity of IRLPs. An ROC curve, including clinical characteristics, was drawn, and the AUC was calculated. Finally, univariate, and multivariate Cox regression analyses were used to investigate whether the prognostic value of the IRLPs was affected by other clinical characteristics.

Construction and Evaluation of Nomograms

We combined the clinical characteristics of the TCGA data set with the IRLPs signature to construct a nomogram. We used the C index to evaluate the discriminative power of the nomogram and drew a calibration chart to evaluate the accuracy of the nomogram. We then compared the decision curve analysis between the clinical characteristics model and the combined model, including gene signature.

Estimation of Immune Infiltration

Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data (ESTIMATE) is a tool for predicting tumor purity and the presence of infiltrating stromal/immune cells in tumor tissues using gene expression data. ESTIMATE algorithm is based on single sample Gene Set Enrichment Analysis and generates three scores.

First, the immune infiltration assessment was performed using the "microenvironment cell population count (MCP-counter)" method (27). Using the normalized FPKM expression matrix converted by log2 as input, the absolute abundance scores of 8 immune cells and 2 stromal cells populations are generated through the "MCP-counter" package. Research shows that immune cell infiltration assessed by the MCP-counter algorithm performs well when comparing between samples (28). Subsequently, CIBERSORT was used to infer the relative proportion of 22 infiltrating immune cells in each sample for supplementation.

Analysis of the Immunosuppressive Molecules Expressing Related to ICIs

To study the relationship between the model and the expression level of genes related to ICIs, we performed ggstatsplot package and violin plot visualization.

Gene Set Enrichment Analysis

GSEA software (version 4.0.1) was used to perform gene set enrichment analysis between high-risk and low-risk groups. Recognized the enriched terms in IMMUNE and KEGG in high-risk group and low-risk group respectively. $P < 0.05$ and False discovery rate (FDR) <0.05 are considered statistically significant.

Statistical Analysis

Except for gene set enrichment analysis, all statistical analyses involved in this research were conducted using the R software (version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria). Unless otherwise stated, $p < 0.05$ is considered statistically significant.

RESULT

Construction and Evaluation of IRLPs Signature

As shown in **Supplementary Figure 1**, we first retrieved the transcriptome analysis data of HNSCC from the TCGA database. Next, we annotated the data with GTF files, and we applied the edgR package for difference analysis, based on the normal and tumor samples in TCGA, A total of 6720 differentially expressed genes was screened, of which 4063 were upregulated and 2657 were down-regulated (**Figure 1A**). (|log2FC|>1.5, FDR <0.05). Further, the lack of clinical information and duplicate samples were removed, and a total of 499 cases were included in survival-related analysis. We crossed the lncRNAs (2391) related to HNSCC immunity obtained by Immlnc with the lncRNAs highly expressed in HNSCC to obtain 167 immunologically related differential lncRNAs (Because the expression value of lncRNAs in tumor samples is very low and cannot provide valuable reference in subsequent experiments, we chose the highly expressed lncRNAs for the study.) The differential expression of 167 lncRNAs was visualized in **Figure 1B**. Then paired analysis of these lncRNAs, a total of 7719 valid differential expression IRLPs were identified. These gene pairs were subjected to univariate COX analysis ($p < 0.0001$). Finally, 30 IRLPs related to prognosis were screened out (**Figure 1C**). To prevent overfitting, these prognostic lncRNA pairs were

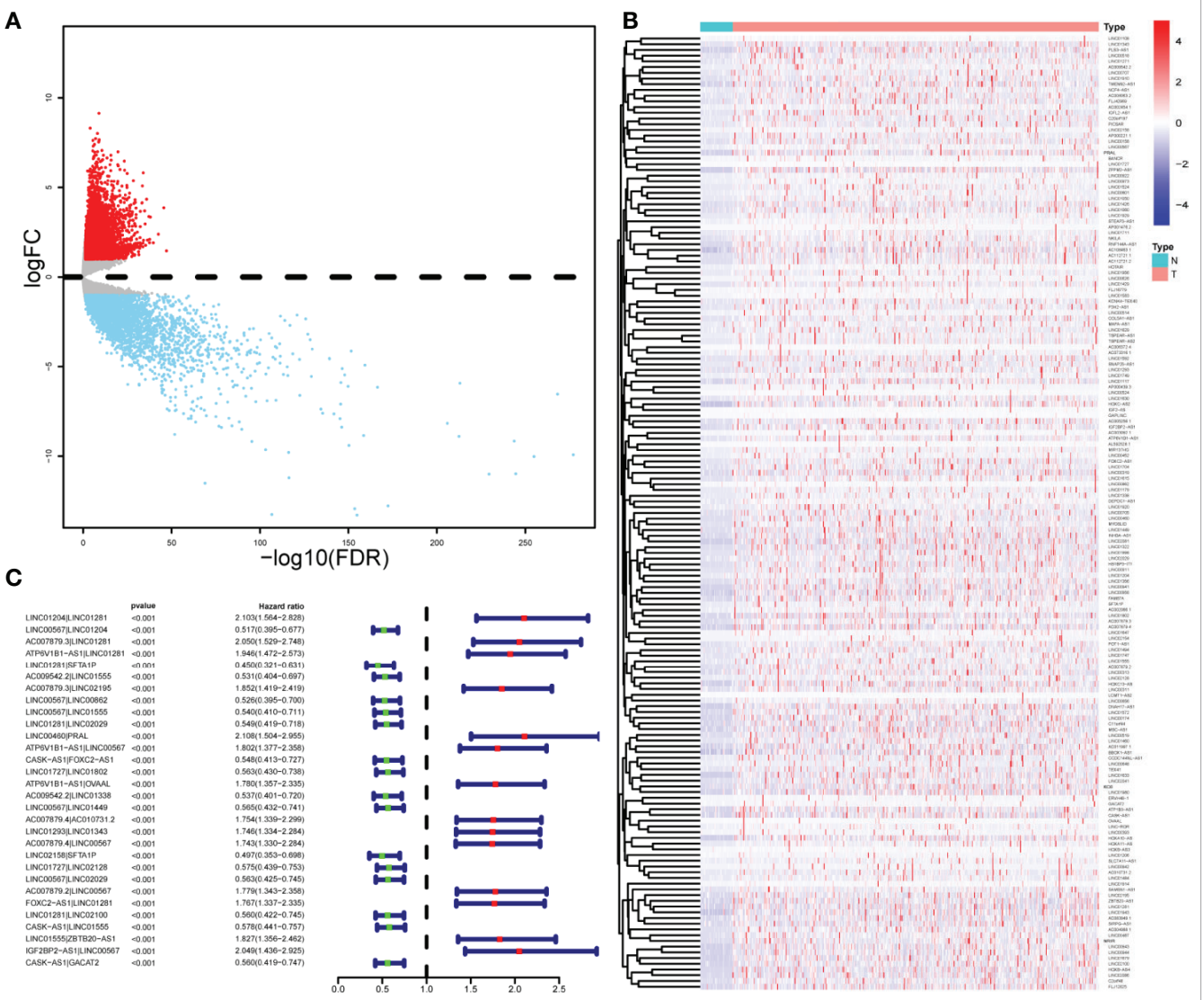

FIGURE 1 | Identification of DEirlncRNAs and IRLPs using TCGA datasets. **(A)** For the volcanic map of 6720 differentially expressed genes, red points represent log2FC >1.5; blue points represent log2FC<-1.5, FDR < 0.05. **(B)** Heat maps of 167 immune related differential lncRNAs in normal and tumor samples. **(C)** Forest map showing 30 DEirlncRNA pairs related to prognosis identified by univariate COX analysis.

subjected to Lasso Cox regression analysis, and 21 IRLPs were obtained (**Figures 2A, B**). The 21 IRLPs (**Table 1**) were selected to construct the signature. (The list of lncRNA pairs, immune pathways and coefficients are shown in **Table 1**). We use the time-dependent receiver operating characteristic (ROC) curve to determine the cutoff value for the best IRLPs signature. The optimal cutoff value for IRLPs signature is -0.433 (**Figure 2C**). According to the cutoff value, patients were divided into the high-risk group and the low-risk group. We find that compared with patients in the low-risk group, patients' overall survival rate in the high-risk group was significantly lower (**Figure 2D**). With the increase of the risk score, the patient's survival time shortened gradually, and the mortality rate gradually increased (**Figures 2E, F**).

Correlation Between IRLPs and Clinical Characteristics

We construct the ROC curve of the IRLPs signature, TNM stage, age, sex, and smoking. The area under the curve (AUC) of the IRLPs signature is 0.721 (**Figure 3A**), which shows that our

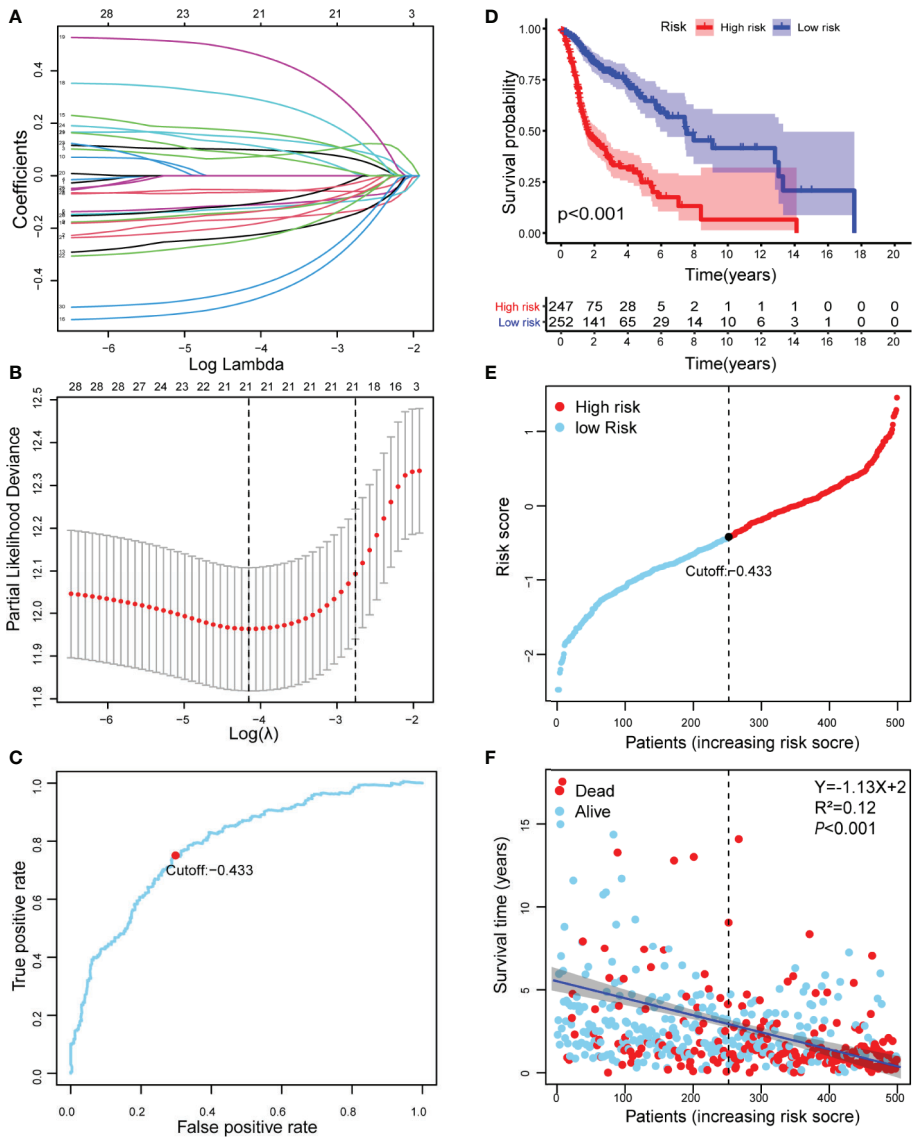

FIGURE 2 | Risk assessment model for prognosis prediction. **(A)** Validation was performed for tuning parameter selection through the Lasso regression model for OS. **(B)** Elucidation for LASSO coefficient profiles of prognostic IRLPs. **(C)** Time-dependent ROC curve of the IRLPs signature in the TCGA cohort. The optimal cutoff value of the IRLPs signature is -0.433; patients are divided into the high-risk group and the low-risk group according to the cutoff value. **(D)** Patients were sorted by increasing risk score in the HNSCC set. **(E)** The Kaplan-Meier survival curve with log-rank test was drawn to demonstrate the relationship between risk model and OS. Compared with the high-risk group, patients in the low-risk group experienced a longer survival time. **(F)** The survival time and survival status of patients with HNSCC worsened as the risk score increased (Y=-1.13X+2, R^2=0.12).

TABLE 1 | Information of 21 IRLPs.

IRLPs	LncRNA Pair1	Immune pathway*	LncRNA Pair2	Immune pathway*	Coefficient
LINC00567|LINC01204	LINC00567	Cytokine Receptors	LINC01204	Cytokines	-0.16528
AC007879.3|LINC01281	AC007879.3	Antigen Processing and Presentation	LINC01281	Natural Killer Cell Cytotoxicity	0.071247
LINC01281|SFTA1P	LINC01281	Natural Killer Cell Cytotoxicity	SFTA1P	Natural Killer Cell Cytotoxicity	-0.12353
AC009542.2|LINC01555	AC009542.2	Cytokines	LINC01555	Antigen Processing and Presentation	-0.11187
AC007879.3|LINC02195	AC007879.3	Antigen Processing and Presentation	LINC02195	Natural Killer Cell Cytotoxicity	0.099465
LINC00567|LINC00862	LINC00567	Cytokine Receptors	LINC00862	Cytokines	-0.05372
LINC00567|LINC01555	LINC00567	Cytokine Receptors	LINC01555	Antigen Processing and Presentation	-0.13669
LINC00460|PRAL	LINC00460	Cytokines	PRAL	Cytokine Receptors	0.145737
CASK-AS1|FOXC2-AS1	CASK-AS1	Antigen Processing and Presentation	FOXC2-AS1	Cytokines	-0.22806
LINC01727|LINC01802	LINC01727	Chemokine Receptors	LINC01802	Cytokines	-0.13905
ATP6V1B1-AS1|OVAAL	ATP6V1B1-AS1	Cytokines	OVAAL	Cytokines	0.167943
AC009542.2|LINC01338	AC009542.2	Cytokines	LINC01338	Cytokines	-0.47794
AC007879.4|AC010731.2	AC007879.4	Cytokines	AC010731.2	Antigen Processing and Presentation	0.297284
LINC01293|LINC01343	LINC01293	Cytokines	LINC01343	Interleukins Receptor	0.469817
LINC02158|SFTA1P	LINC02158	Antimicrobials	SFTA1P	Natural Killer Cell Cytotoxicity	-0.18683
LINC01727|LINC02128	LINC01727	Cytokine Receptors	LINC02128	Antimicrobials	-0.2438
AC007879.2|LINC00567	AC007879.2	Cytokines	LINC00567	Cytokine Receptors	0.117046
LINC01281|LINC02100	LINC01281	Cytokine Receptors	LINC02100	Antimicrobials	-0.10826
CASK-AS1|LINC01555	CASK-AS1	Antigen Processing and Presentation	LINC01555	Antigen Processing and Presentation	-0.06256
IGF2BP2AS1|LINC00567	IGF2BP2-AS1	Cytokines	LINC00567	Cytokine Receptors	0.081704
CASK-AS1|GACAT2	CASK-AS1	Antigen Processing and Presentation	GACAT2	Cytokines	-0.42858

**Immune pathway was annotated by website Immlnc (http://biobigdata.hrbmu.edu.cn-/ImmLnc/index.jsp).*

signature has excellent predictive power. Then we draw the time-dependent ROC curve of the IRLPs signature. We find that the area under the IRLPs signature curve respectively: 1 year: 0.759; 3 years: 0.788; 5 years: 0.777 (**Figure 3B**). This shows that our model has a good predictive ability for patients with 5-year survival, 3-year survival, and 1-year survival. Next, we assessed the prognostic value of the HNSCC risk score. In the univariate analysis, we find that the risk score was significantly correlated

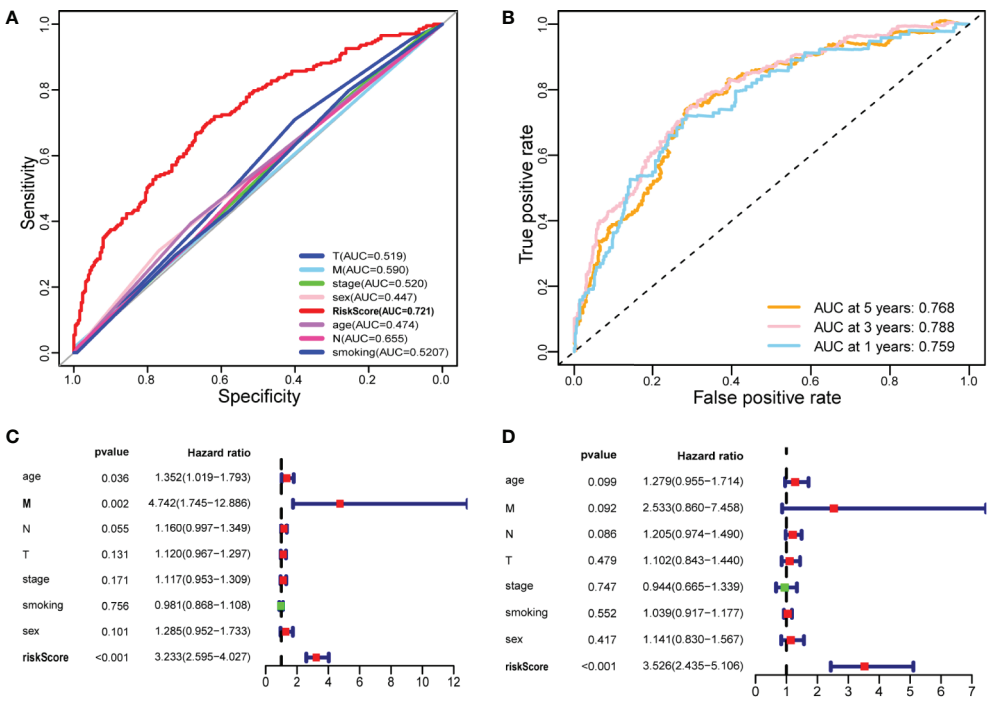

FIGURE 3 | Evaluate the predictive ability of the IRLPs signature. **(A)** A comparison of ROC curves with other common clinical characteristics showed the superiority of the IRLPs signature. **(B)** The 1-, 3-, and 5-year ROC curve of the optimal model suggested that all AUC values were over 0.75. **(C)** Forest plot of univariate Cox regression results shows that risk score ($P < 0.001$) and Metastasis ($P < 0.05$) are prognostic related factors. **(D)** Forest plot of multivariate Cox regression results shows that risk score ($P < 0.001$) is an independent influencing factor for prognosis.

with the overall survival (OS) (HR = 3.233, 95% CI = 2.233 - 4.027, P < 0.001). Multivariate analysis shows that the risk score is an effective independent prognostic predictor of OS (HR = 3.526, 95% CI = 2.435 - 5.106, P < 0.001) (**Figures 3C, D**). In order to further improve the accuracy of the prediction, we constructed a new nomogram based on the IRLPs signature (**Figure 4A**). The nomogram C-index is 0.729. By calculating the total score, oncologists can easily obtain the probability of OS predicted by the nomogram of a single patient. We also use the calibration curve to evaluate the model's prediction accuracy (**Figure 4B**). The results show that the prediction calibration curve of the three calibration points in 1, 3, and 5 years is close to the standard curve, which indicates that the model has good predictive performance. In addition, we also use the DCA (decision curve) to evaluate the reliability of the model (**Figure 4C**). It can be seen that the profit of this model is significantly higher than the limit curve, so it has good reliability.

The Relationship Between IRLPs Signature and Immune Cell Infiltration

We explore the difference in immune cell infiltration between the two groups. Based on the ESTIMATE algorithm, we first calculate the Immune score and ESTIMATE score of each HNSCC sample. As shown in **Figures 5A, B**, compared with the low-risk group, the Immune score (190.71 *vs* 608.83, p < 0.001) and ESTIMATE score (-213.51 *vs* 402.27, p < 0.001) of the high-risk group are lower and negatively correlated with the risk score (correlation coefficients are -0.22 and -0.29, p < 0.001) (**Figures 5C, D**). Next, we used the MCP-counter method to calculate the abundance of 8 immune cells and 2 stromal cells. Significant differences were observed between the two groups of patients. Compared with patients in the high-risk group, the eight cell populations in the low-risk group are more abundant (B cell lineage, CD8(+) T cells, cytotoxic lymphocytes, monocyte lineage cells, myeloid dendritic cells, medium Sex granulocytes, NK cells, T cells) (**Figure 5E**).

We further explored the relationship between the immune cell infiltration and the risk score. The result show that the degree of immune cell infiltration is negatively correlated with the risk score (**Figure 5F**). Subsequently, we used CIBERSORT to further supplement the relative proportion of 22 immune infiltrating cells in each sample (**Figure 6A**). The relative proportions of B cells naive, mast cells resting, plasma cells, T cells CD4 memory activated, T cells CD8, T cells follicular helper, and T cells regulatory (Tregs) in the low-risk group are higher. The relative proportions of dendritic cells activated, eosinophils, T cells CD4 naive, macrophages M0, mast cells activated, and NK cells resting were relatively high in the high-risk group. This indicates that there are great differences in immune cell infiltration between high- and low-risk groups. It is worth noting that the radar chart shows that T cells CD4 memory resting and M0 macrophage infiltration rate are higher in all patients.

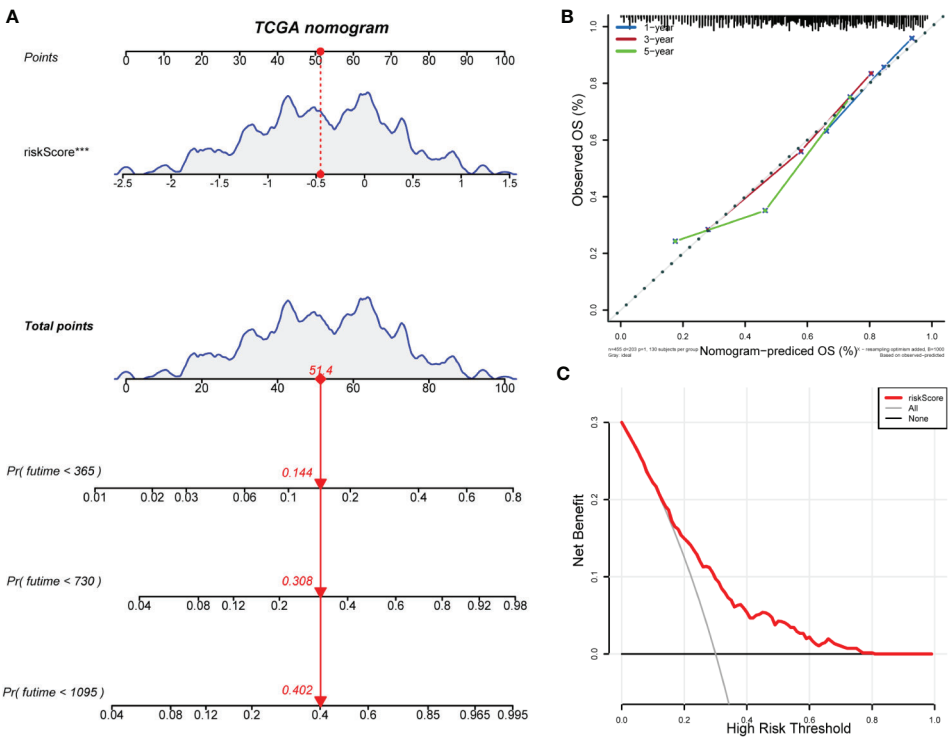

FIGURE 4 | Establishment and evaluation of nomogram model. **(A)** Nomogram predicting the probability of TCGA patient's mortality based on IRLPs and clinical variables. **(B)** Calibration curves of the nomogram for 1, 3 and 5 years. **(C)** Decision curve analysis of the nomograms based on the IRLPs signature.

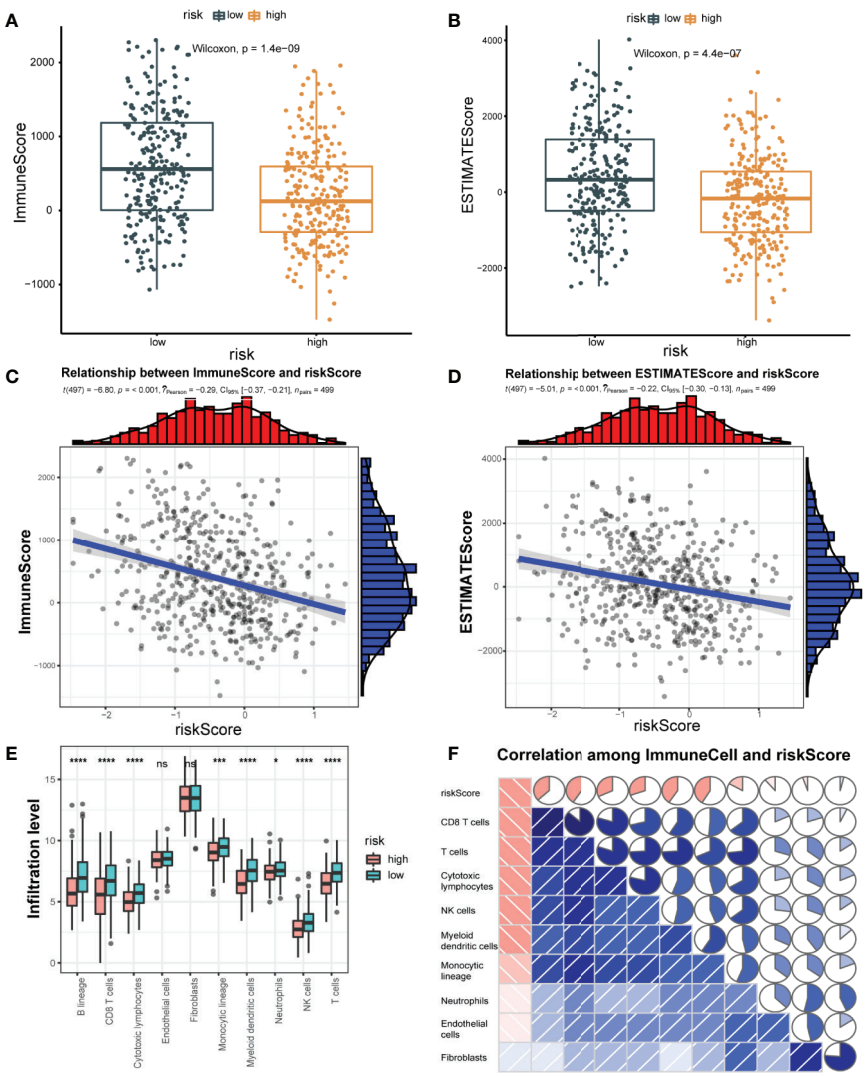

FIGURE 5 | Correlation of 21 IRLPs features with immune cell infiltration and immune scores. **(A)** The Wilcoxon rank-sum test was used to compare differences in immune scores between low- and high-risk groups. (P < 0.001) **(B)** The Wilcoxon rank-sum test was used to compare differences in ESTIMATE scores between low- and high-risk groups. (P < 0.001) **(C)** Spearman's correlation coefficients were computed to investigate the potential relationship between risk score and immune scores. **(D)** Spearman's correlation coefficients were computed to investigate the potential relationship between risk score and ESTIMATE scores. **(E)** The Wilcoxon rank-sum test compared the absolute abundance scores of 8 immune cells and 2 stromal cells populations in two groups of patients. **(F)** Spearman's correlation coefficients were computed to investigate the potential relationship between absolute abundance scores of immune cells and stromal cells and risk score. The area of fan represents the degree of correlation (Red represents a negative correlation and blue represents a positive correlation). ns, no significance.

The Relationship Between IRLPs Signature and Immune Checkpoint

Tumor immunotherapy using ICIs has become a promising treatment for advanced HNSCC (29). To further study the relationship between IRLPs and immunity, we explored the risk score and ICIs-related biomarkers correlation. The results showed that in the low-risk group, the expression levels of PDCD1, CTLA4, LAG3, and HAVCR2 were upregulated (all P < 0.001), and the risk score was negatively correlated to PDCD1 (r = -0.35, P < 0.001), CTLA4 (r = -0.26, P < 0.001), LAG3 (r = -0.22, P < 0.001) and HAVCR2 (r = -0.2, P < 0.001), indicating that the low-risk group had benefited more from immunotherapy (**Figures 6B, C**).

The Relationship Between Risk Score, MMR Gene and HLA Gene Family

Solid tumors lacking the mismatch repair (MMR) genes are usually immunogenic and exhibit extensive infiltrating T cells, making them highly sensitive to ICIs (30). We evaluated the correlation between IRLPs signals and four key MMR genes (MSH6, MLH1, PMS2, MSH2). The expression levels of PMS2

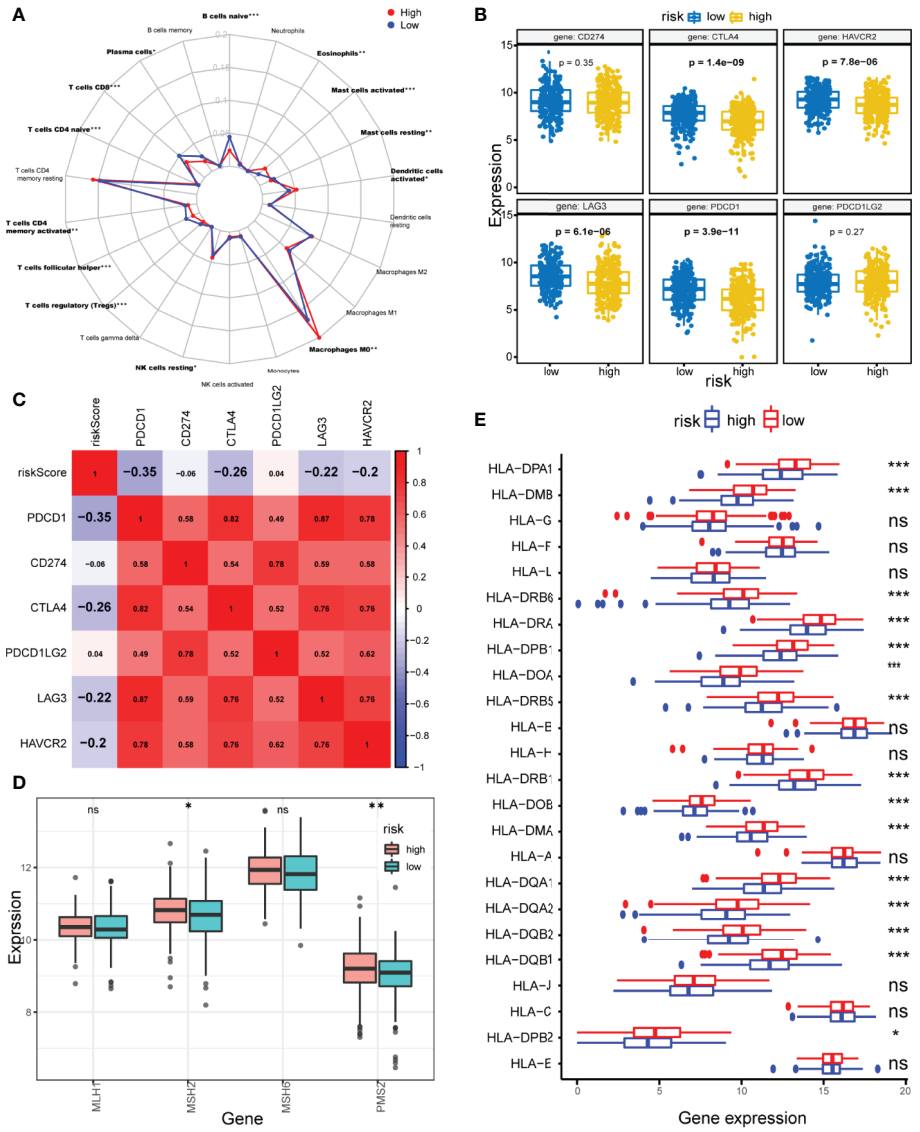

FIGURE 6 | Assessments of the relationship between immune cells, HLA, MMR genes, ICIs, and IRLPs signature. **(A)** Radar chart of the relationship between 22 immune cell infiltration and IRLPs signature grouping. (Wilcoxon test). **(B)** The different expressions of ICIs among risk groups as defined by the 21 IRLPs signature. Results revealed that CTLA4, HAVCR2, LAG3 and PDCD1 were overexpressed in low-risk group (all P<0.001). **(C)** Spearman's correlation coefficients were computed to investigate the potential relationship between our IRLPs signature and ICIs. The highlighted ones represent P<0.001. **(D)** The different expressions of MMR genes among risk groups as defined by the 21 IRLPs signature. (Wilcoxon test). **(E)** The box plot showed that most of the HLA gene families were highly expressed in the low-risk group. (Wilcoxon test). *P < 0.05, **P < 0.01, ***P < 0.001. ns, no significance.

and MSH2 in the high-risk group were upregulated (PMS2 and MSH2 were $p < 0.05$ and $p < 0.05$, respectively), suggesting that high-risk group did not benefit from immunotherapy as much (**Figure 6D**). Furthermore, immune escape is a hallmark of cancer, and the ability to present new antigens through the loss of human leukocyte antigen (HLA) may help immune escape (31). We find that HLA family plays a certain role in the sensitivity difference of immunotherapy, as shown in **Figure 6E**, HLA family was downregulated in the high-risk group, which led to tumor immune evasion, and may be related to immunotherapy insensitivity.

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was performed to determine the gene sets enriched in different IRLPs subgroups. The gene sets of the IRLPs-low samples were enriched in nucleotide excision repair and CD4 T cell, TNF, IL6, etc. (**Figure 7**).

DISCUSSION

HNSCC is a solid malignant tumor with strong immunogenicity, its incidence increasing rapidly worldwide. Advances in surgical

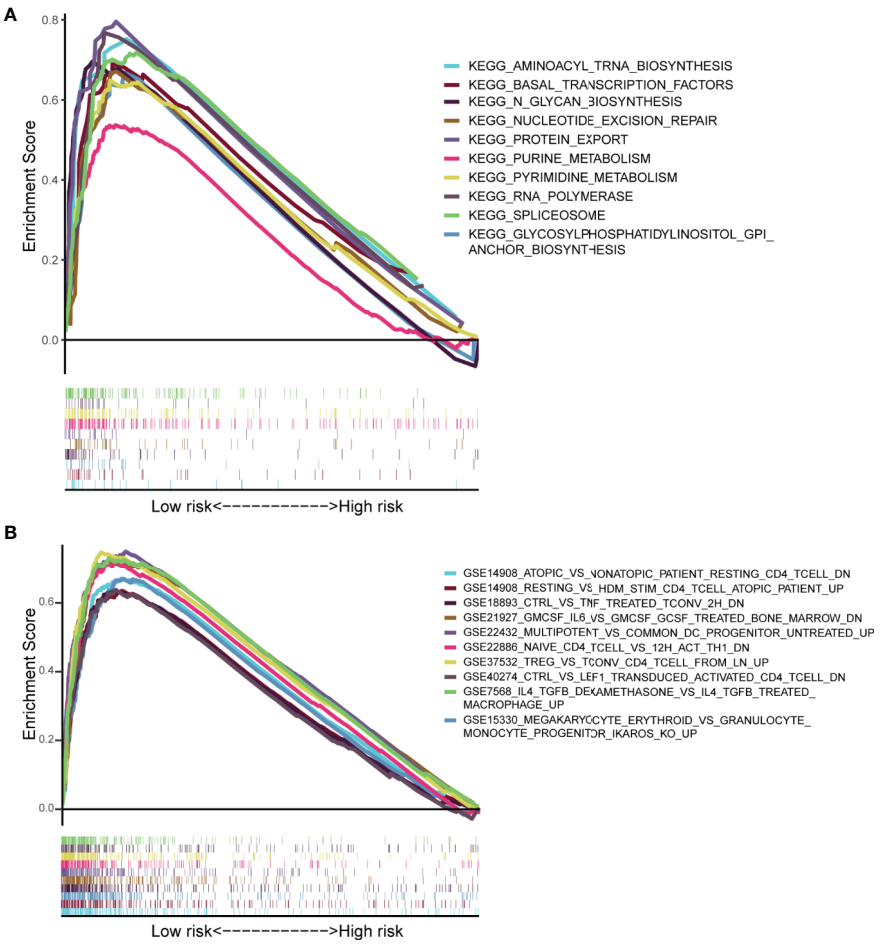

FIGURE 7 | Results of gene set enrichment analysis in the TCGA cohort. **(A)** The significantly enriched KEGG subset of canonical pathways by GSEA. **(B)** The significantly enriched Immunologic gene sets by GSEA.

techniques and comprehensive treatment techniques have improved the local control rate and quality of life of HNSCC patients. Still, in recent decades, the survival rate has not increased significantly. In addition, the 5-year survival rate of patients with this disease is only 40% - 50% (32). Platinum-based chemotherapy, combined with cetuximab, is the standard treatment for relapsed or metastatic HNSCC. However, there are problems such as easy relapse and short median survival after treatment (33–35). ICIs based on PD-1/PDL-1 monoclonal antibodies have become a new clinical treatment option for advanced HNSCC. Both pembrolizumab and nivolumab have been approved by the FDA for relapsed or metastatic HNSCC that have failed platinum-based therapy (36). Important studies based on prognostic signals of immune gene expression have shown that gene expression scores can predict the risk of recurrence and the effect of immunotherapy (37, 38).

Given that the results of single antibody drugs are limited, and there are many connections between the occurrence and development of HNSCC and the immune microenvironment, the strategy of multiple immunotherapies may have better prospects (39). Therefore, it is necessary to use IRLPs to establish prognostic indicators. Reliable prognostic biomarkers can identify patients with poor prognosis and inform patients who may benefit from other systemic treatments. Hence, they have more direct clinical significance.

In our study, based on the HNSCC immune-related lncRNAs data set, IRLPs that significantly affect the OS of patients were constructed. These IRLPs can help identify candidate immune-related biomarkers or therapeutic targets. Unlike traditional prognostic models, the pairwise comparison and score calculation of each IRLPs are based entirely on the lncRNA expression of the same patient, so our IRLPs signature does not have to be standardized on the gene expression profile sequencing platform from different patients. Previous research has proved the effectiveness of this method (40). Therefore, the prognostic signature can overcome the batch effect of different platforms and does not require data scaling and normalization. This approach has been reported to be robust in other cancer-related studies (20, 41).

First, we retrieved raw data of lncRNAs from TCGA, performed a differential co-expression analysis to classify the

differentially expressed irlncRNAs (DEirlncRNAs), and validated lncRNA-pairs using an improved method of cyclically single pairing along with a 0-or-1 matrix. Second, we performed univariate analysis combined with a modified Lasso Cox regression, including procedures of cross-validation, multi-times repeat, and included 21 IRLPs with prognostic significance. Third, we calculated each AUC value of ROC curve to obtain the best model and determined the best cutoff value of the IRLPs signature according to the ROC curve differentiate the high or low risk-group among patients with HNSCC. Fourth, we evaluated this novel model under various clinical settings, including survival, clinicopathological characteristics, tumor-infiltrating immune cells, chemotherapy, and checkpoint related biomarkers. Among the 21 IRLPs, a total of 28 lncRNAs were included. These lncRNAs participate in the occurrence and development of HNSCC. Specifically, CASK-AS1 | GACAT2 may play an important role in the screening and prognosis of HNSCC. The experimental results of Tan et al. showed that the preoperative plasma GACAT2 levels of gastric cancer patients were significantly higher than that after surgery (P=0.031). Therefore, they believed that plasma GACAT2 could be used as a tumor marker for the screening and prognosis prediction of cancer patients (42). The results of Liu et al. identified 5 lncRNAs (TSPEAR-AS, CASK-AS1, MIR137HG, Part1, LSAMP-AS1) as potential prognostic markers and therapeutic targets for laryngeal cancer, which is consistent with our results (43). In addition, the LINC00460 promotes tumor progression through sponge miR-4443 in HNSCC (44). The loss of major histocompatibility complex I (MHC I) molecules is an important mechanism for HNSCC cells to evade immune surveillance. However, LINC02195 is a crucial regulator of MHC I molecules (45). Moreover, novel lncRNA SFTA1P promotes tumor growth by downregulating miR-4766-5p via the PI3K/AKT/mTOR signaling pathway in hepatocellular carcinoma (46). However, studies have shown that IL-22 can induce lncRNA H19 to activate mTOR signal transduction, thereby preventing liver damage. This shows that the mTOR signaling pathway has potential and broad therapeutic prospects (47). Linc01555 promotes proliferation, migration, and invasion of gastric carcinoma cells by interacting with the Notch signaling pathway. LncRNA FOXC2-AS1 enhances FOXC2 mRNA stability to promote colorectal cancer progression via activation of the Ca-FAK signal pathway (48). These studies support that our risk scoring model can be used as an indicator to predict the prognosis of HNSCC patients. In addition, our scoring system has strong predictive power for OS: the AUC values for predicting 1-year, 3-year, and 5-year overall survival rates are 0.759, 0.788, and 0.768, respectively.

Survival analysis and univariate/multivariate Cox proportional hazard analysis proved the prognostic value and that our IRLPs signature is an independent prognostic factor. It is worth noting that the TNM staging, smoking, and age prognostic models did not show good predictive values. Therefore, our risk scoring model may be more helpful for clinicians to predict the survival of HNSCC patients.

In our study, correlation analysis showed that 21 IRLPs signatures were positively correlated with MMR genes MSH2 and PMS2, indicating that these patients had poor responses to ICIs. Immune escape is an important mechanism for the occurrence and development of malignant tumors. Losing the ability to present neoantigens through human leukocyte antigen (HLA) loss may facilitate immune evasion (31). The HLA family plays a certain role in the sensitivity difference of immunotherapy. The results show that the HLA family is downregulated in the high-risk group, which leads to tumor immune evasion, which may be related to immunotherapy insensitivity. Next, we explored the relationship between IRLPs and known predictive biomarkers for immunotherapy. According to the signature characteristics of 21 IRLPs, ICIs, including PDCD1, CTLA4, LAG3, and HAVCR2, were highly expressed in the low-risk group (P<0.001), whose survival rate was higher. When exploring the correlation between the 21 IRLPs values and PDCD1, CTLA4, LAG3, and HAVCR2, the 21 IRLPs signatures were significantly negatively correlated with ICIs expression. There is strong evidence that patients with low-risk scores may benefit more from immunotherapy. Generally, PDCD1+ tumors respond better to anti-PD-1 therapy than PDCD1-tumors (49, 50). However, we found inconsistent results in HNSCC. That is, when evaluating anti-PD-1 therapy in the setting of platinum-refractory relapsed or metastatic HNSCC, CHECKMATE-141 failed to show a significant correlation between PD-L1 expression and tumor response or survival (51). The main reason may be the lack of uniformity in the measurement and the variability used to define the PD-1 positivity threshold. Moreover, we believe that the intensity and location of PD-1 expression detected by immunohistochemistry are more valuable than the PD-1 expression value measured by the transcriptome data. Therefore, further research is needed to clarify the relationship between PD-1 and IRLPs. In summary, our findings reveal the important value of HNSCC immunotherapy.

Understanding the overview of the tumor microenvironment (TME) may help find new ways to treat HNSCC or change TME to improve the effectiveness of immunotherapy. Macrophages that reside within the TME are known as tumor-associated macrophages (TAMs) (52). TAMs and other TME members make up the tumor ecosystem. In most situations, all members of the TME consume oxygen and nutrients from the host for their phenotypic and functional performance (53, 54). Thus, metabolites are accumulated in the TME and recycled from cell to cell. In particular, the metabolites, as messengers for cell-cell contact, which are derived from the TME (tumor cells, T cells, mast cells, cancer-associated fibroblasts, adipocytes, except TAMs), are ingested by TAMs to change their phenotype and function. In turn, TAMs promote tumor progression via metabolic reprogramming, which is triggered by the metabolites that are shuttled in the TME (55). Given the importance of TAMs in tumorigenesis and development, there has been considerable interest in therapeutic strategies that target macrophages, which can be roughly divided into depletion or alteration of TAM protumoral activities (56). Most clinical studies believe that combination therapy is necessary to maximize the benefit of cancer patients (57), these strategies are currently in evaluation either to augment tumor immunity

during standard chemotherapy or radiation therapy, or in combination with T cell-directed immunotherapy (58). Therefore, the assessment of the immune status in the tumor microenvironment is necessary for a comprehensive understanding of the real-time status of the tumor.

First, we verified the correlation between the ESTIMATE score, the immune score, and the risk score. Between the two different risk groups, significant differences were observed in the relative fraction of immune cells infiltrating the tumor tissue. The composition of immune cells between the two subgroups of IRLPs was further analyzed. We find that dendritic cells activated, macrophages M0, mast cells activated, and NK cells resting in the high subgroup of IRLPs are more abundant, while B cells naive, mast cells resting, plasma cells, T cells CD4 memory activated, T cells CD8, T cells follicular helper and T cells regulatory (Tregs) are more common. A large number of studies have shown that dense infiltration of T cells, especially T cells CD8, predicts a good prognosis (59–61). In most tumors, M2 macrophages are the main subtype of macrophages and have been shown to be involved in tumor growth and development with chronic inflammation and aggressive phenotypes. These cells are found in breast, bladder, and ovarian cancer. The prognosis is poor in gastric cancer and glioma (62–66). Our research results support these conclusions.

In addition, our results show differences in biological processes and immune infiltration between the two groups. The results of GSEA show that many immune-related pathways are enriched in the low-risk group. From this perspective, patients in the low-risk group are also more likely to benefit from immunotherapy.

It should be admitted that our research still has some limitations. First, conclusions about the efficacy of immunotherapy have not been confirmed in patients with HNSCC, and further research is needed to verify our results. Finally, although the signature we constructed has excellent performance in predicting immunotherapy and prognosis, it is still the tip of the iceberg in the current immunotherapy field, and a lot of research is needed to enrich and improve.

In short, IRLPs is a promising immune-related prognostic biomarker. IRLPs grouping may help distinguish immune and molecular characteristics and predict patient prognosis. IRLPs may be a potential prognostic indicator of immunotherapy. This may open a new chapter in HNSCC 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 authors.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

XW and KW conceptualized the project, all data analysis, and wrote the first draft of the manuscript. EG, XM, LG, CZ, JG, and GW contributed to processing, analysis, and interpretation of the data. JS and SM contributed to guide the data analysis, and manuscript writing. All authors contributed to the article and approved the submitted version.

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IL-21 Is an Accomplice of PD-L1 in the Induction of PD-1-Dependent Treg Generation in Head and Neck Cancer

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Regulatory T cells (Tregs) are immunosuppressive cells involved in antitumor immunity. However, the regulation of Treg generation by inflammation in the tumor microenvironment has not been carefully investigated. Here, we demonstrated that IL-21-polarized inflammation was enriched in the tumor microenvironment in head and neck squamous cell carcinoma (HNSCC) and that IL-21 could promote PD-L1-induced Treg generation in a PD-1-dependent manner. Moreover, generated Tregs showed a greater ability to suppress the proliferation of tumor-associated antigen (TAA)-specific T cells than naturally occurring Tregs. Importantly, an anti-PD-1 antibody could inhibit only Treg expansion induced by clinical tumor explants with high expression of IL-21/PD-L1. In addition, neutralizing IL-21 could enhance the anti-PD-1 antibody-mediated inhibitory effect on Treg expansion. Furthermore, simultaneous high expression of IL-21 and PD-L1 was associated with more Treg infiltrates and predicted reduced overall and disease-free survival in patients with HNSCC. These findings indicate that IL-21 in the tumor microenvironment may promote PD-L1-induced, Treg-mediated immune escape in a PD-1-dependent manner and that an IL-21 neutralization strategy may enhance PD-1 blockade-based antitumor immunotherapy by targeting Treg-mediated immune evasion in patients with high expression of IL-21 and PD-L1.

Keywords: interleukin-21, regulatory T cells, programmed death-ligand 1, tumor microenvironment, head and neck squamous cell carcinoma

INTRODUCTION

The interaction between cancer cells and their surrounding microenvironment could modulate the immunoediting process, leading to tumor immune privilege (1–3). In this regard, regulatory T cells (Tregs), which infiltrate a variety of solid tumor microenvironments, are considered a key element in the promotion of immune evasion due to their ability to suppress tumor-specific immune

responses and hamper cancer immunotherapy (4–7). In head and neck squamous cell carcinoma (HNSCC), we reported that Treg depletion can repress tumor growth and evoke antitumor immunity and that the accumulation of Tregs predicts poor survival in HNSCC patients (8–10). However, factors that impact Treg generation in the tumor microenvironment are still poorly studied.

We recently found that tumor-associated inflammation (TAI) was positively related to HNSCC tumor-infiltrating Treg generation, which in turn promoted immunosuppression (10, 11). Thus, it would be interesting to further elucidate the potential mechanisms of TAI that are responsible for Treg generation in the tumor microenvironment.

The cytokine Interleukin (IL)-21 is an immune modulator with pleiotropic effects on multiple immune cell type. Driving inflammation by enhancing the generation and functions of cytotoxic T or NK cells is its classical function, importantly, IL-21 also exhibits immunosuppressive properties (12–15). It has been described to mediate the expression of IL-10 by cytotoxic cells and B cells and polarization of tumor-associated macrophage. Furthermore, the effects of IL-21 on immune cells have been reported depending on additional signals (16). The inhibitory checkpoint programmed death-ligand 1(PD-L1) is widely expressed in human malignancies. It interacts through its receptor modulating local immune contexture and serving an inhibitory signal to attenuate anti-tumor immune response. However, little information is currently available regarding interactions between IL-21, PD-L1 and Tregs in local tissue.

In the present study, we identified increased IL-21-polarized inflammation in HNSCC. Moreover, IL-21 could upregulate PD-1 expression on $CD4^+$ T cells and boost PD-L1-induced Treg generation through upregulating PD-1 expression. Neutralizing IL-21 could enhance the blockade effect of an anti-PD-1 antibody on Treg generation induced by $IL\text{-}21^{high}/PD\text{-}L1^{high}$ clinical tumor explants. High expression of IL-21/PD-L1 significantly predicted reduced survival in 102 patients with HNSCC. Our findings provide evidence that IL-21-associated inflammation might be modulated within the tumor microenvironment and negatively influence the antitumor immune response.

MATERIALS AND METHODS

Patients and Healthy Donors

One hundred thirty-seven patients diagnosed with laryngeal squamous cell carcinoma at the First Affiliated Hospital of Sun Yat-sen University were recruited for the present study. In detail, immunohistochemistry (IHC) was performed to evaluate the clinical relevance of tumor microenvironment factors in 102 patients, and 35 patients were selected for other *in vitro* studies. None of these 137 selected patients received palliative surgery or neoadjuvant chemo- and/or radiotherapy before sampling. Clinical staging was classified according to the criteria of the seventh edition of the Union for International Cancer Control (UICC). The freshly resected tumor and adjacent non-tumor tissues were kept in cold PBS for downstream analysis. The adjacent non-tumor tissues were confirmed cancer cells infiltration free by pathological examination. Peripheral blood mononuclear cells (PBMCs) were obtained from 14 healthy donors. All samples were collected after receiving informed consent from the patients, and the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University approved this study (Approval No. 2012-349).

IHC and Staining Evaluation

Paraffin-embedded, formalin-fixed, 5-μm-thick tissue sections (**Table 1**) were incubated with antibodies against human IL-21 (10 μg/ml, NBP1-02706, Novus), FOXP3 (5 μg/ml, ab20034, Abcam), PD-L1 (1:200, 13684, Cell Signaling Technology), and anti-p16/INK4a (1:250, 10883-1-AP, Proteintech) and then stained using the Dako Envision System (DakoCytomation) according to the manufacturer's instructions. The procedure for immunohistochemical staining evaluation was described in our previous studies (11). Briefly, for the categorization of samples by IL-21 or PD-L1 expression, specimens with a number of positive cells greater than the median were defined as 'high', and those with a number lower than the median were defined as 'low'. The median level of $IL\text{-}21^+$ cells was 4.5 cells per field. The expression of PD-L1 was scored semiquantitatively based on the staining intensity and distribution using the immunoreactive score (IRS). The IRS was calculated as staining intensity (SI) × percentage of positive cells (PP) (IRS= SI × PP). The SI was defined as negative (score 0), weak (score 1), moderate (score 2), and strong (score 3). The PP was defined as 0-5% (score 0), 6-25% (score 1), 26-50% (score 2), 51-75% (score 3), and 76-100% (score 4). The cutoff point between low and high PD-L1 expression was 3. P16 was considered positive if there was strong and diffuse nuclear and cytoplasmic staining present in greater than 70% of tumor cells and believed to correlated with Human papilloma virus (HPV). Cells stained with the indicated antibodies were imaged using Zeiss imaging systems (Axio Scan Z1, Carl Zeiss) at 100× and 400× magnification, and at least 5 fields of view per section at 400× magnification were evaluated.

TABLE 1 | Clinicopathological characteristics of patients.

Variable		No. cases	%
Gender	Male	95	93.1
	Female	7	6.9
Age (year)	<55	48	47.1
	≥55	54	52.9
Tumor site	Oral cavity	12	11.8
	Nasopharynx	5	4.9
	Larynx	75	73.5
	Hypopharynx	10	9.8
HPV status	p16+	12	11.8
	p16-	90	88.2
Tumor status	T_{1-2}	75	73.5
	T_{3-4}	27	26.5
Nodal status	N_0	83	81.3
	N_{1-2}	18	18.7
Stage	I+II	70	68.7
	III+IV	32	31.3

Tissue-Infiltrating Lymphocyte Isolation

Lymphocytes were isolated from tissue as previously described (17). Briefly, fresh surgical specimens (n=17) were minced into small pieces and subsequently digested with Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), 1 mg/ml collagenase I, 0.5 mg/ml collagenase II, 1 mg/ml hyaluronidase, and 100 U/ml DNase (all from Sigma-Aldrich) at 37°C for 20 mins. After being filtered through 70 μm cell strainers, the resulting cell suspensions were processed with density gradient centrifugation using a lymphocyte separation medium (MP Biomedical).

Flow Cytometry

Single-cell suspensions were stained with antibodies against CD45, CD3, CD4, PD-1, CD25, interferon (IFN)-γ, IL-17, IL-9, IL-4, FOXP3 (eBioscience), and IL-21 (BD Biosciences) according to the manufacturers' instructions. The dose of each of the above antibodies was 5 μl/test. To detect the cytokine profile, cells were stimulated with Leukocyte Activation Cocktail (2 μl/ml, BD Biosciences) for 5 h before flow cytometric analysis. For intracellular staining, cells were stained with the surface markers listed above and fixed in Fix/Perm Buffer (eBioscience), followed by permeabilization in the presence of antibodies specific for intracellular markers. All the reagents were purchased from BioLegend unless otherwise indicated. The stained cells were acquired on a Cytoflex flow cytometer (Beckman Coulter), and the analysis was performed using FlowJo software (TreeStar).

Cell Isolation and Culture

$CD4^+$ T cells were purified from PBMCs from healthy donors using a $CD4^+$ T cell isolation kit (Miltenyi Biotec). Following isolation, the cells were plated at 2×10^5 cells per well in flat-bottomed 96-well plates in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, and 100 U/ml penicillin and streptomycin. The $CD4^+$ T cells were activated using a tetrameric complex of anti-CD3 and anti-CD28 beads (ImmunoCult human T cell activator, STEMCELL). Dendritic cell (DC) preparation was performed as previously described (17), Briefly, $CD14^+$ cells isolated from PBMCs were cultured at a density of 1×10^5 cells per well in RPMI 1640 medium supplemented with GM-CSF (50 ng/ml, 300-03, PeproTech) and recombinant human IL-4 (20 ng/ml, 200-04, PeproTech) for 6 days.

Preparation of Tumor-Associated Antigen (TAA)-Specific T Cells

Autologous TAA-specific T cells were prepared using antigen-loaded DCs as we previously described (17). Briefly, soluble SNU899 cell line lysate antigens prepared by four freeze-thaw cycles (-140°C/42°C/60°C) were added to DC cultures at day 6 at a ratio of 3:1 (SNU899 cells: DCs). Lipopolysaccharide (LPS, 1 μg/ml, Sigma-Aldrich) was added to induce DC maturation. The maturation of the DCs was determined by measuring the expression of HLA-DR, CD80, CD86, and CD83 by flow cytometry. Antigen-loaded DCs were added to autologous T cells as stimulators at a ratio of 1:20.

Treg Generation Asay

$CD4^+$ T cells were activated using a tetrameric complex of anti-CD3/CD28 beads (ImmunoCult human T cell activator, STEMCELL) with or without recombinant human IL-21 (25 ng/ml, R&D Systems). Recombinant human PD-L1 (rhPD-L1, R&D Systems) was incubated at a concentration of 100 ng/ml in 96-well plates overnight in the presence of a goat anti-human IgG Fc antibody for dimerization and plate immobilization. Tregs were generated by stimulating $CD4^+$ T cells with anti-CD3/CD28 beads for 6 days with or without the presence of immobilized PD-L1 or IL-21. To study Treg generation, which may be influenced by the tumor microenvironment, tumor slices from clinical specimens (n=10) were laid on the bottom of the wells of a 96-well plate and cocultured with $CD4^+$ T cells isolated from PBMCs in the presence of anti-CD3/CD28 beads. For neutralization studies, recombinant human IL-21 R Fc Chimera (10 μg/ml, R&D Systems) and an anti-human PD-1 antibody (10 μg/ml, R&D Systems) were added at the start of the coculture. Cells were harvested at the indicated time and then analyzed with a Cytoflex flow cytometer.

Treg-Mediated Suppression of TAA-Specific T Cells

Using the Regulatory T Cell Isolation Kit (Miltenyi Biotec), generated $CD4^+CD25^+CD127^{low}$ Tregs were isolated from $CD4^+$ T cells stimulated with anti-CD3/CD28 beads and seeded together with autologous TAA-specific T cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 1 μM, eBioscience) at a ratio of 1:2 with a total of 30,000 cells per well.

Statistical Analysis

All statistical analyses were performed using IBM SPSS software version 22.0 (IBM Corporation). The Kaplan-Meier method was used to evaluate survival distributions, and differences between groups were assessed by the log-rank test. The correlation between variables was evaluated using Spearman's correlation test. Two-sided Student's t tests and ANOVA were used to analyze data from IHC and flow cytometry experiments, and the Bonferroni adjustment for multiple comparisons was used for between-group comparisons. Data are presented as the mean ± SD. P-values <0.05 were considered statistically significant.

RESULTS

IL-21-Polarized Inflammation Is Enriched in the HNSCC Tumor Microenvironment

We previously showed evidence that tumor-associated inflammation (TAI) was enriched in the HNSCC tumor environment (10, 17). However, the type of tumor environment inflammation that dominates the regulation of tumor immunity in HNSCC is unknown. In the present study, HNSCC tumor-infiltrating lymphocytes (TILs), which secrete typical cytokines associated with Th1-, Th2-, Th9-, Th17-, and Th21-polarized inflammation (including IFN-γ, IL-4, IL-9, IL-17, and IL-21), were identified using flow cytometric analysis. Our results showed that the frequencies of IFN-γ^+CD4^+ (26.2 ±

4.2%) and IL-21$^+$CD4$^+$ T cells (8.03 ± 4.37%) in tumor tissue were higher than those of IL-4$^+$CD4$^+$ (0.8 ± 0.5%, P< 0.01 and P< 0.01, respectively), IL-9$^+$CD4$^+$ (1.2 ± 0.7%, P< 0.01 and P< 0.01, respectively), and IL-17$^+$CD4$^+$ T cells (3.2 ± 2.6%, P< 0.01 and P< 0.01, respectively), which indicated that IFN-γ and IL-21 may be the dominant secreted cytokines of tumor-infiltrating Th cells (**Figures 1A, B**, **Supplementary Figure 1**).

We next compared the differences in the IFN-γ$^+$CD4$^+$ and IL-21$^+$CD4$^+$ T cell frequencies between tumor and adjacent non-tumor tissues. Notably, our results showed that despite the high frequency of IFN-γ$^+$CD4$^+$ T cells in the tumor tissues, the frequency of IFN-γ$^+$CD4$^+$ T cells was not considerably different between the tumor and adjacent non-tumor tissues (26.2 ± 4.2% *vs.* 22.8 ± 4.8%, respectively, P > 0.05) (**Figures 1A, B**). However, the frequency of IL-21$^+$CD4$^+$ T cells was significantly increased in the tumor tissues compared with the paired adjacent non-tumor tissues (8.03 ± 4.37% *vs.* 2.4 ± 1.4%, respectively, P < 0.01) (**Figures 1A, B**). Moreover, the IL-21-producing T cells in the tumor tissues were FOXP3 negative, and the majority of these cells (84.5 ± 3.3%) were CD3 and CD4 positive (**Figures 1C, D**). Finally, we found that approximately half of the IL-21$^+$CD4$^+$ T cells in the tumor tissues were IFN-γ positive (45.6 ± 5.2%) and that IL-21$^+$CD4$^+$ T cells were rarely IL-4 (0.8 ± 0.3%) or IL-17 (1.6 ± 0.5%) positive (**Figures 1E, F**).

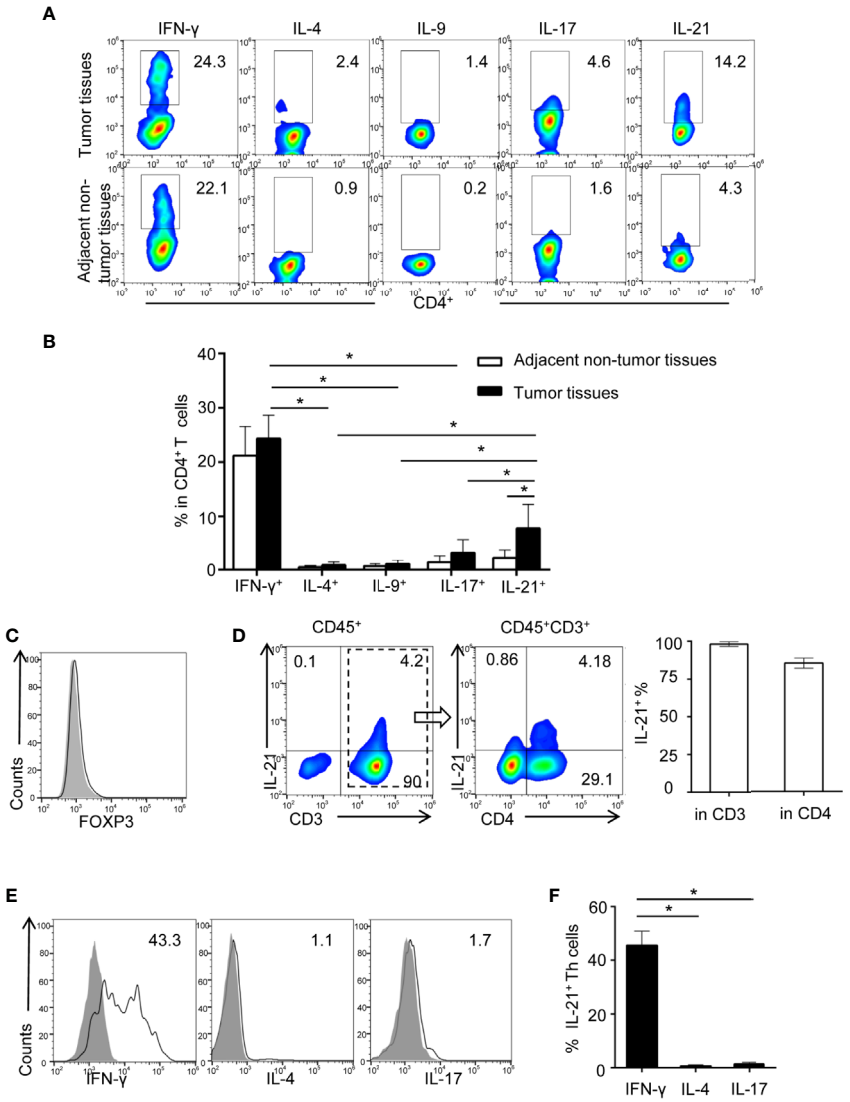

FIGURE 1 | Identification of increasing tumor-infiltrating IL-21 producing Th cells in HNSCC. **(A)** Representative flow cytometric plots of Th subsets isolated from tumor and adjacent non-tumor tissues. **(B)** Quantification of proportions of Th subsets (mean ± SD, n = 9, *P < 0.05 by Student's t test). **(C, D)** Flow cytometric analysis of IL-21 frequency in gated CD45$^+$ cells and CD45$^+$CD3$^+$ cells in tumor infiltrating lymphocytes. **(E, F)** Expression profile of IFN-γ, IL-4, and IL-17 in IL-21$^+$ Th cells from HNSCC tumor tissues, n = 5. Gating strategy was provided in **Supplementary Figure 1**.

IL-21$^+$ Cells Predict Poor Survival in Patients With HNSCC

We subsequently evaluated the clinical relevance of the increased frequency of tumor-infiltrating IL-21-producing cells in 102 HNSCC patients using IHC. Our results showed that IL-21$^+$ cells accumulated in the tumor stroma but not in the tumor nest (**Figure 2A**). The levels of IL-21$^+$ cell infiltration in patients with stage IV disease were higher than those with stage I, II or III disease (8.9 ± 3.6 *vs.* 4.0 ± 2.7, P < 0.001, *vs.* 4.1 ± 2.9, P < 0.001, *vs.* 5.6 ± 3.1, P < 0.05, counts per field, respectively), Additionally, those with stage III disease was higher than stage I (5.6 ± 3.1 *vs.* 4.0 ± 2.7, P < 0.05, counts per field) (**Figures 2A, B**).

To determine whether IL-21$^+$ cell infiltration in HNSCC correlates with disease prognosis, one hundred and two HNSCC patients were divided into two groups according to the median value of their IL-21$^+$ cell density. The patients with a high level of infiltrating IL-21$^+$ cells had worse overall and disease-free survival than the patients with a low level (**Figures 2C, D** and **Table 2**). Cox regression analysis revealed that the IL-21$^+$ counts and TNM stage could be independent predictive factors for overall survival in the HNSCC patients (**Table 3**). Taken together, our results identified that the accumulation of IL-21$^+$ cells was associated with disease progression and poor prognosis in patients with HNSCC.

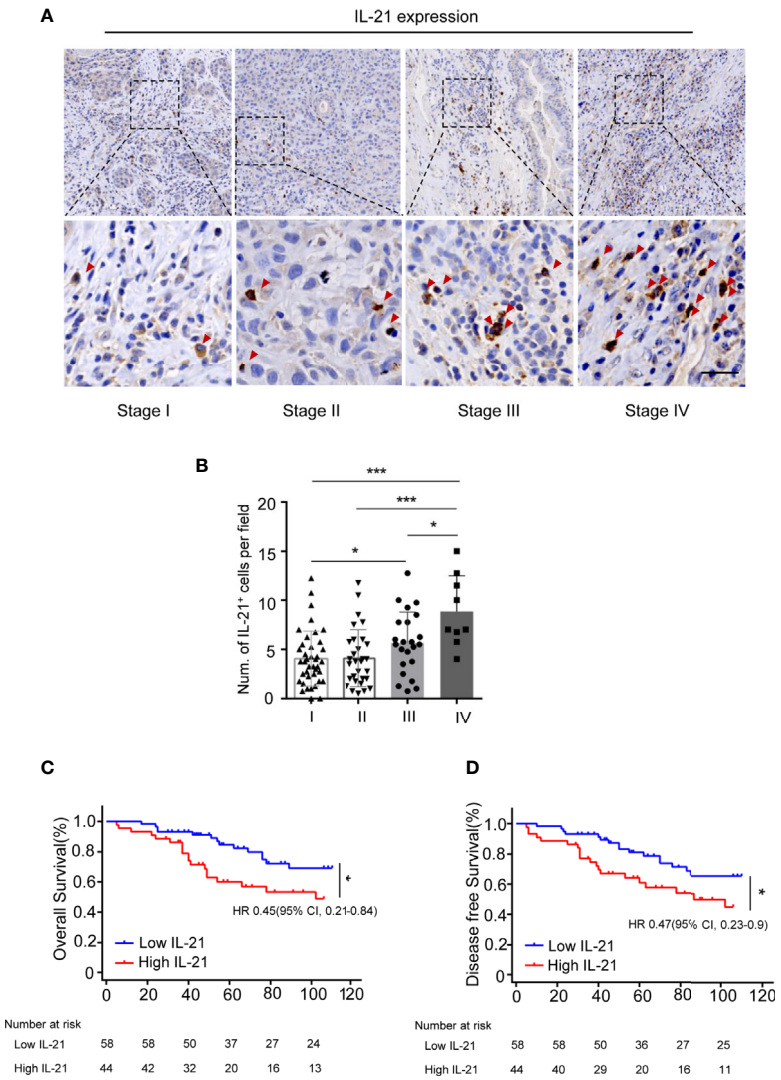

FIGURE 2 | Accumulation of IL-21$^+$ cells are associated with poor prognosis of patients with HNSCC. **(A)** Representative images showing staining of IL-21$^+$ cells within the tumor stroma. Positive cells are stained brown. (Red arrowheads, Scale bar, 50μm). Bottom panels (400×)are magnified images of the boxed area in the corresponding upper panel (100×). **(B)** IL-21$^+$ cell infiltration in patients with each of the stage I, II, III and IV (n=102, *P < 0.05, ***P < 0.001, by One way ANOVA.). **(C, D)** Kaplan-Meier survival curve of HNSCC patients with low and high numbers of tumor infiltrating IL-21$^+$ cells, compared by the log-rank test. Patients with high IL-21$^+$ cells levels had significantly poorer overall survival **(C)** and disease-free survival **(D)** compared with individuals with low.

Abundance of IL-21$^+$ Cells Is Positively Correlated With Treg Infiltration in HNSCC

Considering that Tregs are known to suppress antitumor immunity and that this suppression results in disease progression, we next determined whether the level of IL-21$^+$ cells was related to Treg infiltration. We first examined the prevalence of tumor-infiltrating Tregs and IL-21$^+$ cells using immunohistochemical staining for FOXP3 and IL-21. The results showed that FOXP3$^+$ and IL-21$^+$ cells collocated in the same area in the tumor stroma (**Figure 3A**) and that the increased density of the IL-21$^+$ cells was positively correlated with that of the FOXP3$^+$ cells (**Figure 3B**). Next, flow cytometric analysis showed that the frequency of tumor-infiltrating IL-21$^+$CD4$^+$ T cells positively correlated with that of FOXP3$^+$CD25$^+$CD4$^+$ T cells (**Figures 3C, D**), which supported our immunohistochemical results. We further assessed IL-21R expression on Treg cells and conventional T cells (CD45$^+$CD3$^+$CD4$^+$CD8$^-$CD25$^-$FOXP3$^-$), showing that Tregs have remarkably more IL-21R expression (**Supplementary Figure 2**). These results implied that IL-21$^+$ cells may be involved in Treg infiltration in the HNSCC tumor microenvironment.

IL-21 Promotes PD-L1-Induced Treg Generation in a PD-1-Dependent Manner

To determine whether tumor-infiltrating IL-21$^+$ cells contribute to Treg generation, recombinant human IL-21 was added to cultured CD4$^+$ T cells. However, the unexpected results showed that the frequency of Tregs markedly declined in the CD4$^+$ T cells + IL-21 group compared with the CD4$^+$ T cells alone group (4.8 ± 0.8% *vs.* 7.2 ± 0.9%, respectively, $P < 0.05$) (**Figures 4A, B**), and this result was inconsistent with our clinical data. We speculated that IL-21 may have the effect of antagonizing Treg when treated alone, which was supported by literature demonstrating the pathogenic role of IL-21 in human inflammatory diseases, and may act in concert with other immunosuppressive factors in Treg generation in the tumor

TABLE 2 | Relationships between tumor stromal IL-21$^+$ cells and clinical variables.

Variable		stromal IL-21+ cells		P value
		Low (cases)	High (cases)	
Gender	Male	54	41	0.073
	Female	1	6	
Age, years	<55	25	23	0.879
	≥55	30	24	
Tumor site	Oral cavity	7	5	0.707
	Nasopharynx	4	1	
	Larynx	41	34	
	Hypopharynx	5	5	
HPV status	p16+	8	4	0.346
	p16-	47	43	
Tumor status	T_{1-2}	46	29	**0.023**
	T_{3-4}	9	18	
Nodal status	Negative	50	33	**0.044**
	Positive	6	13	
Stage	I+II	44	26	**0.013**
	III+IV	11	21	

The bold value indicates statistical significance.

TABLE 3 | Univariate and multivariate analyses of factors associated with survival and recurrence.

	OS				DFS			
	Univariate		Multivariate		Univariate		Multivariate	
	P	HR	95%CI	P	P	HR	95%CI	P
Gender(Male)	0.366				0.532			
Age(≥55)	0.431				0.739			
Tumor site(Larynx)	0.541				0.675			
HPV status(p16+)	0.673				0.714			
Tumor status (T_3-T_4)	**0.041**	1.43	0.73-2.32	0.837	0.065			
Nodal status (Positive)	**0.012**	1.52	1.18-2.76	0.203	**0.026**	1.23	0.76-1.91	0.253
Stage (III-IV)	**0.003**	2.03	1.32-3.28	**0.023**	**0.007**	1.17	0.62-1.89	**0.035**
IL-21^{high}	**0.013**	1.83	1.06-2.72	**0.007**	**0.032**	1.28	0.87-1.50	0.112
IL-21^{high}PD-L1^{high}	**<0.001**	2.71	1.24-4.85	**0.004**	**<0.001**	1.62	1.02-2.46	**0.019**

The bold value indicates statistical significance.

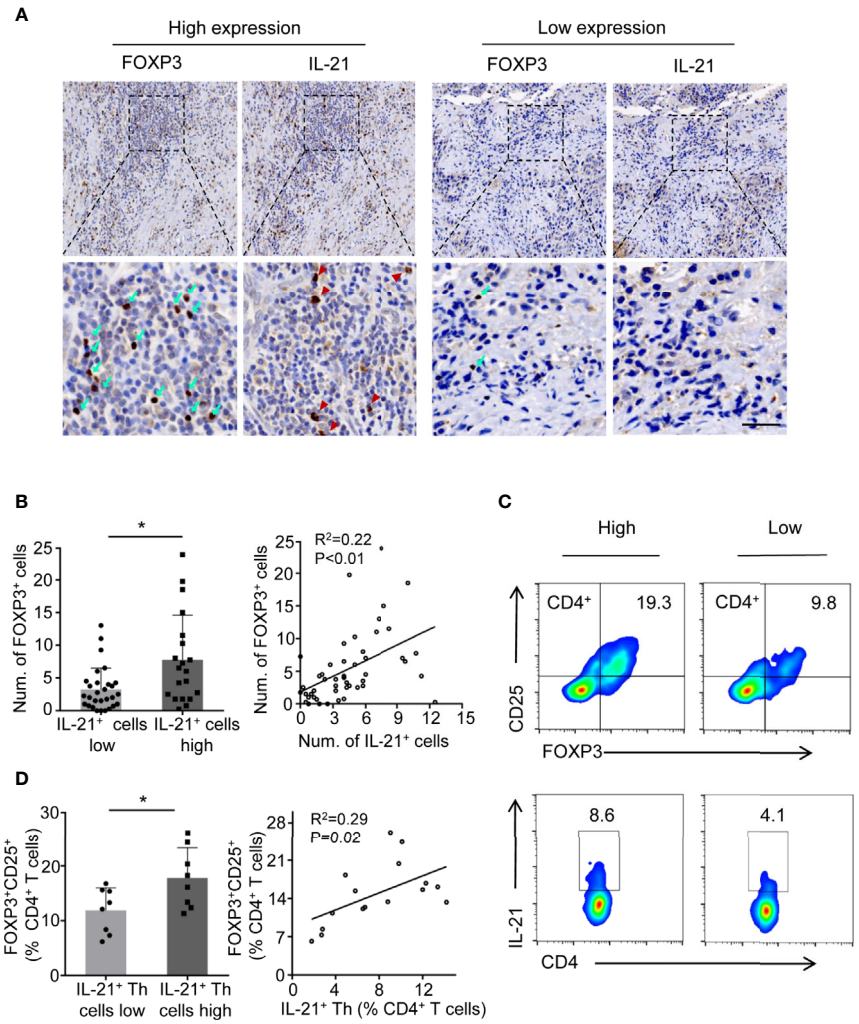

FIGURE 3 | IL-21$^+$ Th cells accumulation is positively correlated with Treg infiltration in HNSCC tissues. **(A)** High and low expression of FOXP3 (cyan arrows) and IL-21 (red arrowheads) positive cells at the same area of stroma. Scale bar, 50μm, magnification, bottom panels 400×, upper panel 100×. **(B)** Correlation between FOXP3$^+$ and IL-21$^+$ cells using immunohistochemical analysis. n=49. **(C)** Representative flow cytometric plots of tumor infiltrating FOXP3$^+$ CD25$^+$CD4$^+$ and IL-21$^+$CD4$^+$ T cells. **(D)** Correlation between FOXP3$^+$ and IL-21$^+$ cells using flow cytometric analysis. n=16. In immunohistochemical analysis, the median level of IL-21$^+$ cells was 4.5 cells per filed. In flow cytometric analysis, the median percent of IL-21$^+$ Th in CD4$^+$ T cells was 7.75%. (*P < 0.05 by Student's t-test).

microenvironment. As the PD-L1/PD-1 pathway has been recognized as a molecular checkpoint in immune evasion in various cancers (18–22), we next examined the effect of IL-21 on Treg generation from CD4$^+$ T cells in the presence of PD-L1. Excitingly, our results showed that the frequency of Tregs was not only higher in the CD4$^+$ T cells + PD-L1 group than in the CD4$^+$ T cells alone group (13.2 ± 3.2% *vs.* 7.2 ± 0.9%, respectively, P < 0.05) but also higher in the CD4$^+$ T cells + PD-L1 + IL-21 group than in the CD4$^+$ T cells + PD-L1 group (17.9 ± 4.1% *vs.* 13.2 ± 3.2%, respectively, P < 0.05) (**Figures 4C, D**), which meant that IL-21 could enhance PD-L1-induced Treg generation. We next examined whether this Treg generation was PD-1 dependent, and the results showed that compared with PD-L1 (23.5 ± 3.5%, P < 0.05) or IL-21 alone (19.4 ± 5.1%, P < 0.05), the combined use of PD-L1 and IL-21 (32.0 ± 4.4%) markedly upregulated PD-1 expression on CD4$^+$ T cells (**Figures 4E, F**), suggesting a synergistic effect of IL-21 and PD-L1 on PD-1 induction. Most importantly, blocking PD-1 with a neutralizing antibody abrogated the effects of IL-21 and/or PD-L1 on Treg generation (*vs.* IL-21+PD-L1: 5.7 ± 1.5% *vs.* 17.9 ± 4.1%, P < 0.05; *vs.* PD-L1: 8.1 ± 1.5% *vs.* 13.2 ± 3.2%, P < 0.05) (**Figures 4G, H**), which indicated that IL-21 and PD-L1-induced Treg generation was PD-1 dependent.

PD-L1 and IL-21-Induced Tregs Show a Stronger Ability to Suppress the Proliferation of TAA-Specific T Cells Than Naturally Occurring Tregs

For the analysis of the functionality of the generated Tregs, Tregs were isolated from different groups and cocultured with

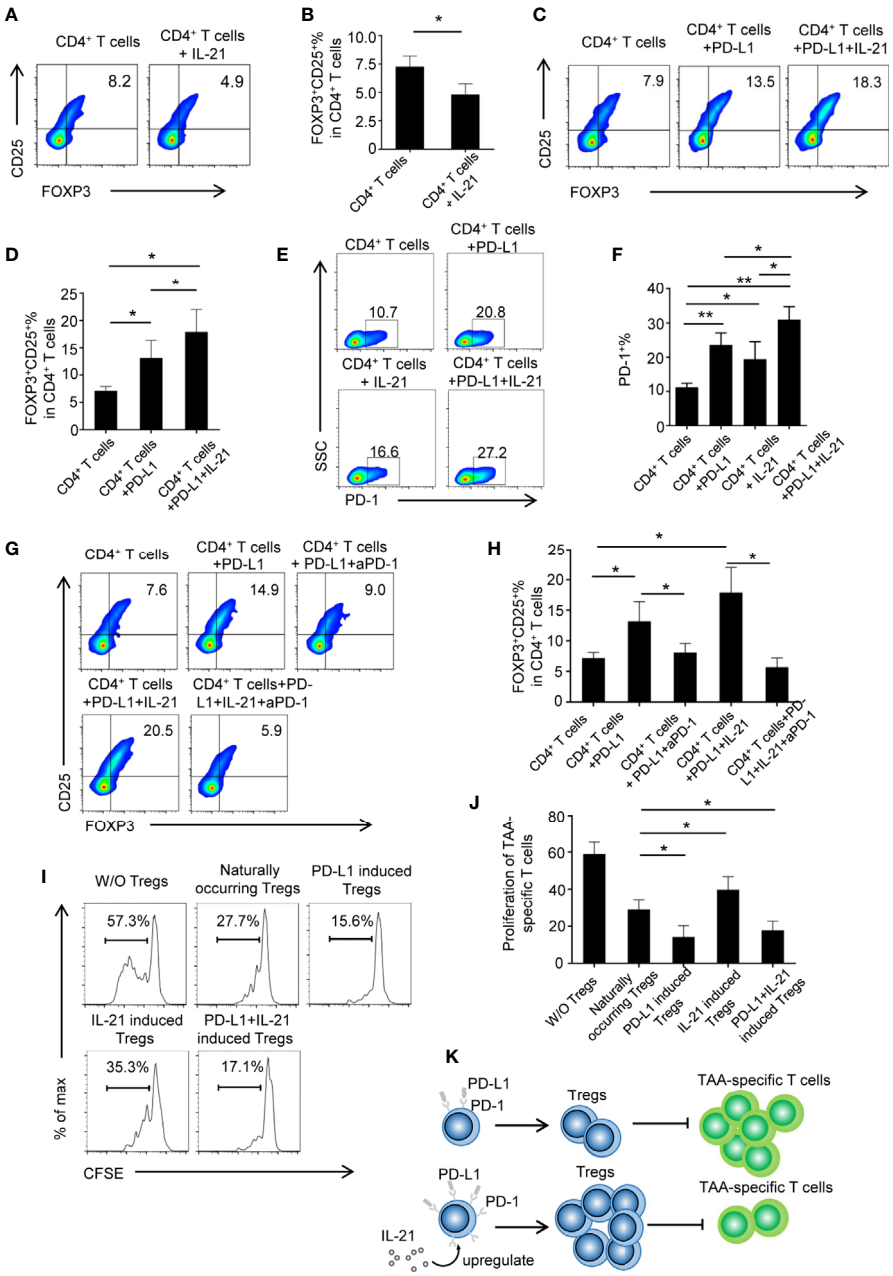

FIGURE 4 | IL-21 contribute to PD-L1 induced Tregs generation in PD-1 dependent manner. **(A, B)** $CD4^+$ T cells from human peripheral blood were stimulated with anti CD3/28 antibody coated beads with or without IL-21. The percentage of $FOXP3^+CD25^+$ Tregs were quantitated and shown. (n = 5, *P < 0.05, by Student's t-tests.) **(C, D)** CD4+ T cells were treated with or without PD-L1 or/and IL-21. Percentage of $FOXP3^+CD25^+$ Tregs was shown (n = 5, *P < 0.05, by Student's t-tests). **(E, F)** Proportion of PD-1 expressing $CD4^+$ T cells in the presence or absence of IL-21 or PD-L1 (n = 4, *P < 0.05, **P < 0.01 by one way ANOVA). **(G, H)** CD4+ T cells were treated with or without PD-L1 or/and IL-21 in the presence or absence of PD-1 neutralization antibody. Percentage of $FOXP3^+CD25^+$ Tregs was shown (n = 5, *P < 0.05, by Student's t-tests) **(I, J)** Induced Tregs were cocultured with CFSE labeled TAA-T responder cells at ratios of 1:2 and in the presence of anti-CD3/anti-CD28 antibodies. Proliferation of responder cells were assessed after 72h by flow cytometry. Plots are representative of five separate experiments, *P < 0.05, compared with naturally occurring control Tregs by Student's t-tests. **(K)** Schematic figure illustrating that IL-21 promoted PD-L1-induced Treg generation in a PD-1-dependent manner, IL-21 and PD-L1-induced Tregs inhibited TAA-specific T cell proliferation at a greater degree than naturally occurring Tregs.

CFSE-labeled TAA-specific T cells. Our results showed that the generated Tregs from the $CD4^+$ T cells + PD-L1 + IL-21 group (17.8 ± 4.9%, P<0.05) or the $CD4^+$ T cells + PD-L1 group (14.3 ± 6.1%, P<0.05) had stronger suppressive effects on TAA-specific T cell proliferation than those from the $CD4^+$ T cells alone group (29.0 ± 5.2%), whereas compared with those from the $CD4^+$ T cells alone group, the Tregs isolated from the $CD4^+$ T cells + IL-21 group showed an impaired suppressive

ability (39.7 ± 7.0% *vs.* 29.0 ± 5.2%, respectively, P<0.05) (**Figures 4I, J**). In addition, TAA-specific $CD8^+$ T cells were assessed as well (**Supplementary Figure 3**). It revealed that PD-L1 and IL-21-induced Tregs show expected suppressive effects as on TAA-specific $CD8^+$ T cells. Taken together, schematic figure (**Figure 4K**) illustrates that IL-21 promoted PD-L1-induced Treg generation in a PD-1-dependent manner, and IL-21 and PD-L1-induced Tregs could inhibited TAA-specific T cell proliferation at a greater degree than naturally occurring Tregs.

Neutralizing IL-21 Enhances the Blockade Effect of an Anti-PD-1 Antibody on the Treg Generation Induced by IL-21^{high}/PD-$L1^{high}$ Tumor Explants but Not on that Induced by IL-21^{low}/PD-$L1^{low}$ Tumor Explants

To test whether the HNSCC tumor environment is capable of potentiating Treg generation through IL-21 and PD-L1, we cocultured $CD4^+$ T cells with tumor explants from HNSCC patients with or without IL-21 and/or an anti-PD-1 neutralizing antibody. Accordingly, tumor explants from 10 patients with HNSCC were divided evenly into 2 groups according to the cutoff points for PD-L1 and IL-21 described in the methods section. There were 5 samples with high expression of both IL-21 and PD-L1 (IL-21^{high}/PD-$L1^{high}$) and 5 samples with low expression of both IL-21 and PD-L1 (IL-21^{low}/PD-$L1^{low}$). Our results showed that the frequency of Tregs in the group of IL-21^{high}/PD-$L1^{high}$ tumor explants + $CD4^+$ T cells was higher than that of the group of IL-21^{low}/PD-$L1^{low}$ tumor explants + $CD4^+$ T cells (38.8 ± 8.5% *vs.* 25.8 ± 2.4%, respectively, P < 0.05) (**Figures 5A, B**). The anti-PD-1 antibody showed an inhibitory effect on Treg generation when the $CD4^+$ T cells were cultured with the IL-21^{high}/PD-$L1^{high}$ tumor explants (31.3 ± 5.0% *vs.* 38.8 ± 8.5%, P < 0.05). In addition, the combination of IL-21 and the anti-PD-1 antibody had a stronger inhibitory effect on Treg generation than the anti-PD-1 antibody alone (26.6 ± 4.7% *vs.* 31.3 ± 5.0%, respectively, P < 0.05), whereas blocking IL-21 alone had no significant inhibitory effect on Treg generation (38.1 ± 7.1% *vs.* 38.8 ± 8.5%, P > 0.05). Conversely, the Treg generation induced by the IL-21^{low}/PD-$L1^{low}$ tumor explants failed to be reversed by neutralizing IL-21 (24.4 ± 3.7% *vs.* 25.8 ± 2.4%, P > 0.05), PD-1 (22.8 ± 2.4% *vs.* 25.8 ± 2.4%, P > 0.05), or both IL-21 and PD-1 (22.5 ± 3.1% *vs.* 25.8 ± 2.4%, P > 0.05), indicating that Treg generation in the IL-21^{high}/PD-$L1^{high}$ tumor microenvironment may be regulated by mechanisms distinct from those in the IL-21^{low}/PD-$L1^{low}$ tumor microenvironment (**Figures 5A, B**). Treg generation induced by IL-21^{high}/PD-$L1^{high}$ tumor explant was further validated by cocultured with TAA-specific T cells (both CD4 and CD8) (**Supplementary Figures 4, 5**). Using intracellular staining, the cytokine-producing ability of responder $CD4^+$ T cells and $CD8^+$ T cells was assessed. Our results showed that the stimulated responder $CD4^+$ and $CD8^+$ T cells exhibited strong proliferation in both, high IFN-γ, IL-2 levels in responder $CD4^+$ T cells, high GranzymeB and Perforin levels in responder $CD8^+$ T cells. Adding the induced Treg by tumor explants with or without anti-PD-1 or/and anti-IL-21 treatment strongly inhibited the proliferation of responder T cells (both CD4 and CD8) (**Supplementary Figures 4A, 5A**), reduced the IFN-γ, IL-2 levels of $CD4^+$ T cells (**Supplementary Figures 4B, D**), and cytotoxicity, GranzymeB, Perforin levels of $CD8^+$ T cells (**Supplementary Figures 5B, E**). However, no significant differences were observed between tumor explant with no antibody, with anti-PD-1, with anti-IL-21 group and with the combination anti-PD-1 and anti-IL-21. (**Supplementary Figures 4D, 5E**).There data suggested the Tregs induced by tumor explant with the treatment of anti-PD-1 or/and anti-IL-21 are expectedly suppressive as the Tregs induced by tumor explant alone.

The above data suggested that IL-21 neutralization may enhance the effect of PD-1-targeted tumor immunotherapy in only HNSCC patients with high expression of both IL-21 and PD-L1.

Simultaneous High Expression of IL-21 and PD-L1 Is Associated With More Treg Infiltrates and Worse Survival

Given that IL-21 plays a positive role in PD-L1-induced Treg generation, we next investigated the relationships between the expression patterns of IL-21, PD-L1, and FOXP3 in tumor specimens. Our results showed that the tumors with high expression of IL-21 or PD-L1 had more $FOXP3^+$ cells than those with low expression of both IL-21 and PD-L1 (6.8 ± 3.6 *vs.* 3.2 ± 2.7 counts per field, respectively, P < 0.05) (**Figures 6A, B**). Furthermore, the tumors with high expression of both IL-21 and PD-L1 had more $FOXP3^+$ cells (9.6 ± 4.9 counts per field) than the tumors with low expression of both IL-21 and PD-L1 (3.2 ± 2.7 counts per field, P < 0.05) or the tumors with high expression of either IL-21 or PD-L1 (6.8 ± 3.6 counts per field, P < 0.01) (**Figures 6A, B**). Moreover, in the tumor stage analysis, the PD-L1 expression score in patients with stage IV disease was considerably higher than that in those with stage I, II or III disease (5.8 ± 1.9 *vs.* 2.0 ± 1.6, P <0.001, *vs.* 3.1 ± 2.2, P < 0.01, *vs.* 3.2 ± 1.8, P <0.05, respectively), (**Figure 6C**). Fifteen cases (47%) with simultaneous high expression of IL-21 and PD-L1 were observed among the stage III or IV samples, and 20 cases (29%) were observed among the stage I or II samples, while simultaneous low expression of IL-21 and PD-L1 was observed in 8 cases (25%) among the stage III or IV samples and in 36 cases (51%) among the stage I or II samples. Moreover, high expression of either PD-L1 or IL-21 was observed in 9 cases (28%) among the stage III or IV samples, and 14 cases (20%) were observed among the stage I or II samples (**Figure 6D**). We finally evaluated whether the simultaneous high expression of IL-21 and PD-L1 correlated with the clinical prognosis of HNSCC patients. As expected, the patients with simultaneous high expression of IL-21 and PD-L1 had worse overall and disease-free survival than those with simultaneous low expression of PD-L1 and IL-21 (OS: P < 0.01, DFS: P < 0.01), worse overall survival than high expression of either PD-L1 or IL-21 (P < 0.05, DFS not significant) (**Figures 6E, F**). Overall survival was still significantly different between IL-21^{high}/PD-$L1^{high}$ and IL-21^{low}/PD-$L1^{low}$ group at stages I + II (P < 0.05) and III + IV (P < 0.05), respectively (**Figures 6G, H**). Disease-free survival

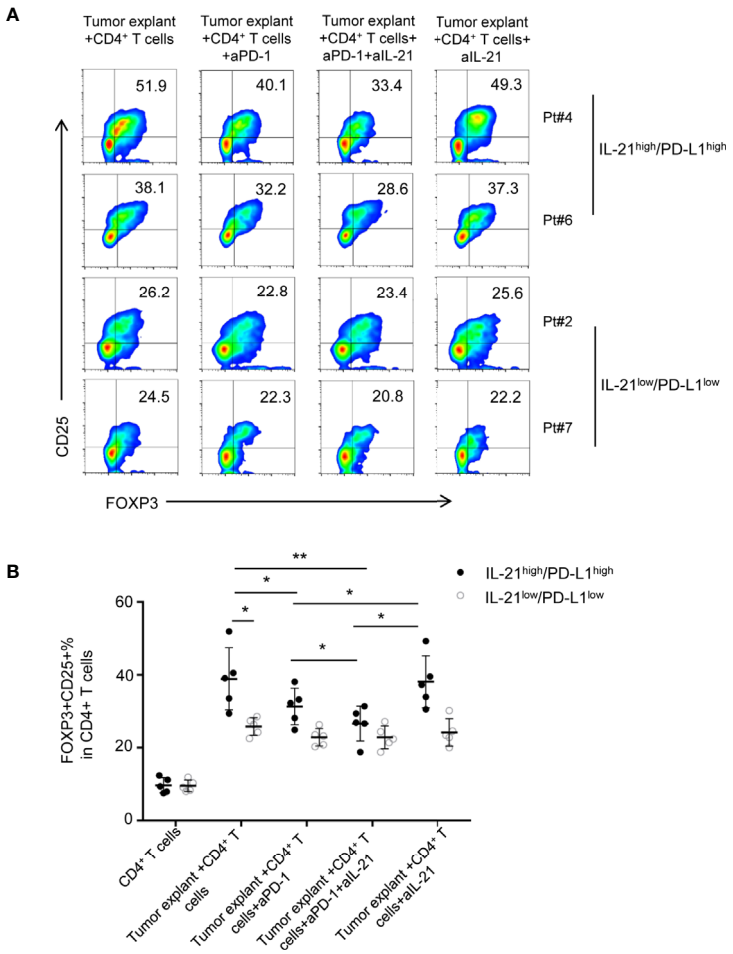

FIGURE 5 | Neutralizing IL-21 enhances the blocked effect of PD-1 antibody on Treg generation induced by IL-21^{high}/PD-L1^{high} tumor explants. **(A)** Tumor explants from 10 patients with HNSCC were divided into 2 groups according to the cutoff points of PD-L1 and IL-21. Effect of HNSCC tumor explant on Treg induction was assessed by culturing tumor explants and CD4$^+$ T cells isolated from peripheral blood in the presence or absence of IL-21 and/or PD-1 neutralization antibody. **(B)** Quantification of percentage of FOXP3$^+$CD25$^+$ Tregs was shown (n = 5, *P < 0.05, **P < 0.01, by Student's t-test).

was significantly different between IL-21^{high}/PD-L1^{high} and IL-21^{low}/PD-L1^{low} group at stages I + II ($P < 0.05$), but not stages III + IV ($P > 0.05$) (**Figures 6I, J**). Cox regression analysis revealed that the simultaneous high expression of PD-L1 and IL-21 was an independent prognostic marker for overall and disease-free survival in HNSCC patients (**Table 3**).

DISCUSSION

Substantial evidence shows that immune cells are habitually recruited into the tumor microenvironment, where they create inflammatory responses and play a critical role in the establishment of the immunosuppressive milieu (23, 24). The present study demonstrated that the tumor microenvironment inflammatory factor IL-21 may promote PD-L1-induced, Treg-mediated tumor immune escape in a PD-1-dependent manner. These findings provide evidence that the tumor microenvironment inflammation represented by IL-21 plays an important role in inhibiting antitumor immune responses *via* Treg generation.

To date, few studies have shown that IL-21-associated inflammation exists in human solid tumors, and the immune significance of IL-21 in the tumor microenvironment remains controversial. Some studies have revealed that IL-21 may enhance the cytotoxic activity of CD8$^+$ T cells and natural killer (NK) cells, suggesting that IL-21 functions as an antitumor agent (25, 26), while other studies have reported the immunosuppressive significance of IL-21 in promoting tumor-associated macrophage polarization and the expression of IL-10 by cytotoxic cells and B cells (12, 14, 15). We speculated that these inconsistencies could be attributed to the different tumor microenvironment contexts, IL-21 has been proposed acting in a context-dependent manner (27), therefore while presence of IL-21 provide a valid explanation for the activation of T or B cells in tumor tissue, a variety of cofactors e.g., PD-L1, CTLA-4 and tumor-derived factors may influence IL-21 actions. Moreover,

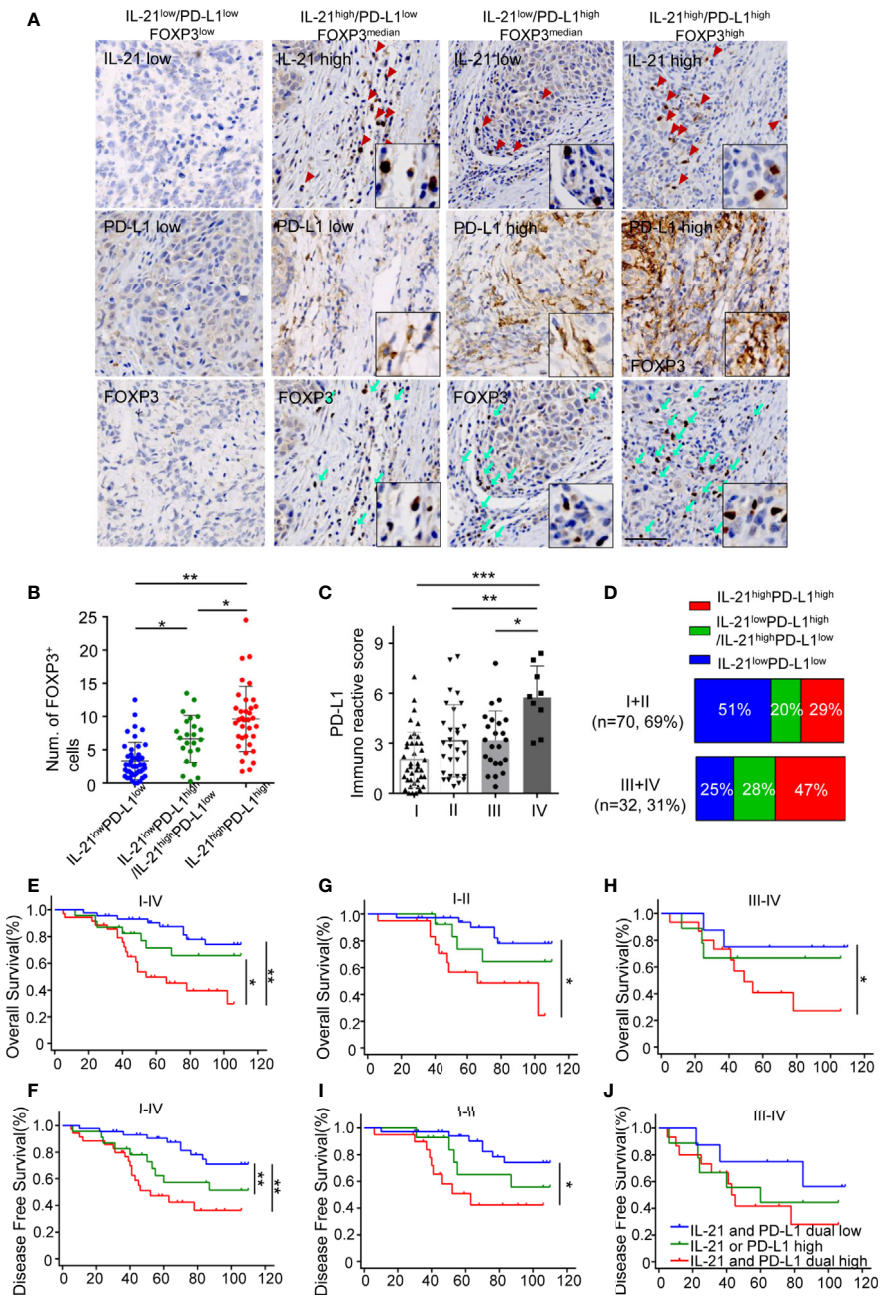

FIGURE 6 | Simultaneous high expression of IL-21 and PD-L1 was associated with more Treg infiltrates and worse survival. **(A)** Representative images of IHC staining showed IL-21, B7-H1 and FOXP3 in serial sections. Scale bar represent 100μm, magnification, 200×. **(B)** Quantitative histogram showed that tumors with simultaneous high expression of IL-21 and B7-H1 have more FOXP3$^+$ cells than those with simultaneous low expression of IL-21 and PD-L1 and those with high expression of either IL-21 or PD-L1. **(C)** B7-H1 expression in tumors with each of the stage I, II, III and IV, *P < 0.05. **P < 0.01, ***P < 0.001, by One way ANOVA **(D)** Comparison of the percentage of PD-L1 and/or IL-21 expression with stage I and II compared to those with stage III and IV. **(E–J)** Simultaneous high expression of IL-21 and B7-H1 predicted worse overall and disease-free survival in patients at stages I–IV **(E, F)**, stages I and II only **(G, I)**, or stages III and IV only **(H, J)** with HNSCC. (n (IL-21 and B7-H1 dual low expression) = 44, n (IL-21 or B7-H1 high expression) = 23, n (IL-21 and B7-H1 dual low expression) = 35, *P < 0.05. **P < 0.01). Number at risk and hazard ratio for survival plots has been provides in supplementary table section.

local presence of IL-21 within tumor microenvironment is likely to interact with a variety of cell types or environmental factors than its systemic occurrence, for PD-1 ligation was observed to skew TCR repertories (28). It is thus possible that IL-21 selectively support a local immunosuppressive environment, whereas systemic application of IL-21 activates cytotoxic T or NK cells with anti-tumor activity at tumor-distant sites. Our hypothesis is supported by the proposal that tumor immune

evasion is selectively modulated in local tumor microenvironment (22). Therefore, it likely depends on the status and type of immune infiltrates and the presence of environmental cofactors such as PD-L1 whether anti-tumor immune response is suppressed or supported by IL-21 in tumor microenvironment.

Recent studies reported the discrepant transcriptional and functional properties between tumor infiltrating Tregs, tissue resident Tregs and peripheral blood Tregs, indicating that tumor microenvironment may play an essential role in the regulation of the phenotype and immunosuppressive functionality of Tregs (29, 30). We recently showed that HNSCC-associated inflammation can augment the tumor microenvironment Treg population, which implied the inflammatory significance of Treg generation (10). However, the specific relationship between TAI and Treg generation is currently unclear. The present study aimed to evaluate the mechanisms by which IL-21-mediated inflammation regulates the generation and function of Tregs in the tumor microenvironment.

To our knowledge, this is the first study to propose significant crosstalk between IL-21 and PD-L1 in the generation of the tumor microenvironment Tregs that are responsible for the enhanced suppression of TAA-specific T cell proliferation. In detail, we first examined the effect of IL-21 alone on Treg generation, and the results showed that IL-21 inhibited Treg generation, which was inconsistent with our immunohistochemical data that showed that Treg infiltrates positively correlated with the counts of IL-21-positive cells. The above results implied that other tumor microenvironment elements may be involved in the regulation of Treg generation. As PD-L1/PD-1 signaling has recently been shown to modulate the Treg homeostasis and facilitate tumor immune tolerance (18–21), we hypothesized that PD-L1/PD-1 signaling may be involved in the process of Treg generation regulated by IL-21. Hence, we next added PD-L1 to IL-21-treated $CD4^+$ T cells and found that IL-21 significantly promoted Treg generation in the presence of PD-L1. The potential mechanism is that IL-21 can induce the expression of PD-1 for PD-L1/PD-1 signaling, which is responsible for Treg generation. The mechanism of the upregulation of PD-1 expression induced by IL-21 might be a TCR-triggered calcineurin signaling cascade that leads to the activation of the transcription factors NFATc1 and STAT family proteins (31, 32). Moreover, although a recent study reported that cancer cells produced TGF-β1 in tumor supernatants and when $CD4^+$ T cells were cultured in tumor supernatants, $FOXP3^+$ Tregs generation was promoted. While IL-21 was able to inhibit cancer cell-mediated FOXP3 induction in the context of tumor supernatants (33), which was contrary to our study. We believe that this inconsistency may be attributed to that study ignoring the involvement of the tumor microenvironment, and IL-21 may exert double sword effects on Tregs at the context of other immunosuppressive component derived from tumor microenvironment such as PD-L1.

To validate the synergistic effect of IL-21 and PD-L1 on Treg generation in the tumor microenvironment, we used tumor explants freshly isolated from cancer patients to induce Treg generation from $CD4^+$ T cells and to test whether neutralizing IL-21 and PD-1 was capable of blocking Treg generation. Our results showed that compared with either treatment alone, IL-21 neutralization combined with an anti-PD-1 neutralizing antibody could effectively inhibit IL-21^{high}/PD-$L1^{high}$ tumor explant-induced Treg generation, whereas this effect was not observed with IL-21^{low}/PD-$L1^{low}$ tumor explant-induced Treg generation, indicating that other mechanisms might be involved in Treg generation in tumor microenvironments with low expression of IL-21 and PD-L1. The potential mechanisms that may be involved in Treg generation involve other mediators, such as TGF-β, IL-10, and IDO, and interactions with tolerogenic DCs or stromal cells in the tumor milieu (34–38). The above clinical data suggest that the IL-21 neutralization strategy may represent a promising immunotherapeutic approach that may enhance PD-1 blockade-based tumor immunotherapy by targeting Treg-mediated immune evasion in only patients with high expression of IL-21 and PD-L1.

To expand the understanding of the correlations between IL-21, PD-L1, and Tregs in the tumor microenvironment and their prediction of disease prognosis, we detected the expression of IL-21, PD-L1, and FOXP3 in 102 newly diagnosed HNSCC patients. Our results showed that enriched IL-21^+ cells and PD-L1 expression were positively associated with $FOXP3^+$ cell infiltration and that high expression of IL-21 and PD-L1 correlated with advanced tumor stage and poor overall and disease-free survival in patients with HNSCC.

In conclusion, our results suggest that increased IL-21-associated inflammation in the tumor milieu may favor Treg-mediated immune evasion through the PD-L1/PD-1 pathway and that the IL-21 neutralization strategy may enhance PD-1 blockade-based tumor immunotherapy by targeting Treg-mediated immune evasion in patients with high expression of IL-21 and PD-L1. Understanding the mechanisms of the interactions between Tregs and tumor microenvironment inflammation may be beneficial for optimizing Treg-targeted antitumor strategies.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception and design: WS and WW. Acquisition of data: YZ, ZZ, YW, LC, KL, HL, YX, RM, and FW. Analysis and interpretation of data: WS, YHW, WL, and FJ. Writing, review,

and/or revision of the manuscript: YZ, ZZ, WS, and WW. Study supervision: WS and WW. 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.2021.648293/full#supplementary-material

Supplementary Figure 1 | Gating strategy of flow cytometric plot for tumor infiltrating lymphocytes.

Supplementary Figure 2 | (A) Representative flow cytometric histograms of the expression of IL-21R on Treg cells and conventional T cells (CD45+CD3+CD4+ CD8-CD25-FOXP3-) in tumor are shown; **(B)** The frequencies of IL-21R+ Treg cells were significantly higher compared with conventional T cells, n=5, *P < 0.05.

Supplementary Figure 3 | Proliferation of CD8+ T responder cells were assessed after 72h by flow cytometry. **(A, B)** Induced Tregs were cocultured with CFSE labeled TAA- specific CD8+ T responder cells at ratios of 1:4 and in the presence of anti-CD3/anti-CD28 antibodies. n=5, *P < 0.05.

Supplementary Figure 4 | Induced Treg cocultured with TAA-specific CD4+ T cells. Tregs induced by IL-21high/PD-L1high tumor explants were cocultured with CD4+ T responder cells at ratios of 1:4 and in the presence of anti-CD3/anti-CD28 antibodies. **(A)** Proliferation of CFSE labeled TAA CD4+ T responder cells were assessed after 72h by flow cytometry. Production of IFN-γ **(B)**, IL-2 **(C)** in cocultured CD4+ T responder cells was detected by intracellular staining. **(D)** Quantification of proliferation and percentage of IFN-γ, IL-2 of CD4+ T responder cells. n=5, n.s., not significant.

Supplementary Figure 5 | Induced Treg cocultured with TAA-specific CD8+ T cells Tregs induced by IL-21high/PD-L1high tumor explants were cocultured with CD8+ T responder cells at ratios of 1:4 and in the presence of anti-CD3/anti-CD28 antibodies. **(A)** Proliferation of CFSE labeled TAA CD8+ T responder cells were assessed after 72h by flow cytometry. **(B)** TAA-specific cytotoxicity was determined by flow cytometry. Production of GranzymeB **(C)**, Perforin **(D)** in cocultured CD8+ T responder cells was detected by intracellular staining. **(E)** Quantification of proliferation, TAA-specific cytotoxicity and percentage of GranzymeB, Perforin of CD8+ T responder cells. n=5, n.s., not significant.

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Combination of Immunotherapy and Radiotherapy for Recurrent Malignant Gliomas: Results From a Prospective Study

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Background: World Health Organization (WHO) grade IV glioma remains one of the most lethal tumors with a dismal prognosis and inevitable recurrence. We evaluated the safety and efficacy of immunotherapy with radiotherapy in this population of patients.

Methods: This study was a single-arm, open-label, phase I trial based on patients with recurrent WHO grade IV glioma. Patients were treated with intracranial and systemic immunoadjuvants in combination with low-dose reirradiation. The primary endpoint of the present trial was safety. Secondary endpoints were overall survival (OS) and progression-free survival (PFS). This trial is registered at ClinicalTrials.gov, NCT03392545.

Results: Thirty patients were enrolled. The most common adverse events (AEs) were fever (66.7%), vomiting (33.3%), headache (30.0%), and fatigue (23.3%). Only a single patient experienced grade 3 fever, and no grade 4 AEs or deaths related to treatment were observed. Of the 30 patients, 1 (3.3%) had a complete response, 5 (16.7%) had a partial response, 9 (30.0%) had stable disease, and 15 (50.0%) had progressive disease, resulting in an objective response rate of 20.0%. The median PFS of the entire cohort was 88.0 (61.0-254.0) days, and the median OS was 362.0 (197.0-601.0) days. Patients could be divided into responders and non-responders, and these groups exhibited a significant difference in terms of survival time, T lymphocyte subsets, frequency of cell division cycle 27 (CDC27) mutation status, and CD15 and CD68 expression ($P<0.05$).

Conclusion: The combination of immunotherapy and radiotherapy is well tolerated and may provide clinical benefit for patients with recurrent WHO grade IV glioma. A prospective phase II study is needed to further validate the efficacy of our therapeutic regimen.

Keywords: malignant gliomas, immunotherapy, reirradiation, immunoadjuvant, immuno-oncology

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INTRODUCTION

World Health Organization (WHO) grade IV malignant glioma, including glioblastoma (GBM) with wild-type or mutant isocitrate dehydrogenase (IDH) and diffuse midline glioma (DMG) with H3K27M mutation, is the most common primary central nervous system tumor and confers a poor prognosis (1). The current treatment for patients with GBM involves maximal safe resection, radiotherapy, temozolomide (TMZ) based chemotherapy, and even the latest tumor treating field (TTF) therapy (2–4). Despite these multimodal approaches, the median survival of GBM is still less than 24 months and relapse after therapy is inevitable (2, 5). During recurrence, treatment options are less well defined and no interventions have shown encouraging efficacy (6). Hence, there is an urgent need for more effective therapies for recurrent WHO grade IV gliomas.

Immuno-oncology, which has prominently transformed the management of many cancers, has indicated that immunotherapy is the most promising treatment for grade IV gliomas (7–10). Since the discovery of central nervous system lymphatic vessels (11), immunotherapies, including immune checkpoint inhibitors (12), chimeric antigen receptor (CAR) T cell therapy (13), vaccines (14), and oncolytic virus (15), have been attempted to treat malignant gliomas. However, due to some challenging factors (16), such as the existence of intact blood-brain barrier (BBB) of tumor region, intratumor heterogeneity, and the unique immunosuppressive microenvironment, no significant benefit of these regimens has been observed in clinical practice (6, 17).

Recently, radiotherapy concurrent with immunotherapy has made great strides in the treatment of various tumors (18–20). It has been reported that radiotherapy in combination with immunotherapy can induce a synergistic effect *via* immunomodulation (18, 21). Radiotherapy can enhance the immunologic response to tumors by creating an *in situ* vaccine by eliciting antigen released from dying tumor cells (22, 23). In this study, to obtain more favorable antitumor activity, radiotherapy was delivered in conjunction with intracranial and systemic immunoadjuvants, a combination which has been shown to strengthen the efficacy of tumor antigen vaccination (24). Therefore, the present trial was designed to evaluate the safety and immunological efficacy of low-dose reirradiation in combination with polyinosinic:polycytidylic acid (poly I:C) and granulocyte-macrophage colony stimulating factor (GM-CSF) in adult patients with recurrent WHO grade IV glioma.

MATERIALS AND METHODS

Study Design and Participants

This study was a single-arm, open-label, phase I trial in patients with recurrent WHO grade IV glioma. Patients were enrolled in Beijing Tiantan Hospital, an affiliate of Capital Medical University, on the basis of the following inclusion criteria: aged 18-65 years, pathologically confirmed recurrent WHO grade IV glioma by resection or biopsy, amount of corticosteroids was no more than 2 mg/day, Karnofsky performance scale (KPS) score of 70 or higher, and adequate hematological, hepatic, renal, and coagulation function. Exclusion criteria included previous medical treatment for other malignancies, systemic inflammatory and immune system diseases, allergy to immunoadjuvants, and pregnancy or lactation.

Procedures

The study procedures are elaborated in detail in **Figure 1**. Patients received low-dose cyclophosphamide (CTX) intravenously 24 hours before immunoadjuvant treatment to eliminate regulatory T cells (25, 26). Then, intracranial and systemic adjuvants were successively administered. Intracranial immunoadjuvant was poly I:C, which was infused into a surgical cavity or ventricle with a dose of 1-2 mg per shot, qd, for a total of

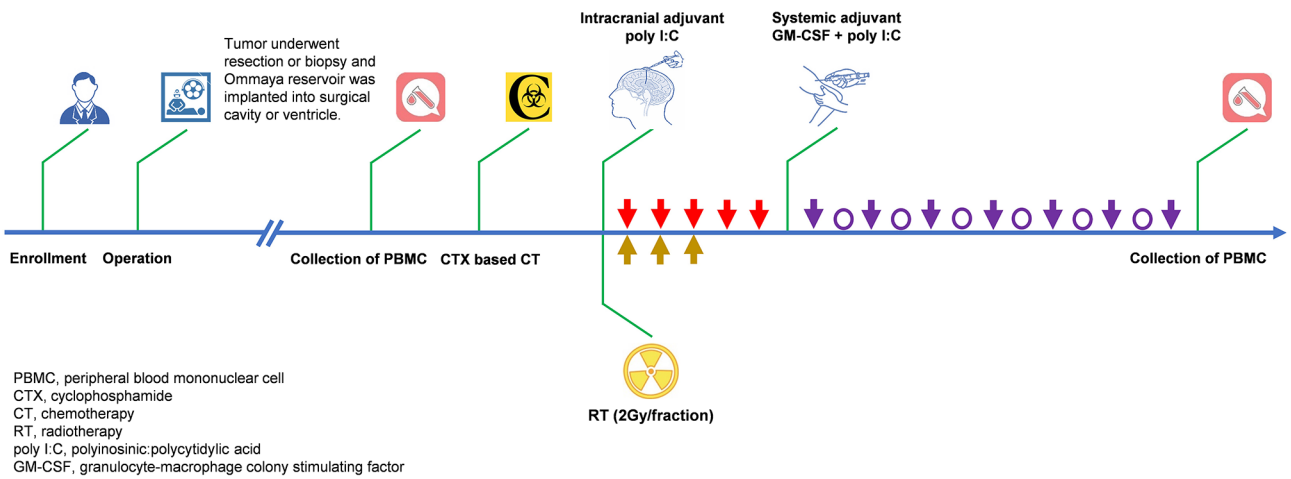

FIGURE 1 | Treatment scheme for patients enrolled in the present study.

5 shots, with the first three shots concomitant to radiation (2.0 Gy/fraction). Systemic immunoadjuvants consisted of poly I:C (50 μg/kg per shot, qod, 7 shots, intramuscular) and GM-CSF (125μg/m^2 per shot, qod, 7 shots, subcutaneous). This treatment continued until disease progression or onset of intolerable toxic effects. Patients could restart this treatment after the first evidence of progression, until confirmed by follow-up magnetic resonance imaging (MRI) within 12 weeks if there was evidence of clinical activity and adequate tolerability. Tumor assessments were performed with contrast-enhanced MRI at an interval of 8 weeks. Treatment response was defined as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) by the investigators based on immunotherapy response assessment in neuro-oncology (iRANO) criteria (27). Adverse events (AEs) were evaluated according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) (version 4.0). Immunological response was also assessed one day prior to CTX-based chemotherapy and one day post the last shot of systemic immunoadjuvant by flow cytometry assays on peripheral blood mononuclear cells (PBMCs).

Outcomes

The primary endpoint of this study was safety. Patients were monitored continuously for AEs at each clinic visit and AEs were graded according to CTCAE Version 4.0. Secondary endpoints included progression-free survival (PFS) and overall survival (OS). OS was defined as the time from the date of CTX-based chemotherapy to the date of death or last follow-up. PFS was defined as the time from the date of CTX-based chemotherapy to the date of progression or last follow-up.

Flow Cytometry Assay

Patient blood samples were collected by venipuncture. All peripheral blood samples (5 ml per subject) were collected in vacutainer tubes (BD Biosciences, San Jose, CA, USA) containing ethylenediaminetetraacetic acid (EDTA). For surface staining, 100 μL of heparinized peripheral blood was added to tubes containing 10 μL of mouse anti-human monoclonal antibodies (mAbs), including Peridinin Chlorophyll Protein Complex (PerCP)-conjugated anti-CD45, FITC-conjugated anti-CD3, APC-conjugated anti-CD4, PE-conjugated anti-CD8, and PE-conjugated anti-CD16+CD56, which were provided by ACEA Biosciences (San Diego, CA, USA). Isotype-matched mouse anti-human IgG antibodies served as negative controls for all fluorescein-conjugated IgG mAbs. Thereafter, the blood was mixed with the cocktail monoclonal antibody solution and incubated for 15 min at room temperature. Then, a lysing solution (OptiLyse C, Beckman Coulter) was added, and the mixture was incubated for another 15 min. To detect the percentage and absolute cell numbers of different subsets in peripheral blood, cells were collected and analyzed on a NovoCyte Flow Cytometer (ACEA Biosciences, San Diego, CA, USA) using NovoExpress Software (ACEA Biosciences, San Diego, CA, USA).

Whole Exome Sequencing (WES)

Tumor and matched blood samples from patients were collected. Then DNA was extracted using Qiagen DNeasy Blood & Tissue Kit (#69504), and quantified by means of Nanodrop ND-100 (Thermo Scientific, Waltham, MA) and Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). DNA was captured and amplified with Agilent Technologies SureSelect Human All Exon version 5 (Agilent Technologies, Santa Clara, CA, USA), followed by paired-end sequencing (2 × 125 cycles) on a HiSeq2500 platform (Illumina Inc., San Diego, CA, USA), according to the manufacturer's protocol. Raw image analysis and base calling were performed using the Illumina onboard RTA3 program with default parameters. After removing adapters and low-quality reads, the remaining reads were aligned to NCBI human genome reference assembly hg19 using the Burrows-Wheeler Aligner (BWA) tool and further processed using the Genome Analysis Toolkit (GATK, version 3.5), including the GATK Realigner Target Creator to identify regions that needed to be realigned. Single-nucleotide variants (SNVs), Indels, and copy number variation (CNV) were assessed using ANNOVAR, VarscanIndel, and CNVnator software, respectively (28). During mutation calling, the reads from the tumor sample were compared with those from the paired blood from the same patient to generate a list of somatic mutations. The called somatic mutations were then filtered and annotated using the Variant Effect Predictor (VEP) package (hg19 version) (29).

Imaging Mass Cytometry (IMC)

Metal-labeled antibodies were prepared according to the Fluidigm protocol. The antibody panel included lymphocyte types, cytokine expression, lymphocyte activation, vascular and spatial structure of cells from other tissues. Metal-conjugated primary antibodies were prepared with a Maxpar labeling kit (Fluidigm). The antibodies were diluted in antibody stabilization solution (Candor Bioscience GmbH, Wangen, Germany) for long-term storage at 4°C. Descriptions of the antibodies, isotope tags, clones, and concentrations used for staining are shown in **Table S1**.

Tumor samples were fixed in formalin and embedded in paraffin. Sections with a thickness of 5 μm were baked at 60°C for 2 hours, deparaffinized in xylene, and hydrated in a graded series ethanol (100%, 95%, 80%, 70%) for 5 min each. Next, 40 mL Antigen Retrieval Reagent-Basic (R&D Systems, diluted from 10× to 1×) was added to conical tubes, and the tubes were further incubated on a heating block (97°C) with loose lids. After immediate cooling to 60°C for 20 min, the sections were then blocked with 3% bovine serum albumin (BSA) for 45 min at room temperature. For staining, the sections were incubated overnight at 4°C with an antibody master mix. Samples were washed twice in 0.1% Triton X-100 in PBS for 8 min with slow agitation in Coplin jars. Sections were then stained with Intercalator-Ir (Fluidigm; cat. no. 201192A) in PBS for 30 min at room temperature. Slides were air dried and stored at 4°C for ablation.

According to hematoxylin-eosin staining, we selected the appropriate 500 × 500 μm location for laser-based cell ablation

and imaging. IMC images were acquired using a Hyperion Imaging System (Fluidigm). The largest square area was laser-ablated in a rastered pattern at 200 Hz, and preprocessing of the raw data was completed with commercial acquisition software (Fluidigm). IMC acquisition stability was monitored by interspersed acquisition of an isotope-containing polymer (Fluidigm). All successful image acquisitions were processed using MCDViewer, CellProfilor, and HistoCAT. R scripts were used to quantify cell number, generate t-distributed stochastic neighbor embedding (t-SNE) plots and perform neighborhood analysis.

Statistical Analysis

Patients who experienced CR, PR, or SD were regarded as responders, while those with PD were regarded as non-responders. Categorical comparisons between responders and non-responders were performed using the chi-square test or Fisher's exact test, as appropriate. Differences in age at diagnosis were evaluated by Student's t-test. The survival rate was estimated using the Kaplan-Meier method, and differences between subgroups were compared by the log-rank test. For the assessment of immunological response after receiving intracranial and systemic immunoadjuvants, paired t tests were used to compare the numbers of $CD4^+$ T, and $CD8^+$ T cells and natural killer (NK) cells before and after the immunoadjuvant treatments. All tests were two-sided, and a P value less than 0.05 was considered to indicate statistical significance. All analyses were performed with SPSS Statistics software for Windows version 26.0 (IBM Corporation, Armonk, NY, USA) and R software (https://cran.r-project.org).

RESULTS

Demographics and Clinical Characteristics

Between January 2018 and December 2019, thirty patients with a diagnosis of recurrent WHO grade IV glioma were enrolled in the present study. Of these patients, there were 21 males and 9 females, and the mean age was 43.0 ± 13.2 (range, 18-65) years. The baseline characteristics are shown in **Table 1**. Fourteen (46.7%) patients had tumors located in a single lobe. After chemoradiotherapy, 10 (33.3%) patients experienced local recurrence and 20 (66.7%) patients experienced distant recurrence (**Figure S1**). All diagnoses of tumor recurrence were confirmed by operation and histopathology, including 13 (43.3%) surgical resections and 17 (56.7%) biopsies. According to the 2016 WHO classification scheme, there were 19 (63.3%) IDH-wildtype GBM, 6 (20.0%) IDH-mutant GBM, and 5 (16.7%) H3K27M-mutant DMG. The frequencies of KPS scores of 70, 80, 90, and 100 during recurrence were 33.3%,

TABLE 1 | Comparison of clinicopathologic data of responders and non-responders.

Variable	Responder (n = 15)	Non-responder (n = 15)	P value
Age at diagnosis (years)	48.3 ± 10.6	37.8 ± 13.9	**0.028**
Gender (n, %)			0.427[#]
Male	12(80.0%)	9(60.0%)	
Female	3(20.0%)	6(40.0%)	
Tumor location			0.143
Single lobe	9(60.0%)	5(33.3%)	
Multiple lobes	6(40.0%)	10(66.7%)	
Previous chemoradiotherapy			
Yes	15(100.0%)	15(100.0%)	NA
No	0(0.0%)	0(0.0%)	
KPS score			0.672
70	4(26.7%)	6(40.0%)	
80	6(40.0%)	3(20.0%)	
90	3(20.0%)	4(26.7%)	
100	2(13.3%)	2(13.3%)	
Recurrence pattern			**0.020**
Local	8(53.3%)	2(13.3%)	
Distant	7(46.7%)	13(86.7%)	
Extent of resection			0.269
Resection	8(53.3%)	5(33.3%)	
Biopsy	7(46.7%)	10(66.7%)	
Pathology subtypes			0.632
IDH-wildtype GBM	9(60.0%)	10(66.7%)	
IDH-mutant GBM	4(26.7%)	2(13.3%)	
H3K27M-mutant DMG	2(13.3%)	3(20.0%)	
MGMT promoter			0.439
Methylated	6(40.0%)	4(26.7%)	
Unmethylated	9(60.0%)	11(73.3%)	

KPS, Karnofsky performance scale; DMG, diffuse midline glioma; IDH, isocitrate dehydrogenase; GBM, glioblastoma; MGMT, O^6-methylguanine-DNA-methyltransferase; NA, not applicable.

[#]Fisher exact test.

Bold values mean a p value less than 0.05.

30.0%, 23.3%, and 13.3%, respectively. All patients were available for the assessment of O^6-methylguanine-DNA-methyltransferase (MGMT), and 10 (33.3%) patients were identified to have a methylated MGMT promoter.

Safety

Patients who received at least one dose of immunoadjuvants and reirradiation were included in the analysis. The treatment-related AEs are summarized in **Table 2**. Overall, the treatment was safe and well tolerated. The most common AEs were flu-like symptoms including fever (66.7%), vomiting (33.3%), headache (30.0%), and fatigue (23.3%). Only a single patient experienced grade 3 fever possibly related to the immunoadjuvants. All these symptoms could be controlled with routine supporting therapies and symptomatic treatments. There were no grade 4 AEs or deaths attributable to this regimen. PD was the most common cause of treatment discontinuation.

Clinical Efficacy and Immunological Response

Among the 30 patients, 1 (3.3%, patient 25) experienced CR and 5 (16.7%) experienced PR, which contributed to an objective response rate (ORR) of 20.0%. Nine (30.0%) patients had SD for 40 days to 118 days after the first infusion of intracranial immunoadjuvant. Fifteen (50.0%) patients experienced PD with a median time to progression of 52.0 (95% confidence interval [CI]: 43.5-60.5) days. The time-on-study for all enrolled patients is shown in **Figures 2A, B**. Notably, patient 2 and patient 22 showed radiological responses that were categorized as PD because of the occurrence of new lesions (**Figures S2**, **S3**). At a median follow-up of 693.0 days, a total of 29 (96.7%) patients progressed, and 23 (76.7%) patients died. The median PFS of the entire cohort was 88.0 (95% CI: 61.0-254.0) days, and the median OS was 362.0 (95% CI: 197.0-601.0) days (**Figure 2C**).

On the basis of treatment response, patients who had CR, PR, or SD were defined as responders in this study, while those with PD were defined as non-responders (**Figure 3A**). The following subgroup analyses showed that both the PFS and OS of responders were significantly longer than those of non-responders (PFS: 266.0 *vs.* 52.0 days, $P<0.0001$; OS: 601.0 *vs.* 187.0 days, $P=0.008$) (**Figures 3B, C**).

Moreover, we further investigated alterations in immune cell subsets in patients who received immunoadjuvant therapy. Twenty-four patients had PBMCs from peripheral blood samples available for immunological analysis. In the subgroup of responders, the counts of $CD8^+$ T cells and NK cells were significantly increased after immunoadjuvant infusion ($P<0.05$). In contrast, the counts of $CD8^+$ T cells and NK cells unexpectedly decreased in the subgroup of non-responders ($P<0.05$), while no obvious correlation was observed between treatment response and $CD4^+$ T cell counts (**Figure 4**).

Factors Associated With Treatment Response

We considered the prominent survival benefit of responders achieved after receiving immunoadjuvants and reirradiation and compared the baseline characteristics between responders and non-responders to explore potential factors associated with patients' treatment response. The final results showed that responders had an older age at diagnosis (48.3 ± 10.6 *vs.* 37.8 ± 13.9 years, $P=0.028$) and a higher rate of local recurrence (53.3% *vs.* 13.3%, $P=0.020$) than non-responders (**Table 1**).

WES was performed on 13 patients, including 4 responders and 9 non-responders. We then compared the frequency of the mutations between responders and non-responders. Apart from that of the known molecular marker IDH1, we found that the status of cell division cycle 27 (CDC27), podocon (PODN), α-thalassemia/mental retardation syndrome X-linked (ATRX) and

TABLE 2 | Adverse events of patients enrolled in this study.

Variable	Grade 1	Grade 2	Grade 3	Grade 4
Hematologic toxicity				
Anemia	1(3.3%)	0(0.0%)	0(0.0%)	0(0.0%)
Thrombocytopenia	1(3.3%)	0(0.0%)	0(0.0%)	0(0.0%)
Neutropenia	2(6.7%)	1(3.3%)	0(0.0%)	0(0.0%)
Lymphopenia	1(3.3%)	0(0.0%)	0(0.0%)	0(0.0%)
Nervous system disorder				
Hypersomnia	0(0.0%)	2(6.7%%)	0(0.0%)	0(0.0%)
Seizure	1(3.3%)	1(3.3%)	0(0.0%)	0(0.0%)
Headache	6(20.0%)	3(10.0%)	0(0.0%)	0(0.0%)
Gastrointestinal disorder				
Nausea	2(6.7%)	3(10.0%)	0(0.0%)	0(0.0%)
Vomiting	8(26.7%)	2(6.7%)	0(0.0%)	0(0.0%)
Diarrhea	0(0.0%)	1(3.3%)	0(0.0%)	0(0.0%)
General disorder				
Fever	14(46.7%)	5(16.7%)	1(3.3%)	0(0.0%)
Chills	3(10.0%)	0(0.0%)	0(0.0%)	0(0.0%)
Fatigue	3(10.0%)	4(13.3%)	0(0.0%)	0(0.0%)
Others				
Arthralgia	1(3.3%)	0(0.0%)	0(0.0%)	0(0.0%)
Rash maculo-papular	0(0.0%)	3(10.0%)	0(0.0%)	0(0.0%)

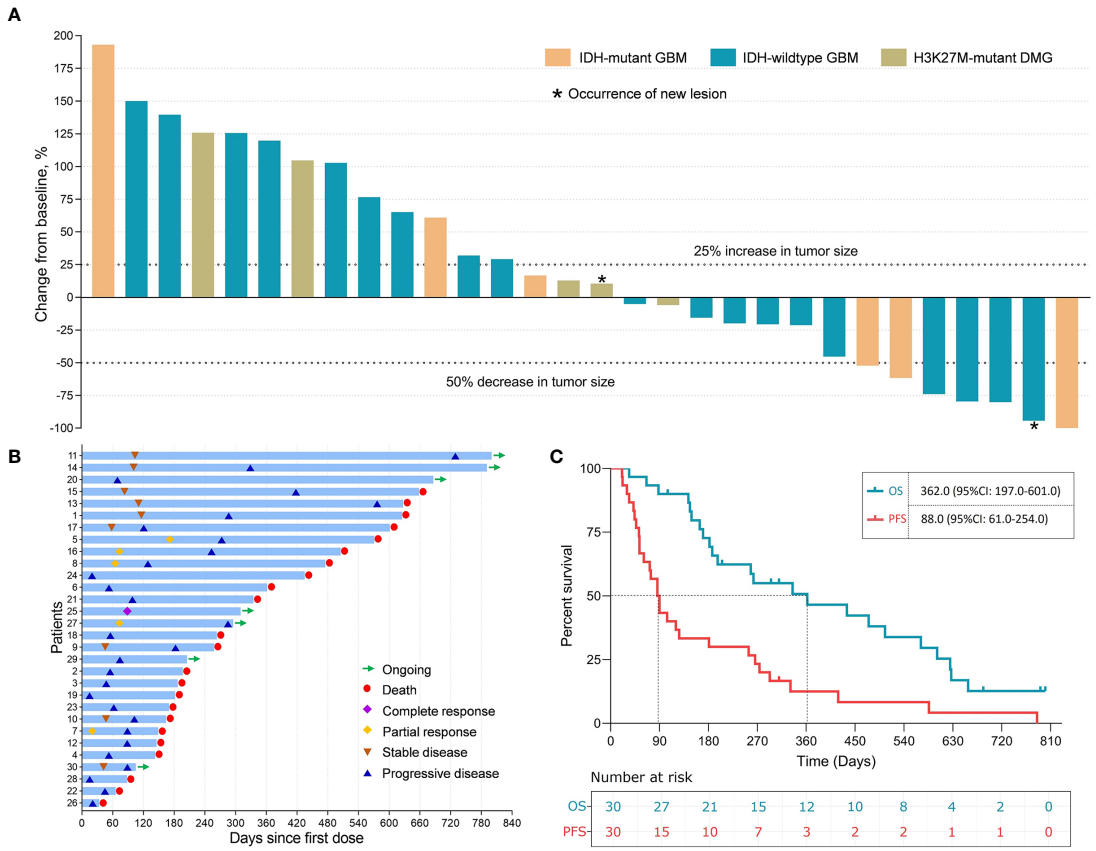

FIGURE 2 | The clinical efficacy of immunoadjuvant treatment and reirradiation in patients with recurrent WHO grade IV gliomas. **(A)** Waterfall plot showing the best tumor response in patients treated with immunoadjuvant therapy and reirradiation. **(B)** Swimmer plot showing disease status and survival time in 30 patients treated with immunoadjuvant therapy and reirradiation. **(C)** The median progression-free survival and overall survival of patients treated with immunoadjuvant therapy and reirradiation.

ryanodine receptor type 1 (RYR1) was significantly different between the two subgroups (P<0.05) (**Figure 5A**). In particular, all four patients with CDC27 mutations responded, and the others without this mutation were confirmed as non-responders, reminding us of the significance of CDC27 in predicting the treatment response to immunotherapy. We also compared the frequency of CNVs and cytobands, and found several potential biomarkers, including carbohydrate sulfotransferase 7 (CHST7), 15q21.3 and 15q22.2, which were associated with treatment response (P<0.05) (**Figure 5A**).

Ten patients, including 5 responders and 5 non-responders, were successfully assessed with IMC. We identified 43071 single cells and quantified the expression of marker genes of each cell. Clustering with PhenoGraph identified 29 diverse cell phenotypes (**Figures 5B–D**). According to the comparison of cell clusters between responders and non-responders, we found that the percentage of $CD15^+$ and $CD68^+$ cells in the subgroup of non-responders was higher than that in the subgroup of responders (P<0.001, **Figure 5E**). These data suggest that $CD15^+$ or $CD68^+$ cells may play an important role in the tumor immune microenvironment of patients who receive immunotherapy.

Survival Analysis

We then conducted univariate and multivariate survival analyses to better understand factors associated with patient prognosis. The univariate analysis confirmed treatment response (P<0.0001), age at diagnosis (P=0.007), and recurrence pattern (P=0.002) as prognostic factors for PFS, while treatment response (P=0.008), KPS score (P=0.033), and recurrence pattern (P=0.035) were confirmed as prognostic factors for OS (**Figures 3** and **S4**). The extent of resection showed potential for predicting survival, but it did not reach statistical significance (P=0.058 for PFS and P=0.051 for OS) (**Figure S4**). In the included Cox proportional hazard model, which shows all these prognostic factors screened by univariate analysis, treatment response was identified as an independent prognostic factor. The adjusted hazard ratio (HR) was 0.022 (95% CI: 0.004-0.126, P<0.001) for PFS and 0.323 (95% CI: 0.132-0.785, P=0.013) for OS.

DISCUSSION

Developments in the field of immunotherapy have recently provided new options for patients with GBM, especially when

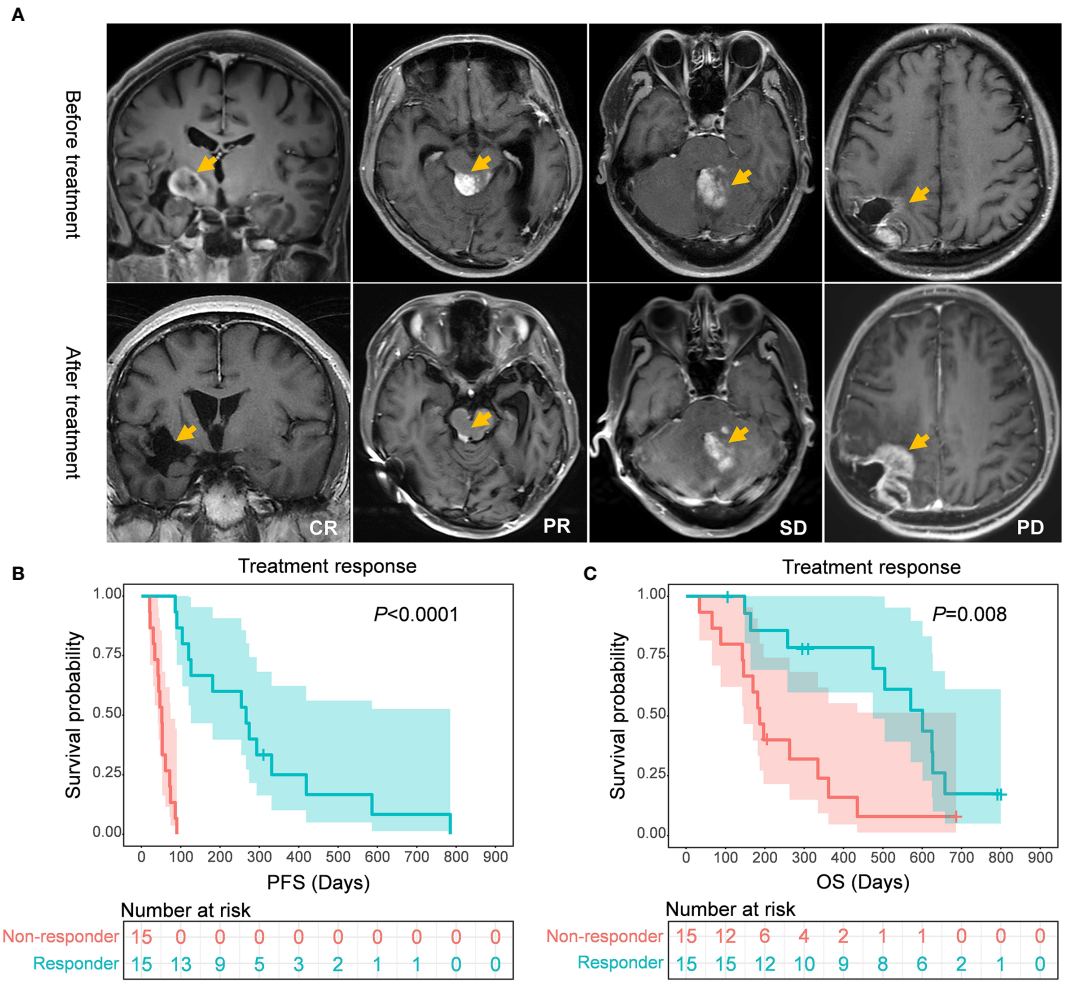

FIGURE 3 | **(A)** Representative MR images of patients with different treatment responses. **(B, C)** Comparisons of survival rates between responders and non-responders. Responders showed a significantly longer progression-free survival ($P < 0.0001$) and overall survival ($P=0.008$) than non-responders.

tumors progress after conventional treatments (30). However, to date, all phase III clinical trials of immunotherapy against GBM have reported unsuccessful results, largely blamed on tumor heterogeneity and an immunosuppressive microenvironment (6, 16). Previous studies suggested that radiation could prime the immune system to enhance the effciency of immunotherapy and that a combination of radiation with immunotherapy is more effective than monotherapy (18, 21, 23). In the present study, we evaluated the safety and efficacy of reirradiation plus immunoadjuvants in adult patients with recurrent WHO grade IV glioma and found that it was well tolerated and seemed to be effective. Patients who received this protocol achieved a median PFS of approximately three months and a median OS of approximately one year without any severe treatment-related adverse events, which appeared to be no significant survival advantage over previous salvage therapies (31). But of note, the median PFS and OS of responders was 266 and 601 days, respectively, which has been remarkably prolonged.

To our knowledge, this is the first study based on the combination of reirradiation and intracranial and systemic immunoadjuvants for recurrent malignant gliomas. It is believed that radiation can, to a certain extent, kill tumor cells and consequently result in the release of tumor neoantigens (18, 22). These antigens serve as "*in situ* vaccines" that can be recognized by the immune system and stimulate the infiltration of T cells. Reynders et al. systemically reviewed a rare clinical event: the abscopal effect, which was induced by radiation (23). The abscopal effect was defined as a phenomenon of tumor regression at nonirradiated, distant tumor sites (18, 23). In our study, we found that three (10.0%) patients had undergone the abscopal effect. For example, the tumors in the septum pellucidum, brainstem, and left temporal lobe of patient 2 simultaneously regressed when radiation was performed in the field of the septum pellucidum (**Figure S2**).

It should be noted that the success of immunotherapy depends on two simultaneous prerequisites: the availability of

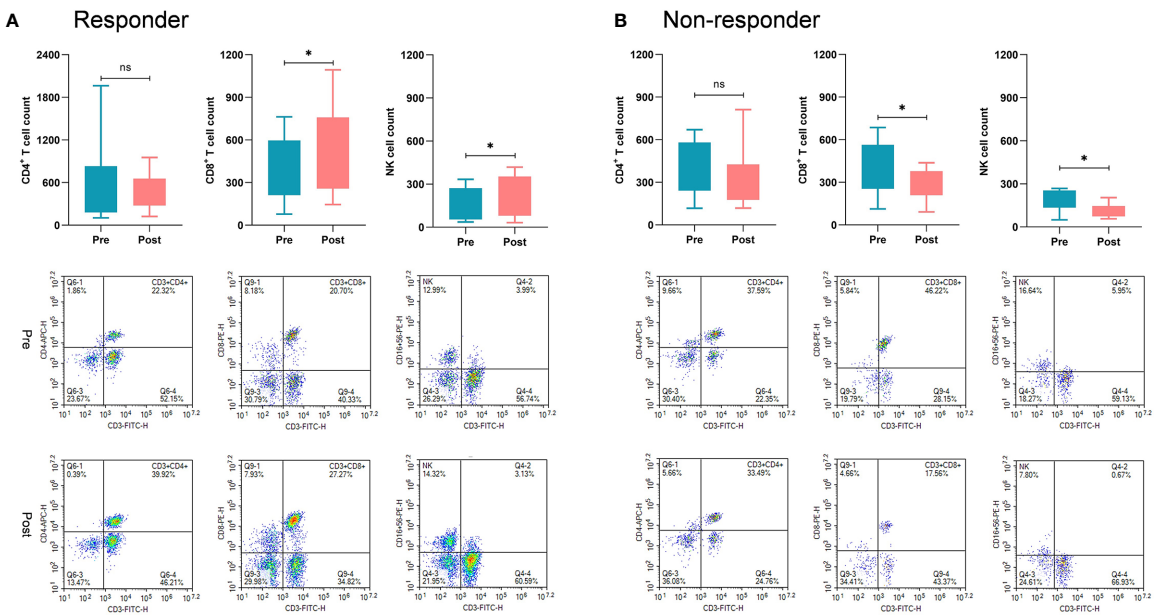

FIGURE 4 | Comparisons of immunological response between responders and non-responders. **(A)** In the subgroup of responders, the counts of $CD8^+$ T cells and NK cells were significantly increased after receiving immunoadjuvant infusion ($P < 0.05$). **(B)** In the subgroup of non-responders, the counts of $CD8^+$ T cells and NK cells were markedly decreased after receiving immunoadjuvant infusion ($P < 0.05$). *$p < 0.05$; ns, not significant.

identifiable tumor antigens and adequate infiltration of effector T cells (32). Therefore, we introduced the application of intracranial and systemic immunoadjuvants concurrent with radiation. Poly I:C and GM-CSF are considered immunoadjuvants with high potential to boost immunological activity (14, 33), and have been widely used in the treatment of many tumors, including GBM (14, 34, 35). Traditionally, immunoadjuvants are given *via* systemic administration, such as intramuscular administration, which may only induce limited immunoactivity due to the existence of the BBB (36, 37). Thus, poly I:C was directly infused into the surgical cavity or ventricle in our study to achieve a maximal immunological response. Poly I:C is known to be a toll-like receptor 3 (TLR-3) agonist, which can facilitate maturation of dendritic cells (38). It has been reported poly I:C can prolong the survival of CD4+ T cells and enhance the proliferation of activated T cells, and that it is involved in the reactivation of tumor-infiltrating CD8+ T cells (39). Our results showed that poly I:C enhanced higher activation of $CD8^+$ T and NK cells, but a less extent of $CD4^+$ T cells in responders, which led to a more favorable $CD8^+$:$CD4^+$ T cell ratio. Notably, the increased counts of $CD8^+$ T and NK cells were positively correlated with patient survival, which was in accordance with previous findings (40). In contrast, the counts of $CD8^+$ T and NK cells in non-responders were decreased even after infusion of immunoadjuvants, which implied that the immune cells had already been exhausted thereby indicating a poor outcome in these patients.

With respect to the safety profile for immunoadjuvants, we found that the most common AEs were fever, vomiting, headache, and fatigue. Fortunately, no severe AEs have been observed, consistent with the results reported in other clinical trials (36, 37). All these data suggested that our regimen was safe and well-tolerated. Aside from the safety and efficacy of this regimen, identifying the subgroup of patients who would obtain clinical benefits is also of great significance. Therefore, in this study, we systematically explored the characteristics of responders and non-responders. The final results demonstrated that the responders had an older age at diagnosis and a lower rate of distant recurrence. Generally, older patients tended to show a relatively worse treatment response than younger patients because of decreased immune system effectiveness (41, 42). In this study, non-responders seemed to be more common in the younger patient group, which could be partly explained by the different frequencies of H3K27M-mutant DMG between non-responders and responders (20.0% *vs.* 13.3%). As we all know, H3K27M-mutant DMG is a malignancy predominately found in children and young adults and is concurrent with a poor immune response (43). Distant recurrence is regarded as a sign of late stage disease in patients whose immune systems have declined and tumoral immune escape has enhanced (44). Hence, patients with local recurrence are more likely to exhibit a favorable immune response.

In addition, our results showed that CDC27, PODN, ATRX and RYR1 status was significantly different between responders and non-responders. In particular, CDC27, a gene correlated with tumor progression and programmed death ligand-1 expression (45), was mutated in all responders and wild-type in all non-responders, which indicated great significance in predicting the treatment response of immunotherapy. We also found that CHST7, 15q21.3 and 15q22.2 could serve as potential

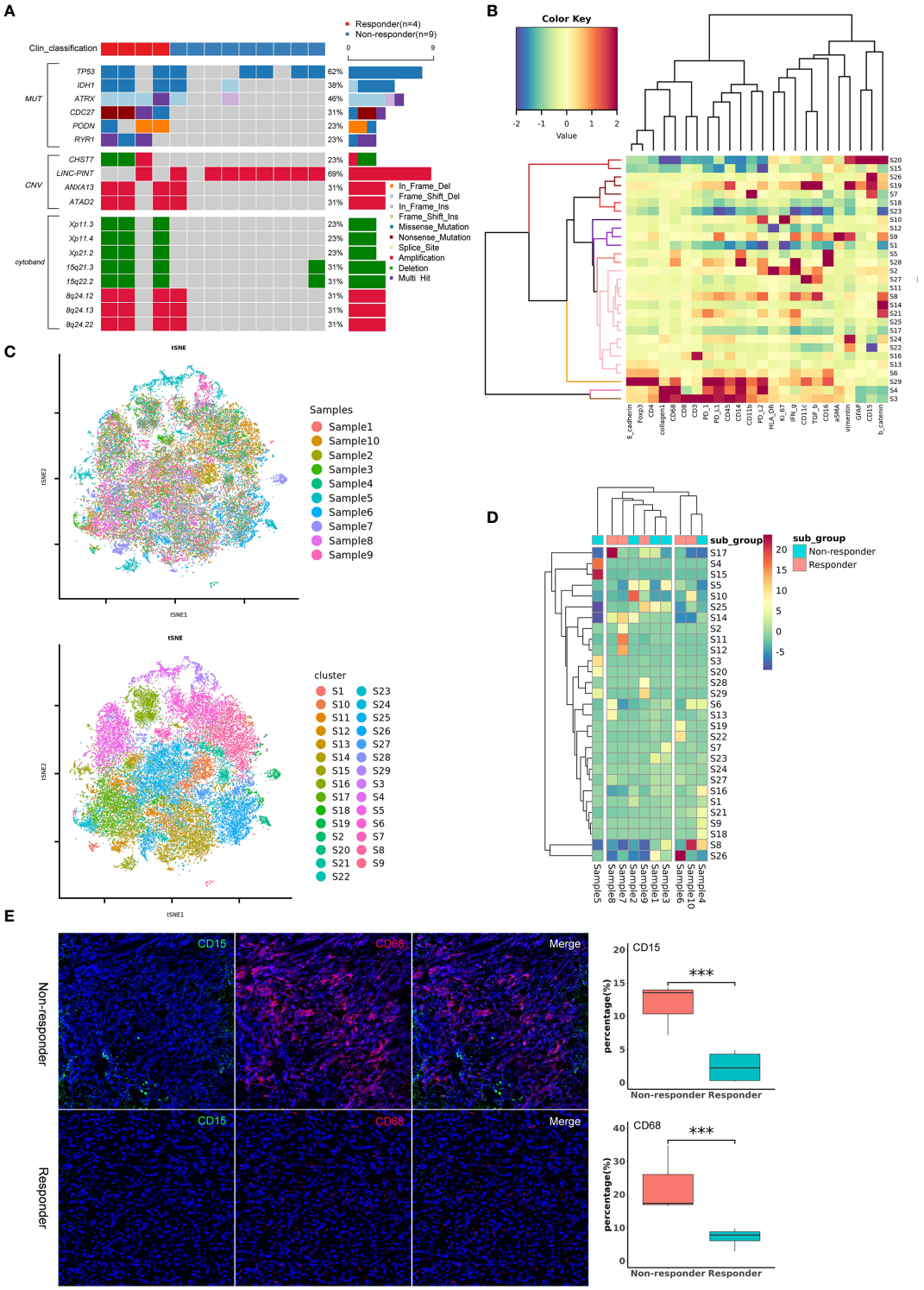

FIGURE 5 | **(A)** Oncoplot of significantly different mutations, CNVs and cytobands between the responder and non-responder subgroups. **(B)** Heatmap showing the z-scored mean marker expression of the panel markers for each PhenoGraph cluster. Clusters and markers are grouped by expression profiles. **(C)** t-SNE plots of 43071 subsampled single cells from each PhenoGraph cluster identified in the heatmap image. Cells are colored by samples and clusters. **(D)** Heatmap showing the z-score of the mean percentage of single-cell clusters in each sample. Clusters and patients are grouped by the densities of single-cell clusters. **(E)** Imaging mass cytometry analysis of the tumor immune microenvironment between responders and non-responders. The non-responders showed higher percentages of $CD15^+$ (green) and $CD68^+$ (red) cells than the responders ($P < 0.001$). ***p value < 0.001

biomarkers predicted for treatment response. Furthermore, by analyzing the features of the tumor immune microenvironment of patients, we found that the percentages of $CD15^+$ and $CD68^+$ cells in non-responders were higher than those in responders. It has been reported that both CD15 and CD68 are immunosuppressive markers that play an important role in suppressing T-cell-mediated immunity (46, 47). CD68 is a major biomarker for the quantification of tumor-associated macrophages (TAMs) (48). As we all know, TAMs are a well-recognized core element of tumor microenvironment (TME) and generally characterized as M2-like macrophages which are associated with tumor progression and poor prognosis (49, 50). This is the reason that high tumor CD15 and CD68 expression indicates a limited response and poor survival. Therefore, all these biomarkers might be exploited as potential therapeutic targets for malignant gliomas in the future.

Limitations do exist in our study. First, it is a study from a single institution, which to some extent decreases the stability of the conclusion. Second, the potential molecular mechanism of patients who responded to this treatment protocol has yet to be clarified. We collected the cerebral spinal fluid of patients before, during, and after treatment and made some interesting observations. We believe the mechanism can be elucidated in the near future. Third, the amount of Tregs has not been detected in our research, which was important in assessing the effect of CTX in ablating Tregs. Finally, we should continue this study until the last patient has reached the endpoint because there are still 7 patients alive at the current stage.

CONCLUSIONS

In summary, this trial demonstrated that the combination of immunotherapy and radiotherapy was well tolerated. Low-dose reirradiation plus intracranial and systemic immunoadjuvants has shown promising immunological responses and clinical benefits in patients with recurrent WHO grade IV gliomas. These data support a larger phase II study of this regimen in patients with recurrent GBM, in which feasibility will be assessed in multicenter settings and efficacy will be evaluated in comparison with that of controls.

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 Institutional Review Board of Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Acquisition of data: HJ, ML, CY, and XZ. Analysis and interpretation of data: HJ, KY, YC, and XR. Statistical analysis: HJ and KY. Drafting of the article: HJ and SL. Funding acquisition: SL and YC. Conception and design: YC and SL and study supervision: SL. 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.2021.632547/full#supplementary-material

Supplementary Figure 1 | Representative images of patients with local and distant recurrence.

Supplementary Figure 2 | The pre- and posttreatment MR images of patient 2. The patient experienced both local **(A)** and distant **(B, C)** recurrence. After one cycle of treatment, the lesion in the left temporal lobe **(E)** and metastases in the septum pellucidum **(F)** and brainstem **(G)** were in remission. But a new lesion was found in the left cerebellum **(D, H)**. Therefore, the treatment response of this patient was defined as PD.

Supplementary Figure 3 | The pre- and posttreatment MR images of patient 22. The patient had a recurrent lesion in the midbrain **(A)**. After one cycle of treatment, the lesion disappeared **(C)**. However, new lesions occurred in the bilateral thalamus and basal ganglia **(B, D)**. Therefore, the treatment response of this patient was defined as PD.

Supplementary Figure 4 | Univariate survival analyses of the prognostic factors.

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CASE REPORT
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Case Report: Combined Intra-Lesional IL-2 and Topical Imiquimod Safely and Effectively Clears Multi-Focal, High Grade Cutaneous Squamous Cell Cancer in a Combined Liver and Kidney Transplant Patient

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Cutaneous squamous cell carcinoma (cSCC) is the second most common non-melanoma skin cancer worldwide, with ever increasing incidence and mortality. While most patients can be treated successfully with surgical excision, cryotherapy, or radiation therapy, there exist a subset of patients with aggressive cSCC who lack adequate therapies. Among these patients are solid organ transplant recipients who due to their immunosuppression, develop cSCC at a dramatically increased rate compared to the normal population. The enhanced ability of the tumor to effectively undergo immune escape in these patients leads to more aggressive tumors with a propensity to recur and metastasize. Herein, we present a case of aggressive, multi-focal cSCC in a double organ transplant recipient to frame our discussion and current understanding of the immunobiology of cSCC. We consider factors that contribute to the significantly increased incidence of cSCC in the context of immunosuppression in this patient population. Finally, we briefly review current literature describing experience with localized therapies for cSCC and present a strong argument and rationale for consideration of an IL-2 based intra-lesional treatment strategy for cSCC, particularly in this immunosuppressed patient population.

Keywords: intralesional immunotherapy, intralesional IL-2, organ transplant recipient, imiquimod, cutaneous squamous cell carcinoma (cSCC), intralesional, interleukin-2 (IL2), aldara

INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is the second most common non-melanoma skin cancer worldwide (1, 2), and its incidence is steadily increasing yearly (3). While the mortality rates for almost all other forms of cancer decline, the age-standardized mortality rate of cSCC continues to rise. Despite being vastly outnumbered in incidence by basal cell carcinoma (BCC) (4), cSCC is associated with a significantly higher mortality (5). Indeed, in the United States alone, mortality figures for cSCC are now comparable to those of melanoma; a less common disease which is far more lethal (5, 6). Of the nearly 1 million new cases of cSCC each year, there are an estimated 15,000 deaths, compared to 9730 in the case of melanoma (7, 8).

Initial treatment strategies for cSCC include electrodessication and curettage, surgical excision, cryotherapy, or radiation treatment (9). Surgical excision is considered the standard treatment of cSCC, and is able to cure 90% of cSCC cases with a 5-year recurrence rate of 8% and 5-year metastasis rate of 5% (9). Surgery, however, is not always possible, as on occasion, a cSCC is unresectable or is confined to cosmetically or functionally sensitive area. In addition to inoperable cSCCs, a small percentage of cSCCs are aggressive and refractory to standard dermatologic therapies (5, 10, 11). This subset of cSCC, known as aggressive (or high-risk) SCC, has a substantially higher rate of metastasis and associated morbidity and mortality (12, 13). Features consistent with aggressive cSCC include tumor size ≥ 2cm, evidence of perineural invasion, bone invasion or erosion and invasion beyond subcutaneous fat (14). It is noteworthy that high grade histology is also still considered a feature of aggressive disease in the BWH cSCC classification system but no longer in the AJCC 8th edition of cSCC classification (15). High risk cSCC in the context of a history of local recurrence or immunosuppression predicts a significantly higher risk of disease recurrence, metastasis and disease specific mortality.

Herein, we describe the novel utilization of two immunomodulatory local therapies, intralesional IL-2 and topical 5% imiquimod, used in conjunction to achieve complete remission in a double solid order transplant recipient with no evidence of inducing immune-mediated organ rejection.

Case Description

A Case of High Grade, Multi Focal, Rapidly Progressing Cutaneous Squamous Cell Carcinoma in a Double Solid Organ Transplant Recipient

Our patient, a 72-year-old male with a past medical history of polycystic kidney disease, received a liver and kidney transplant from a single donor in January 2006; however, this first kidney immediately failed. He subsequently received a second living-related (i.e. from a living family member) donor kidney in January 2008. His initial anti-rejection medications included tacrolimus (4 mg daily), mycophenolate (2 g daily) and prednisone (10 mg daily). From a transplantation perspective, he tolerated this regimen well. In 2009, however, he developed a small area of cSCC just above his right eyebrow. This was locally excised with clear margins. Cutaneous squamous cell cancer recurred at the same site over the right eye in 2014 and again in 2015. His second recurrence was characterized as well-differentiated cSCC, resected with clear margins but associated with perineural invasion. He received targeted radiation of 5000cg to the surgical site. In Aug 2017, cSCC recurred in the skin along the supraorbital rim, again with perineural invasion. Due to the extent of this recurrence, he received a radical excision with right eye enucleation and radial forearm skin graft to repair the resulting facial skin defect. All margins were reported negative. By this point, the patient's immunosuppression medications had been modified and included sirolimus (1 mg oral daily), tacrolimus (1 mg oral daily), and prednisone (5 mg oral daily). Following his surgery, the tacrolimus dose was reduced (to 0.5mg every two days), and the sirolimus dose increased (to 2 mg oral daily) in hopes of reducing the risk of developing further cSCC. However, he went on to develop a small cSCC on the right side of the nose; treated with excision and local radiation. He subsequently developed two new lesions in April and May of 2019, both excised with narrow margins. In January 2020 he developed a further two new lesions, one in the skin overlying the right zygoma and a second at the margin of the radial forearm skin graft on the right cheek. Margins were now involved. The area was treated with targeted radiation, taking care to avoid significant radiation field overlap from previous treatments. In February 2020 five new lesions were identified (**Figure 1A**). Pathology now demonstrated poorly differentiated cSCC with lymphovascular invasion and positive margins (**Figure 1B**). At least one of the lesions was felt to be metastatic. Consultation was made for consideration of systemic immunotherapy; however, being a liver and kidney transplant recipient, it was felt that systemic immunosuppression would confer significant risk to failure of both transplants. Intra-lesional immunotherapy was offered as a potentially safer experimental alternative. After careful consideration of the options and associated potential risks and benefits, weekly injections of intra-lesional IL-2 (8M IU total dose per session, divided among multiple lesions and sites of injection) was initiated. Initially, he experienced partial regression in some lesions but clinical progression in others, with developmental of a new submandibular nodule deep to the skin. This subcutaneous nodule was treated intra-lesional injections of IL-2 (bringing the total dose administered to 10M IU per session). Additionally, at this time imiquimod (a topical TLR-7 agonist) was added to all facial lesions, administered as a thin film once daily for five out of seven days per week, by the patient. This was done in the hopes of augmenting an IL-2-mediated anti-tumor immune response. Over the course of the following six weeks, all facial lesions completely clinically responded. In addition, the subcutaneous, submandibular nodule had significantly diminished in size (**Figure 2A**). For a number of reasons, including patient-reported severe pain with injections particularly at the site of the submandibular lesion, ongoing cost of medications, and costs associated with travelling back and forth for weekly treatment, we elected to proceed with excision of the residual submandibular lesion; at the same time, a representative biopsy of the right facial skin was taken as well. The submandibular lesion

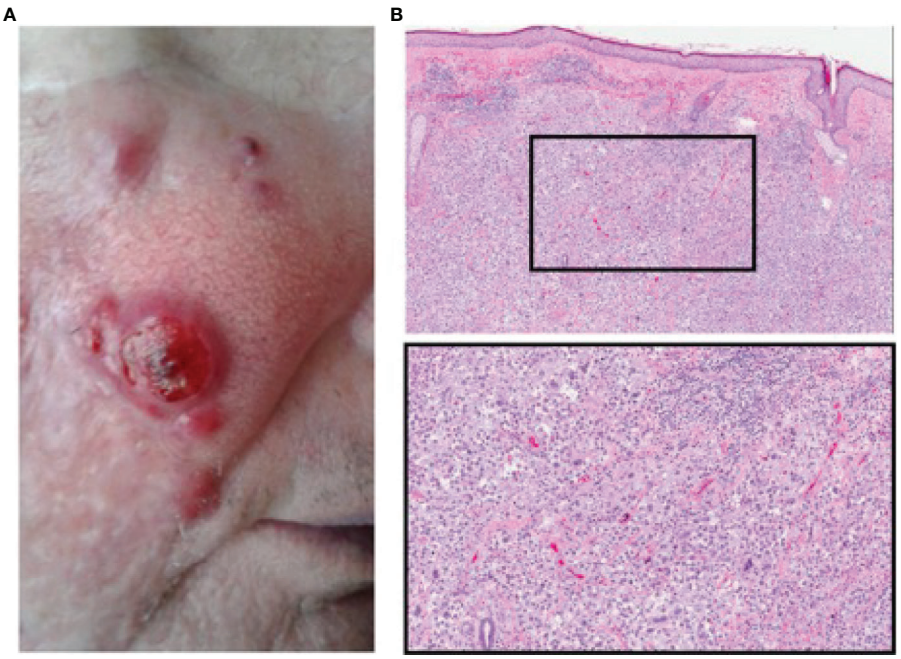

FIGURE 1 | Pre-treatment recurrence of facial cSCC. **(A)** Extent of disease on the right cheek prior to starting treatment with intra-lesional IL-2. **(B)** Histological profile of facial lesions prior to starting intra-lesional IL2 (top 40X, bottom 100X magnification), showing poorly differentiated cSCC.

demonstrated residual high-grade cSCC with necrosis and a pronounced lymphocytic infiltrate but no evidence of nodal tissue (**Figure 2C**). Histological analysis of the right cheek skin revealed no evidence of any residual cSCC (**Figure 2D**). Margins were clear and no lymphovascular invasion or perineural invasion was identified. The area remains disease free 3 months post-treatment (**Figure 2B**). During the course of treatment, liver and kidney function was closely monitored and unaffected by the localized treatment strategy. Overall, he experienced no decline in either (kidney or liver) graft function and had no signs of rejection.

DISCUSSION

The Immunopathological Basis of cSCC in Solid Organ Transplant Recipients

The immune system plays a vital role in the pathogenesis and progression of cSCC (16). Indeed, the major risk factors for cSCC development include genetically defined skin type, chronic UV exposure, chronic skin damage, and immunosuppression (17–21). The impact of immunosuppression on cSCC development has been studied most thoroughly in the context of solid organ transplant recipients. While immunosuppression is necessary to prevent transplant rejections, lifelong use of these agents has been shown to promote carcinogenesis, with cSCC being one of the most common in these patients (22).

The prominence of cSCC development in patients with iatrogenic immunosuppression strongly suggests that cSCC may have an inherent ability – that other cancers lack – to circumvent cancer immune surveillance. It has been shown in a large series of renal transplant patients that immunosuppression increases cSCC formation up to 250-fold in comparison to immunocompetent patients (23–25). The degree of immunosuppression may correspond to cSCC incidence, where reduction of immunosuppression reduces the total number, and rate of formation of cSCC (26). Numerous immunosuppressive drugs have been linked to cSCC development, namely calcineurin inhibitors (26, 27), glucocorticoids (28–30), and biologics (infliximab (31, 32), etanercept (32–34), adalimumab (32)). Furthermore, when solid organ transplant recipients do develop cSCC, their tumors tend to be more aggressive and carry a higher risk for metastasis (35). Indeed, iatrogenic immunosuppression *via* calcineurin inhibitors inhibit Langerhan's cells (36, 37), dermal dendritic cells (38, 39), and T-cell signaling and proliferation, and cyclosporine directly promotes tumor development (40–42). Calcineurin inhibitors effectively disrupt IL-2 production, and as such are able to dampen immune response to allogenic antigens – a desired effect when trying to persevere tolerance towards solid organ transplants; however, this iatrogenic immunosuppression comes at a price, and significantly impairs cancer immunosurveillance (23–27). Unlike calcineurin inhibitors, the mammalian target of rapamycin (mTOR) inhibitors block IL-2 induced signal transduction, and does not abrogate IL-2 production completely, thereby allowing some functions of IL-2 to remain intact (43). As such, recently mTOR inhibitors, which include sirolimus (rapamycin), temsirolimus, and everolimus, have garnered favour because they have a lower association with *de novo* skin malignancies, and may in-and-of themselves have a direct anti-tumor effect. In retrospective analyses

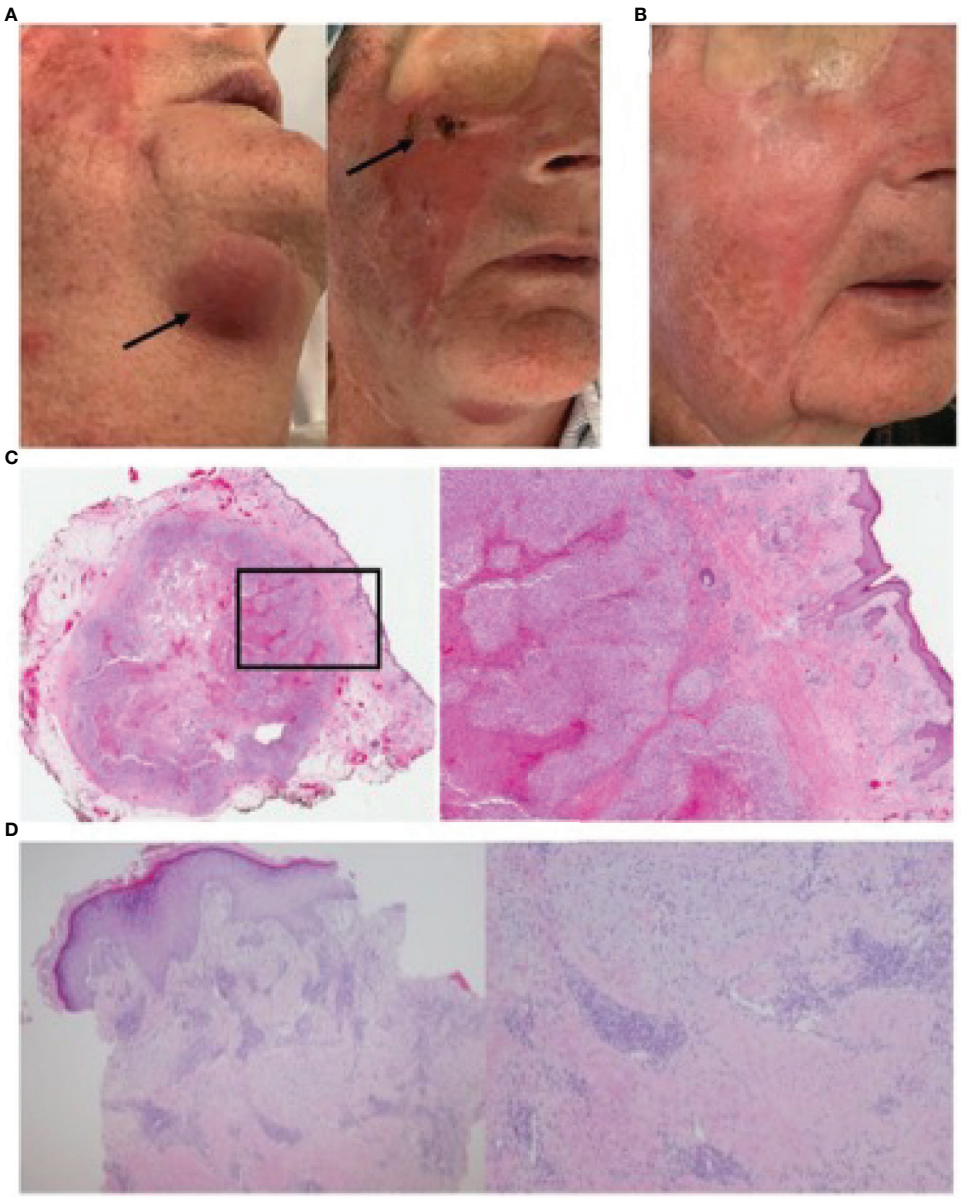

FIGURE 2 | Post-treatment course with intra-lesional IL2 and imiquimod. **(A)** Complete response of facial lesions with resolving submandibular nodule. Arrows identify nodule and infraorbital area of healing (biopsied) skin. **(B)** Sustained complete resolution of facial cSCC three months following completion of treatment. **(C)** (left) Histological profile of excised submandibular nodule low magnification; (right) 20X magnification reveals residual high-grade cSCC with necrosis, a pronounced lymphocytic infiltrate, with no evidence of nodal tissue **(D)** (left) 40X magnification of biopsied, healing infraorbital skin showing ulceration and complete clearance of cSCC; (right) 100X magnification.

renal transplant recipients who received either sirolimus or everolimus without cyclosporine had a reduced number of *de novo* skin malignancies (44), and some patients experienced regression of skin cancers such as Kaposi's sarcoma (KS) and SCC that were present prior to initiation of mTOR therapy (45–49). For these reasons, in our high-risk patient presented above, recurrent cSCC was the major factor in deciding to switch him from tacrolimus to sirolimus early on. However, he unfortunately continued to present with recurrent cSCC.

The substantially higher incidence of cSCC in immunosuppressed patients underscores the impact of the immune system in cSCC susceptibility and pathogenesis. Indeed, variations in immunological makeup may influence the ability of human hosts to recruit adaptive immune responses needed to prevent cSCC development (50). Class I and class II HLA genes encode major histocompatibility complex (MHC) proteins which allow for presentation of antigenic peptides such as tumor antigens to CD8+ and CD4+ T-cell lymphocytes,

respectively. Variations in MHC proteins have been implicated in multiple cancers by influencing host defenses against tumorigenesis (50). Aberrant expression of both class I and class II HLA proteins on the surface of cSCC cancer cells is reported in both immunocompetent and immunosuppressed patients (51–55). Abnormalities in class I and class II HLA proteins are well documented in cSCC cells, reinforcing the notion that cSCC pathogenesis is inherently connected to faulty immune regulation. Several clinical studies have indicated that specific class I HLA germlines may predispose the development of cSCC in immunosuppressed patients (56–58). It has also been proposed that aberrant expression of class II HLA proteins on cSCC cancer cells may facilitate tumor escape from host defense mechanisms, as seen in other cancers including HNSCC and acute myeloid leukemia (59, 60). Furthermore, cSCC has the ability to downregulate the presentation of highly immunogenic neoantigens to TCRs (61). Thus, an important facet in the mechanism of cSCC immune escape is HLA and neoantigen dysregulation; targeting mechanisms that improve tumor-associated antigen presentation may thus be useful in the immunotherapy of cSCC.

Another avenue through which cSCC mediates immune escape is through local cytokine dysregulation. For example, cSCC significantly downregulates CCL27, a chemokine that promotes T cell homing to skin, throughout its progression from AK to malignant cSCC (62). cSCC tumors that tend to be deeper and more advanced also significantly upregulate CXCR7, which signals through CXCL12 to promote ERK signaling, thus prolonging tumor cell survival (63). Cytokine profiling of tumors reveals that in the progression to malignant cSCC, precancerous lesions dramatically upregulate production of IL-6 (64), a proinflammatory cytokine that has previously been shown to augment cSCC growth through modulation of pro-tumorigenic cytokines and angiogenic factors (65). Therefore, therapies that modulate the local cytokine and chemokine profile of cSCC may be of benefit.

Systemic Treatment of cSCC

Aside from surgical methods, there is a paucity of treatment modalities for aggressive cSCC. Systemic therapies for cSCC have shown limited success, although rigorous assessment of systemic therapies has been limited (66). To date, a number of systemic therapies have been used to treat cSCC, including: chemotherapeutics (cisplatin (67–69), 5-fluorouracil [5-FU] (67, 68, 70, 71), bleomycin (67), and doxorubicin (69)), 13-cis-retinoic acid (13cRA (72)), immunotherapies (interferon-α2a [IFN-α] (72)), gefitinib (73) and cetuximab (74) (agents targeting epidermal growth factor [EGFR]), and more recently nivolumab (75) and cemiplimab (76) (PD-1 immune checkpoint inhibitors).

Although chemotherapeutics have enjoyed modest success in treating surgically unresectable, and metastatic cSCC, they are accompanied by a wide range of – sometimes intolerable – gastrointestinal, hematologic, and metabolic side effects (67–69). Studies using 13cRA and IFN-α to treat SCC are conflicting; in the only trial using these compounds as adjuvant therapies in cSCC, they were ineffective (66, 72). In recent years targeting EGFR has shown some promise; indeed, EGFR is implicated in a variety of cancers including non-small cell lung cancer, colorectal cancer, pancreatic cancer, and head and neck squamous cell carcinoma (HNSCC) (77–81). Insofar as treating cSCC, two candidates have recently made it through phase II clinical trials: cetuximab, a humanized monoclonal antibody targeting EGFR, and gefitinib, which inhibits ATP-binding to EGFR (73, 74). Both compounds showed modest complete response rates, albeit with moderate toxicity.

The moderate to severe toxicities of systemic agents used to treat aggressive cSCC makes it challenging to use these drugs in the context of high-risk patients with significant comorbidities and chronic conditions, such as SOTRs. Additionally, the use of any systemic immune modifying agents may trigger immune-related adverse events (irAEs) (82) that could manifest as life-threatening (i.e. organ rejection) in patients who are iatrogenically immunosuppressed; thus, they are generally not recommended for use in this clinical context (83). Therefore, it is imperative that therapies that minimize systemic toxicities while maximizing the local tumor clearance be studied further.

Intra-Lesional and Topical Therapies

The serious toxicity profile associated with systemic therapies may be altogether avoided by treating instead with intra-lesional injections. Prior studies have revealed lowered rates of toxicity associated with intra-lesional injections when compared with systemic administration. Furthermore, by delivering an increased concentration of the active agent at the site of action, increased rates of efficacy are observed (84, 85). To date, only a handful of intra-lesional agents have been used for treatment of cSCC, although this method of delivery has been extensively studied in melanoma wherein systemic adverse events are minimized and the local immune response is maximized (86).

Several case reports and small trials have been reported where actinic keratosis (AK) has been treated successfully using intra-lesional therapies (87, 88). Furthermore, 5-FU (70), methotrexate (MTX) (89), several INFs (90–93), and bleomycin (94) have all shown some utility in treating both AK and cSCC, although the data for cSCC is somewhat limited. The vast majority of reports of intra-lesional treatments of cSCC's are case reports; however, most show good response rates and limited side effects, with the majority of side effects reported including erythema, pain and swelling at the site of injection, and occasionally, mild fever and chills. Indeed, when reflecting on our patient presented above, he experienced no immune-related adverse events. Remarkably, in a patient who cannot tolerate T cell activation (due to risk of graft failure), local injection of the potent T cell activator IL-2 was sufficient to maximize the anti-tumor immune response and did not cause any further toxicities. The patients' side effects were limited to pain and swelling at the injection site with a short period of chills following injection, further demonstrating the benefit of intra-lesional immune therapies compared to systemic therapies.

Topical therapies that are applied locally can also mitigate the risk of systemic adverse events. A number have achieved moderate success in the treatment of AK, such as topical 5-FU, imiquimod, ingenol mebutate, and diclofenac, which are all FDA approved for this indication (95). With regards to cSCC,

topical 5-FU is also commonly used (96). Additionally, early randomized controlled trials show a high degree of clinical benefit from topical imiquimod in the context of cSCC, with approximately a 70% complete response rate (97, 98). Of particular importance, imiquimod is a potent Toll-like receptor 7 (TLR7) agonist that induces local cytokine changes to cause a shift in the immunological balance intratumorally (99).

These therapies individually or in combination therefore represent a possible treatment modality in the context of high risk cSCC. In solid organ transplant recipient patients who cannot tolerate other therapies, or have failed standard local treatment with surgery and/or radiation, use of intra-lesional and/or local therapies may provide substantial clinical benefit. As iatrogenic immunosuppression plays a role in mediating immune escape of these patients' tumors, identifying local therapies that can counteract that process is paramount.

Augmenting the Anti-Tumor Immune Response in cSCC to Prevent Immune Escape

As briefly discussed above, one of the avenues through which cSCC mediates immune escape is by downregulation of cytotoxic T cells. Thus, local therapies that promote T cell proliferation and activity are of particular interest. Outside of case reports, there are very few studies examining the immunological response to intra-lesional therapies in cSCC. Neoadjuvant intra-lesional MTX is able to induce lymphocytic inflammatory infiltrate, although the cell types and their role within the infiltrate are unclear (100). IFNα-2β;, another immunological treatment that was used intra-lesionally in a number of early studies achieved moderate clinical success, with a 88.2% complete response rate, but the specific mechanism of action is still unclear (101). However, extrapolating from its function in other contexts, it is likely upregulating a T cell-mediated anti-tumor response through the JAK1/STAT1 pathway (102). Unfortunately, in the years since these early studies, IFNα-2β; has fallen out of clinical investigation.

Interestingly, other potent T cell activating immunotherapies such as IL-2, have not yet been studied in the context of cSCC, to our knowledge. This is despite its extensive investigation as an intra-lesional agent in melanoma (86, 103–105) and HNSCC (106–109), where it mediates a shift to CD8+ T cell-mediated tumor clearance. The excellent response demonstrated by this case warrants further investigation into the possible role intra-lesional IL-2 may have in the treatment of cSCC.

Imiquimod, the other local agent used in this case, applied topically to cSCC is able to induce numerous changes targeted at augmenting T cell effector function. First, imiquimod causes dense CD8+ T cell infiltration into treated tumors, which produce significantly higher amounts of IFNγ, perforin, and granzyme compared to untreated tumors (99). In addition, treatment with imiquimod causes a shift to a polarized Th1 cytokine response (110). Production of IL-10 and TGF-β, which are known cytokines responsible for cSCC immune evasion (111), were significantly downregulated following imiquimod treatment. Imiquimod also antagonizes cSCC-mediated vascular remodeling, by upregulating E-selectin to promote cytotoxic T cell homing to the tumor (112). Furthermore, it significantly decreases FOXP3+ Treg cell levels in treated tumors, and also inhibits their function (112). Most importantly, imiquimod-treated tumors exhibit clonally expanded CD8+ T cell repertoires (112), suggesting a specific anti-tumor immune response and possibly adaptive immunity.

CONCLUSION

The case presented herein provides an excellent example of the complexity of the pathobiology and clinical behavior of cSCC in the immunosuppressed patient population. The primary objective of immunosuppressive regimens in solid organ transplantation is prevention of acute allograft rejection through IL-2 blockade. Calcineurin inhibitors such as tacrolimus prevent IL-2 production while sirolimus inhibits IL-2 receptor signal transduction *via* action on mTOR. These complementary mechanisms of action align to effectively preventing acute allograft rejection while at the same time compromising both innate and adaptive immune pathways.

Carcinogenesis in cSCC may be associated with a number of cellular modifications that facilitate immune escape including aberrant HLA expression, downregulation of important chemokines associated with T-cell homing and production of other cytokines such as IL-6, a cytokine having a myriad of functions including promoting angiogenesis, tumor cell growth and an overall proinflammatory and pro-tumorigenic response. In the setting of an immunocompromised host, cSCC is therefore 'facilitated' to evade the body's natural cancer immune surveillance mechanisms *via* the aforementioned mechanisms. Our patient began experiencing cSCC within a year of his second kidney transplant. His initial lesions were small, well differentiated cSCC's. However, early on in his disease he developed a high-risk feature, that being perineural invasion. Despite achieving clear surgical margins and receiving adjuvant radiation to the field, the disease recurred. Over time, the recurrences became more frequent, with increasing numbers of high-grade features including lesions greater than 2cm, perineural invasion and eventually transformation to high grade histology. At this point, further surgery was deemed futile and with no further options for radiation an alternate treatment strategy needed to be considered.

With recent randomized evidence to support systemic immunotherapy in cSCC, the option of systemic treatment with a PD-1 inhibitor was initially discussed. Given that there is currently a paucity of evidence to support safe delivery of systemic immune therapy in the solid organ transplantation population, and with both a liver and a kidney allograft at risk of rejection, we decided against systemic immunotherapy. However, we have developed local experience and success with IL-2 based intra-lesional treatment of cSCC in some of our kidney transplant patients. Our rationale for an IL-2 based treatment strategy in this population is based on the knowledge that current immunosuppressive regimens target IL-2 production or its effects systemically. Our hypothesis is that local re-introduction of IL-2 into skin bearing cSCC and

specifically into the tumor microenvironment will serve to restore both the innate immunity, and effector T-cell function lost through iatrogenic IL-2 suppression thereby re-establishing effective cytotoxic immunity against cSCC. The initial observed response to IL-2 monotherapy was mixed with some lesions regressing while others progressed. We were concerned that significantly increasing the local IL-2 dose beyond the 10M IU (total injected dose) might systemically impact immunity and potentially initiate allograft rejection. Therefore, we added topical imiquimod with the intention of potentiating the local cytokine response promoting T-cell and NK-cell homing and effector function. The combination proved highly effective with clearance of all facial lesions and marked regression of the subcutaneous, submandibular nodule making surgical excision much easier to achieve with a clear margin. To help reduce the development of further cSCC's, the patient's calcineurin inhibitor dose was further reduced.

The foundational concepts of cSCC tumorigenesis in the immunocompromised population are aberrant HLA expression, alteration of chemokine and cytokine profiles, and T cell dysfunction, as discussed prior. In our patient, we used two immunomodulatory agents in conjunction in an attempt to modulate some of the aforementioned pathways in the favor of an anti-tumor response, while mitigating systemic immune toxicity. We acknowledge that it is not fully clear how IL-2 and imiquimod may act in conjunction to mediate these anti-tumor immune responses; this merits further mechanistic study.

Herein we demonstrate, for the first time to our knowledge, that an IL-2 based intra-lesional treatment strategy safely and effectively treated multiple high grade cSCC lesions in an immunocompromised, multi-organ transplant patient. It is arguable that given the biological propensity for cSCC to evade immune systems in the setting of iatrogenic immunosuppression, an IL-2 based intra-lesional immunotherapy treatment strategy should be first line consideration for all cSCC lesions. Furthermore, from a patients' perspective, offering patients a minimally invasive, localized therapy gives them the opportunity to forego complex surgical management, which in some cases can be undesirable cosmetically or may carry major perioperative risks in this population.

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

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DV and CG contributed to conception and design of this manuscript, and wrote sections of the manuscript. GS wrote sections of the manuscript. SP provided data for figures within the manuscript. MW, KP, SJ, and LH contributed to project conception and design. All authors contributed to the article and approved the submitted version.

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EVA1C Is a Potential Prognostic Biomarker and Correlated With Immune Infiltration Levels in WHO Grade II/III Glioma

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Background: Immunotherapy is an effective therapeutic approach for multiple human cancer types. However, the correlations between *EVA1C* and patients' prognosis as well as immune infiltration remain obscure. Herein, we employed transcriptomic and clinical data extracted from two independent databases to systematically investigate the role of *EVA1C* in the oncological context.

Methods: The differential expression of *EVA1C* was analyzed *via* TCGA and Oncomine databases. We evaluated the influence of *EVA1C* on clinical prognosis using Kaplan-Meier plotter. We then used the expression profiler to calculate stromal score, immune score, and ESTIMATE score based on the ESTIMATE algorithm. The abundance of infiltrating immune cells was calculated *via* TIMER. The correlations between *EVA1C* expression and immune infiltration levels were analyzed in two independent cohorts.

Results: In patients with World Health Organization (WHO) grade II/III glioma, high *EVA1C* expression was associated with malignant clinicopathological features and poor overall survival in both cohorts. *EVA1C* expression was positively associated with immune infiltration levels of B cell, CD4+ T cell, neutrophil, macrophage, and dendritic cells (DCs). Besides, *EVA1C* expression strongly correlated with diverse immune marker sets. And the predictive power of *EVA1C* was better than that of other indicators in predicting high immune infiltration levels in glioma.

Conclusions: For the first time, we identified the overexpression of *EVA1C* in glioma, which was tightly correlated with the high infiltration levels of multiple immune cells as well as poor prognosis. Meanwhile, *EVA1C* might be a potential biomarker for predicting high immune infiltration in WHO grade II/III gliomas.

Keywords: *EVA1C*, immune infiltration, biomarker, glioma, microenvironment

INTRODUCTION

Human brain is derived from the neural ectoderm and accounts for about half of all intracranial tumor incidences (1). In China, there are 106,207 new cases and 59,120 glioma-related deaths each year (2). Diffuse WHOII/III glioma is a lethal threat to young adults, which tends to have a wide range of genetic and transcriptional heterogeneity (3). Compared with WHOIV gliomas, the course of WHOII/III gliomas is very slow. Recent studies have mainly classified gliomas based on two genetic markers, such as isocitrate dehydrogenase (IDH) mutation and codeletion of chromosome arms 1p and 19q (codeletion) (4). In the vast majority of WHOII/III gliomas, IDH mutation is present in 84% of cases, and 1p/19q codeletion is present in 35% of cases. IDH mutation tends to occur in the early stage of gliomas (3). Although adjuvant therapeutics have improved the prognosis of glioma to some extent, the overall survival (OS) of glioma patients remains poor (5, 6). Thus, novel strategies are in urgent need for the hope to improve the unpleasing outcomes.

Immunotherapy has emerged as one of the most important therapeutic means for tumor in the past decades (7, 8). Especially, approaches targeting recognized immune checkpoints, such as anti-PD-1 and anti-CTLA4, have been approved for clinical utilization and achieved encouraging outcomes (9, 10). However, there are still some limitations in existing T cell-based immunotherapies, which are attributed to the extremely complex immunosuppressive processes of tumor microenvironment (TME) and its regulatory networks (11). Importantly, many patients didn't respond well or acquired rapid resistance to the immune checkpoint blockers in clinical practice. Therefore, it is necessary to further explore the immunosuppressive essence and the underlying mechanism within TME (12). Existing studies have confirmed that local TME is composed of various cell types, including tumor cells, infiltrating cells and stromal cells, as well as soluble factors that support tumor growth and progression (13). TME usually confers a high degree of immunosuppression, preventing the clearance of malignant cells by immune components, which negatively impacts cancer immunotherapy (14). Therefore, seeking novel immune checkpoints and overcoming immunosuppressive processes are very critical for the improvement of effective immunotherapies against tumors.

EVA1C (aliases *C21orf63*), first identified in 2001, is a membrane protein encoding-gene (15). *EVA1C* protein has been found in a variety of human tissues. Kanae Mitsunaga (16) identified *EVA1C* protein possessing two repeats of putative 'galactose-binding lectin domains' that bind heparin. Although the role of EVA1C has not been reported in tumor, Manas Kotepui et al. reported that *ADGRL3* (*LPHN3*), an important paralog of *EVA1C* gene, was upregulated in breast cancer and was correlated with axillary lymph node metastasis (17). In addition, Inna M. Yasinska et al. found that the PKCa pathway could be activated by *FLRT3 via LPHN1*, *LPHN2* and *LPHN3* (18). Collectively, their findings indicated *FLRT3-LPHN-Tim-3-galectin-9* pathway plays a key role in escaping systemic immunosurveillance across various cancer types. However, the specific expression and function of *EVA1C*, especially in the context of immuno-oncologic interactions, remain poorly understood.

Herein, our study was aimed to identify the *EVA1C* expression and its correlation with clinicopathological factors, and survival prognosis of patients with WHO grade II/III glioma. Meanwhile, we focused on the correlation between *EVA1C* expression and abundance of immune infiltrates by immune profiles, and further investigate whether *EVA1C* could act as a new immune marker for assessing immune microenvironment of glioma patients.

MATERIALS AND METHODS

Data Extraction

The mRNA sequencing data and clinicopathological data of all cases in this study were extracted from the Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/) and The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/) databases. Patients with incomplete follow-up data were excluded. Finally, a total of 182 patients with WHO grade II/III glioma were included from the CGGA (Dataset ID: mRNAseq_325) database as the CGGA cohort, and 457 patients with WHO grade II/III glioma from TCGA database were defined as validation cohort. The detailed demographics of enrolled glioma patients in both cohorts were included in **Supplementary Tables 1** and **2**, respectively. The Estimation of Stromal and Immune cells in gliomas using Expression data (ESTIMATE) and Tumor Immune Estimation Resource (TIMER) algorithms were used to explore the immune infiltration landscapes. Since all the data are from public databases, the Ethics Committee of Nanfang Hospital granted ethical approval for the study, but waived the requirement for informed consents.

Differential Expression of *EVA1C* in Tumors

The GEPIA (http://gepia.cancer-pku.cn/) is an online tool for dynamic analysis of gene expression profile data. GEPIA analyzed the RNA sequencing data of 9736 tumors and 8587 normal samples from TCGA and GTEx projects. The expression data of TCGA and GTEx were recalculated under the same pipeline, which can be used for very comprehensive expression analysis directly. Therefore, we employed the GEPIA webtool to examine the *EVA1C* expression profile and its correlation with patients' prognosis. We further verified the differential expression of *EVA1C* at the mRNA level in glioma *via* the Oncomine database (www.oncomine.com). Normalized mRNA expression data for CCLE human cancer cell lines were extracted from the CCLE portal (https://portals.broadinstitute.org/ccle). The expression of *EVA1C* protein was obtained online from the Human Protein Atlas (HPA, www.proteinatlas.org).

GO and KEGG Pathway Enrichment Analyses

To understand the potential biological functions of *EVA1C*, including molecular function, biological processes, and cellular components, we employed the DAVID database (Version 6.8, http://david.abcc.ncifcrf.gov/) to perform the GO and KEGG enrichment analyses. At first, we used the co-expression scores to obtain the top 1000 genes (19) co-expressed with *EVA1C*, which were used for subsequent functional and pathway enrichment analyses including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) analyses ($P < 0.05$ and false discovery rate (FDR) < 0.05). And the potential protein-protein interaction (PPI) networks were developed using the STRING database (Version 11; https://string-db.org/), which is an online search database for protein interaction relationship. Additionally, GO analyses were further analyzed with Coexpdia (http://www.coexpedia.org/) that was based on GEO datasets.

Statistical Analysis

Statistical analysis was performed by SPSS (version 23.0, Corp., Armonk, NY, USA) and R programming language (Version 3.6.1). The ESTIMATE immune score and stromal score were computed by the ESTIMATE algorithm. Different immune cells infiltration levels were calculated by TIMER algorithm. The chi-square tests were performed to calculate the difference of categorical data. Spearman's correlation analyses were used to gauge the degree of correlation between certain variables. And the survival plots were generated by the Kaplan-Meier method. Time-dependent receiver operating characteristic (ROC) curves were constructed using the R programming language. All tests were two-sided, and P value <0.05 was the significance threshold in this study.

RESULTS

The mRNA and Protein Levels of *EVA1C* Were Upregulated in Glioma

Firstly, we compared the differences in *EVA1C* expression between glioma and normal brain tissues by GEPIA website, and found that the mRNA levels of *EVA1C* were upregulated in glioblastoma (GBM) (**Figure 1A**). Meanwhile, immuno histochemistry results showed that *EVA1C* protein was strongly over-expressed in GBM compared with normal brain tissues (**Figure 1B**). The upregulation of *EVA1C* mRNA in GBM was also verified in two independent cohorts ('Sun Brain' and 'Murat Brain') from the Oncomine database (**Figures 1C, D**). The *EVA1C* expression in different tumors cell lines was obtained from CCLE database, it was also confirmed that *EVA1C* was highly expression in glioma cell lines (**Supplementary Figure 1A**). Additionally, we also found that, compared with normal tissues, *EVA1C* mRNA levels were higher in other cancer types including kidney renal clear cell carcinoma (KIRC), acute myeloid leukemia (LAML), pancreatic adenocarcinoma (PAAD), and thymoma (THYM)

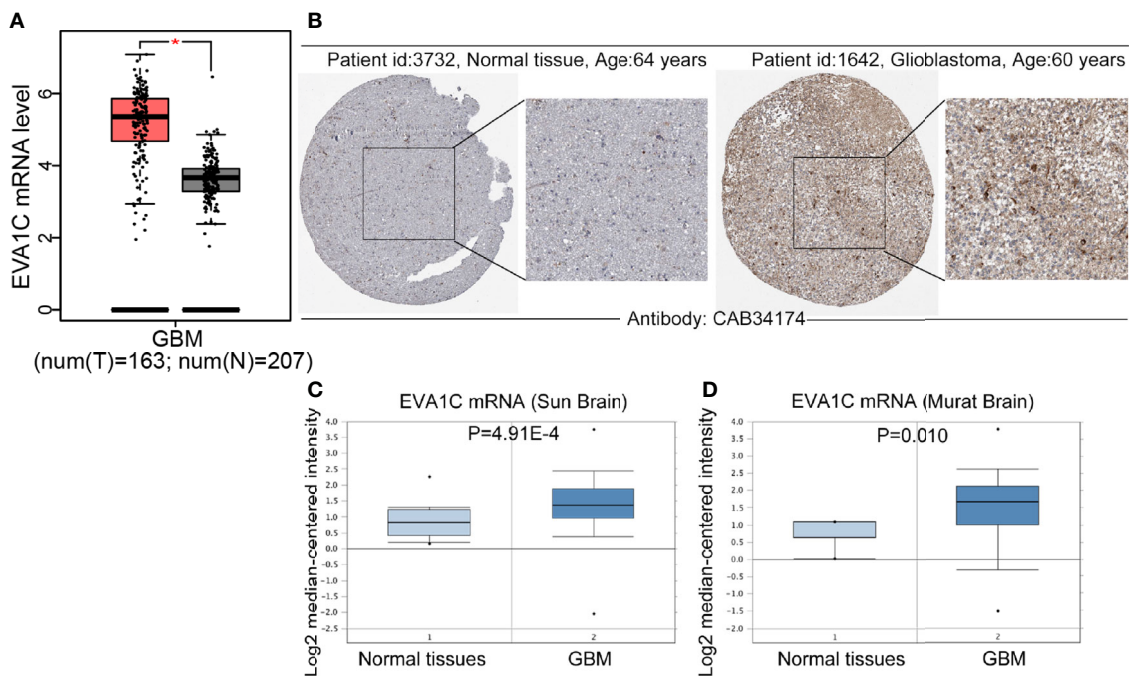

FIGURE 1 | Compared with normal brain tissues, *EVA1C* expression was upregulated in glioma. **(A, B)** Compared with those in the normal brain tissues, the *EVA1C* mRNA and protein levels in GBM were upregulated. **(C, D)** The mRNA levels of *EVA1C* were upregulated in GBM (Oncomine database). T, Tumor tissues; N, Normal tissues.

(**Supplementary Figure 1B**), and *EVA1C* protein levels were upregulated in renal cancer and pancreatic cancer (**Supplementary Figure 1C**).

Elevated *EVA1C* Expression Correlated With Malignant Clinicopathological Features and Poor Prognosis in Patients With WHO II/III Glioma

To identify the role of *EVA1C* in glioma, we statistically analyzed the correlation between *EVA1C* expression and clinicopathological features as well as prognosis in the CGGA cohort of 182 patients with WHO grade II/III glioma. The 182 patients were divided into low and high *EVA1C* expression groups based on the median value of *EVA1C* mRNA level. Results of chi-square tests revealed that high *EVA1C* expression was significantly correlated with several malignant features including WHO grade, histopathological type, IDH mutation status, and 1p/19q non-codeletion (**Table 1**, **Supplementary Figure 2**). However, the correlations were not significant between *EVA1C* expression and age, gender, history of radiotherapy or chemotherapy, *MGMT* promoter methylation, as well as tumor recurrence (**Table 1**). Critically, the expression level of *EVA1C* was significantly and positively correlated with that of vimentin (r = 0.51, P = 2.33e-13, **Figure 2A**). These results suggested that high *EVA1C* expression was positively associated with malignant properties of glioma. Furthermore, *EVA1C* was considered as a risk factor for glioma patients as Kaplan-Meier curves showed that the high *EVA1C* expression group presented poorer prognosis than the low expression group (**Figure 2C**).

Next, a larger TCGA cohort of 457 glioma patients was used to validate the above results. Similarly, the analysis results indicated that *EVA1C* expression was correlated with WHO grade, histopathological type, *IDH* mutation status and 1p/19q codeletion (**Supplementary Table 3**). The robust correlation between *EVA1C* and vimentin was also confirmed in the validation cohort (r = 0.657, P < 0.0001, **Figure 2B**). Moreover, survival analysis verified the significant association between elevated *EVA1C* expression and poorer prognosis in the TCGA cohort (**Figure 2D**). Additionally, the ROC curves revealed that the AUCs of *EVA1C* for predicting the 1-, 3-, and 5-year survival were 0.810, 0.751, and 0.656, respectively (**Figure 2E**). Finally, we also analyzed the correlation between *EVA1C* expression and the prognosis of patients with other solid tumors, including KIRC, LAML, PAAD and THYM. However,

TABLE 1 | The correlation between *EVA1C* expression level and clinicopathological features of patients in the CGGA cohort (n = 182).

Characteristics	*EVA1C* expression		*P* value
	Low expression	High expression	
Age (years)			
≥40	53	41	0.075
<40	38	50	
Sex			
Male	57	54	0.648
Female	34	37	
WHO grade			
WHO II	64	39	<0.0001
WHO III	27	52	
Histopathology			
O	41	11	<0.0001
A	27	29	
AO	9	3	
AA	14	48	
IDH			
Mutation	87	46	<0.0001
Wildtype	3	45	
1p/19q			<0.0001
Codeletion	48	12	
Non-codeletion	42	78	
MGMT promoter			
Methylation	52	37	0.066
Unmethylation	34	43	
Radiotherapy			
Yes	74	68	0.592
No	15	17	
Chemotherapy			
Yes	45	46	0.810
No	39	37	
Recurrence			
Yes	15	23	0.145
No	76	68	

O, oligodendroglioma; A, astrocytoma; AO, anaplastic oligodendroglioma; AA, anaplastic astrocytoma.

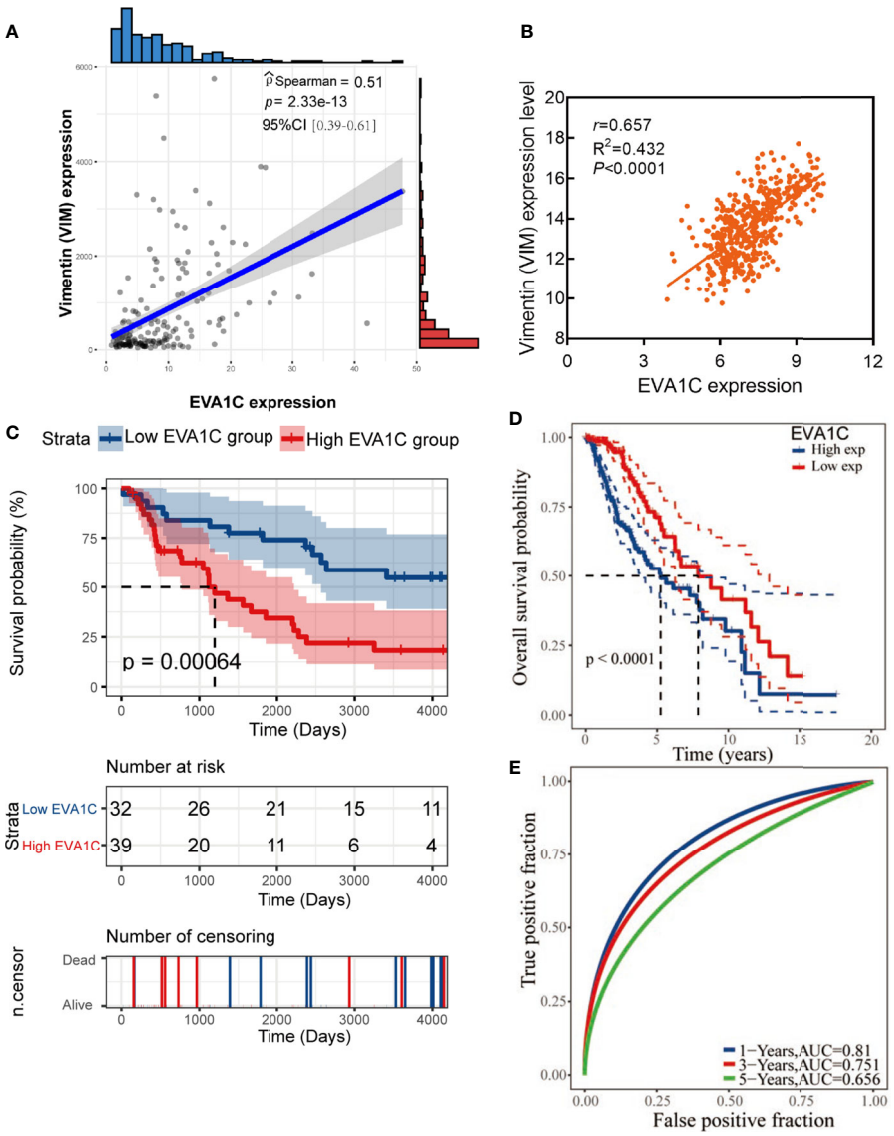

FIGURE 2 | High *EVA1C* expression was associated with poor prognosis in WHO grade II/III glioma. **(A, B)** *EVA1C* expression was strongly correlated with vimentin expression in the CGGA and TCGA cohorts. **(C, D)** Patients with high *EVA1C* expression had a poor prognosis in the CGGA and TCGA cohorts. **(E)** The predictive power of *EVA1C* for predicting the 1-, 3-, and 5-year survival rate was relatively strong (TCGA cohort).

the significant correlation between high *EVA1C* expression and poor prognosis was only observed in patients with LAML (**Supplementary Figure 3**).

EVA1C Was Associated *W*ith Immune-Related Biological Functions

To identify the potential functions of *EVA1C* in glioma, the top 1000 co-expressed genes of *EVA1C* were extracted and subsequently inputted for enrichment analysis. As shown in **Figure 3A**, genes that co-expressed with *EVA1C* were enriched in several immune-related GO terms, including "innate immune response", "antigen processing and presentation", "B cell activation", and "platelet degranulation". Meanwhile, the significantly enriched KEGG pathways included "staphylococcus aureus infection", "viral myocarditis", "intestinal immune network for IgA production", "cell adhesion molecules", "antigen processing and presentation", and "allograft rejection" (**Figure 3B**). These findings suggested that *EVA1C* might regulate the immune microenvironment through various immune processes such as antigen processing and presentation, complement and coagulation cascades, and intestinal immune network for IgA production. Next, we also used the Coexpedia online website, which based on 384 human GEO datasets and 248 mouse GEO datasets, to analyze the biological functions of *EVA1C* gene. The results also showed that *EVA1C* gene was associated with the activation of NF-KB signaling pathway, macrophage activation involved in immune

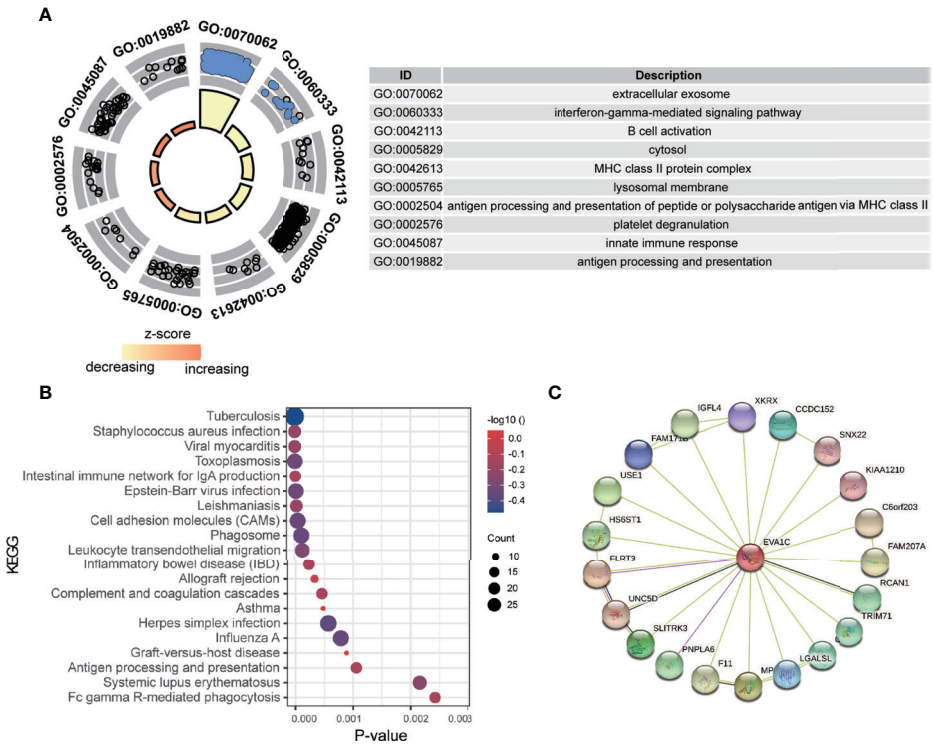

FIGURE 3 | The enrichment analysis of *EVA1C* co-expression genes indicated *EVA1C* was involved in inflammatory and immune biological processes. **(A)** The top 10 GO enrichment terms. **(B)** The top 20 KEGG pathways enriched. **(C)** The protein-protein network was constructed *via the* STRING database.

response, cellular response to interleukin-6 (**Supplementary Figure 4**). Previous study also showed that the activation NF-KB signaling pathway in breast cancer cells could upregulate interleukin-6 expression, and further promote cancer cell metastasis (20).

In addition, the proteins interacting with *EVA1C* (**Figure 3C**) were enriched in terms including 'complement and coagulation cascades' and 'thyroid hormone signaling pathway', showing the potential role of *EVA1C* in affecting the processes of innate and acquired adaptive immune responses, which might impact tumor initiation and progression.

Association Between *EVA1C* Expression and Microenvironment of Glioma

We used the expression profiler to calculate stromal score, immune score and ESTIMATE score by the ESTIMATE algorithm, and calculate the infiltration abundance of immune cells. The immune landscape illustrated that various immune infiltration levels in the *EVA1C* high expression group were higher than those in the *EVA1C* low expression group (**Figure 4A**). Specifically, the immune score, stromal score, and ESTIMATE score were all significantly higher in the *EVA1C* high expression group (**Figures 4B–D**). Furthermore, we calculated the enrichment scores based on the TIMER algorithm and found that the scores of B cell, CD4+ T cell, neutrophil, macrophage, and DCs (except for CD8+ T cell) in the *EVA1C* high expression group were higher than those in the *EVA1C* low expression group (**Figures 4E–J**). The immune landscape illustrated the proportions of different immune cell subpopulations in CGGA and TCGA cohorts, and the findings were quite consistent (**Figure 5A**). Subsequent scatter plots showed the similar results that *EVA1C* expression had significant correlations with the infiltration levels of B cell (r = 0.262, $P = 0.004$), CD4+ T cell (r = 0.418, $P < 0.0001$), neutrophil (r = 0.335, $P < 0.0001$), macrophage (r = 0.608, $P < 0.0001$), and DCs (r = 0.645, $P < 0.0001$), except for CD8+ T cell (r = 0.037, $P = 0.620$) (**Figures 5B–G**).

We also used the TCGA validation cohort to verify the above positive correlations. Similar results were obtained that elevated *EVA1C* expression was associated with higher abundance of various immune infiltrates (**Supplementary Figure 5**). Likewise, significant correlations were observed between *EVA1C* expression and infiltration levels of B cell (r = 0.394, $P < 0.0001$), CD4+ T cell (r = 0.402, $P < 0.0001$), CD8+ T cell (r = 0.312, $P < 0.0001$), neutrophil (r = 0.301, $P < 0.0001$), and macrophage (r = 0.527, $P < 0.0001$) in the TCGA validation cohort (**Figures 5H–L**). These findings strongly indicated the important role *EVA1C* played in immune infiltrating processes in the context of WHO grade II/III glioma. Finally, the correlation between *EVA1C* expression and the mRNA levels of chemokines, interleukins, interferons and other important cytokines and their receptors in the microenvironment of WHO

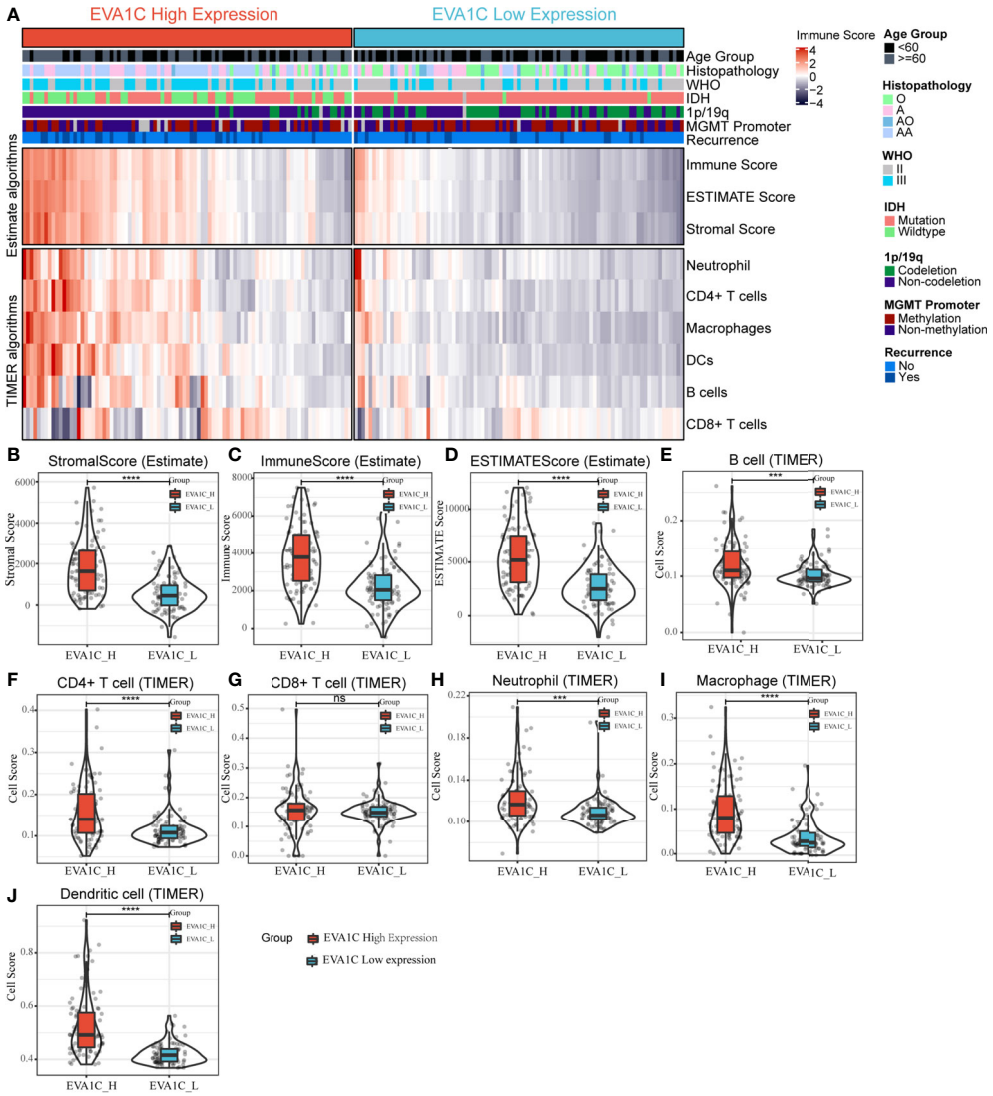

FIGURE 4 | The relationship between *EVA1C* expression and immune infiltration in the CGGA cohort. **(A)** The heatmap represents cell type enrichment score of each immune cell type for the 182 samples. **(B–D)** The comparison of stromal score, immune score, and ESTIMATE score between the high and low *EVA1C* expression groups. **(E–J)** The comparison of the abundance of B cell, CD4+ T cell, CD8+ T cell, neutrophil, macrophage, and dendritic cell between the high and low *EVA1C* expression groups.

grade II/III glioma by GEPIA database (**Figure 6**). These results suggested that, in the microenvironment of glioma with high *EVA1C* expression, there were not only a variety of immune cells, but also high expression of many chemokines including *CCR5, CCL5, CXCL10*, and *CXCL9*, which have been shown to attract DCs, T cell.

Correlation Analysis Between *EVA1C* Expression and Immune Marker Sets

Given the correlation between *EVA1C* expression and immune infiltration levels, we further analyzed the relationship between *EVA1C* expression and the marker genes of essential immune cells in glioma. **Figure 7A** shows the correlations between *EVA1C* expression and various immune markers in the CGGA cohort. Interestingly, *EVA1C* expression was associated with gene markers (21) of B cell, CD8+ T cell, M2 macrophage, DCs, Th2 cell, exhausted T cell, and neutrophil in WHO II/III glioma (**Supplementary Table 4**). These results suggested that *EVA1C* might play a specific role in regulating macrophage polarization in WHO II/III glioma. In addition, *EVA1C* expression was related to the markers of tumor associated macrophage (TAM), such as CCL2, CD68 and IL10. These findings further revealed a robust interaction between *EVA1C* and TAM infiltration. Furthermore, a significant relationship was detected between *EVA1C* expression and DCs markers (*HLA-DPB1, HLA-DRA, HLA-DPA1, CD1C* and *ITGAX*). In

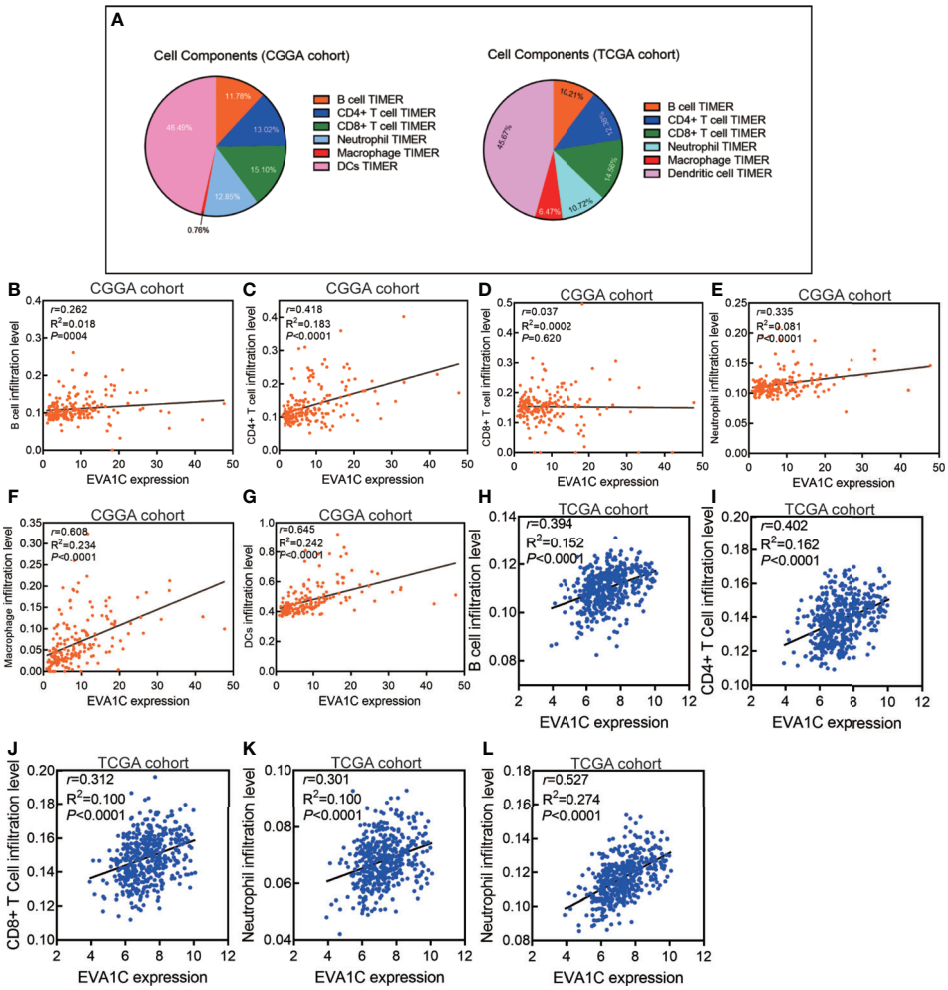

FIGURE 5 | High *EVA1C* expression was positively correlated with immune infiltration levels. **(A)** The heatmap shows the proportions of different immune cell subpopulations in CGGA and TCGA cohorts. The scatter plots show correlations between *EVA1C* expression with the abundance of various immune infiltrates in the CGGA cohort **(B–G)** and the TCGA cohort **(H–L)**.

addition, significant correlations were found between *EVA1C* and TGFβ (TGFB1, marker of Treg cell), as well as TIM-3 (HAVCR2, T cell exhaustion) (**Supplementary Table 4**). These results were further confirmed in the TCGA validation cohort (**Figure 7B**), suggesting that *EVA1C* participated in immune escape within the tumor microenvironment of WHO II/III glioma.

The Performance of *EVA1C* in Predicting a High Immune Score in Glioma

To determine whether *EVA1C* could be considered as a potential biomarker to discriminate the immune infiltration levels in glioma, we applied the ROC curve to evaluate the ability of *EVA1C* in predicting a high immune score for glioma. As shown by **Figure 8A**, the sensitivity and specificity of *EVA1C* in predicting a high immune score in the CGGA cohort were 74.7% and 70.3%, respectively. The area under the curve (AUC) was 0.785. Additionally, we also compared the predictive performance between *EVA1C* and other commonly utilized indicators including PD-1, LAG3, CTLA-4 and Siglec15. In terms of AUC, *EVA1C* demonstrated the highest predictive performance in predicting a high immune score within glioma (**Figure 8B**). The consistent results were obtained in the TCGA cohort (**Figures 8C, D**).

DISCUSSION

Nowadays, immune-oncological microenvironment has become the focus of cancer researches (22). Immunosuppressant began to be gradually applied to clinical patients. Although PD-1 and CTLA-4 antibodies have achieved a sustained response in some patients (23), most patients with glioma demonstrated poor responses to them. Such phenomena could be attributed to the

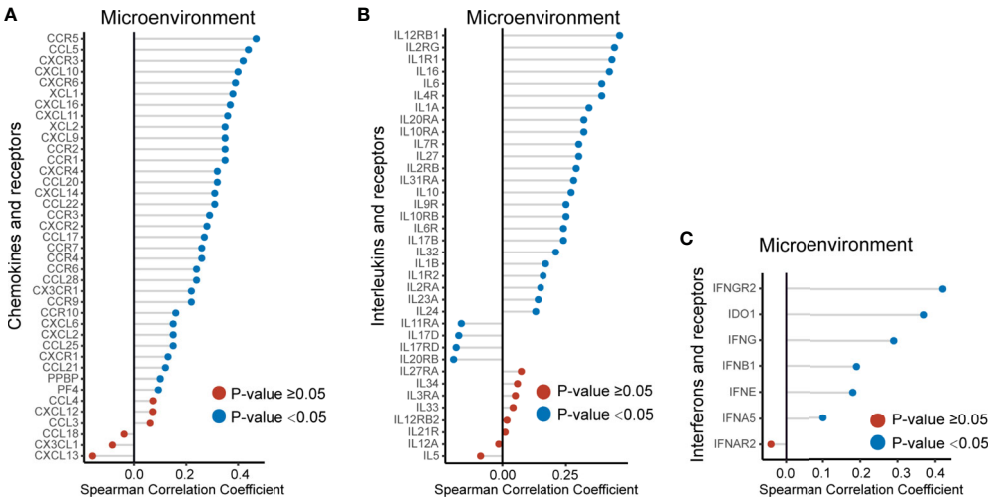

FIGURE 6 | The *EVA1C* expression was correlated with the expression of cytokines, including chemokines **(A)**, interleukins **(B)** and interferons **(C)**, and their receptors in the microenvironment of WHO grade II/III glioma.

essential and complicated immune escape processes in tumor microenvironment (24). There are multiple macrophages infiltrating in the glioma TME, which could prevent the immune system from eliminating malignant cells effectively (25). Thus, a deepening understanding of the interplay between TME and immunotherapy not only helps to explore the mechanism of immune escape, but also provides new approaches to improve the immunotherapeutic efficacy.

In this study, we observed for the first time that *EVA1C* was significantly overexpressed in glioma, and significantly correlated with malignant clinicopathological features. In this study, *EVA1C* high expression might be a potential poor prognostic factor. KEGG and GO enrichment analyses showed that *EVA1C* expression correlated with immune and inflammation related biological processes.

In mammals, the EVA1 family mainly includes three members: *EVA1A, EVA1B* and *EVA1C*. Previous studies showed that *EVA1A* (*TMEM166*) protein which was located in cell membrane could induce cell autophagy and apoptosis (26, 27). Ming Tao et al. found that, compared with normal pancreatic acinar cells, the *EVA1C* expression was remarkably higher in pancreatic acinar carcinoma, and it was mainly located in cell membrane and cytoplasm (28). Interestingly, Ziyi Wang et al. reported that *EVA1A* could inhibit *NLRP3* activation to reduce liver hypoxia-reperfusion damage *via* inducing autophagy in Kupffer cells (29). On the other hand, Bang-Yi

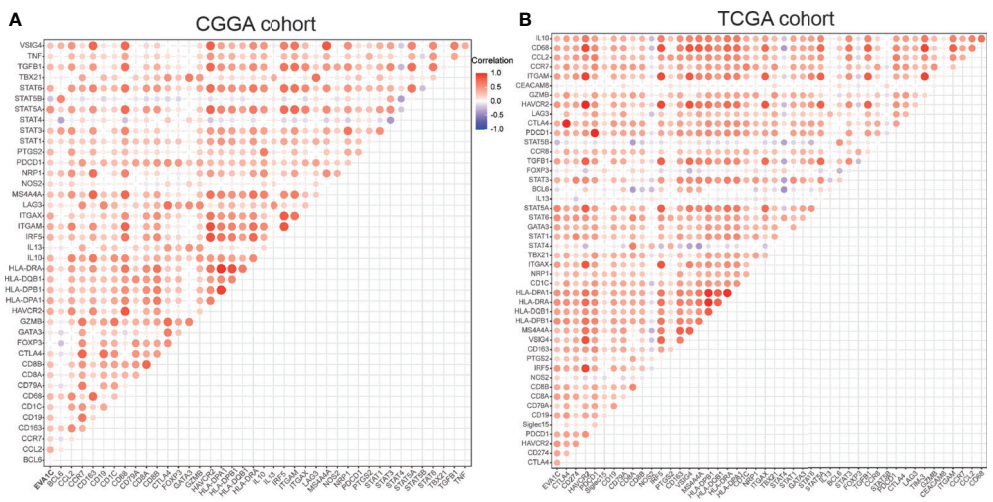

FIGURE 7 | Correlation between *EVA1C* and marker gene sets of immune cells in the CGGA cohort **(A)** and the TCGA cohort **(B)**.

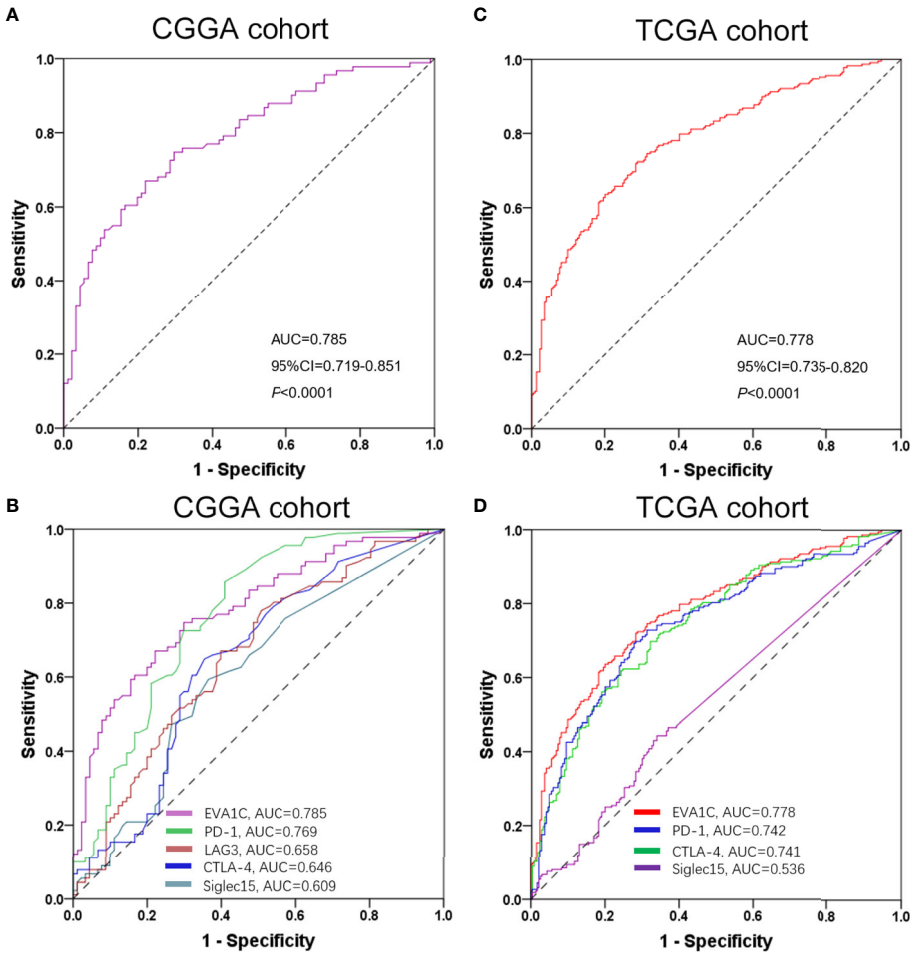

FIGURE 8 | ROC curves of *EVA1C* gene for predicting high immune infiltration showed excellent power. ROC curves of *EVA1C* and other indicators in predicting high immune infiltration levels in the CGGA cohort **(A, B)** and the TCGA cohort **(C, D)**.

Lin et al. found that *EVA1A* promoted papillary thyroid cancer progression and EMT by the Hippo signaling pathway (30). As a core kinase of the Hippo signaling pathway in cancers, *MST1/2* participates in the development and function of Treg and Th17 cells (31). The imbalance of these two cell types is a leading cause of multiple inflammatory and autoimmune diseases (32). The *EVA1C* is mainly mapped to critical region chromosome 21 (21q21-21q22.3) associated with Down syndrome. Intriguingly, Gregory James et al. found that the expression pattern of *EVA1C* was consistent with an axon guidance role in the mouse nervous system (33). However, what is the role of EVA1C plays in glioma is not reported. This is a subject that needs to be further explored.

A key finding in our study is that high *EVA1C* expression correlates with the high abundance of immune infiltrates including B cells, CD4+ T cells, neutrophils, macrophages and DCs in WHO grade II/III glioma. There is no statistical correlation between EVA1C and CD8+ T cell infiltration level in CGGA cohort. The possible reason is low statistical power due to small sample size, which need to further explore. Significant correlations between the *EVA1C* and the markers of TAMs as well as M2 macrophages suggest that *EVA1C* possesses the potential in regulating the polarization of TAMs, which is crucial for cancer development and metastasis (34). Adam Wu et al. found that cancer stem cells promote immunosuppression by M2 macrophages secreting many kinds of cytokines, such as TGF-β1 and IL-10 (35). IL-10 and TGF- β 1 has been shown to promote tumor cell immune escape by inhibiting T cell proliferation (36). Another intriguing finding in our study is the relationship between *EVA1C* and immune markers of DCs, Treg cells, and exhausted T cells. DCs can induce tumor cell metastasis by favoring Treg cells and reducing CD8+ T cell cytotoxicity (37). Additionally, TGF-β secreted by M2 macrophages could induce the shift from immature CD4+ T cells to Treg cells, and promote the proliferation of them (38). These outcomes reveal that *EVA1C* protein may increase the recruitment of immune cells in WHO grade II/III glioma. Notably, the predictive performance of *EVA1C* in predicting high immune infiltration levels was excellent, demonstrating its

potential in predicting immune profiles in WHO II/III glioma. However, it is still unclear whether the expression of *EVA1C* is related to the efficacy of immunotherapy and chemotherapy for glioma, and there is no data in this regard at present.

Although we revealed an immune-related biomarker and target for the first time in the patients with glioma, this study has some limitations. Firstly, the aim of this study was to elaborate the findings from the perspective of genomics, and the analysis of gene transcription levels could only reflect some aspects of immune status, but not the overall changes. In this study, Estimate and Timer algorithms were adopted, and conventional statistical methods were used for analysis. Secondly, although the above results could be validated in TCGA cohort with 457 patients, they need to be verified by another retrospective single-center cohort. Thirdly, the functions and in-depth mechanisms of *EVA1C* were explored *in vitro*. This study is only an exploratory discovery, and lays a foundation for our next functional mechanism experiments.

In summary, we found that *EVA1C* expression was upregulated in glioma compared with normal brain tissues, and the elevated expression level was significantly associated with malignant features and poor prognosis of glioma patients. Importantly, high *EVA1C* expression correlated with high immune infiltration levels and chemokines, interleukins, interferons and their receptors in WHO grade II/III glioma. In addition, *EVA1C* expression was significantly correlated with gene expression of M2 macrophages, TAMs, DCs, exhausted T cells and Treg cells markers. These findings suggest that *EVA1C* may be not only a potential immune-related biomarker, but also a key modulator in governing tumor microenvironment.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/) and The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/) databases.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Nanfang Hospital. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

Conception and design: SQ and ZH. Data analysis: SQ. Writing and revising: ZH. 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.2021.683572/full#supplementary-material

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CASE REPORT
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Immunotherapy-Based Therapeutic Strategies for Recurrent Advanced Squamous Cell Carcinoma of the Head and Neck: A Case Report and Literature Review

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We present a patient with locoregionally advanced laryngeal carcinoma, who experienced recurrence 2 months after surgery. We exploratively treated this patient with immunotherapy combined with targeted therapy with or without radiation therapy. The patient exhibited a significant and durable response. Thus far, there are no standard or effective second-line therapeutic modalities for recurrent locoregionally advanced laryngeal carcinoma. The efficacy of conventional chemotherapy with anti-epidermal growth factor receptor (anti-EGFR) remains unsatisfactory. The addition of immunotherapy resulted in substantial improvement in the progression-free survival (PFS) and overall survival (OS) of this patient. In this case, immunotherapy combined with anti-EFGR was administered, leading to good tumor response; based on this observation, radiotherapy was added to further intensify tumor control. This therapeutic strategy may be a novel option for recurrent locoregionally advanced squamous cell carcinoma of the head and neck.

Keywords: case report, locoregionally advanced squamous cell carcinoma of head and neck, targeted therapy, immunotherapy, radiotherapy

INTRODUCTION

Over 800,000 new cases of head and neck squamous cell carcinoma (HNSCC) occur annually worldwide, and the mortality rate associated with this disease is approximately 40–50% (1). Particularly for patients with recurrent or metastatic (R/M) HNSCC, the expected survival periods tend to be <1 year due to limited treatment options. Most patients with R/M HNSCC undergo palliative systematic treatment and best supportive care, with only a minority having the chance to receive radical local treatment (e.g., surgery and radiotherapy). According to the EXTREME protocol and Chinese CHANGE-2 study, the recommended first-line therapy involves platinum and 5-fluorouracil (5-FU)-based chemotherapy combined with cetuximab. It has been shown that this regimen significantly enhances the tumor regression rate, reduces the progression rate, and extends overall survival, thereby, improving the quality of life of patients. In recent years, an immunotherapy based on checkpoint inhibitors has been linked to important developments in the treatment of advanced HNSCC. The U.S. Food and Drug Administration

approved the use of nivolumab and pembrolizumab for the salvage treatment of R/M HNSCC. Based on the results of the KEYNOTE-048 study, the National Comprehensive Cancer Network has listed immunotherapy (as monotherapy or in a combination regimen) as one of the first-line treatment options for R/M HNSCC. However, we observed that, at the initial stage, the objective response rate (ORR) and progression-free survival of patients undergoing immunotherapy are similar to those reported in the EXTREME strategy. Herein, we present a case of R/M HNSCC who was treated with the exploratory combination of immunotherapy and targeted therapy, followed by radiotherapy, achieving encouraging results. Furthermore, we also reviewed the relevant literature.

CASE PRESENTATION

In July, 2019, a 64-year-old male was admitted to the Guangdong Provincial People's Hospital complaining of a lump found on the left side of his neck. Neck magnetic resonance imaging (MRI) revealed an irregular mass in the left glottic region involving the vocal cords, posterior commissure, left epiglottis, and thyroid cartilage, which indicated glottic carcinoma with left neck lymph node infiltration. A pathological analysis through a laryngoscopic biopsy indicated squamous carcinoma of the larynx. The definitive diagnosis was larynx squamous cell carcinoma cT4N3M0, stage IV. On July 17 and August 9, 2019, the patient underwent two cycles of induction chemotherapy with docetaxel and cisplatin combined with 5-FU, which resulted in a stable disease. Subsequently, the patient underwent a radical laryngectomy and a cervical lymph node dissection in September, 2019, but he refused to receive adjuvant chemoradiotherapy. In November, 2019, the patient was admitted to our hospital with enlarged left neck lymph nodes. A head and neck computed tomography (CT) following admission revealed a left neck lymph node enlargement (10 × 12 cm) (**Figures 1A–C**). A thorax and abdomen CT

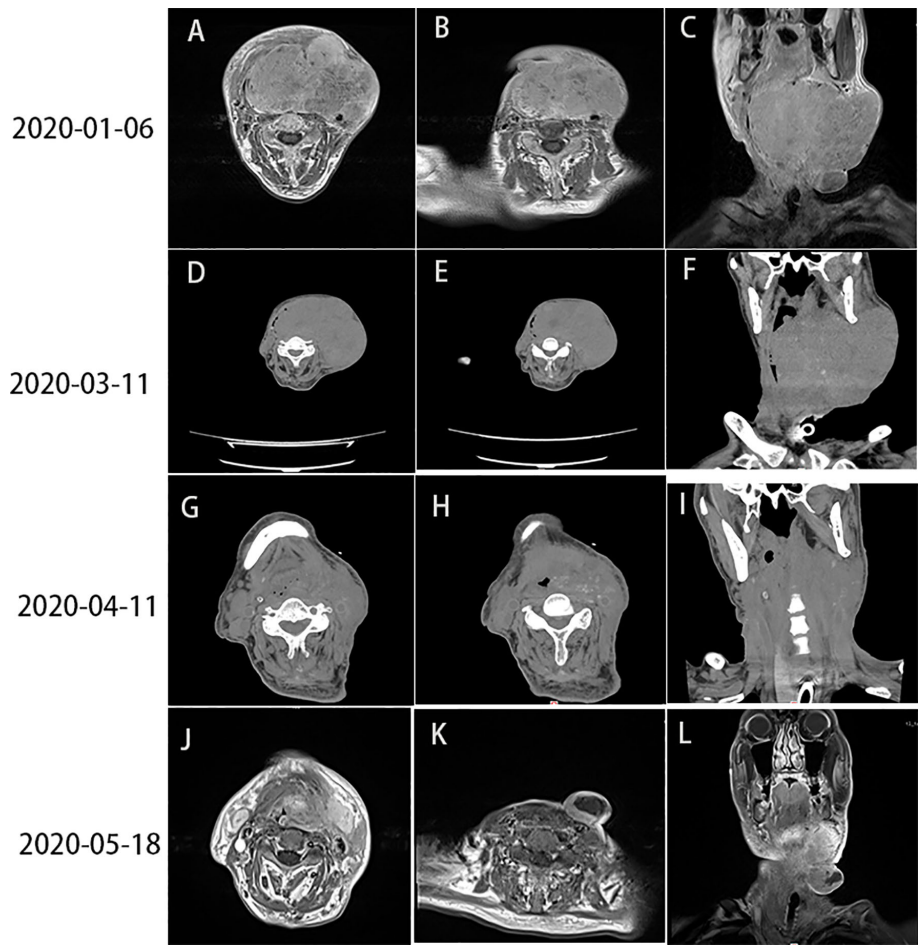

FIGURE 1 | **(A–C)** Head and neck CT showed lymph node enlargement (10 × 12 cm) on the left side of the neck (January 6, 2020). **(D–F)** Head and neck CT, performed 10 days after the first cycle of treatment, showed progression of the tumor mass (13 × 10 cm) (March 11, 2020). **(G–I)** PR was detected after the first course of combination therapy with sintilimab and nimotuzumab (April 11, 2020). **(J–L)** Head and neck MRI revealed PR (May 18, 2020). CT, computed tomography; MRI, magnetic resonance imaging; PR, partial response.

(on January 6, 2020) did not show lung or liver metastasis. A biochemical analysis of blood revealed increased levels of creatinine (228 umol/L), sodium (156 mmol/L), and calcium (4.02 mmol/L). The patient was diagnosed with larynx squamous carcinoma cT4N3M0, stage IV (according to the eighth edition of the American Joint Committee on Cancer TNM staging); this was recurrent after surgery and accompanied by renal insufficiency and hypercalcemia.

The patient had difficulty in food intake due to tumor compression. On January 8, 2020, gastrostomy was performed to relieve this symptom and improve the patient's state of nutrition. Treatment selection was based on the National Comprehensive Cancer Network guidelines, the results of the EXTREME and CHANGE-2 studies, and the poor general condition of the patient. Owing to its good safety profile, albumin paclitaxel in combination with nimotuzumab 200 mg was the selected regimen. The patient underwent two cycles of treatment between January 15 and February 17, 2020. A testing of electrolytes in blood suggested intractable hypernatremia and hypercalcemia. Hence, the patient underwent continuous renal replacement therapy. A reexamination after two courses of the treatment revealed significant enlargement of the neck mass, which indicated progressive disease.

To further seek other treatment options, the patient consented to undergo next-generation sequencing, which covered 428 genes and an immunohistochemical analysis of programmed death-ligand 1 (PD-L1). The outcome of next-generation sequencing showed a low tumor mutational burden (11.5 mut/Mb), and mutations in genes F-box and WD repeat domain containing 7 (*FBXW7*), *PKHD1*, and FAT atypical cadherin 1 (*FAT1*). The results of the immunohistochemical analysis yielded a combined positive score (CPS) of 95 and a tumor proportion score of 95% for PD-L1, which indicated positivity (**Figure 2A**).

Based on the results of two major clinical trials, namely KEYNOTE-048 and KEYNOTE-040, an immunotherapy was considered for this patient. On February 29, 2020, the patient received the first cycle of treatment with albumin paclitaxel (100 mg) in combination with sintilimab (200 mg). However, a reexamination using a head and neck CT on March 11, 2020 revealed progression of the tumor mass (13 × 10 cm) (**Figures 1D–F**).

Despite previous treatment, the tumor remained uncontrolled. Moreover, the general condition of the patient was deteriorating. We reviewed clinical trials involving combinations of immunotherapy and targeted therapy using the PubMed database

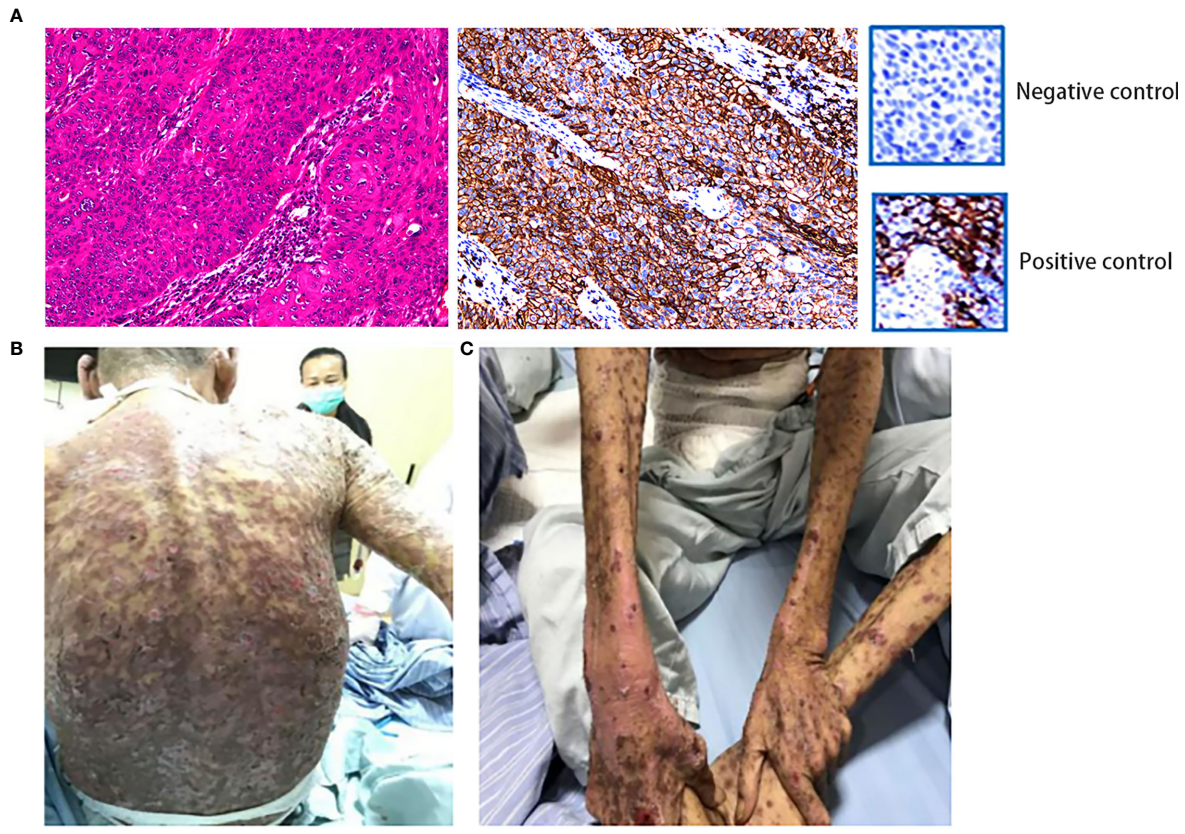

FIGURE 2 | **(A)** IHC showed positivity for PD-L1. (Dako 22C3 antibody was used to provide positive control and negative control.) This specimen was sliced from formalin-fixed paraffin-embedded tissue. **(B, C)** The patient developed grade IV drug-induced dermatitis after the first course of combination therapy with sintilimab and nimotuzumab. HE, hematoxylin–eosin; IHC, immunohistochemistry; PD-L1, programmed death-ligand 1.

(National Institutes of Health, Bethesda, MD, USA). Most of them were phase II studies without declared preliminary results. We communicated with the family of the patient and suggested using the combination of immunotherapy and targeted therapy. On March 20, 2020, the patient received sintilimab (200 mg, once every 3 weeks) and nimotuzumab (200 mg, once weekly). Four days after the treatment, the patient developed grade IV drug dermatitis (**Figures 2B, C**). The patient recovered after treatment with glucocorticoids, antiallergic agents, and other symptomatic treatments (e.g., relief of itching and promotion of mucosal repair).

On April 11, 2020, a head and neck CT revealed regression of the tumor mass (6 × 7 cm) and partial response (PR) to the therapy (**Figures 1G–I**).

The general condition of the patient improved 1 month later. Subsequently, he received two additional cycles of sintilimab (200 mg, once every 3 weeks) and nimotuzumab (100 mg once weekly) from April 24 to May 18, 2020. Considering that the dermatitis had not been completely resolved, the dose of nimotuzumab was reduced by half. The patient was reexamined on May 18, 2020; head and neck MRI showed a maximum tumor mass diameter of 3 × 4 cm and PR to therapy (**Figures 1J–L**).

On June 12, 2020, the patient underwent a routine review. Unfortunately, physical examination revealed an enlargement of the tumor mass (**Figures 3A–C**).

Subsequently, radiotherapy of the cervical lesions was performed. The intensity-modulated radiation therapy plans were adopted (dose total: planning gross tumor volume: 64 Gy/31 F; planning clinical tumor volume: 56 Gy/31 F). On June 16, 2020, the patient underwent concurrent treatment with sintilimab (200 mg, once every 4 weeks) and nimotuzumab (200 mg, once weekly). From July 7 to August 14, 2020, he received two cycles of treatment with sintilimab (200 mg, once every 4 weeks) and nimotuzumab (200 mg, once every 2 weeks). On August 13, 2020, a boost radiotherapy dose (dose total: planning gross tumor volume: 10 Gy/4 F) was administered for residual lesions. The patient completed the radiotherapy on August 20, 2020. Compared to baseline imaging data before radiotherapy

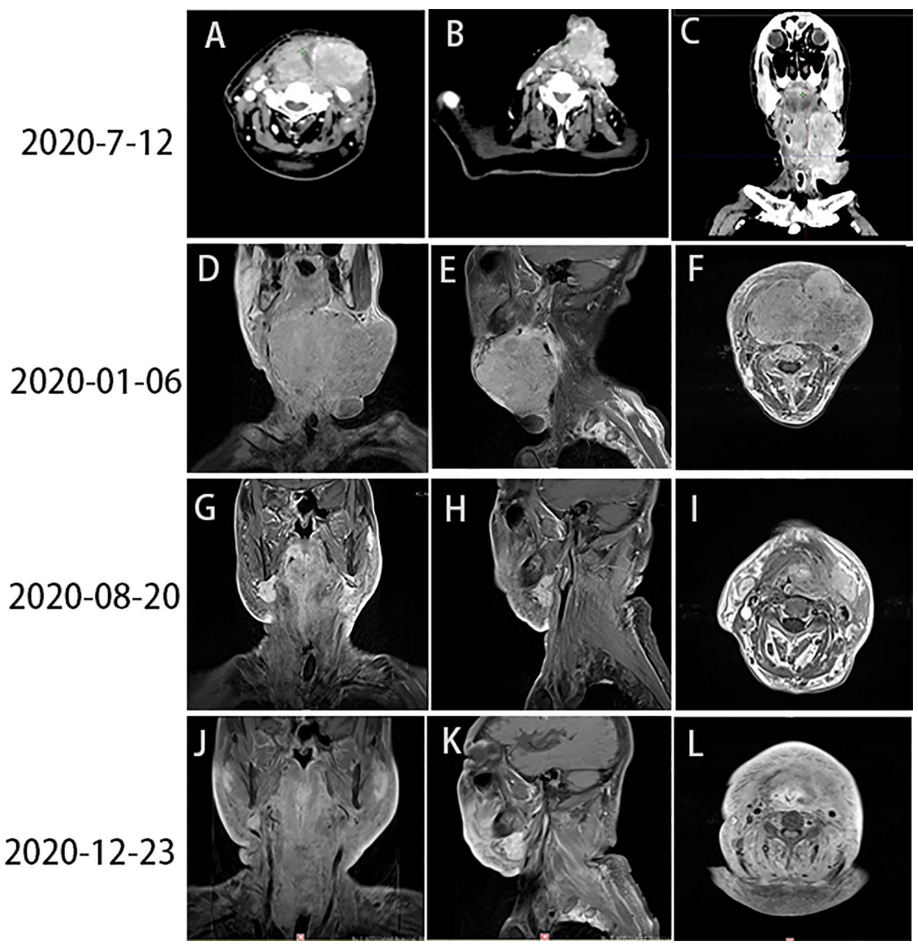

FIGURE 3 | **(A–C)** Head and neck CT revealed PD (June 12, 2020). **(D–F)** Images captured prior to radiotherapy (January 6, 2020). **(G–I)** Images captured after the end of radiotherapy (August 20, 2020). **(J–L)** The tumor mass had almost completely regressed by December 23, 2020. CT, computed tomography; PD, progressive disease.

(**Figures 3D–F**), reexamination revealed that the size of the neck mass was significantly reduced (**Figures 3G–I**).

The last follow-up visit of this patient was conducted on December, 2020. His general state was markedly improved, and the Karnofsky Performance Status score was 80. A head and neck MRI revealed that the neck mass had nearly disappeared (**Figures 3J–L**). The entire treatment process and disease status of the patient are summarized in **Figure 4**.

DISCUSSION

Overall Prognosis of R/M HNSCC

Head and neck carcinoma is the seventh most common type of cancer worldwide, with approximately 800,000 new cases and >500,000 deaths reported annually (1). More than 65% of patients with this disease may develop R/M HNSCC, which was considered incurable in the past (2).

Interpretation of the Classic EXTREME and Chinese CHANGE-2 Studies *Versus* the Outcome of the Present Case

Before the advent of immunotherapy, the EXTREME trial was the first to investigate the addition of targeted therapy to the traditional chemotherapy regimen. This study established the combination of cetuximab (EGFR monoclonal antibody) with 5-FU and platinum for the first-line treatment of R/M HNSCC. Following the addition of the EGFR monoclonal antibody, the OS of patients was extended from 7.4 to 10.1 months,; the PFS was also extended by 2.3 months compared with chemotherapy alone (5.6 *vs.* 3.3 months, respectively). The CHANGE-2 study, involving a Chinese population, lowered the drug dose on the basis of the EXTREME study. The results of both studies were similar. The PFS and OS of patients in the CHANGE-2 study were significantly prolonged (5.5 months *vs.* 4.2 months and 10.2 months *vs.* 8.4 months, respectively). The ORR of patients who underwent chemotherapy combined with cetuximab was 50%; this rate was significantly higher than that of patients who underwent chemotherapy alone (26.6%). With reference to the results of the international EXTREME study and the Chinese CHANGE-2 study, the patient in this case was treated with nimotuzumab (EGFR monoclonal antibody) combined with chemotherapy (albumin paclitaxel). However, the effect was not significant, and progressive disease was detected after approximately 1 month.

Interpretation of Advances in Immunotherapy for HNSCC *Versus* the Present Case

In the 10 years following the approval of targeted therapy, the emergence of immunotherapy has further improved the survival and prognosis of patients with R/M HNSCC. The early KEYNOTE-012 phase Ib study and CheckMate-141 phase III clinical trial showed that single-agent immunotherapy as second-line treatment significantly prolonged the OS of patients with R/M HNSCC (3, 4), thereby supporting the use of immunotherapy in this setting. The KEYNOTE-048 study included 882 untreated R/M HNSCC patients with positive PD-L1 expression (CPS ≥1/ CPS ≥20). The study compared single-agent immunotherapy, immunotherapy combined with chemotherapy, and the classic regimens used in the EXTREME study. The results showed that single-agent immunotherapy with pembrolizumab was associated with fewer adverse reactions compared with the EXTREME study regimens. Moreover, both single-agent immunotherapy and the combination therapy of chemotherapy and immunotherapy showed a longer OS *versus* the EXTREME study regimen. Among the study population, the patients with a CPS ≥20 exhibited a higher 4-year OS than those with a CPS ≥1 (28.6% *vs.* 19.4%, respectively), suggesting that higher expression of PD-L1 may be associated with longer OS. The present patient had a CPS of 95, which indicated high expression of PD-L1; hence, the patient belonged to the population that can benefit from immunotherapy. Therefore, treatment with sintilimab (200 mg) combined with albumin paclitaxel (100 mg) was initiated. However, the tumor was enlarged after 2 weeks, possibly due to insufficient activation of the immune system. The present case demonstrates that patients with high expression of immune biomarkers may have better disease control following treatment with the combination of chemotherapy and immunotherapy.

Interpretation of the Progress in the Feasibility and Safety of Clinical Trials of Immunotherapy Combined With Targeted Therapy for Advanced HNSCC

Regimens without chemotherapeutic agents, which can reduce the serious adverse reactions caused by chemotherapy, have attracted considerable attention in clinical treatment. Nevertheless, it is important to investigate the safety challenges associated with immunotherapy and targeted therapy. According to the KEYNOTE-040 trial, treatment with pembrolizumab significantly reduced the risk of death compared with cetuximab monotherapy (hazard ratio = 0.56). The safety profile of immunotherapy combined with targeted single-drug therapy is currently being investigated, with limited published clinical data thus far. Among them, the median PFS of immunotherapy-naïve patients treated with nivolumab combined with cetuximab was 6.0 months (5). The overall safety was good, with fatigue (13%) and skin rash (4.4%) being the most common adverse reactions. The preliminary results of another phase II clinical trial (NCT03082534) showed that pembrolizumab combined with cetuximab exerts a considerable therapeutic effect on platinum-refractory patients with R/M HNSCC, with a median PFS of 8.2 months (6). Similarly, rash was the most commonly recorded immune-related adverse reaction. In the present case, the severity of dermatitis also increased during the combination therapy. As an immunoglobulin G1 (IgG1) molecule, cetuximab can induce antibody-dependent cellular cytotoxicity, in addition to blocking the activation of EGFR. Subsequently, it generates specific T cells to produce a sustained immune response. This effect is thought to be tumor immune infiltration induced by cetuximab, thereby

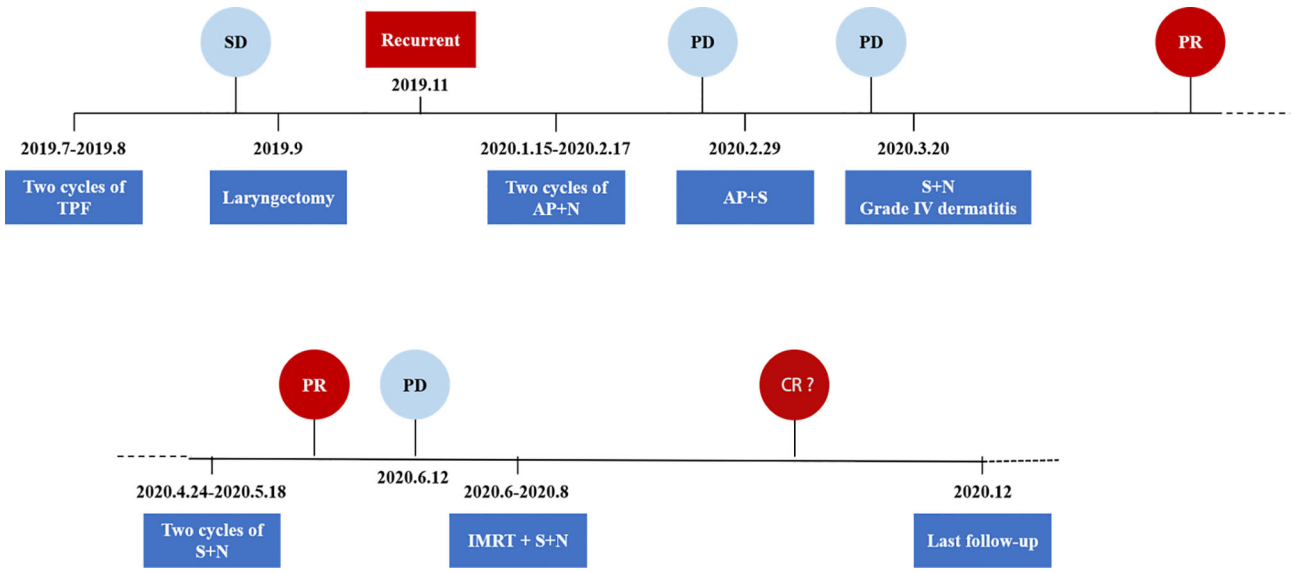

FIGURE 4 | Schematics and timeline of treatment. AP, albumin paclitaxel; CR, complete response; N, nimotuzumab; PD, progressive disease; PR, partial response; S, sintilimab; SD, stable disease; TPF, docetaxel, cisplatin, and 5-fluorouracil.

restoring the immune suppression of the HNSCC tumor microenvironment (7, 8). The nimotuzumab and cetuximab used in this case are anti-EGFR extracellular IgG1 antibodies shown to induce the production of EGFR-specific T cells (9).

The subsequent intense immunotherapy-related response of the patient (including adverse reactions and tumor control) may be related to the activation of the immune system and promotion of the immunotherapy response by nimotuzumab. Zhou et al. retrospectively analyzed the efficacy and survival data of 4,971 patients who had received immunotherapy. They found that patients who developed adverse reactions benefited from the treatment in terms of OS and PFS (hazard ratios = 0.54 and 0.52) compared with those who did not report adverse effects (10). It is thought that adverse reactions related to immunotherapy may be linked to immune initiation triggered by tumor antigens released after treatment or humoral immune disorders (11, 12). More basic and prospective clinical research studies are warranted to further investigate the mechanism involved in this process.

How to Improve the Anti-Tumor Activity of Immunotherapy? How Can Immunotherapy and Other Treatments Be Combined to Release More Antigens for the Activation of the Immune System?

In the present case, the combination of radiotherapy, immunotherapy, and targeted therapy eventually resulted in an excellent curative effect, thereby preventing the risk of disease hyperprogression. Could this be a new approach to immunotherapy?

Improving the immune microenvironment and activating the immune response are currently the main methods used to enhance the anti-tumor activity of immunotherapy. In this case, the addition of an EGFR monoclonal antibody induced the production of specific T cells, which could stimulate the response of the patient to immunotherapy. In a recent study, the combination of indoleamine 2,3-dioxygenase-1 (IDO1) inhibitors and PD-L1 also achieved some initial promising results in the treatment of HNSCC (13).

In addition, the combination of immunotherapy with radiotherapy or chemotherapy is also widely used. Studies have shown that PD-L1 is upregulated within 24–48 h after radiotherapy, activating the immune microenvironment (14, 15). At present, research studies on chemoradiotherapy combined with immunotherapy using different PD-1/PD-L1 monoclonal antibodies are ongoing. Among them, KEYNOTE-412 (NCT02586207), which is study combining pembrolizumab, radiotherapy, and chemotherapy, has demonstrated the safety and feasibility of this regimen. Furthermore, large-scale phase III clinical studies are also underway.

CONCLUSION

In the present case, the combination of immunotherapy with radiotherapy resulted in better tumor remission. These results suggest that patients with HNSCC may benefit from a therapeutic strategy combining immunotherapy with radiotherapy or chemotherapy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Fifth Affiliated Hospital of Guangzhou Medical University. 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

Case report design: all authors. XS and HN contributed to the content of the article. Data collection and patient follow-up: HN and TC. Drafting of the manuscript: HN and TC. KH, CJL, and WG reviewed the literature and clinical data. All authors contributed to the article and approved the submitted version.

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REVIEW
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Immunotherapy in Recurrent/Metastatic Squamous Cell Carcinoma of the Head and Neck

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Head and neck cancer is the 6th most common cancer worldwide with the most common histology being squamous cell carcinoma (HNSCC). While the majority of patients present at a stage where curative intent therapy is possible, when patients recur and/or develop metastatic disease, outcomes are generally poor, especially with systemic therapy alone, and they lag behind other solid tumors. Over the last decade immunotherapy has revolutionized the field of oncology, and anti-PD-1-based therapy has changed the standard of care in recurrent/metastatic (R/M) HNSCC as well. With these gains have come new questions to continue to move the field forward. In this review, we discuss the tumor immune microenvironment and predictive biomarkers and current status and future directions for immunotherapy in recurrent/metastatic head and neck cancer.

Keywords: head and neck cancer, HNSCC, recurrent, metastatic, systemic therapy, immunotherapy, PD-1, PD-L1

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INTRODUCTION

Head and neck cancer is the 6th most common cancer worldwide, and while it includes many histologies, squamous cell carcinoma represents 90% of diagnosis, with the most common primary sites being oral cavity, hypopharynx, larynx, and oropharynx (1). In addition to traditional risk factors of smoking and alcohol, there are two virally driven cancers, the Epstein Barr Virus (EBV) in the nasopharynx and the Human Papillomavirus (HPV) in the oropharynx, with the latter associated with a significantly better prognosis (2). While the majority of squamous cell carcinoma of the head and neck (HNSCC) patients present at a stage where therapy is definitive, with only 10% presenting with distant metastatic disease, a large proportion of patients, especially HPV negative HNSCC, will recur. In the recurrent/metastatic (R/M) setting there is a great need for improvement in outcomes, especially when treatment is with systemic therapy alone. Immunotherapy has changed our standard-of-care approach and improved outcomes in this setting, but there is still more work to do to continue to move the needle forward. In this review we detail the current status of immunotherapy in R/M HNSCC, predictive biomarkers, and future directions in the field.

THE TUMOR IMMUNE MICROENVIRONMENT IN HNSCC

Antitumor immunity is a back-and-forth duel between the immune system and the cancer. The cancer immunoediting theory hypothesizes that at first the immune system recognizes and

eliminates all cancer cells, then the cancer evades the immune system such that only equilibrium is achieved in which tumor growth is controlled but not eradicated, followed by the "escape" phase whereby the tumor fully eludes the immune system and progresses clinically (3). Numerous steps need to occur in order for the immune system to achieve effective cancer killing. The process begins with the release of cancer neoantigens and their uptake by antigen-presenting cells such as dendritic cells (DC) with subsequent required signaling to move forward with presentation on MHC I and MHC II molecules to T cells (4). Next, effector T cells are activated and then migrate to and infiltrate the tumor microenvironment (5, 6). Then finally T cells bind to the target cancer cells *via* their T cell receptors (TCR) and kill them *via* multiple mechanisms (7, 8). HNSCC, like other cancers, can evade or suppress the immune response at each of these steps. For example, in HNSCC, the tumor-infiltrating T cells can be compromised *via* functional defects leading to decreased proliferation in response to cytokines, impaired ability to kill tumor cells, and suppressed IL-1 and/or IFN-γ production (9–13). Moreover, the cytotoxic properties of NK cells are inhibited *via* TGF- β1 overexpression that leads to reduced expression of NK cell receptors KHG2D and CD16 (14). HNSCC can modulate the immune response to favor induction and conversion to immunosuppressive cells such as Tregs, which are abundant in the tumor microenvironment (TME) as well as peripheral blood, exerting their immunosuppressive function by inducing apoptosis of CD8+ T cells and inhibiting proliferation of CD4+ T cells (15–17). Additionally, Myeloid-Derived Suppressor Cells (MDSCs) can inactivate T cells *via* production of arginase-1 and inducible NO synthase (18, 19). Finally, stromal fibroblasts as well as non-cellular components of TME including growth factors, glycoproteins, and structural proteins produced by Extracellular Matrix (ECM) further enhance tumor invasion, migration, and progression (20–23).

Another important mechanism the tumor uses to modify the immune response and block antitumor immunity is *via* manipulation of co-signaling molecule signaling. Co-signaling molecules can be stimulatory or inhibitory on immune function. This includes the most studied and clinically relevant programmed cell death protein 1 (PD-1): Programmed death ligand 1 (PD-L1) pathway. PD-1 is a member of the CD28 family of T-cell costimulatory receptors and is expressed on activated T cells, B cells, and monocytes (24–26). In addition to tumor cells, PD-L1 is expressed on activated T cells, B cells, NK (natural killer) cells, dendritic cells, macrophages, and non-hematopoietic cells (27, 28). Importantly, PD-L1 can be upregulated in tumor cells *via* inflammatory signals, mainly under the influence of IFN-γ produced by immune cells and activation of downstream pathways such as EGFR, MAPK, or PI3K-Akt (29–34). Even before monoclonal antibodies made it into the clinic, PD-L1 expression was observed in HNSCC, ranging from 46 to 100% in primary, recurrent, and metastatic settings (34–40). The ligation of PD-1 by PD-L1 or PD-L2 suppresses antitumor response *via* effector T-cell exhaustion and/or apoptosis (26). In addition to the effector T cell tumor interface, antitumor immunity can be induced by blockade of the PD-L1:PD-1 pathway on dendritic cells, resulting in increased CD8-positive T cell infiltration of the tumor and suppression of the inhibitory ability of Tregs either directly or indirectly through augmentation of CTL proliferation (41, 42). Moreover, PD-L1 can ligate B7-1 (CD80), a costimulatory molecule found on T cells, that regulates the downstream immune responses through the PD-1 pathway (43). Other relevant inhibitory co-signaling molecules expressed in HNSCC that are already the target of therapeutic intervention include CTLA4, LAG3, B7-H3, TIGIT, TIM3, and stimulatory OX40, ICOS, GITR, and 4-1BB (44, 45).

In HNSCC the tumor immune microenvironment (TIME) has been analyzed *via* various methods ranging from immunohistochemistry to genomic and transcriptomic analysis, examining the effect of HPV, molecular smoking signatures, and other genomic predictors (**Figure 1**). Using bulk RNA sequencing data from The Cancer Genome Atlas (TCGA), HNSCC tumors showed high levels of immune infiltration, including NK cells, with the highest infiltration by Tregs and Treg/CD8 ratio, compared to nine other solid tumors including NSCLC, RCC, melanoma, and breast (46). Delineating a T cell inflamed phenotype (TCIP) using a validated chemokine gene expression signature, 34% of HNSCC tumors were characterized as high, 32% intermediate, and 34% low. TCIP high phenotype correlated with increased CD8 T cell infiltration and mesenchymal subtype but also increased exhaustion/cytotoxic CD8 T cell ratio and higher inhibitory co-signaling molecule expression of PD-L1, PD-1, CTLA4, TIM3, and LAG3 compared to TCIP-low. TCIP high tumors were enriched in pathways including JAK-STAT, NFkB, TNF, RAS, PI3K/AKT, and MAPK, whereas Hedgehog and WNT/B-catenin signaling was associated with TCIP low (47).

Multiple studies have compared the TIME by HPV status. Mandal and colleagues observed that HPV positive HNSCC was associated with a higher immune infiltrate and activation status by a cytolytic score, as well as increased Treg and Treg/CD8 ratio compared to HPV negative. Specifically, in regard to Tregs, other studies have similarly found an increase in HPV positive HNSCC, while other analysis have not shown a difference by HPV status (46, 48, 49). The TIME of HPV positive HNSCC has been observed to have increased NK cells, M1 macrophages (as compared to M2), and CD8 T cells, with more limited studies showing no difference in MDSCs by HPV status (47, 49–54). Using single-cell RNA sequencing, Cillo and colleagues found HPV-positive tumors to be enriched in CD4 conversion cells with different differentiation trajectories and have increased germinal center B cells with increased ligand/receptor interactions between these B cells and T follicular helper cells, compared to HPV negative (48). Other studies have also shown an increase in B cells and that the B cells or more activated in HPV-positive tumors (49, 55, 56). HPV-positive HNSCC was observed to have a higher percentage of tumors with TCIP high phenotype compared to negative, with 51 *vs.* 21% TCIP high, respectively. Dividing HNSCC into previously established molecular subtypes atypical, basal, classical, and mesenchymal, the atypical and mesenchymal subtypes had the highest degree of immune infiltration and activity, with HPV-positive HNSCC

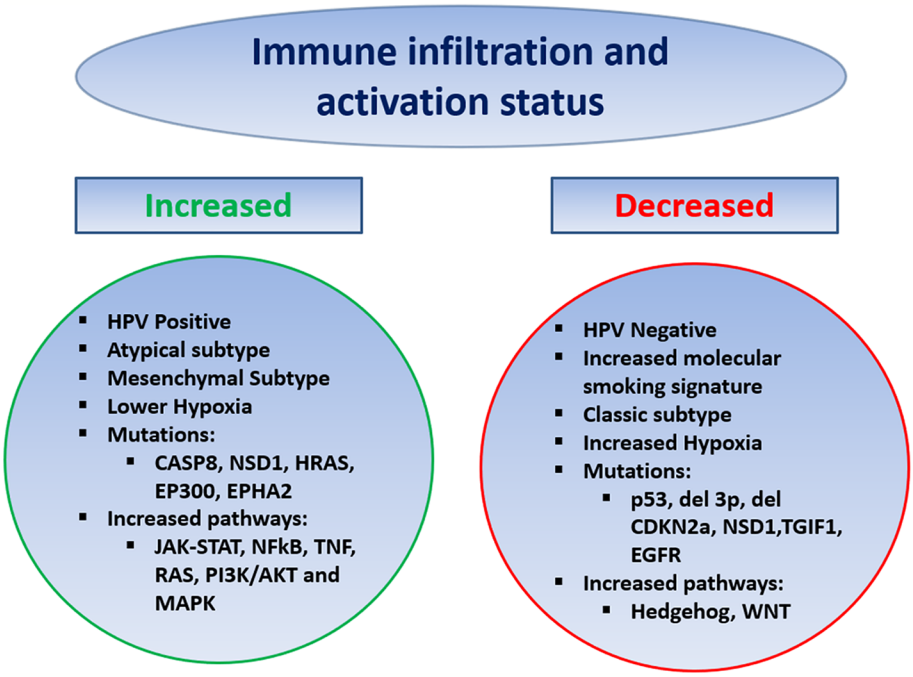

FIGURE 1 | Predictors of the Tumor Immune Microenvironment in HNSCC.

making up the majority of the atypical subtype, while the classical subtype, which most resembles SCC of the lung, had the lowest (46).

Tobacco remains a risk factor for HNSCC, and multiple studies have found an increased molecular smoking signature is associated with a significantly higher mutational burden but lower immune infiltration and activation, independent of HPV status, and was a stronger predictor than reported smoking history. The smoking signature was higher in p53 mutated patients and larynx primary site (46, 57). Interestingly, the opposite was seen in SCC of the lung where higher smoking signature was associated with increased immune infiltration, potentially driven by increased inflammatory response in the lungs compared to the mucosa of the head and neck (57). Various mutations have correlated with the TIME including p53 mutations, deletion of chromosome 3p, deletion of CDKN2a, as well as NSD1, TGIF1, and EGFR mutations associated with lower immune infiltration/activation status, whereas mutation in CASP8, NSD1, HRAS, EP300, and EPHA2 has been associated with increased immune infiltration and activation (46, 47, 57). Increased intratumoral hypoxia has been associated with a more immune-suppressed TIME in HNSCC (58, 59).

Taken together, HNSCC while heterogeneous is generally associated with a TIME that may be infiltrated with immune cells but also one in which immune regulatory mechanisms are abundant. Amongst HNSCC, including HPV positive, there is a range of immune infiltration and activation status. Numerous studies have looked at the prognostic implications of various features of the TIME including cellular populations and co-signaling molecule expression, with overall conflicting data on prognostic implications, likely owing the limitations of looking at static isolated features amongst a dynamic immune response (49). It should be noted that most of our studies in HNSCC that guide our understanding of the TIME, including the important genomic and transcriptomic studies highlighted above, analyzed tumors mostly from the upfront locally advanced setting. Data are significantly limited on the changes in the TIME at recurrence after curative intent therapy. Waterman et al. uniquely compared paired primary and recurrent tumors and found a significant decrease in B cells, CD8 T cells, and NK cells, and a downward trend in CD8/Treg ratio in recurrent tumors. Additionally, receipt of adjuvant chemoradiation was associated with a significant decrease in B cells and a greater decrease in CD8/Treg ratio, an increase in macrophages and neutrophils of myeloid lineage, as well as downregulation of genes associated with cytokines and B cell immune response (60). Thus, the tumor microenvironment of recurrent/metastatic patients likely represents a more immune-suppressed phenotype compared to initial presentation.

Immunotherapy seeks to reverse the tumor-driven evasion and downregulation and use the immune system to eradicate cancer. In HNSCC, like other solid tumors, this has mostly been in the form of agents targeting co-signaling molecules, especially the PD-1:PD-L1 pathway which have changed standard of care. However, there is also ongoing evaluation of other checkpoint inhibitors, vaccines, as well as T cell therapy and additionally how chemotherapy and radiation can enhance immunotherapy.

IMMUNOTHERAPY AS STANDARD OF CARE IN R/M HNSCC

Prior to immunotherapy, platinum-based chemotherapy with or without cetuximab had been the standard systemic treatment for R/M HNSCC for over a decade (61). Unfortunately, the prognosis of patients receiving chemotherapy was poor, especially in the platinum failure setting (62). After promising efficacy of anti-PD-1/PD-L1 mAbs in smaller single-arm trials, randomized phase III trials first in the platinum failure setting and then in the frontline setting were conducted and have changed the standard of care systemic treatment for R/M HNSCC patients. Results of these phase III trials are summarized in **Table 1**.

Nivolumab and Pembrolizumab, both IgG4 anti-PD-1 monoclonal antibodies, were evaluated in phase III trials in R/M HNSCC patients with oral cavity, oropharynx, larynx, or hypopharynx primary after failure of platinum-based chemotherapy, and compared to investigator choice chemotherapy (Docetaxel, Cetuximab, or Methotrexate). Platinum failure was defined as progression within 6 months of platinum-based chemotherapy for R/M disease or within 6 months of platinum-based chemoradiation given in the curative intent setting. CHECKMATE-141 was the first phase III clinical trial to report efficacy and demonstrated significantly longer OS with nivolumab compared to chemotherapy (hazard ratio [HR] for death 0.70, 95% CI [0.51, 0.96], p=0.01). Importantly, nivolumab was better tolerated (G3/4 AEs 13.1 *vs.* 35.1% for nivolumab *vs.* chemotherapy respectively) and improved quality of life (63, 64). With these positive results, Nivolumab became the first therapeutic to significantly improve overall survival in R/M HNSCC patients that had failed platinum-based chemotherapy (63). In KEYNOTE 040, a similarly designed trial, pembrolizumab also improved overall survival compared to chemotherapy (65). Notably, both trials did not require PD-L1 expression for entry, and the primary endpoint was not powered by PD-L1 status. Neither study showed a significant difference in progression-free survival (PFS). Similar to other solid tumors, prolongation of overall survival but not PFS was likely driven by most patients on anti-PD-1 mAb progressing at first imaging evaluation, with the durability of the therapeutic effect for responders, and also a proportion of those with stable disease, driving the OS benefit. For example, while only 13% had a response with Nivolumab, the duration of response was a median of 9.7 months (2.8 to 32.8+), more than double that of chemotherapy (66). Based on the findings of CHECKMATE-141 and KEYNOTE-040, the FDA approved nivolumab and pembrolizumab monotherapy in patients with R/M HNSCC who had failed platinum-based chemotherapy in 2016.

Anti-PD-L1 mAbs as monotherapy and in combination with anti-CTLA4 mAb have also been evaluated in the platinum failure setting. After initial phase II trials with durvalumab in PD-L1 high (HAWK) and durvalumab, durvalumab plus tremelimumab, or tremelimumab alone in PD-L1 low patients (CONDOR), the phase III EAGLE trial was initiated and randomized platinum failure R/M HNSCC patients to durvalumab plus tremelimumab, durvalumab monotherapy, or investigator choice standard of care chemotherapy. This trial was dually powered for OS comparison of durvalumab and combination durvalumab plus tremelimumab separately, compared to chemotherapy. There was no difference in OS with durvalumab (HR 0.88, 95% CI [0.72, 1.08], p=0.20) or durvalumab plus tremelimumab. (HR 1.04, 95% CI [0.85, 1.26], p=0.76) compared to chemotherapy. Accepting the limitations of cross-trial comparisons, it is notable that while the median OS with durvalumab was similar to nivolumab in Checkmate 141 (7.6 *vs.* 7.5 months, respectively), the median OS of the control arm was numerically longer in EAGLE compared to CHECKMATE 141 (8.3 months *vs.* 5.1 months respectively). Exploratory analysis from EAGLE suggests that this higher-than-expected OS in the control group may have come from imbalance in baseline characteristics (higher percentage of ECOG PS 0 and distant metastasis only in the control arm), increased usage of paclitaxel in the control arm, which was not a choice in CHECKMATE 141 or KEYNOTE 040, and subsequent receipt of anti-PD-1 mAb therapy (67). Notably, there are differences between anti-PD-1 and PD-L1 mAbs. Both block the interaction of PD-1:PD-L1, but anti-PD-L1 mAb's block the interaction of PD-L1:CD80, whereas anti-PD-1 mAbs inhibit the ligation of PD-1 by PD-L2. However, whether this difference has a clinically relevant effect is not known.

Success in the platinum failure setting led to evaluation of immunotherapy in the frontline systemic treatment of R/M HNSCC patients. The phase III randomized trial KEYNOTE-048 evaluated pembrolizumab monotherapy and platinum/5FU/pembrolizumab each separately compared to the EXTREME regimen (platinum/5FU/cetuximab) for the total population, PD-L1 combined positive score (CPS) ≥1, and ≥ 20 (**Table 1**). CPS was defined as the number of PD-L1–positive cells [tumor cells, lymphocytes, macrophages] divided by the total number of tumor cells × 100. Pembrolizumab monotherapy significantly improved OS in patients with CPS ≥1 and ≥20. While the response rate was lower than chemotherapy (19–21 *vs.* 36%, respectively), the median duration of response with pembrolizumab monotherapy was fivefold higher (median 20.9 *vs.* 4.5 months, respectively). Chemotherapy plus pembrolizumab significantly improved OS for all three populations. There was no significant difference in response rate and PFS between chemotherapy plus pembrolizumab and the EXTREME regimen. As expected, pembrolizumab monotherapy was associated with less toxicity, while a similar rate of adverse events occurred with platinum/5FU/pembrolizumab as compared to EXTREME (68). This led to the FDA approval in 2019 of platinum/5FU plus pembrolizumab for all patients and pembrolizumab monotherapy only for patients with a PD-L1 CPS ≥1. The phase III KESTREL trial randomized patients 2:1:1 to durvalumab alone, durvalumab plus tremelimumab, and the EXTREME regimen. The primary endpoint was OS for durvalumab monotherapy *vs.* EXTREME in PD-L1 high expressers (tumor cell expression of >50% or tumor-infiltrating lymphocyte expression >25%) and secondary endpoint of OS for durvalumab plus tremelimumab *vs.* EXTREME for all patients. While the data are not available yet from the trial, by press release it was announced that the trial had failed to meet these endpoints.

KEYNOTE 048 importantly represents the first change in frontline therapy since the EXTREME regimen in 2009; however,

TABLE 1 | Completed Phase III studies of anti-PD-1/PD-L1 mAb therapy in Recurrent/Metastatic HNSCC.

Study	Study agents	Setting		ORR[1]	OS, HR, (95% CI), P value[2]	PFS, HR, 95% CI, P value[3]	FDA approval
CHECKMATE 141	(1) Nivolumab	Platinum Failure		(1) 13.3%	(1) 7.5, HR 0.70 (0.51, 0.96), p:0.01	(1) 2.0, HR 0.89 (0.70, 1.13)	Platinum Failure
	(2) CONTROL: MTX, Docetaxel, or Cetuximab			(2) 5.8%	(2) 5.1, reference	(2) 2.3, reference	
KEYNOTE 040	(1) Pembrolizumab	Platinum Failure		(1) 14.6%	(1) 8.4, HR 0.80 (0.65, 0.98), p:0.0161	(1) 2.1, HR 0.96 (0.79, 1.16)	Platinum Failure
	(2) CONTROL: MTX, Docetaxel, or Cetuximab			(2) 10.1%	(2) 6.9, reference	(2) 2.3, reference	
EAGLE	(1) Durvalumab	Platinum Failure		(1) 17.9%	(1) 7.6, HR 0.88 (0.72, 1.08)	(1) 2.1, HR 1.02 (0.84, 1.25)	No
	(2) Durvalumab + Tremelimumab			(2) 18.2%	(2) 6.5, HR 1.04 (0.85, 1.26)	(2) 2.0, HR 1.09 (0.90, 1.33)	
	(3) CONTROL: MTX, Taxane, Cetuximab, 5-FU, Capecitabine, TS-1			(3) 17.3%	(3) 8.3, reference	(3) 3.7, reference	
KEYNOTE 048	(1) Pembrolizumab	First line	Total population	(1) 17%	(1) 11.6, HR 0.85 (0.71, 1.03)	(1) 2.3, HR 1.34 (1.13, 1.59)	First Line Treatment
	(2) Pembrolizumab + Platinum + 5-FU			(2) 36%	(2) 13.0, HR 0.77 (0.63, 0.93), p:0.0034	(2) 4.9, HR 0.92 (0.77, 1.10)	1. Pembrolizumab plus platinum/ 5-FU for all patients
	(3) CONTROL: Cetuximab + Platinum + 5-FU			(3) 36%	(3) 10.7, reference	(3) 5.2, reference	
			PD-L1 CPS ≥1	(1) 19%	(1) 12.3, HR 0.78 (0.64, 0.96), p:0.0086	(1) 3.2, HR 1.16 (0.96, 1.39)	2. Pembrolizumab monotherapy for CPS ≥1
				(2) 36%	(2) 13.6, HR 0.65 (0.53, 0.80), p:<0.0001	(2) 5.0, HR 0.82 (0.67, 1.00)	
				(3) 36%	(3) 10.4, reference	(3) 5.0, reference	
			PD-L1 CPS ≥20	(1) 31%	(1) 14.8, HR 0.58 (0.44, 0.78), p:0.0007	(1) 3.4, HR 0.99 (0.76, 1.29)	
				(2) 54%	(2) 14.7, HR 0.60 (0.45, 0.82), p:0.0004	(2) 5.8, HR 0.76 (0.58, 1.01)	
				(3) 44%	(3) 11.0, reference	(3) 5.3, reference	
			PD-L1 CPS <1[4]	(1) 2%	(1) 7.9, HR 1.51 (0.96, 2.37)	(1) 2.1, HR 4.31 (2.63, 7.08)	
				(2) 12%	(2) 11.3, HR 1.21 (0.76, 1.94)	(2) 4.7, HR 1.46 (0.93, 2.30)	
				(3) 19%	(3) 11.3, reference	(3) 6.2, reference	
			PD-L1 CPS 1-19[4]	(1) 18%	(1) 10.8, HR 0.86 (0.66, 1.12)	(1) 2.2, HR 1.25 (0.96, 1.61)	
				(2) 34%	(2) 12.7, HR 0.71 (0.54, 0.94)	(2) 4.9, HR 0.93 (0.71, 1.21)	
				(3) 45%	(3) 10.1, reference	(3) 4.9, reference	
JUNIPER-02[5]	(1) Cisplatin + Gemcitabine + Camrelizumab	First Line		(1) 88%	(1) Not Reached, HR 0.67 (0.41,1.11)	(1) 10.8,HR 0.51 (0.37,0.69), p<0.001	Pending
	(2) Cisplatin + Gemcitabine + Placebo			(2) 81%	(2) 22.6, reference	(2) 6.9, reference	

[1]*Overall Response Rate.*

[2]*Overall survival in months (median), Hazard ratio, 95% Confidence interval, P value shown if significant.*

[3]*Progression-free survival in months (median), Hazard ratio, 95% Confidence interval, P value shown if significant.*

[4]*Data retrieved from exploratory post-hoc analysis of KEYNOTE 048, p values are not applicable.*

[5]*JUNIPER-02 included nasopharyngeal carcinoma only. All other studies listed included squamous cell carcinoma of the oral cavity, oropharynx, larynx, hypopharynx.*

MTX, Methotrexate; 5-FU, 5-Fluorouracil; CPS, Combined Positive Score.

questions that effect day-to-day practice remain. One is whether patients with a CPS ≥20, which represented 44% of PD-L1 expressers, drove the benefit with pembrolizumab monotherapy in the CPS ≥1 group. Put another way, is pembrolizumab monotherapy enough for a patient with a PD-L1 CPS 1-19? Exploratory subgroup analysis from KEYNOTE 048 showed there was still a benefit from pembrolizumab compared to EXTREME for CPS 1-19 (HR 0.86 95% CI [0.66–1.12]) albeit less benefit relative to CPS ≥20 patients (HR 0.58 95% CI (0.44–0.78)) (69). In practice, the decision to choose pembrolizumab monotherapy *versus* chemotherapy plus pembrolizumab for a patient with CPS 1-19 depends on multiple patient and disease factors, such as tumor and symptom burden, comorbidity, and performance status. In patients with a PD-L1 CPS 1-19 with high tumor burden and/or significant symptoms that can tolerate chemotherapy, we favor chemotherapy plus pembrolizumab as a standard of care treatment, to maximize potential response, which can translate directly into a quality-of-life benefit. Additionally, the total population is not the same as PD-L1 negative patients, which accounted for only 15% of the patients in the trial. In practice most providers will know if a patient has a PD-L1 CPS <1. Subgroup analysis for PD-L1 negative patients treated with chemotherapy plus pembrolizumab favored the EXTREME regimen with a HR of 1.22 (95% CI [0.76–1.94]) (69); however, this should not affect practice given very small patient numbers in this cohort, and chemotherapy plus pembrolizumab is still the new standard of care for a patient with known PD-L1 negative status.

In summary, current frontline standard of care systemic therapy options for R/M HNSCC of the oral cavity, oropharynx, larynx, and hypopharynx include pembrolizumab monotherapy or platinum/5FU plus pembrolizumab for PD-L1 expressers by CPS or platinum/5FU plus pembrolizumab for all patients. While this change in frontline systemic therapy has limited the applicability of nivolumab and pembrolizumab monotherapy for platinum failure patients, it is notable that patients that fail platinum-based chemoradiation within 6 months still meet criteria for anti-PD-1 monotherapy regardless of PD-L1 status.

Owing to some differences in biology and higher risk of distant metastasis, nasopharyngeal carcinoma has been evaluated in trials separately from other HNSCC sites. Both Pembrolizumab and Nivolumab were evaluated in single-arm phase II trials with treatment with single agent nivolumab in the platinum failure setting associated with an RR of 20.5% with a median PFS and OS of 2.8 months and 17.1 months, respectively, in the 44 patients enrolled in the trial (70, 71). These trials led to a category 2B NCCN recommendation for pembrolizumab and nivolumab as an option after failure of first-line Cis/Gem, as a randomized phase III trial will not be conducted in the platinum failure setting for nasopharyngeal carcinoma. Combination Ipilimumab and Nivolumab was studied in 40 patients with EBV-positive nasopharyngeal carcinoma with an RR of 35% and median PFS and OS of 5.3 and 17.6 months, respectively (72). This compares favorably to an RR of 18% observed with combination anti-CTLA4 plus anti-PD-L1 in non-nasopharyngeal HNSCC (67). The first phase III randomized trial in the frontline setting, JUPITER-02, was presented at the ASCO 2021 annual meeting. This trial randomized patients to Cisplatin plus Gemcitabine plus anti-PD-1 mAb camrelizumab *vs.* Cisplatin plus Gemcitabine plus placebo. The study met its primary endpoint of PFS with a significant improvement in PFS with the addition of camrelizumab with a median PFS of 10.8 months *vs.* 6.9 months in the control arm (HR 0.51[95% CI 0.37 to 0.69], P<0.0001). Notably, 82% of patients had undifferentiated carcinoma, and patients were enrolled regardless of PD-L1 status without stratification. It is expected that this will be a practice-changing trial, and this new regimen has received breakthrough therapy designation by the FDA.

PREDICTIVE BIOMARKERS

While the approvals of Pembrolizumab and Nivolumab have been a great stride for the field, only a minority of patients benefit from blockade of the PD-1:PD-L1 pathway. As such there continues to be a need for predictive biomarkers for efficacy. Biomarkers evaluated in R/M HNSCC include PD-L1, immune gene expression, tumor mutational burden, as well as the effect of viral etiologies such as HPV.

By far the most vetted biomarker across solid tumors and in R/M HNSCC is PD-L1 expression with higher expression predictive of increased efficacy. In Checkmate 141, using a cut point of ≥1% tumor membranous PD-L1 expression, there was a greater reduction in the risk of death with Nivolumab *versus* standard therapy in positive patients (HR for death: 0.55; 95% CI: 0.36–0.83) compared to PD-L1 negative (HR for death: 0.89; 95% CI: 0.54–1.45). Updated analysis after extended follow-up showed that the benefit of Nivolumab in PD-L1 negative patients increased over time, with a reduction in the HR for death to 0.73, while benefit was maintained in PD-L1 expressing patients (63, 66). The addition of PD-L1 expression on tumor-infiltrating lymphocytes (TIL) has shown better predictive value compared to tumor PD-L1 expression alone in HNSCC. For example, in a retrospective analysis of patients treated with pembrolizumab, there was no significant difference in response by tumor PD-L1 expression alone (defined as ≥1%), but when combination tumor plus TIL PD-L1 expression was used, PD-L1 positive HNSCC patients had a significantly higher RR, PFS, and OS (73). Added predictive value with inclusion of TIL PD-L1 was also shown in exploratory subgroup analysis of checkmate 141 and Keynote 048 (68, 74).

Immune gene expression profiles (GEP) have also shown predictive value with anti-PD-1 mAb treatment (75–78). For example, in HNSCC, a composite score based on six Interferon gamma related genes (CXCL9, CXCL10, IDO1, IFNG, HLA-DRA, and STAT1) was predictive of response and PFS with pembrolizumab. It showed a high negative predictive value (95%) as only 5% of patients below the Youden index had a response compared to 40% with a score above (77). First observed in melanoma, higher tumor mutational burden (TMB) has also been associated with increased efficacy with anti-PD-1 mAb therapy in R/M HNSCC patients (79, 80). Other new potential biomarkers include intratumoral hypoxia, which has been associated with immunosuppression. Evaluation of anti-PD-1 treated R/M HNSCC patients showed lower intratumoral hypoxia was associated with increased efficacy and was independently associated with clinical

benefit rate and PFS in multivariate analysis, which included tumor infiltrating CD8 T cells, the latter of which has also shown predictive value with anti-PD-1 mAb therapy (81, 82). Hypoxia as a biomarker is promising because it also has the potential to be modulated by therapeutics. While the oral microbiome was not predictive in HNSCC patients treated with Nivolumab, the intestinal microbiome has not been evaluated to date (83).

In HNSCC, there are two relevant viral etiologies, EBV for nasopharyngeal carcinoma and HPV for oropharyngeal. While HPV-positive oropharyngeal SCC is associated with a better prognosis in the R/M setting, the magnitude is much less as compared to the locally advanced setting, and systemic therapy alone is still only palliative (84). HPV-positive oropharyngeal SCC has made up approximately 20% of patients enrolled in anti-PD-1/L1-based trials, with conflicting data on whether HPV status is associated with increased efficacy with these agents. Subgroup analysis of Checkmate 141 showed a similar magnitude of OS benefit with nivolumab compared to chemotherapy in HPV-positive oropharyngeal cancer (9.1 *vs.* 4.4 months, HR 0.60; 95% CI, 0.37–0.97) *versus* negative (7.7 *vs.* 6.5 months, HR 0.59; 95% CI 0.38–0.92). However, this comparison of the difference in efficacy relative to chemotherapy within the same group is different than the question as to whether an HPV-positive patient is more likely to respond and have better efficacy from anti-PD-1/L1 mAb blockade compared to an HPV-negative patient. Analysis of the tumor microenvironment shows a spectrum of T cell activation status in both HPV-positive and HPV-negative patients, with a higher percentage of a T cell inflamed phenotype in HPV-positive patients, 51 *vs.* 21%, respectively (54). When compared by HPV status directly, some analysis show higher efficacy and some no difference. These analyses are challenged however by low sample sizes and also lack of controlling for PD-L1 status. For example, analysis of patients from Keynote 055, which included both PD-L1-positive and -negative patients, showed similar response rates, while analysis of the HAWK trial and Keynote 012, both of which included only PD-L1-positive patients, showed increased response rate and OS for HPV-positive oropharyngeal compared to HPV negative (77, 85, 86). While data are somewhat conflicting, what is clear is that both HPV-positive and HPV-negative patients benefit from anti-PD-1/L1 mAb therapy. Outside of the oropharynx there is not a defined role for HPV in oncogenesis or prognosis. However, interestingly, in the phase II HAWK study, both HPV-positive oropharyngeal and HPV-positive non-oropharyngeal patients had similarly higher efficacy with durvalumab compared to HPV negative. This suggests that perhaps the effect of HPV on the tumor microenvironment even as a bystander in non-oropharyngeal SCC may be associated with increased efficacy (86). However, this needs to be validated before any conclusions can be made.

Less is known about the predictive value of EBV as most reported prospective trials have included only EBV-positive patients. Reduction of plasma EBV DNA after initiation of nivolumab showed some trend in responders but was not significantly associated with efficacy in a small subgroup (70). A small retrospective analysis showed a numerically higher RR in EBV positive compared to negative, but it was similarly not statistically significant (87).

The interaction of PD-L1, HPV, GEP, and TMB has been analyzed in HNSCC. In 258 R/M HNSCC patients treated with pembrolizumab, response and PFS were significantly associated with TMB, GEP, and PD-L1 expression, as well as OS for the latter two biomarkers. Amongst HPV-positive patients, there was a suggestion that TMB was less predictive compared to GEP and PD-L1 (80). The reason for this may be that viral etiology was enough for immune activation, resulting in less dependence on a higher number of mutations and resulting increased neoantigens to drive immune recognition. While there was moderate correlation between PD-L1 and GEP, TMB did not correlate with either. TMB, GEP, and PD-L1 were independently associated with response, with those with high TMB and PD-L1 or high GEP and high TMB having the greatest likelihood of response (34%) (80). This important analysis highlights that even with two favorable biomarkers, the response rate was still only 34%, speaking to the complexity of the immune microenvironment. However, an unfavorable combination of these biomarkers was associated with a high negative predictive value.

While the predictive value of these biomarkers is not absolute, a high negative predictive value for anti-PD-1 monotherapy is important, especially in the frontline setting when considering adding chemotherapy or recommending clinical trial. Another important question is how much the tumor immune microenvironment changes over time in an individual patient including after various therapeutic interventions. The aforementioned analysis all include archival tissue of various durations as well as some patients with a new biopsy before treatment. There is direct data that the predictive value of PD-L1, for example, is similar in archival *vs.* tissue samples immediately prior to anti-PD-L1 treatment, and lack of a significant change in PD-L1 expression in paired primary and recurrent tumors (60, 88). This brings up the question as to whether a patient's immune phenotype and thus likelihood of efficacy is relatively fixed over their treatment course. Further analysis of changes over time in these biomarkers are needed.

FUTURE DIRECTIONS

The success of Checkmate 141, Keynote 040, and more recently Keynote 048 represents great progress for patients with R/M HNSCC. With progress comes important new questions and goals in order to continue to improve outcomes. This includes the integration of immunotherapy earlier in the recurrent setting with salvage surgery and/or reirradiation, improving efficacy in the frontline setting and the role of immunotherapy after failure of anti-PD-1 mAb-based therapy. Ongoing trials for each of these categories are shown in **Table 2**.

While salvage resection is generally considered the most aggressive option for locoregionally recurrent HNSCC, long-term survival is still poor (89). Similarly, there is a need for improvement in outcomes with reirradiation plus concurrent chemotherapy, including with reduced toxicity. Preclinical data suggest radiation has pro-immunogenic as well as immunosuppressive effects (90), and it will take clinical trials to best determine how to maximize the former in patients. Trials combining reirradiation and immunotherapy in the recurrent setting

TABLE 2 | Ongoing immunotherapy trials in Recurrent/Metastatic HNSCC*.

NCT	Trial name	Phase	Experimental Arm	Control Arm	Primary Endpoint
Adjuvant immunotherapy after surgical resection of recurrent HNSCC					
04671667	ECOG 3191	II	1. Adjuvant Reirradiation plus Pembrolizumab 2. Adjuvant Pembrolizumab monotherapy	Reirradiation plus platinum	Overall Survival
03355560		II	Neoadjuvant and adjuvant Nivolumab plus Lirilumab		Disease-Free Survival
03406247		II	1. Adjuvant Nivolumab 2. Adjuvant Nivolumab plus Ipilimumab		Disease-Free Survival
03355560		II	Adjuvant Nivolumab		Toxicity
02769520		II	Adjuvant Pembrolizumab		Disease-Free Survival
Reirradiation plus immunotherapy					
03546582	KEYSTROKE	II	SBRT reirradiation plus Pembrolizumab	SBRT alone	Progression-Free Survival
02289209		II	Hyperfractionated reirradiation plus Pembrolizumab		Progression-Free Survival
03521570		II	Re-irradiation plus Nivolumab (definitive or adjuvant)		Progression-Free Survival
03803774		I	BAY1895344 plus SBRT and Pembrolizumab		Toxicity
Frontline Systemic Therapy Trials (PD-L1 positive)					
Combination immunotherapy					
04634825		II	Enoblituzumab plus Retifanlimab		Response Rate
04633278		II	CMP-001 plus pembrolizumab		Response Rate
04260126	VERSATILE002	II	PDS0101 plus pembrolizumab		Response Rate
04398524		II	Cemiplimab plus ISA101b	Cempilimab + Placebo	Response Rate
04034225		I/II	SNS-301 Intra-tumor injection + Pembrolizumab		Toxicity
04453046		I	Hemopurifier plus pembrolizumab		Safety
04408898	SPEARHEAD 2	II	ADP-A2M4 plus pembrolizumab		Response Rate
Molecularly targeted therapy plus immunotherapy					
04199104	LEAP-010	III	Pembrolizumab plus Lenvatinib	Pembrolizumab plus Placebo	Overall Survival, Response Rate, Progression-Free Survival
04114136		II	1. Metformin plus Pembrolizumab 2. Rosiglitazone plus Pembrolizumab	Pembrolizumab	Response Rate
Frontline Systemic Therapy Trials (regardless of PD-L1 status)					
Combination immunotherapy					
02741570	Checkmate 651	III	Ipilimumab plus Nivolumab	EXTREME	Overall Survival
04634825		II	Enoblituzumab plus tebotelimab (PD-L1 negative)		Response rate
Molecularly targeted therapy plus immunotherapy					
03468218		II	Pembrolizumab plus Cabozantinib		Response Rate
03498378		I	Avelumab plus Cetuximab plus Palbociclib		Toxicity
Cytotoxic chemotherapy plus immunotherapy					
04489888	KEYNOTE B10	IV	Pembrolizumab plus Platinum plus Paclitaxel		ORR
04282109	NIVOTAX	II	Paclitaxel plus Nivolumab	Paclitaxel plus cetuximab	Overall Survival
Immunotherapy failure trials					
Immunotherapy Combination					
04590963	INTERLINK-1	III	Monalizumab + Cetuximab	Placebo + Cetuximab	Overall survival
04326257		II	1. Nivolumab plus Relatlimab 2. Nivolumab plus Ipilimumab		Response Rate
04150900		II	Pembrolizumab plus Bavituximab		Response Rate
04408898		II	ADP-A2M4 T cells plus pembrolizumab		Response Rate
03769467		I/II	Tabelecleucel plus pembrolizumab		Toxicity/Response Rate
04196283		I	1.ABBV-368 plus Tilsotolimod plus Nab-paclitaxel plus ABBV-181 2. ABBV-368 plus Tilsotolimod plus Nab-paclitaxel 3. ABBV-368 plus Tilsotolimod		Toxicity
Molecular targeted therapy plus Immunotherapy					
04428151	LEAP-009	II	Pembrolizumab plus Lenvatinib	1. Chemotherapy (Taxane, cetuximab, or capecitabine) 2. Lenvatinib	Response Rate
03019003		I/II	Decitabine plus Durvalumab		Toxicity/Response Rate
04624113	–	I/II	Tazemetostat plus Pembrolizumab		Toxicity/Response Rate

**Trials included are those that are focused entirely in HNSCC.*

are ongoing. A phase II trial with hyperfractionated reirradiation (1.2 Gy twice daily for a total of 60 Gy) plus pembrolizumab for patients with locoregional recurrence without a surgical option, was first to report acute toxicity, without unexpected adverse events, with the trial now accruing towards its primary endpoint of PFS (NCT02289209) (91). The NRG foundation Keystroke trial is ongoing in the same setting comparing reirradiation with SBRT alone *vs.* SBRT plus pembrolizumab (NCT03546582). Nivolumab is being combined with daily radiation in another single-arm phase II trial that includes both definitive and adjuvant reirradiation patients (NCT03521570).

While a randomized phase II trial showed adjuvant reirradiation plus concurrent chemotherapy significantly improved DFS post-salvage resection, there was no difference in OS, providing clinical equipoise for challenge, including with the evaluation of immunotherapy alone in the adjuvant setting (92). Multiple smaller studies are evaluating anti-PD-1 mAb monotherapy after salvage resection. One such single-arm trial reported a pre-planned interim analysis at ESMO 2019 passing its futility boundary for efficacy with estimated DFS of 55% at 10.2 months, and continues on towards its primary endpoint (93). An ECOG trial has recently opened for patients that undergo salvage resection of recurrent or second primary HNSCC that have high-risk features of ENE and/or positive margins and PD-L1 CPS ≥1. Patients in this trial are randomized to pembrolizumab monotherapy for 12 months, reirradiation (2 Gy daily to total 60 Gy) plus pembrolizumab for 12 months, or control arm of reirradiation plus concurrent weekly platinum chemotherapy. Both experimental arms are being compared to control separately with a primary endpoint of OS (NCT04671667). This trial enriches for those more likely to benefit from anti-PD-1 mAb monotherapy and in combination with radiation by including only PD-L1 expressers. Notably, PD-L1-positive patients were the only subgroup that benefited from the addition of avelumab to chemoradiation in exploratory analysis of the Javelin trial in the definitive locally advanced setting (94).

The FDA approval of frontline pembrolizumab alone and in combination with chemotherapy has driven new trials trying to build upon this new standard of care. This has come in the form of combination immunotherapy, molecularly targeted therapy plus immunotherapy, and additional combinations of cytotoxic chemotherapy plus immunotherapy. One of the key questions is whether we can increase the efficacy of pembrolizumab monotherapy with another immunotherapy or targeted agent and avoid the added toxicity from cytotoxic chemotherapy. GSK3359609 is an inducible T cell co-stimulatory receptor (ICOS) agonist. ICOS is a member of the CD28 co-receptor family. Preliminary data with GSK3359609 plus pembrolizumab in immunotherapy naïve patients, 53% of which had received at least one prior line of therapy, showed an RR of 26% with four complete responses. The median PFS and OS of 4.2 months and 13.1 months respectively. This led to a phase II/III trial of Pembrolizumab +/− GSK3359609 in the frontline setting in patients with PD-L1 expression; however, after a planned interim analysis of efficacy, the decision was made to not transition to the phase III component (95). Promising efficacy with anti-B7H3 mAb Enoblituzumab plus pembrolizumab with a response rate of 33% in platinum failure anti-PD-1 naive HNSCC patients has led to a phase II study with enoblituzumab plus anti-PD-1 retifanlimab in PD-L1-expressing patients (96). While the phase III Kestrel trial was reported as negative, fully accrued is Checkmate 651 evaluating Ipilimumab plus Nivolumab in the frontline setting *versus* EXTREME, and we await these results. Specifically, in HPV-positive oropharyngeal SCC, based on an RR of 33% with combination nivolumab and ISA 101, a synthetic long-peptide HPV-16 vaccine, a randomized phase II trial is ongoing including frontline and platinum failure patients (NCT03669718). Given the morbidity and mortality driven by local disease in HNSCC, immunotherapy injected directly into the tumor could be a potentially clinically meaningful option for the subset of patients with accessible lesions. Early data on stimulator of interferon genes (STING) agonist ADU100 plus pembrolizumab in the frontline PD-L1-expressing setting showed tolerability and PR in 5/8 patients (97). Promising data with TLR9 agonist CMP001 in melanoma has led to the exploration of this agent plus pembrolizumab also in the frontline R/M HNSCC setting (NCT04633278) (98).

In terms of combination therapies targeting molecular pathways, LEAP-010 is a phase III placebo-controlled, randomized study of Pembrolizumab with or without Lenvatinib as first-line therapy in PD-L1-expressing patients. Additionally, promising efficacy has been observed with IgG1 mAb cetuximab plus anti-PD-1 mAb. For example, cetuximab plus pembrolizumab in immunotherapy naïve patients showed an RR of 45% with a median duration of response of 14.9 months (99). Different chemotherapy backbones are also being evaluated, as well as adding additional immunotherapy to chemotherapy. For example, KEYNOTE B10 is an ongoing study of Pembrolizumab with Carboplatin and Paclitaxel as first-line treatment for R/M HNSCC (NCT04489888).

Driven by all R/M HNSCC patients now receiving anti-PD-1 mAb-based therapy in the frontline setting, there is great and growing need for better therapeutics after anti-PD-1 failure. The majority of immunotherapy-based trials are in early phase with most combinations being tested in phase I trials with expansion cohorts, some of which include HNSCC. Preliminary data have been reported for two cetuximab-based combinations. Cetuximab plus nivolumab showed RR of 17% and SD in an additional 17% in 23 patients that had failed prior anti-PD-1 mAb therapy (100). Natural Killer Group 2A (NKG2A) inhibitor Monalizumab plus cetuximab was associated with an RR of 20%, SD in 37.5%, and median duration of response of 5.2 months (95% CI; 3.9-not reached). This combination is currently being compared to cetuximab alone in a phase III clinical trial for patients that have failed prior anti-PD-1 and platinum (NCT04590963) (101). Additionally, Lenvatinib plus pembrolizumab is also being tested in the anti-PD-1 failure setting compared to standard of care chemo and Lenvatinib monotherapy in a randomized phase II Trial (NCT04428151).

Adoptive T cell therapy especially CAR T cells have shown significant efficacy in hematologic malignancies. In solid tumors, challenges to adoptive T cell therapy include appropriate antigen targets and adequate penetration into the tumor microenvironment. While headway has been made in some solid tumors such as melanoma, data in HNSCC are more preliminary with trials ongoing. Preliminary data using pan-ErbB targeted CAR-T cells showed tolerability and SD in 60% (3/5) at 6 weeks (102). Autologous TIL therapy Lifileucel in combination with pembrolizumab in anti-PD-1 naïve R/M HNSCC patients showed a response rate of 44% in nine patients with responses ongoing in three out of the four patients at a median follow up of 8.6 months (103). A trial with ADP-A2M4 targeting MAGE-A4-positive HNSCC in combination with pembrolizumab, also in anti-PD-1 naïve patients, is currently accruing. A number of studies have focused on viral antigens including EBV and HPV. Ten patients with R/M NPC positive for EBV encoded RNA and/or EBV-LMP1 refractory to multiple lines of therapy received autologous T cell therapy weekly ×4 doses then every 2–4 weeks. The clinical benefit rate was 60% with a PR in two

patients and SD in four patients (104). Smith and colleagues reported a phase I trial with T cells generated by an adenovirus-based vector, AdE1-LMPpoly, which expands LMP1&2- and EBNA1-specific T cells also in EBV-positive advanced NPC. Out of 14 patients treated, SD was seen in 10 patients with a median time to progression of 66 days (range 38–420) (105). A larger phase II trial evaluated 35 patients treated with frontline carboplatin plus gemcitabine ×4 cycles followed by autologous EBV cytotoxic lymphocytes. While there was only minimally enhanced response beyond what is expected with chemotherapy alone, the median OS of 29 months compares favorably to the expected median OS of 22 months with platinum/gemcitabine alone (106). A phase III trial with this regimen is ongoing (NCT02578641). Tabelecleucel, an allogeneic T cell immunotherapy, is currently being evaluated with pembrolizumab in EBV positive NPC (NCT03769467). Small studies have evaluated targeting HPV E6 and E7. Twelve patients with HPV16-positive advanced cancer were treated with autologous genetically engineered T cells expressing a TCR against HPV16 E6. Two patients (anal SCC) had a PR, and the one oropharyngeal SCC patient experienced SD lasting 4 months (107). Another study evaluated targeting HPV16 E7 also with T cells with engineered TCR. This study included 12 patients, of which six patients achieved a PR and four SD. The study included four HNSCC patients, all of which had failed platinum and anti-PD-1. In the HNSCC patients there were two PRs and two SD with response/stability lasting for 3–4 months (108). While small sample sizes preclude conclusions, the higher efficacy in the latter study suggests that E7 may be a better target than E6 for HPV-positive patients. These trials highlight the feasibility of T cell therapy in HNSCC with larger trials needed to establish its efficacy. Similar to checkpoint inhibition, continued study of predictive biomarkers specifically for T cell therapy will be critical to guide selection of patients for this type of therapy.

With numerous frontline combination trials underway in patients with PD-L1 expression, we must strive not only for better efficacy but also concurrent knowledge on how to select the best therapy. This will be critically important if multiple new regimens improve OS in phase III trials. For example, meaningful to integration into everyday practice would be powering trials by CPS score subgroups so we would know whether a combination is effective in just CPS >20 or also CPS 1-19 patients. While only a select group of patients will have lesions amenable to intratumoral injection, consistent response of injected lesions could prove important in reducing at minimum morbidity. In addition to local effects, the key question is whether intratumoral injection in combination with anti-PD-1 will also enhance response in non-injected sites and ultimately improve mortality.

Important to making progress with combination cytotoxic chemotherapy in the immunotherapy era is a better understanding of the effect of chemotherapy on the tumor microenvironment. Preclinical data show immunogenic effects of numerous cytotoxic agents active in HNSCC such as Cisplatin, 5FU, and Taxane; however, there is suggestion that with repetitive doses, immunosuppressive effects can also occur (109, 110). While chemotherapy plus immunotherapy has improved OS in HNSCC and other solid tumors, the effect is additive at best. For example, in Keynote 048, while the RR was higher with chemotherapy plus pembrolizumab compared to pembrolizumab monotherapy (36 *vs.* 19% in PD-L1 expressers), the median duration of response was three times lower. One explanation for this is that the response in the additional 17% was driven by solely the chemotherapy with no benefit from the pembrolizumab. This highlights the need to examine the best type and sequence of cytotoxic chemotherapy and immunotherapy with the goal of achieving not only a higher response but a prolonged duration of response. Additionally, an open question is whether anti-PD-1 monotherapy should be continued after progression with subsequent addition of chemotherapy. A better understanding is also needed as to whether systemic agents can change a patient's tumor microenvironment so that they may be more likely to benefit from immunotherapy with re-challenge either with anti-PD-1 again or novel combination immunotherapy.

With a seemingly infinite number of immunotherapy combination options being developed and tested, especially in the anti-PD-1 failure setting, we are searching for the next big step for the field. It is unlikely that any combination will work in 90% or even 50% of patients, but rather that a more personalized approach using a tumor microenvironment–driven selection strategy to choose the best combination may be the only way to get the majority of patients to benefit from immunotherapy. To do this we must start evaluation of selection strategies prospectively where clinical equipoise allows.

CONCLUSION

Immunotherapy has transformed the field of oncology over the last decade including in head and neck cancer with a current standard of care role in the frontline and platinum failure setting in R/M HNSCC. It is an exciting time for both patients and providers with an explosion of new agents and clinical trials. While rare, it is amazing to see the durable benefit with anti-PD-1 mAb-based therapy achieved in some patients. But as a field we are also at a critical juncture as to how to take the next big leap after anti-PD-1 mAb therapy to help more patients benefit from immunotherapy. Undoubtedly, we will have to rein in our approaches focused and tailored by an increased understanding of the tumor immune microenvironment in patients, with the ultimate goal of a more personalized approach leading to benefit with immunotherapy in the majority of patients. While we have a lot more work to do, the future is brighter for our R/M HNSCC patients.

AUTHOR CONTRIBUTIONS

All authors participated in the development, writing, and editing of the review article. All authors contributed to the article and approved the submitted version.

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Immunotherapy for Head and Neck Cancer: A Paradigm Shift From Induction Chemotherapy to Neoadjuvant Immunotherapy

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Neoadjuvant immunotherapy has the potential to enhance clinical outcomes by increasing anti-tumor immune responses in the presence of abundant tumor-derived antigen in an immune microenvironment that has not been exposed to previous therapy. The current mainstay of advanced head and neck squamous cell carcinoma (HNSCC) treatment remains surgery and radiotherapy with/without conventional chemotherapy. Despite this multi-modality treatment, advanced human papillomavirus (HPV)-negative HNSCC shows poor prognosis. Treatment intensification with neoadjuvant (induction) chemotherapies with platinum drugs are insufficient to significantly prolong overall survival. Although only 15-20% of patients benefit, immunotherapies have been approved and widely used for recurrent and metastatic HNSCC. These successes have led to checkpoint blockade therapies being testing in earlier treatment settings. Recent clinical trials of neoadjuvant immunotherapy show promising results and this methodology has the potential to change the treatment algorithm of HNSCC. This overview examines the treatment history of neoadjuvant approaches for HNSCC, and especially focuses on the recent topics of neoadjuvant immunotherapy for HNSCC.

Keywords: head and neck squamous cell carcinoma, neoadjuvant immunotherapy, clinical trial, biomarker, pathological tumor response

INTRODUCTION

Squamous cell carcinoma (SCC) is the predominant malignant histology of the mucosal surfaces of the head and neck (HN) region that includes the oral cavity, pharynx, and larynx. Conventional HNSCC is mainly caused by habitual alcohol drinking and smoking, and often occurs in older adults, while human papillomavirus (HPV)-related HNSCC of the oropharyngeal region is rapidly increasing in relatively younger patients (1). The head and neck region is anatomically complex and serves essential functions such as eating, speaking, and breathing. Multi-disciplinary treatments,

integrating surgery, chemotherapy, and radiation, aim to maximize treatment effects but have significant functional impact. Historically, surgery and radiotherapy with/without conventional chemotherapy including platinum, taxanes or fluorouracil, were applied to treat HNSCC. Therapeutically, HPV-positive HNSCC demonstrates sensitivity to chemoradiotherapy, and offers a better prognosis (2).

Post-operative adjuvant treatments for locally advanced HNSCC have been studied for many years as historically surgery alone for locally advanced disease had very poor outcomes. Several landmark trials established the clinical benefit of using cisplatin-based chemoradiotherapy after surgery for locally advanced, high-risk HNSCC patients (3, 4). These early studies led to two randomized Phase III trials, which provided Level 1 evidence supporting the use of concurrent chemoradiotherapy in high-risk HNSCC patients (5–7). Although these Level 1 data established a new postoperative standard of care to treat high-risk HNSCC patients, the five-year survival rate in for these patients remains suboptimal.

However, the five-year survival rate is still below 50% in advanced HPV-negative HNSCC patients (8), and many patients suffer from severe impact on essential functions. Furthermore, although distinct tumor-suppressor mutations including *TP53, CDKN2A, NOTCH* have been reported in HNSCC, cancer-promoting driver oncogenic mutations have not been detected (9–11), which makes it challenging to apply molecular targeted therapies.

Checkpoint inhibitors (CPI) targeting the programmed death 1 (PD-1) pathway have been approved for recurrent and metastatic (R/M) HNSCC patients in the first- and second-line settings (12–14) and have dramatically changed the treatment algorithm of HNSCC. The effects of checkpoint inhibitors are mainly derived from reinvigoration and activation of tumor-oriented antigen-specific T cells (15). HNSCC shows a relatively high tumor-mutational burden (TMB) (16) and immune infiltration (17), consistent with a potential to achieve therapeutic efficacy from cancer immunotherapy.

The landmark phase III CheckMate 141 trial resulted in the approval of nivolumab in the R/M second-line HNSCC setting (12). Following this, the phase III KEYNOTE-048 trial established a new paradigm for first-line R/M HNSCC patients (14). Based on this study and depending on the programmed death-ligand 1 (PD-L1) combined positive score (CPS) either pembrolizumab alone or with chemotherapy represents the first choice for these patients (14). Overall, only 15-20% of patients ultimately benefit from anti-PD-1 in these studies highlighting the need for improving efficacy of CPIs for HNSCC treatment.

These encouraging findings have led to numerous ongoing studies testing combinations to improve CPI response rates and also testing these agents in other settings. We and others have focused on the definitive surgical setting with integration of neoadjuvant immunotherapy and in this review focus on historical and current approaches. Neoadjuvant chemotherapy has a long history in HNSCC where induction chemotherapy (IC) prior to conventional platinum-based chemotherapy has been tested in numerous studies HNSCC (18). The indications for IC are limited to those with significantly advanced disease and may result in a high frequency of severe adverse events. A natural extension of this work has led several groups to test whether neoadjuvant chemotherapy prior to surgery would improve clinical outcomes. However, negative Phase III trials (19, 20) in this setting have reduced enthusiasm for these approaches. Note, there are institution specific protocols where induction chemotherapy prior to surgery is still used for larger tumors to achieve more rapid control (21). The goal of cytotoxic chemotherapy in this setting is to directly attack tumor cells to reduce tumor burden. By contrast, neoadjuvant immunotherapy is fundamentally distinct as it targets the host immune system to attack tumor cells in a durable fashion. In this review, we present a brief overview of the history of neoadjuvant (induction) chemotherapy in the definitive surgical management of HNSCC. Then, we focus on the rationale and clinical trials of neoadjuvant immunotherapy and its potential impact on HNSCC treatment.

INDUCTION CHEMOTHERAPY FOR HNSCC

In addition to the adjuvant chemotherapy, platinum-based neoadjuvant chemotherapy (induction chemotherapy; IC) has also been examined to augment subsequent (chemo) radiotherapy or surgery. The goals of induction chemotherapy are to achieve rapid tumor responses in particular with large volume disease and to "chemo-select" patients prior for definitive (chemo)radiotherapy or surgery. For larynx cancer, this approach was initially focused on reducing metastases, and preserving laryngeal function including speech and swallowing. The landmark VA Larynx study compared IC (cisplatin and fluorouracil) followed by RT versus total laryngectomy followed by RT in advanced laryngeal cancer (22). IC resulted in larynx preservation but did not contribute to improved survival. To test the sequencing of these therapies in the laryngeal cancer setting, RTOG 91-11 compared the clinical efficacy of 1) IC followed by RT, 2) CCRT and 3) RT alone for advanced laryngeal cancer patients (23). The data and subsequent meta-analysis showed the superiority of CCRT to preserve the larynx in advanced laryngeal cancer patients (8, 23).

To determine the survival benefit of IC using docetaxel plus cisplatin and fluorouracil (TPF) regimen followed by CCRT, two-phase III randomized trials were completed: the PARADIGM trial reported in 2013 (19) and DeCIDE trial reported in 2014 (20). Both trials did not show a significant extension of OS and DFS, consistent with the subsequent studies (24, 25). Importantly, phase III clinical trials which examined the clinical efficacy of IC treatment prior to surgery also failed to show suppression of loco-regional relapse and distant metastasis or extend OS (26–28). These results underscore that TPF IC is not recommended for survival benefit. In addition, IC may increase the possibility of severe AEs as compared to CCRT in non-surgical locally, advanced HNSCC treatment. However, IC

remains an attractive approach for specific cases of advanced disease with a high risk for local or distant failure or to "debulk" rapidly growing tumors (19).

IMMUNOTHERAPY FOR R/M HNSCC

Despite these efforts to improve clinical prognosis, the five-year survival rate of locally advanced stage III/IV HNSCC patients is still sub-optimal [53% in postoperative CCRT treated patients (7)], and half of advanced patients show recurrence within three years (8). Immune checkpoint blockade therapies, especially anti-PD-1 and anti-CTLA4, were first approved in advanced melanoma patients (29) and then applied for various cancers (30), which has dramatically impacted the cancer treatment algorithm. In HNSCC, anti-PD-1 agents (nivolumab, pembrolizumab) were first examined and approved in R/M setting. The checkmate 141 phase III trial evaluated the effect of anti-PD-1 (nivolumab) for R/M HNSCC patients (12). Positive results from this study established the application of anti-PD-1 for R/M HNSCC treatment, and proved the existence of actionable, efficient anti-cancer immunity in HNSCC tumors. Similarly, the Keynote-040 randomized phase III trial compared the efficacy of pembrolizumab (anti-PD-1) versus SOC (methotrexate, docetaxel, or cetuximab) (13) for R/M HNSCC patients after platinum-containing treatment. These trials led to US Food and Drug Administration (FDA) approval of the use of anti-PD-1 (nivolumab and pembrolizumab) for second-line for recurrent and metastatic HNSCC patients who had already experienced platinum-based therapies (31).

Subsequently the Keynote-048 study, a randomized multi-center phase III study from 37 countries, examined pembrolizumab alone or with chemotherapy (platinum plus fluorouracil) versus cetuximab with chemotherapy (the EXTREME regimen (32)) for first-line treatment of R/M HNSCC (14). In this trial, pembrolizumab monotherapy significantly improved the OS of PD-L1 positive (CPS ≥20 or CPS ≥1) HNSCC. Additionally, R/M HNSCC patients treated with pembrolizumab plus chemotherapy had significantly prolonged OS compared to the cetuximab with chemotherapy group. This trial highlighted the effectiveness of combination immunotherapy and chemotherapy for subsets of HNSCC patients. Based on KEYNOTE-048, the FDA approved use of pembrolizumab monotherapy in the first-line for R/M HNSCC with CPS ≥1 and pembrolizumab plus platinum-based chemotherapy for those with CPS<1 R/M HNSCC (31).

RATIONALE OF NEOADJUVANT IMMUNOTHERAPY FOR HNSCC

The significant impact of checkpoint inhibitor therapy for R/M HNSCC has proven the existence of anti-cancer immunity in HNSCC (12–14). Thus, targeting immune suppression pathways with checkpoint inhibitors has been broadened to the exploration of therapeutic options in all HNSCC treatment settings. Notably, the timing of immune checkpoint inhibitors may influence the outcome of cancer treatment (33). There are now numerous studies introducing neoadjuvant immunotherapy in diverse cancer types (34–36). Considering the treatment naïve situation and the absence of treatment-resistant cells compared with the R/M setting, neoadjuvant immunotherapy is hypothetically likely able to result in a strong and durable therapeutic effect. In a spontaneous mouse metastatic breast cancer model, neoadjuvant checkpoint inhibitors showed an enhanced survival compared to the adjuvant setting by suppressing metastatic lesions (37). Intriguingly, in preclinical mouse models, a specific interval between neoadjuvant immunotherapy and subsequent surgery was important to establish potent systemic T cell response (33), suggesting that it will be important to establish the optimal duration in the clinical setting.

There are three major potential benefits to use CPIs in the neoadjuvant setting. First, neoadjuvant immunotherapies will enhance systemic T cell responses for tumor-specific antigens before surgery (34). The premise of neoadjuvant immunotherapy is to use the existing tumor mass as an in-situ source of tumor-specific antigens to enhance systemic immunity *via* dendritic cell antigen presentation to rejuvenate T cells and priming especially for cytotoxic T cells (34). This enhanced function acts to destroy micro-metastasis in clinically advanced tumors, decreasing loco-regional or distant metastasis after primary therapies. In support of this, neoadjuvant anti-PD-1 treatment in a mouse HNSCC model resulted in conversion of functional immune-dominance and induced robust anti-cancer responses, supporting the application of neoadjuvant immunotherapy for HNSCC (38). Second, in contrast to conventional chemotherapy, immunotherapy is much better tolerated by patients. Considering the high-frequency of severe adverse events and lack of significant effect OS prolongation with induction chemotherapy, neoadjuvant immunotherapy thus represents an attractive option for advanced HNSCC treatment. Finally, considering the ease of biopsies in the head and neck region, compared to adjuvant immunotherapy, neoadjuvant immunotherapy has the benefit to enable translational efforts such as TCR analysis, gene-expression profiling, and cytokine evaluation in the primary tumor which is not affected by other treatments including chemotherapeutics or radiation. These studies with previously untreated tumors may enable establishment of predictive biomarkers to select appropriate patients and also define mechanistic pathways.

PATIENT SELECTION FOR NEOADJUVANT IMMUNOTHERAPY

An important consideration in neoadjuvant immunotherapy approaches is appropriate patient selection. Completed and ongoing trials have focused on a diverse group of HNSCC patients including early and advanced stage and HPV-positive and negative patients. This diverse patient selection has been used primarily to define a "signal" of activity. However, as

immunotherapy has associated toxicities (see section on this below) and is expensive, careful patient selection to determine who may benefit from these approaches is critical. We and others have focused on HPV-negative, locally advanced disease patients with high-risk pathologic features (positive surgical margins or extra-nodal extension). These patients have the worst prognosis despite multimodality approaches and may benefit from neoadjuvant/adjuvant immunotherapy. As trials mature, patient selection for neoadjuvant immunotherapy will need to be defined further.

BIOMARKER CANDIDATES FOR NEOADJUVANT IMMUNOTHERAPY

Given that CPIs are still expensive drugs and sometimes induce severe immune-related toxicities, it is important to establish the appropriate markers which can predict efficacy of CPIs (39, 40). PD-L1 expression in tumor cells and immune cells remains the most widely used biomarker in HNSCC and other cancers (40, 41). In the KEYNOTE-048 phase III trial, significant survival benefit of pembrolizumab for patients was seen with PD-L1 expression ≥ 1% and ≥ 20% by CPS (14). In addition, in the KEYNOTE-040 phase III study, the correlation of clinical outcome and PD-L1 expression on tumor (PD-L1 tumor proportion score ≥ 50%) was evident (13). However, PD-L1 negative tumors sometimes respond to CPI treatment, suggesting the existence of other mechanisms. The expression level of PD-L1 in the tumor does not necessarily correlate with the response to CPIs. In Checkmate-141 phase III trial, there was no correlation of survival extension and PD-L1 expression on tumors (PD-L1+ >1%, 5% and 10%) (12). These data indicate that PD-L1 expression on tumor cells is not a "perfect" biomarker to predict the clinical outcome. In addition, the dynamic expression change of PD-L1 with tumor heterogeneity also makes it difficult to evaluate the expression of PD-L1 (41). Other work showed that PD-L2 expression was significantly correlated with PD-L1 expression in HNSCC clinical samples (42). Tumors with both PD-L1 and PD-L2 expression responded better than tumors with only PD-L1 expression, indicating that combinatorial scoring may be an attractive approach.

HPV infection might also be a clinical biomarker to predict the response to CPIs. HPV-related oropharyngeal HNSCC shows better survival related to HPV-negative oropharyngeal HNSCCs. HPV infection results in production of virus-related proteins, which may induce *de novo* T cell response and more CD8+ T cell infiltration in tumor (43). In the KEYNOTE-055 phase II trial, the response rate to pembrolizumab was 22% for p16 positive patients and 16% for p16 negative patients (44). A meta-analysis which examined the results of clinical trials including Checkmate 141, KEYNOTE-012, KEYNOTE-055 showed that HPV infection status was associated with the response rate to anti-PD-1 treatment independently of PD-L1 expression and TMB in HNSCC (45). Another meta-analysis showed that HPV positive HNSCC patients display significant improved outcomes with PD-1/PD-L1 axis blockage treatment compared to HPV negative HNSCC patients (46). As further investigation of these intriguing results is needed, the SITC HNSCC immunotherapy guidelines does not recommend using HPV status for anti-PD1 treatments in R/M HNSCC (31).

TMB is a potential predictive biomarker that also needs further exploration. The probability of response to CPIs has at least in part been linked to TMB across cancer types, including HNSCC (16). Patients with high-TMB have more effective clinical responses with improved survival in lung, bladder, and head and neck cancer patients (47, 48). Given that the genomic analyses of HNSCC has not identified widely shared oncogenic driver mutations but shows relatively high TMB (49, 50), the relationship between TMB and response to CPIs is promising. A study in over 300 patients across 22 solid tumor types from four KEYNOTE trials and an observational study of 126 HNSCC patients revealed HNSCC patients with high TMB showed significantly better anti-PD-1 response (51, 52). Intriguingly, TMB was significantly higher among HPV-/EBV- responders and correlated with OS, but not high in HPV+/EBV+ responders who didn't show any correlation between TMB and OS (52). These data suggest that virus infection status impacts TMB as a biomarker. Notably, other work has contradicted the above studies on TMB and concluded that that high TMB failed to predict the effect of ICI (53). Thus, further studies are needed to define the role of TMB as a predictive biomarker.

Immune cells phenotypes in TME may also be important to predict the response to CPIs. HNSCC patients with high CD8+ T cells infiltration showed better anti-PD-1 response in the adjuvant setting (52, 54). In addition, CD8+ T cells with lymphocyte-activation gene 3 (LAG-3) or T cell immunoglobulin domain and mucin domain-3 (TIM-3) co-expression with PD-1 was higher among non-responders (52). Furthermore, tertiary lymphoid structures (TLS) in the tumor bed are suggested to contribute favorable outcome (55). Considering the TME will be dramatically changed after therapeutic treatment, neoadjuvant immunotherapy for HNSCC can provide an opportunity to establish immune markers to predict efficacy of subsequent immunotherapy.

PATHOLOGIC RESPONSE CRITERIA FOR NEOADJUVANT IMMUNOTHERAPY

How to accurately evaluate the effect of neoadjuvant immunotherapy is an evolving area. For example, radiological tumor examination is widely used in Response Evaluation Criteria In Solid Tumors (RECIST) after organ preservation therapy including radiotherapy and chemotherapy. In the neoadjuvant immunotherapy context, immune-modified RECIST (imRECIST) criteria have been proposed (56). However, some immunological therapeutic effects can induce pseudo-progression or development of new lesions because of infiltration of immune cells into the primary tumor or lymph

nodes, which makes it difficult to evaluate the treatment efficacy only with radiographical information (57). In fact, a study evaluating 20 resected non-small cell lung cancer (NSCLC) tumors after neoadjuvant anti-PD-1 treatment showed a discrepancy between radiological and pathological evaluation (58). These findings highlight the clinical importance to establish standard pathological criteria to accurately evaluate the therapeutic effect of neoadjuvant immunotherapy after definitive surgery.

Pathological complete response (pCR) and major pathologic response (MPR) are widely used as surrogate clinical endpoints for long-term survival (59–62). Pathologic complete response means the ablation of all cancer cells in resected tumor after the treatment. On the other hand, MPR represents ≤ 10% of residual viable tumor (63). However, while pCR and MPR are considered the "gold standard", they do not take into account lesser degrees of immunological reaction in the tumor that may still impact clinical outcomes. In fact, meta-analysis of melanoma neoadjuvant immunotherapy trials has shown that any degree of pathologic response and not just MPR/pCR, was correlated with better clinical outcomes (64). We defined pathological tumor response (pTR) as one such approach which is quantified as the proportion of the resection bed with tumor necrosis, keratinous debris, and giant cell/histiocytic reaction were distinct from growing tumor and only seen after therapy (**Figure 1**). We classified pTR into pTR-0 (≤10%), pTR-1 (≤10-49%), and pTR-2 (≥50%) (54). Pathologic treatment effect (PTE) is another similar scale, which is evaluated by the area showing fibrosis or lymphohistiocytic inflammation divided by total tumor area (65). The establishment of the best pathological method to evaluate the response of neoadjuvant immunotherapy is still evolving as the ultimate clinical impact of histologic changes is understood.

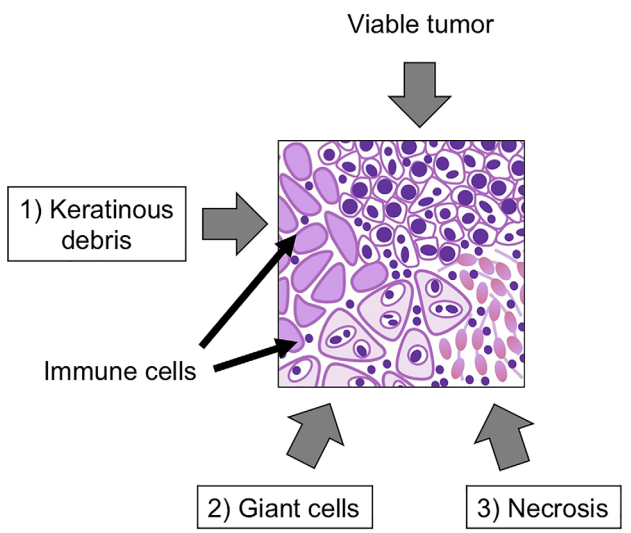

FIGURE 1 | Representative figure of pathological tumor response (pTR). Key pathological findings after neoadjuvant immunotherapy include 1) keratinous debris, 2) giant cells, histiocytic reaction and 3) tumor necrosis. The pTR rate is calculated as the area of regions 1-3/(1-3)+area of any remaining tumor.

CLINICAL STUDIES OF NEOADJUVANT IMMUNOTHERAPY FOR HNSCC

With the positive responses in the R/M HNSCC setting, several trials have reported results with neoadjuvant checkpoint immunotherapy prior to surgery (**Table 1**). The phase II Checkpoint Inhibitors Assessment in Oropharynx cancer (CIAO) trial (NCT03144778) tested a combination of durvalumab (1500 mg) and tremelimumab (75 mg) in the neoadjuvant setting, preceding SOC (surgery with or without radiation therapy) (70). The primary endpoint of this trial was comparison between arms of a change in the CD8+ tumor infiltrating lymphocyte (TIL) density. A total of 28 patients were eligible, and 24 (86%) of patients were HPV positive. The combinatorial therapy group did not significantly increase the CD8+ TILs. Although neither baseline CD8+ T cell infiltration status nor PD-L1 expression level correlated with overall response, there was a trend in which greater CD8+ T cells infiltrated patients tended to show MPR. Note that MPR was observed in 8 (29%) patients in either the primary tumor or lymph node metastasis. The CD8+ T cell data was correlated with preclinical models, where anti-PD-1 and anti-CTLA4 combinatorial therapy increased tumor-infiltrating CD8+ T cells (71).

Schoenfeld et al. examined neoadjuvant 1) nivolumab (N) or 2) nivolumab plus ipilimumab (N+I) in untreated 29 oral cavity cancer patients in a phase II trial (eligible for ≥ T2 or node positive) (NCT02919683) (68). Nivolumab (3 mg/kg) was administered on weeks 1 and 3, while ipilimumab (1 mg/kg) was given on week 1 only. Although a total of 21 patients experienced AEs, including grade 3/4 AEs in 2 (N) and 5 (N+I) patients, there were no surgical delays. In addition, there was evidence of response in both arms. Notably, four patients (N, n=1; N+I, n=3) had major/complete response (greater than 90%). These data suggest clinical tolerability and effectiveness of neoadjuvant immunotherapy.

We reported a phase II trial, in which neoadjuvant/adjuvant pembrolizumab was tested in locally advanced, resectable HPV-negative HNSCC patients (NCT02296684) (54). In this trial, safety, pTR, and relapse rate with pembrolizumab were evaluated. A total of 36 patients (T3/T4; 80%, stage IV; 92%) were enrolled and received one time dose of neoadjuvant pembrolizumab (200 mg) followed by surgery two or three weeks after the immunotherapy. Per standard of care, postoperative RT or CCRT were performed, and adjuvant pembrolizumab treatment was used in high-risk patients with positive surgical margins or extra-nodal extension. Notably, grade 3/4 serious adverse events or delay of surgery didn't occur, underscoring the safety of neoadjuvant immunotherapy. Furthermore, the one-year relapse rate in high-risk patients was 16.7%, which was lower than historical data. The pTR scores were evaluated by two independent pathologists and graded using the following scale: pTR-0 < 10%, pTR-1; 10-49%, pTR-2 ≥ 50%. Any pTR was seen in 44% and pTR-2 was seen in 22% of patients. Notably, any pTR after neoadjuvant pembrolizumab correlated with baseline tumor PD-L1, immune infiltration, and IFN-γ activity, but not TMB. These data suggest the reactivity of

TABLE 1 | Completed neoadjuvant immunotherapy clinical trials.

NCT number (Trial name)	Phase	Title	Protocol	Immunotherapy Drugs	Primary endpoint	Ref.
NCT03238365 (Merlino et al.)	I	Discordant Responses Between Primary Head and Neck Tumors and Nodal Metastases Treated With Neoadjuvant Nivolumab: Correlation of Radiographic and Pathologic Treatment Effect.	neoadjuvant	nivolumab	RVR, PTE	(65)
NCT03247712 (Leidner et al.)	Ib	Neoadjuvant immunoradiotherapy results in high rate of complete pathological response and clinical to pathological downstaging in locally advanced head and neck squamous cell carcinoma.	neoadjuvant/ adjuvant	nivolumab	surgical delay, pCR, MPR, pathological downstaging	(66)
NCT02488759 (Checkmate 358)	I/II	Neoadjuvant nivolumab for patients with resectable HPV-positive and HPV-negative squamous cell carcinomas of the head and neck in the CheckMate 358 trial.	neoadjuvant	Nivolumab	safety and tolerability, response rate, surgical delay	(67)
NCT02919683 (Schoenfeld et al.)	II	Neoadjuvant Nivolumab or Nivolumab Plus Ipilimumab in Untreated Oral Cavity Squamous Cell Carcinoma: A Phase 2 Open-Label Randomized Clinical Trial.	neoadjuvant	nivolumab, ipilimumab	Safety, volumetric response	(68)
NCT02296684 (Uppaluri et al.)	II	Neoadjuvant and Adjuvant Pembrolizumab in Resectable Locally Advanced, Human Papillomavirus-Unrelated Head and Neck Cancer: A Multicenter, Phase II Trial.	neoadjuvant/ adjuvant	pembrolizumab	safety, pTR-2, 1-year relapse rate	(54)
NCT03021993 (Xiong et al.)	II	Immunological effects of nivolumab immunotherapy in patients with oral cavity squamous cell carcinoma.	neoadjuvant	nivolumab	pathological response	(69)
NCT03144778 (Ferrarotto et al.)	II	Impact of Neoadjuvant Durvalumab with or without Tremelimumab on CD8(+) Tumor Lymphocyte Density, Safety, and Efficacy in Patients with Oropharynx Cancer: CIAO Trial Results.	neoadjuvant	durvalumab, tremelimumab	CD8+ TILs density	(70)

RVR, Radiographic volumetric response; PTE, Pathologic treatment effect; pCR, pathological complete response; MPR, major pathological response; pTR, pathological tumor response.

neoadjuvant immunotherapy is related to immunogenic phenotype before treatment and highlights the future possibility to select patients for neoadjuvant immunotherapy before surgery.

Merlino et al. reported on findings from a clinical trial where neoadjuvant nivolumab (240 mg on days 1 and 15) with or without tadalafil was tested. Patients received two cycles of drug therapy. The radiographic volumetric response (RVR) and PTE were evaluated, and the results of RVR and PTE was significantly correlated in primary tumor and lymph nodes. Intriguing findings from this study reported discordant responses between primary tumor and regional metastatic lymph nodes (NCT03238365) (65).

A phase II trial was reported by Xiong et al. (NCT03021993), in which a total of 10 locally advanced OSCC patients were treated with neoadjuvant nivolumab (3 mg/kg on days 1, 14 and 28) (69). The immunological responses were analyzed using blood before and after treatment. Although this study didn't report pathologic responses or clinical efficacy, the proportion of CD8+ T cells, especially granzyme B positive cells, increased after treatment. However, the proportion of CD4+ T cells were decreased while the rate of CD4+FoxP3+ regulatory T cells was increased with treatment. These data highlight the difficulty of interpreting peripheral lymphocyte populations with clinical responses in HNSCC patients treated with neoadjuvant immunotherapy.

The Checkmate 358 phase I/II study examined clinical safety and efficacy of two doses of neoadjuvant nivolumab in HPV positive or negative HNSCC (NCT02488759) (67). No new safety signals were observed and there were no surgical delays. Pathologic responses were evaluated in 34 patients (17 HPV+ and 17 HPV-negative). Major pathological responses were seen in 1 HPV-positive tumor with none in the HPV-negative tumors. Three HPV-positive tumors and one HPV-negative tumor had partial pathologic responses.

ONGOING CLINICAL TRIALS

In addition to the published studies above, several ongoing neoadjuvant immunotherapy trials with subsequent surgery for locally advanced HNSCC have reported results at major oncology meetings (**Table 2**).

Updated results of a phase II neoadjuvant pembrolizumab trial prior to surgery followed by adjuvant concurrent pembrolizumab and radiation along with cisplatin for clinically high-risk (T3/4 stage and/or ≥ 2+ LNs) HPV-negative HNSCC patients (NCT02641093) were recently presented (74). This is multi-institutional trial enrolled 92 patients and 76 patients were evaluable for DFS. They used pathological response (PR) criteria which was defined tumor necrosis and/or histiocytic inflammation and giant cell reaction to keratinaceous debris (74). Of eighty evaluated patients, 32 patients (40%) showed a PR [26 partial PR (≥ 20% and <90%) and 6 with major PR (>90%)]. Notably, patients with PR (partial plus major) showed significantly improved 1-year DFS compared to patients with no PR (100% versus 68%, $p = 0.01$; HR = 0.23). These are the first clear data in HNSCC supporting the finding that neoadjuvant anti-PD1 induced PR is a predictor of clinical outcomes.

The IMCISION study (NCT03003637) presented at ESMO 2020 is examining neoadjuvant nivolumab and ipilimumab for stage II-IVa HNSCC patients. This trial included both definitive and salvage surgery patients. Notably, the treatments were safe and 16/26 patients (61.5%) had pathologic responses (>20%) and 8/26 (31%) of patients experienced complete response (72).

TABLE 2 | Ongoing neoadjuvant immunotherapy clinical trials.

NCT Number (Trial Name)	Phase	Protocol	Drugs	Primary Endpoint	Ref.
NCT03238365	I	neoadjuvant	Nivolumab, Tadalafil	immune cell polarization (Th1/Th2; M1/M2)	
NCT03003637 (IMCISION)	IB/II	neoadjuvant	Nivolumab, Ipilimumab	tolerability, pathological response, hypoxia	(72)
NCT03174275	II	adjuvant/neoadjuvant	Duravalumab, carboplatin, nab-paclitaxel	pCR rate	
NCT03721757 (NICO)	II	adjuvant/neoadjuvant	Nivolumab	DFS (12 months following surgery)	
NCT03107182 (OPTIMA-II)	II	neoadjuvant	Nivolumab, Nab-paclitaxcel, Carboplatin, 5-FU, Paclitaxcel	tumor shrinkage rate with DRR	
NCT03341936	II	neoadjuvant	Nivolumab, Lirilumab	DFS	(73)
NCT03342911	II	neoadjuvant	Nivolumab, Paclitaxcel, Carboplatin	pCR	
NCT03708224	II	neoadjuvant	Atezolizumab, Tiragolumab, Tocilizumab	CD3+ T cells increase rate (≥ 40%)	
NCT03944915 (DEPEND)	II	neoadjuvant	Nivolumab	DRR	
NCT02641093	II	adjuvant/neoadjuvant	Pembrolizumab, Cisplatin	safety and benefit of adding Pembro to SOC	(74, 75)
NCT04080804	II	Neoadjuvant	Nivolumab, Relatlimab, Ipilimumab	safety, AEs rate	
NCT03765918 (Keynote-689)	III	adjuvant/neoadjuvant	Pembrolizumab, Cisplatin	MPR, event free survival (EFS)	(74, 75)
NCT03700905 (IMSTAR-HN)	III	adjuvant/neoadjuvant	Nivolumab, Ipilimumab	DFS (approximately 71 months)	

IMCISION, immunomodulation by the combination of ipilimumab and nivolumab in neoadjuvant to surgery in advanced or recurrent head and neck carcinoma; NICO, Neoadjuvant and adjuvant nivolumab as immune checkpoint inhibition in oral cavity cancer; OPTIMA-II, Chemotherapy and locoregional therapy trial for patients with head and neck cancer; DEPEND, De-escalation therapy for human Papillomavirus negative disease; IMSTAR-HN, Study of Nivolumab alone or in combination with Ipilimumab as immunotherapy vs standard follow-up in surgical resectable HNSCC after adjuvant therapy; pCR, pathological complete response; DFS, disease free survival; DRR, deep response rate; AE, adverse event; MPR, major pathological response; EFS, event free survival; PFS, progression free survival.

As opposed to the CIAO and IMCISION trials where some patients enrolled were undergoing salvage surgery, a third trial recently presented at ASCO 2021 focused exclusively on challenging recurrent, surgically resectable HNSCC patients (NCT03341936) (73). Twenty-nine HNSCC patients with locoregionally recurrent disease who were surgically resectable were treated with neoadjuvant nivolumab and lirilumab, an anti-KIR blocking antibody focused on NK cell checkpoint inhibition. Patients also received 6 months of adjuvant nivolumab and lirilumab. There were no delays to surgery and 3/28 patients had Grade 3 AEs. Pathologic responses were seen in 12/28 (43%) of patients with 4 having MPR. Clinical outcomes were better than historical with 70% 1-year disease free survival and 85% 1-year overall survival.

In addition to ongoing Phase II trials, KEYNOTE-689 is an international phase III study (NCT03765918) where surgically resectable locally advanced HPV-negative HNSCC patients are randomized to receive upfront surgery with SOC adjuvant treatment or neoadjuvant pembrolizumab (two doses) followed by surgery and SOC adjuvant treatment with pembrolizumab (76). This trial aims to enroll 600 patients. In this trial, primary endpoints are rate of major pathological response (≤10% tumor cells in resected primary and lymph nodes on central review) and event-free survival (EFS). Secondary endpoints are OS, complete pathological response, and assessment of safety and tolerability.

Finally, we recently reported a second cohort of our neoadjuvant pembrolizumab trial where instead of one dose, patients received two doses of drug similar to the neoadjuvant phase of the KEYNOTE-689 Phase II trial (75). Compared to our initial cohort with one dose, we found that 50% of patients had any pTR and 44% of patients exhibited pTR2. This was nearly double what we saw with one dose of pembrolizumab. These data show that two doses or the longer neoadjuvant window (3 versus 6 weeks) resulted in an increased rate of pTR but did not increase the total proportion of patients with pTR.

IMMUNE RELATED ADVERSE EVENTS IN NEOADJUVANT IMMUNOTHERAPY TREATED PATIENTS

An important consideration in neoadjuvant immunotherapy approaches is clinical safety as the possibility of lifelong autoimmune complications in the definitive surgical setting needs to be weighed carefully. As mentioned above, to date neoadjuvant immunotherapy has been shown to be safe and has not resulted in surgical delays. In a phase II neoadjuvant immunotherapy clinical trial for oral cavity cancer patients which treated with nivolumab (N, n=14) or nivolumab and ipilimumab (N+I, n=15), two (N) and five (N+I) patients showed grade 3/4 AEs. These included oral mucositis and one patient with autoimmune diabetes (68) and there were no surgical delays. In another phase II neoadjuvant pembrolizumab clinical trial, we reported no severe grade 3/4 AEs and no surgical delays in a total of 36 treated HNSCC patients (54). Recently we reported an extension of this study with an additional 29 HNSCC patients treated with two cycles of neoadjuvant pembrolizumab. In this trial, only one patient showed a grade III AE (rash) while no patients had grade IV AE, consistent with the safety and tolerability of neoadjuvant immunotherapy (75).

CONCLUSION AND FUTURE PERSPECTIVES

The published and ongoing trials described above focused on single agent checkpoint blockade immunotherapy prior to surgery. In addition to this design, immunotherapy is being integrated in several neoadjuvant combinations with radiation or chemotherapy prior to

surgery. The Neoadjuvant Immuno-RadioTherapy (NIRT) phase Ib trial tested neoadjuvant stereotactic body radiation therapy (SBRT) with nivolumab (240 mg, q2 weeks x 3) prior to surgery in HNSCC patients (NCT03247712) (66). There were no treatment related delays thus achieving the primary safety endpoint. There was an 86% MPR rate and a 67% pCR rate. For all cohorts, there was a 90% clinical to pathologic down staging. NIRT did impact healing of wounds that all ultimately resolved. There were excellent clinical outcomes and only one patient required adjuvant chemoradiation. There are several questions about how this approach would integrate with current SOC including whether this treatment intensification is necessary especially in good prognosis HPV+ disease and the role of nivolumab as SBRT alone conferred a high rate of pathologic responses. In addition to radiation and immunotherapy combinations, other trials are testing chemotherapy/immunotherapy combinations. For example, in a phase II trial, platinum combined with immunotherapy (nivolumab) followed by transoral robotic surgery (TORS) or RT/CRT is being examined in oropharyngeal cancer patients (NCT03107182). Using a primary radiation based approach, several ongoing clinical trials aim to de-intensify the treatment impact by adding immunotherapy (77). For example, a phase II/III trial in patients with early-stage HPV-positive HNSCC is testing whether RT plus chemotherapy (cisplatin) or immunotherapy (nivolumab or durvalumab) can be used for de-intensification (NCT03952585, NCT03410615). There are several distinct mechanisms of how radiation and/or chemotherapy can work with immunotherapy and other have covered these topics. These trials will test the important topic of whether there is synergy in combination approaches with RT, immunotherapy and/or chemotherapy.

In conclusion, we provided here an overview of the history of neoadjuvant immunotherapies in HNSCC starting with chemotherapy extending to exciting frontiers using immunotherapy. IC continues to be used at some centers with defined indications including advanced or borderline resectable tumors. Management of toxicities in this setting remains a challenge. However, although IC may help with surgical management, Phase III trial results showed no improvements in survival. It has become clear that neoadjuvant immunotherapy, especially checkpoint inhibitors, are safe and have shown signals of clinical efficacy in HNSCC. These data together support further investigation in Phase III trials such as KEYNOTE-689 to define evidence for survival benefit and identify high-risk patients who may benefit from this approach. In addition, as other checkpoints are testing, further improvements in pathologic responses and clinical outcomes are expected. In conclusion, neoadjuvant approaches provide a potential exciting new treatment paradigm for HNSCC patients.

AUTHOR CONTRIBUTIONS

HS: writing original draft, tables, and figure. SS: editing the manuscript. RU: editing and supervising the manuscript, tables and figure. All authors contributed to the article and approved the submitted version.

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The Immune Landscape of Chinese Head and Neck Adenoid Cystic Carcinoma and Clinical Implication

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Novel systemic agents and effective treatment strategies for recurrence adenoid cystic carcinoma (ACC) of the head and neck are still worthy of further exploration. Here, we analyzed the mutations and expression profiles of 75 Chinese ACC patients, characterized the prognostic value of the immune signature for recurrence or distant metastasis, and explored the potential of immunotherapeutic biomarkers in ACC. In general, MYB fusion and somatic mutations accounted for a high proportion, which was 46.7% (35/75). ACCs displayed an overall low mutation burden and lack of programmed cell death ligand-1 (PD-L1) expression. The antigen-presenting machinery (APM) expression score and immune infiltration score (IIS) were the lowest among ACC patients, compared with other cancer types. For 61 primary cases, the locoregional recurrence-free survival (LRRFS) was statistically significantly correlated with the IIS [univariate analysis; hazard ratio (HR) = 0.32; 95% CI, 0.11–0.92; p = 0.035] and T-cell infiltration score (TIS) (univariate analysis; HR = 0.33; 95% CI, 0.12–0.94; p = 0.037]. Patients with lower IIS (log-rank p = 0.0079) or TIS (log-rank p = 0.0079) had shorter LRRFS. Additionally, solid pattern was also a prognostic factor related to locoregional recurrence, whereas postoperative radiotherapy (PORT) exerted its beneficial effects. We further evaluated the pretreatment immune profile of five ACC patients treated with PD-1 inhibitors. Patients who responded to camrelizumab or pembrolizumab observed elevated APM and TIS, compared with patients with progressive disease. Our study highlights the immune infiltration pattern and messenger RNA (mRNA) signatures of Chinese ACC patients, which has the potential value for prognosis and immunotherapy.

Keywords: immune infiltration, tumor microenvironment (TME), immune checkpoint inhibitors (ICIs), PD-1/PD-L1, adenoid cystic carcinoma (ACC)

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INTRODUCTION

Adenoid cystic carcinoma (ACC) is a rare malignancy predominantly arising from salivary glands, accounting for about 1% of all head and neck malignant tumors (1–3). ACC is characterized by indolent but relentless growing, perineural invasion and perineural spread, high propensity for local recurrence after initial treatment, and common distant metastasis (4). Although postoperative radiotherapy has been shown to increase the local control rate by 89–95% within 5 years (5, 6), the disease-free survival rates decline dramatically at 10 and 15 years (6–13). Therefore, novel systemic agent and effective treatment strategy for recurrence ACC are imperative to explore.

Immunotherapy is an important component of cancer treatment, especially recent advances in immune checkpoint inhibitors (ICIs) have begun to transform clinical cancer care (14). As one of the most successful immunotherapies, ICIs has been approved in a variety of solid tumor types. However, despite immune checkpoint therapy has demonstrated remarkable clinical efficacy in subsets of patients, the majority of patients did not show durable responses (15–17). Therefore, to better understand and overcome the mechanism of resistance, increasing studies have focused on the identification and development of predictive biomarkers of ICI response.

Selected biomarkers involving tumor mutational burden (TMB), microsatellite instability (MSI), programmed cell death ligand-1 (PD-L1) expression, and tumor-infiltrating lymphocytes have shown early promise in predicting response and benefit from ICIs (14, 18). Emerging data suggest that the tumor microenvironment (TME) may be a promising predictive biomarker for the survival benefit and prognosis of immunotherapy (19, 20). TME is complex and continuously evolving, which contains extracellular matrix and diverse cell types, such as fibroblasts, adipose cells, tissue-resident and peripherally recruited immune cells, and endothelial cells (21). Previous researches have suggested that immune cells in TME, as regulators in cancer progression, are becoming alluring therapeutic targets (21–24). For instance, tumor associated macrophages (TAMs) and regulatory T cells (Tregs) have been regarded to be protumor (25, 26), while $CD8^+$ T cells are associated with improved clinical outcomes and response to immunotherapy (27–29). The proliferation and activation of $CD8^+$ T cell rely on their T-cell receptor (TCR) recognizing the peptide antigen presented by major histocompatibility class I (MHC-I) on a target cell, evoking an antigen-specific immune response, thereby killing antigen-bearing cells (30). Antigen-presenting machinery (APM) genes encodes MHC-I subunits and proteins, which are essential for processing antigens and burden them onto MHC-I. Activated $CD8^+$ T cells and other immune infiltrates can secrete type II interferon gamma (IFN-γ), which induces upregulation of APM genes (31). Although the identification of $CD8^+$ T cells may be a predictive biomarker of response to immunotherapy in some contexts (32), it is not adequate to depict the cytotoxic potential of the complex TME.

To date, the molecular mechanism underlying the oncogenic activity of molecular alterations and tumor immune microenvironment in ACC remain elusive. We propose that ACCs own a distinct immune landscape, which might indicate diverse prognoses and treatment responses. Here, we first evaluate the genomic characteristics and the biomarkers currently used for checkpoint immunotherapy in 75 ACC cohort (**Figure 1A**). Then, we employed an APM score, a T-cell infiltration score (TIS), and an overall immune infiltration score (IIS) to highlight the immune infiltration status and their correlation with pathological features. Furthermore, the abundance of immune cells in TME of ACC was analyzed. Finally, in a small series of patients receiving anti-PD-1 agents, we assessed the correlation between immune signatures and the response to checkpoint blockade therapy. This study integrated and analyzed the whole exome, whole transcriptome, and clinical data to improve the understanding of TME in ACC.

METHODS

Clinical Sampling and Processing

We performed a retrospective study on patients with ACC. The study was conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS) and was approved by the Ethics Committee of Shanghai Ninth People's Hospital (approval number: 2016-74-T31). Additionally, a single-center database project involving head and neck cancer was approved in 2020 (approval number: SH9H-2020-T58-1), providing us with partial patient data. An independent cohort of 75 patients with head and neck ACC were included. They enrolled into Shanghai Ninth People's Hospital from 2012 to 2019. All participants obtained written informed consent. The main criteria were as follows: (a) informed consent; (b) no comorbidities (e.g., had suffered from other malignant tumors.); (c) complete and usable follow-up data; (d) a radical surgery performed and postoperative histopathology diagnosis confirmed; and (f) tumor stage classification was carried out according to the 7th edition of the American Joint Committee on Cancer TNM staging system. Pathological data consisted of tumor and nodal stage and subtype according to the 2015 World Health Organization (WHO) classification. Locoregional recurrence-free survival (LRRFS) was calculated from the day of surgery to first local–regional recurrence or death from any cause. Distant metastasis-free survival time (DMFS) was calculated from the day of surgery to the first distant metastases or death from any cause, identified by physical examination, positron-emission tomography–computed tomography (PET-CT) or CT or the most recent follow-up. We ended follow-up in February 2021. The median follow-up time for this cohort was 40.3 months. The 3-year rate LRRFS and DMFS rate in 61 primary cases were 27.9% (17/61) and 49.2% (30/61), respectively. The objective response of five patients treated with PD-1 inhibitors therapy is evaluated according to criteria for measurable disease in Response Evaluation Criteria in Solid Tumors (RECIST). The general data of the 75 patients with ACC are shown in **Table 1**.

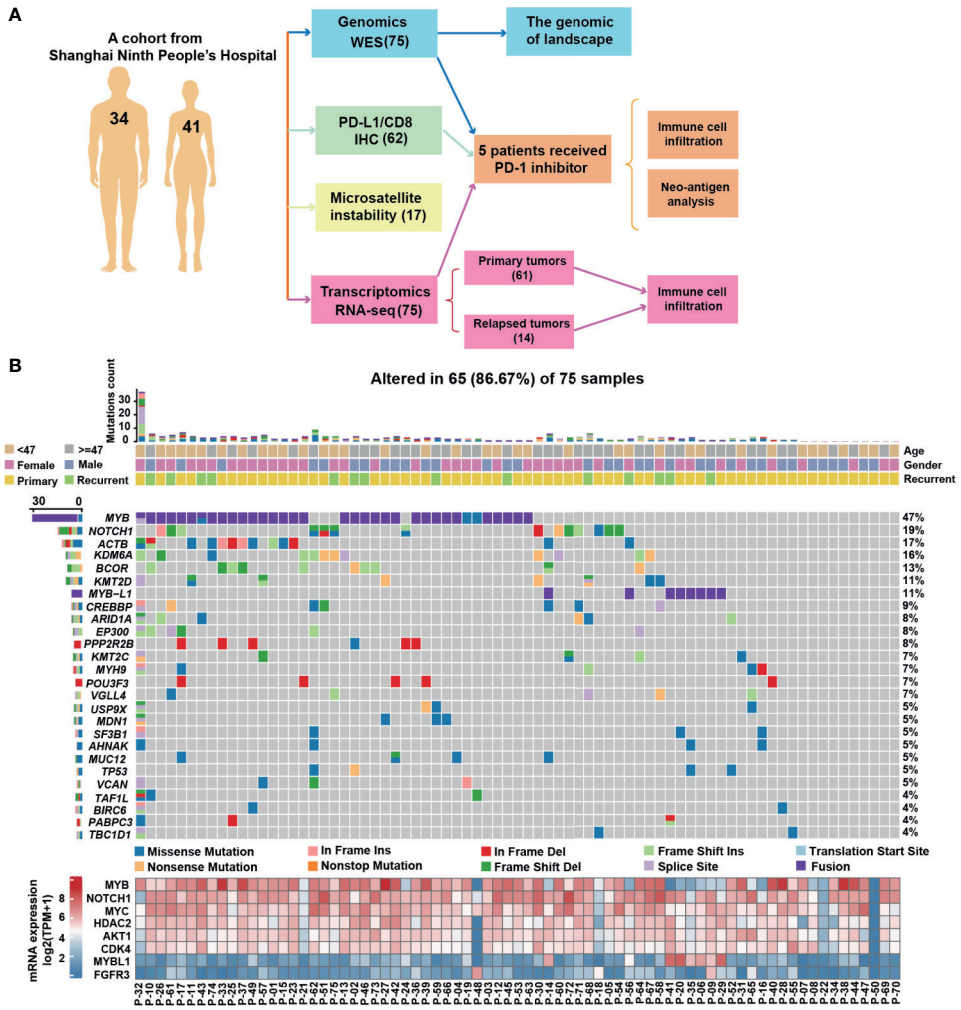

FIGURE 1 | The genomic landscape of adenoid cystic carcinoma. **(A)** Workflow of genetic hallmarks and immune-infiltrate profiling in ACC patients. **(B)** Mutation rate and type, age, gender, and surgical outcomes. Ten tumor samples were undetectable of any variant with allele frequencies (AFs) ≥1%. Bottom panel, RNA expression level for selected genes, expressed as $\log_2$(TPM + 1) for all samples. TPM, transcripts per million (TPM) expression value.

Tumor Infiltrating Lymphocytes, PD-L1, and CD8 Immunohistochemistry

Percentages of stromal tumor-infiltrating lymphocyte (TIL) were estimated in hematoxylin and eosin sections in 62 tumor samples according to the 2014 Guidelines developed by the International TILs Working (33). Immunohistochemistry (IHC) analysis was performed on the BOND-MAX autostainer (Leica, Wetzlar, Germany) with antibodies against the following: PD-L1 (clone 22C3, pharmDx; Dako, Carpinteria, CA, USA) and CD8 (cytotoxic T cells, clone C8/144B, Celnovtebio, China). PD-L1 expression was assessed by tumor proportion score (TPS), which was defined as the percentage of tumor cells with membranous PD-L1 staining. $CD8^+$ T-cell density was defined as the percentage of T cells stained with CD8 in a tumor region (central or marginal). All stained sections were independently reviewed by two pathologists. Any discrepancies were discussed together, and a consensus was achieved under the guidance of another experienced pathologist.

Somatic Variant Calling and Filtering

Somatic mutations from whole exome sequencing data were filtered with the following rules (1): 10 allele reads support (2), allele frequency ≥5% (3), supporting reads should be below 4 in the white blood cells (WBCs) control (4), mutation frequency of tumor should be eight times higher than that of the WBC control (5), the number of mutations in PoN should not exceed 2, and (6) no significant strand bias [GATK parameter FS >60 for single-nucleotide polymorphism (SNP) and FS >200 for indel]. Variants were also functionally filtered to remove those located in non-coding regions and synonymous mutations for downstream analysis. The log2 ratio >0.6 was considered a copy gain event. The log2 ratio less than −0.7 was considered a copy loss event.

TABLE 1 | Demographic data and clinicopathological features of the sample (N = 75).

Characteristics	Number (%)
Age (years)	
≥47	38 (50.7)
<47	37 (49.3)
Gender	
Female	41 (54.7)
Male	34 (45.3)
Histopathology	
Tubular	3 (4.0)
Cribriform	8 (10.7)
Solid	14 (18.7)
Mixed	36 (48.0)
AdCC *ex* PA	1 (1.3)
Unknown	13 (17.3)
TNM stage	
I-II	22 (29.3)
III-IV	35 (46.7)
Unknown	18 (24.0)
Necrosis	
Positive	15 (20.0)
Negative	43 (57.3)
Unknown	17 (22.7)
Solid subtype*	
Yes	20 (26.7)
No	42 (56.0)
Unknown	13 (17.3)
PORT received	
Yes	57 (76.0)
No	15 (20.0)
Unknown	3 (4.0)
Locoregional recurrence	
Yes	31 (41.3)
No	44 (58.7)
Distant metastasis	
Yes	46 (61.3)
No	29 (38.7)
LRRFS	
< 60 months	57 (76.0)
≥ 60 months	18 (24.0)
DMFS	
< 60 months	61 (81.3)
≥ 60 months	14 (18.7)

*Including presence of solid component.

AdCC ex PA, adenoid cystic carcinoma ex pleomorphic adenoma; DMFS, distant metastasis-free survival; LRRFS, locoregional recurrence-free survival; TNM, tumor-nodes-metastases; PORT, postoperative radiotherapy.

Gene Expression Analysis

The raw RNA-sequencing reads were filtered by FastQC and aligned using the spliced read aligner STAR2.0 (34), which was supplied with the Ensembl human genome assembly (GRCh37) as the reference genome. Gene expression levels were estimated by transcripts per kilobase million (TPM). Annotations of messenger RNA (mRNA) in the human genome were retrieved from the GENCODE (v19) database (**Supplementary Table 1**). The pan-cancer raw count gene-level RNA-Seq data were downloaded from The Cancer Genome Atlas (TCGA) Data Portal (35) (https://genome-cancer.ucsc.edu/). These cohorts consisted of adrenocortical carcinoma (tumor case = 79), bladder urothelial carcinoma (BLCA, tumor case = 433), colon adenocarcinoma (COAD, tumor case = 519), kidney chromophobe (KICH, tumor case = 89), lung squamous cell carcinoma (LUSC, tumor case = 551), lung adenocarcinoma (LUAD, tumor case = 594), and skin cutaneous melanoma (SKCM, tumor case = 472). Raw count gene expression data were used for gene expression analysis.

Gene Signatures

Marker genes that characterize immune cell types were acquired from Bindea et al. (36). As previously published by Şenbabaoğlu et al. (31), MHC class I genes (HLA-A/B/C, B2M) and genes involved in processing and loading antigens (TAP1, TAP2, and TAPBP) delineated the seven-gene APM signature; the TIS was defined as the mean of the standardized values of nine T-cell subtypes, and the overall immune infiltration score of a sample was similarly defined as the mean of the standardized values of innate and adaptive immune scores. A subset of genes from an IFN-γ gene expression signature was obtained from Efstathiou et al. (37) (**Supplementary Table 1**). Batch-corrected normalized data was input into Tumor Immune Dysfunction and Exclusion (TIDE) (38).

Implementation of Single-Sample Gene Set Enrichment Analysis

As reported by Şenbabaoğlu et al. (31), single-sample Gene Set Enrichment Analysis (ssGSEA) was applied for quantifying immune infiltration and activity in tumors using bulk RNA-seq data. ssGSEA (39) is a rank-based method, which is implemented using R package GSVA (40). Normalized RNA-Seq or microarray data should be used as input without further processing (i.e., no standardization or log transformation).

Statistical Analysis

One-way ANOVA using Kruskal–Wallis with Dunn's correction for multiple comparisons were performed with GraphPad Prism 7 (GraphPad Software, CA, USA). The unsupervised clustering of tumor samples, immune cell types, and gene expression was performed with hierarchical algorithm, Ward linkage, and Euclidean distance in R. Time-to-event endpoints were estimated using the Kaplan–Meier method. Univariate and multivariate analyses were performed using Cox proportional hazards models. To be assessed in the multivariate analysis, the variable should be significant ($p \leq 0.1$) in the univariate analysis. $p < 0.05$ were considered statistically significant ($^*p < 0.05$).

RESULTS

Patients and Tumor Characteristics

Seventy-five ACC patients were analyzed, including 61 cases of primary disease and 14 cases of recurrent disease (**Figure 1A**). Whole-exome sequencing (WES) of 75 tumors targeted 149,323 exons in 19,397 genes (mean coverage, 254×; 92.2% of target bases >50×). MuTect identified 5,330 somatic mutations, including 3,832 point mutations and 1,498 indels (insertions or deletions). The average and median somatic mutation rates were 4.55 and 0.85 per megabase (Mb), respectively. Nearly half (46.7%, 35/75) of the samples had MYB somatic alterations

(**Figure 1B**), including a total of 33 ACCs with MYB fusion, which were associated with increased mRNA level. Additionally, splice sites and coding mutations involving multiple exons of MYB were also discovered. Twenty-four NOTCH1 mutations were identified in 14 tumors, and six patients harbored more than one NOTCH1 mutation. It is worth noting that we observed relatively higher levels of MYBL1 mRNA in eight tumors with MYBL1 fusions compared with negative cases. Common copy number variances (CNVs) were found in AKT1 (29 cases; 39%), FGFR3 (21 cases; 28%), HDAC2 (16 cases; 21%) and CDK4 (15 cases; 20%) in our cohort (**Supplementary Figure S1A**), which were unassociated with mRNA expression (**Figure 1B**).

ACCs Display Overall Low Mutation Burden and PD-L1 Expression

TMB measured by whole-exome sequencing is associated with clinical benefit of multiple checkpoint inhibitors (41). TMB is defined as the number of non-synonymous mutations per 1 Mbp and divided into tertiles, while indelTMB is composed of small insertions and deletions with frameshifts. The median TMB was 0.85 Muts/Mbp (0–230.33 Muts/Mbp), and median indelTMB was 0.09 Muts/Mbp (0–49.64 Muts/Mbp, **Figure 2A**). Except for case No.P-32 who harbored numerous hotspot mutations (**Figure 1B** and **Supplementary Table 4**), the presented cases exhibit a low mutation burden, which is consistent with previous studies (42–44). Furthermore, the analysis of WES data demonstrated all patients with ACC were MSI negative, and 17 of them were confirmed by routine MSI-PCR testing (**Figure 2B**).

In 75 patients with primary or recurrent malignant neoplasms who underwent surgical treatment, 62 tumor tissues were analyzed for PD-L1 expression by immunohistochemistry, and the presence of $CD8^+$ immune cells was detected. The remaining 13 tumors could not be evaluated because specimens had an inadequate number of tumor cells. The majority of cases (72.6%, 45/62) did not show any membranous expression of PD-L1 (**Figure 2C**, left panel). Only 17 cases (27.4%, 17/62) of ACC displayed components with mild intensity of PD-L1 staining, accounting for approximately 1% of the tumor cells (**Supplementary Figure S1B**). The presence of infiltrating $CD8^+$ immune cells was evaluated in the tumor tissue and the surrounding stroma. In general, CD8+ immune cells in the entire cohort had a low degree of tumor infiltration. Merely 11 tumors

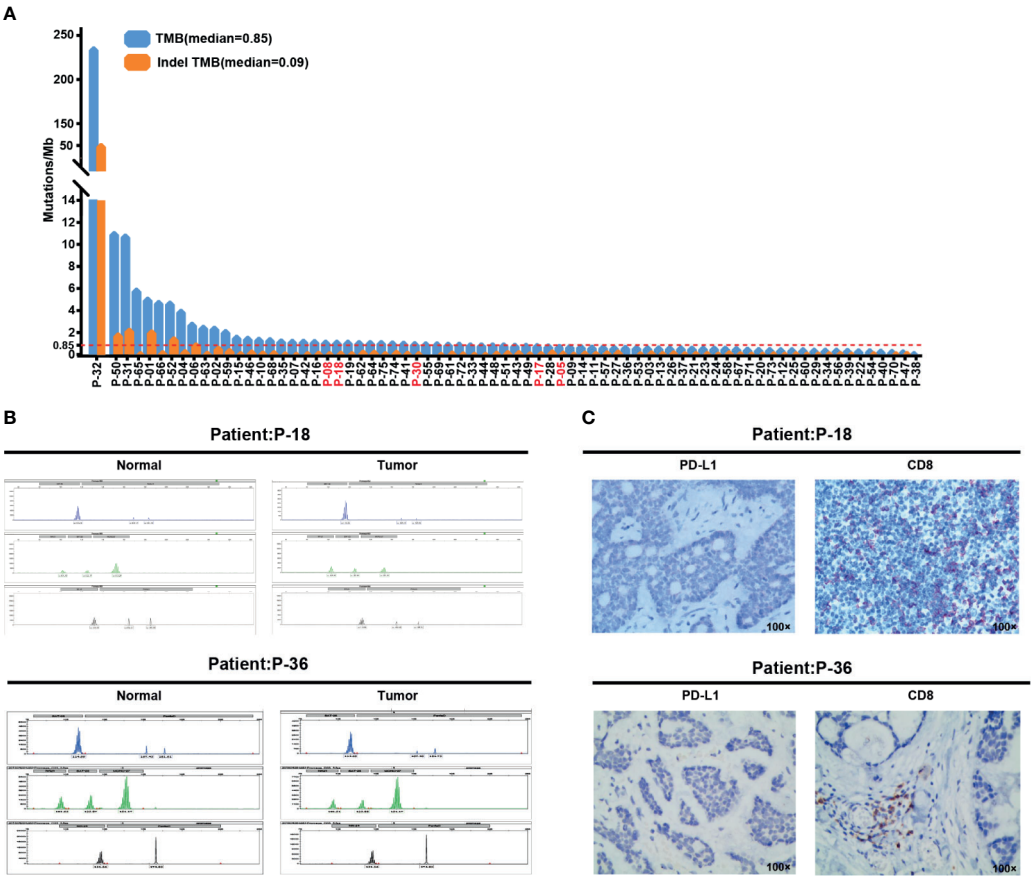

FIGURE 2 | Biomarkers for checkpoint inhibitor immunotherapy in ACC patients. **(A)** Distribution of TMB and indelTMB in 75 ACC samples. The red dotted line indicates the median value of TMB. Samples are arranged in order of decreasing TMB. **(B)** Case No.P-18 and No.P-36, microsatellite stable (MSS) according to conventional MSI-polymerase chain reaction (PCR) test. **(C)** Representative images for PD-L1 and CD8 immunohistochemical staining from case No.P-18 and No. P-36 at 100× original magnification.

(17.7%, 11/62) showed >10% $CD8^+$ prevalence (**Figure 2C**, right panel), and 23 tumors (37.1%, 23/62) were <1% or none (**Supplementary Table 2**).

Immune Infiltration of Tumor Microenvironment may be Used as an Indicator of Primary Tumor Recurrence

Şenbabaoğlu et al. employed mRNA-based scores for immune cell infiltration and the APM signatures, which were computed separately for each sample using ssGSEA (31). The TIS and IIS of each sample in the eight studied cancer types were calculated and used as the sum of the individual scores of the relevant immune subpopulations. Notably, the median APM score or IIS was the lowest in our cohort compared with seven other cancer types (**Figures 3A, B**), and the TIS of our ACC cohort was merely higher than that of TCGA adrenocortical carcinoma (**Figure 3C**). Furthermore, using the ssGSEA scores from the expanded panel of 28 immune-related and inflammation-related gene signatures, we observed that our ACC patients had low infiltration and weak CD8 signal compared with that of the samples of seven TCGA cancer types (**Figure 3D**). The low mutation load in ACC cells combined with the weak activity of

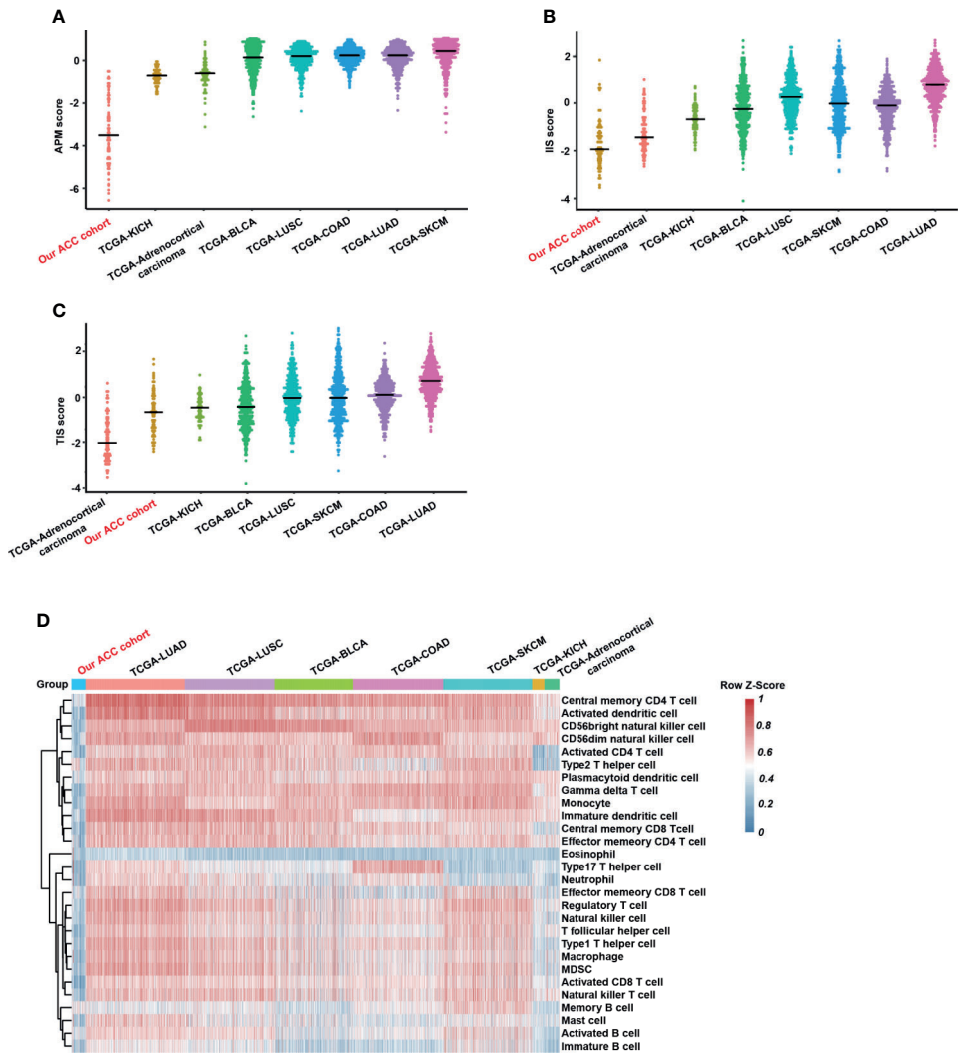

FIGURE 3 | APM, immune infiltration, T cell infiltration, and immune cells in our ACC cohort compared with seven TCGA cohorts. **(A)** Antigen-presenting machinery (APM) score in eight tumor types. Each dot represents an individual tumor sample. Tumor types are ordered from left to right according to increasing median APM (medians indicated by horizontal black bars). **(B)** Overall immune infiltration score (IIS) for eight tumor types. Tumor types are ordered from left to right according to increasing median IIS (medians indicated by horizontal black bars). **(C)** T-cell infiltration score (TIS) in eight tumor types. Tumor types are ordered from left to right according to increasing median TIS (medians indicated by horizontal black bars). Immune cell Score estimated by ssGSEA for our cohort compared with adrenocortical carcinoma, BLCA, COAD, KICH, LUSC, LUAD, and SKCM TCGA samples, representing overall immune infiltration, based on gene level normalized count of respective RNA-Seq data. **(D)** Unsupervised clustering patients from our ACC cohort and 7 TCGA cohorts using ssGSEA scores from 28 immune cell types. ACC, adenoid cystic carcinoma; BLCA, urothelial bladder carcinoma; COAD, colon adenocarcinoma; KICH, kidney chromophobe; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; SKCM, skin cutaneous melanoma.

antigen processing and presentation indicates that the availability of depressed tumor antigens may exist extensively in ACCs and foster an immune-poor tumor microenvironment.

These data lead us to explore whether the tumor microenvironment of primary disease is related to the ability of tumors to evolve locoregional recurrence or distant metastasis after initially definitive therapy. For 61 primary cases, the association of LRRFS or DMFS with APM score, IIS and TIS were analyzed by log-rank test, and the data suggested that APM gene expression alone was not associated with improved outcomes (**Figure 4A** and **Supplementary Figure S2F**), while ACCs with lower IIS (log-rank p = 0.0079) or TIS (log-rank p = 0.0079) score had shorter locoregional recurrence-free survival time (**Figures 4B, C**). However, IIS and TIS were not associated

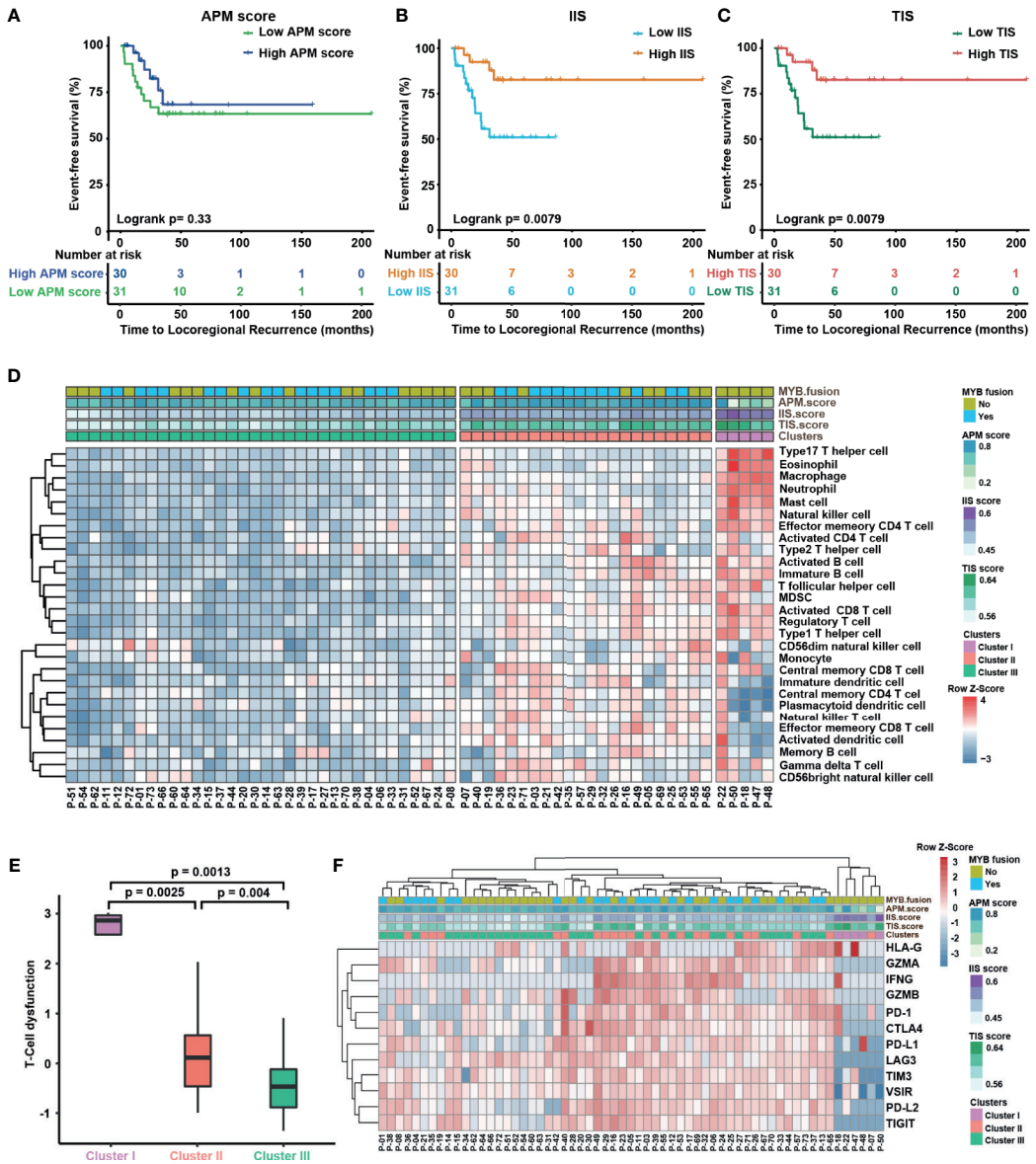

FIGURE 4 | Immune infiltration of tumor microenvironment may be able to exert an indicator of primary tumor recurrence. **(A–C)** Kaplan–Meier curves for distant metastasis-free survival in the above-median and below-median groups for the APM score **(A)**, IIS **(B)**, and TIS **(C)**. The median values for IIS and TIS are able to stratify 65 primary cases into groups with significant locoregional recurrence differences. Statistical significance was assessed by using the log-rank test. **(D)** Unsupervised hierarchical clustering of ACC patients from 65 primary tumors using ssGSEA scores from 28 immune cell types. Hierarchical clustering was performed with Euclidean distance and Ward linkage. We discover three immune infiltration clusters, here termed (1) cluster I (2), cluster II, and (3) cluster III. **(E)** TIDE was used to analyze T-cell dysfunction among three clusters. Kruskal–Wallis test with Dunn correction (nonparametric). **(F)** Heat map showing expression of the inhibitory checkpoint molecules (PDCD1, PD-L1, PD-L2, LAG3, TIM3, CTLA-4, TIGIT, and VISTA) and effector molecules prominently associated with T-cell response (GZMA, GZMB, HLA-G and IFNG) across tumors from 65 primary cases.

with DMFS (**Supplementary Figures S2G, H**), and the stage-specific differences in the expression levels of APM or IIS or TIS were not significant (**Supplementary Figures S2C–E**). To elucidate the underlying mechanism of immune cell infiltration on ACC recurrence, 61 primary cases were clustered into three clusters (cluster I, 5; cluster II, 22; and cluster III, 34) in terms of 28 immune cell types applied by unsupervised clustering. We observed that patients in the cluster II harbored higher APM scores, whereas cluster I have lower APM scores and higher IIS (**Figure 4D**, upper panel). Although there was no significant difference in clinical outcomes between patients in the cluster III and the other two clusters, a faster trend of recurrence was observed (**Supplementary Figures S3A, B**), which could be explained by a scarcity of immune surveillance in a poorly immune microenvironment. We further examined whether any T cells entered a state of dysfunctional or exhausted. A computational method to evaluate T-cell dysfunction, named TIDE, was utilized (38). Our results indicated significant differences in the level of T-cell dysfunction between the three clusters (**Figure 4E**). Furthermore, we analyzed the expression of inhibitory checkpoint molecules (PD-1, PD-L1, PD-L2, LAG3, TIM3, CTLA-4, TIGIT, and VISTA) and effector molecules (GZMB and IFNG) that were prominently associated with T-cell response. Strikingly, patients in the cluster I had lower expression of these inhibitory checkpoints compared to clusters II and III (**Figure 4F**). These results suggested T-cell dysfunction does not play a critical role in the recurrence of ACCs.

Furthermore, the prognostic significance of immune infiltration signatures and other clinicopathological features was examined using univariate and multivariate regression models (see **Table 2** and **Supplementary Table 2**). Factors related to a shorter LRRFS in univariate analysis were high IIS [hazard ratio (HR) = 0.32; 95% CI, 0.11–0.92; p = 0.035], high TIS (HR = 0.33; 95% CI, 0.12–0.94; p = 0.037), with postoperative radiotherapy (PORT, HR = 0.18; 95% CI, 0.06–0.51, p = 0.001), the presence of necrosis (HR=3.91, 95% CI 1.38 to 11.09, p= 0.01), and solid growth pattern (HR = 16.13; 95% CI, 5.80–44.87; p = 0.00). In the multivariate analysis, the solid growth pattern was shown to be an independent prognostic factor related to tumor recurrence (HR = 20.63; 95% CI, 4.35–97.75; p = 0.0001), and postoperative radiotherapy was also confirmed as an excellent independent prognostic factor (HR = 0.07; 95% CI, 0.02–0.31; p = 0.0004). Similarly, the solid growth pattern (HR = 3.4; 95% CI, 1.53–7.54, p = 0.003) and the relapsing disease (HR = 2.0; 95% CI, 1.03–4.01; p = 0.041) were confirmed to be correlated with a higher rate of distant metastasis, and postoperative radiotherapy (HR = 0.36; 95% CI, 0.14–0.94, p = 0.036) was an independent prognostic factor related to improved clinical outcome.

In contrast, all parameters of immune infiltration were not significantly associated with DMFS in 14 cases of recurrent tumors (**Supplementary Figures S4A–C**). Unsupervised clustering of recurrent cases according to 28 immune cell types showed no predominantly separation, except for three cases with high immune infiltration (**Supplementary Figure S4D**). Unexpectedly, immune-rich cases had higher inhibitory checkpoints expression and APM score compared with other patients (**Supplementary Figures S4E, F**), slightly concordant with IFN-related gene expression (**Supplementary Figure S4G**). Although our results may indicated that immunotherapy is a potential choice for effective control of these characteristic recurrent tumors, no significant response was observed in patients with recurrent/metastatic ACC in recently updated trial (45).

TABLE 2 | Univariate and multivariate analyses of factors associated with time to locoregional recurrence and distant metastasis in ACC cohort (N=61).

Variables	Time to locoregional recurrence				Time to distant metastasis			
	Univariate P	Multivariate			Univariate P	Multivariate		
		HR	95%CI	P		HR	95%CI	P
Age, years (≥45 *vs.*<45)	0.653			NA	0.197			NA
Gender (male *vs.* female)	0.094			NA	0.441			NA
APM score (high *vs.* low)	0.301			NA	0.606			NA
IIS (high *vs.* low)	**0.035**	0.21	0.02-1.85	0.160	0.341			NA
TIS (high *vs.* low)	**0.037**	1.08	0.12-9.59	0.942	0.296			NA
TMB (high *vs.* low)	0.894			NA	0.465			NA
IndelTMB (high *vs.* low)	0.35			NA	0.904			NA
TNM stage (III-IV *vs.* I-II, unknown)	0.479			NA	0.252			NA
PORT (yes *vs.* no, unknown)	**0.001**	**0.07**	**0.02-0.31**	**0.0004**	**0.002**	**0.36**	**0.14-0.94**	**0.036**
PNI (positive *vs.* negative, unknown)	0.454			NA	0.384			NA
Necrosis (positive *vs.* negative, unknown)	**0.01**	1.66	0.46-5.97	0.435	0.656			NA
Margin (positive *vs.* negative, unknown)	0.826			NA	0.121			NA
Solid subtype (yes *vs.* no)	**0**	**20.63**	**4.35-97.75**	**0.0001**	**0.003**	2.01	0.65-6.25	0.228
MYB mutation (positive *vs.* negative)	0.156			NA	0.401			NA
State of disease (relapsed *vs.* naive)	NA	NA	NA	NA	**0.041**	0.98	0.38-2.51	0.961

Univariate analysis was calculated by the Kaplan–Meier method (log-rank test). Multivariate analysis was done using the Cox multivariate proportional hazard regression model with stepwise manner. Bolded values have statistic significance.

APM, antigen presenting machinery; IIS, immune infiltration score; TIS, T cell infiltration score; TMB, tumor mutational burden; Indel, insertions and deletions; TNM, tumor-nodes-metastases; PORT, postoperative radiotherapy; PNI, perineural invasion; HR, hazard ratio; CI, confidential interval; NA, not adopted.

Baseline Elevation in Immune Infiltration Signature of ACC Patients Responding to PD-1 Blockade

Given that we had determined the relationship between immune infiltration signatures and clinical status, we next investigated whether there was a correlation between the baseline immune landscape and response to immunotherapy. Since 2016, immunotherapy with anti-PD-1 inhibitors can be used to treat recurrent/metastatic squamous cell carcinomas of the head and neck. We investigated the pretreatment immune profile of patients treated with this agent using a set of five patients (**Table 3**). The mutational landscape of all five tumors revealed that *NOTCH1* mutations were prominent in this set (**Figure 5A**). Moreover, we found that the expression of APM, TIS, and GZMB was elevated in patients who partially responded to camrelizumab, whereas the expression was lower in patients with progressive disease with anti-PD-1 agents (**Figure 5B**). Remarkably, the responding patients displayed reduced tumor volume on all metastases (**Figure 5C**). This correlation should be corroborated in a larger cohort to determine whether it has predictive capability in determining the response to PD-1 blockade.

DISCUSSION

The treatment of ACC has not surpassed surgery and adjuvant radiotherapy in the past decades (8, 46), and no new drugs have been approved for the disease due to the limited understanding of the molecular alterations associated with aggressive disease (47). This comprehensive study of 75 ACCs provides certain novel insights into disease biology and delineates the immune microenvironment of tumors. Several genomic alterations identified in this study, particularly those involving the c-Myb and Notch1 pathways, have been fully confirmed for their potential oncogenic and prometastatic roles in ACC (48, 49). In particular, the Notch signaling blockade, AL101 showed clinical activity in recurrent/metastatic Notch mutant ACC and seems to be well tolerated (50). MYBL1 fusion in a subset of tumors lacking *MYB* alteration indicates that MYB-like signaling may be required for the development of ACC. In addition, we found mutations in genes encoding chromatin-state regulators, such as *KDM6A*, *CREBBP*, and *KMT2D*, which suggests that there is aberrant epigenetic regulation in ACC oncogenesis.

TMB is an emerging independent biomarker of outcomes with immunotherapy for multiple tumor types (51–55). TMB with at least 10 mutations per megabase is an effective biomarker for lung cancer (51). The more mutations the tumor accumulates, the higher likelihood of production and subsequent presentation of neoantigens on major MHC molecules causing a higher tendency of tumor cell cytotoxicity after inhibition of checkpoint signals (56, 57). In our analysis, ACCs displayed an overall low mutation burden and had the lowest APM median, suggesting that antigen generation and presentation were in an inactive state, which may partially explain the low immune infiltration exhibited by ACC as a cold tumor (58). Additionally, we predicted that MHC-I would bind to tumor neo-antigens in patients receiving PD-1 blockade therapy (**Supplementary Methods**), while patients who responded to immunotherapy did not show high abundance of cancer neo-antigens (**Supplementary Figure S3C** and **Supplementary Table 3**). Some indels in the protein coding region may affect the structure or function of the protein (**Supplementary Table 4**). This potential finding should be corroborated in a larger cohort.

Three growth patterns of ACC have been described: cribriform, tubular, and solid. The cribriform and tubular growth patterns are less aggressive (4). Tumors that exhibit a solid pattern or have solid components are more likely to spread and have a worse prognosis (7–9). In our analysis, the solid growth pattern was shown to be an independent prognostic factor related to tumor recurrence (HR = 20.63; 95% CI, 4.35–97.75, p = 0.0001). Notably, our results validated that PORT may exert its beneficial effect by preventing locoregional recurrence and distant metastasis (**Table 2**).

Although the relationship between survival rate and benefit is still controversial (59–61), multiple studies have reported that the local control with PORT is better, regardless of the tumor stage (46, 62). This difference in clinical outcome is probably due to the effect of radiotherapy on TME. Of note, complicated immune responses to irradiated TME are neither utterly immunostimulatory nor immunosuppressive. These reactions involve effects on the cells inherent in TME such as altered in inflammatory cytokine production, antigen presentation, and dendritic cells (DC) priming, and relative expansion in radioresistant immunosuppressive macrophage and T-cell populations (63). A certain degree of PORT-induced immune response in TME may be predicted based on the baseline information of the primary lesion; it may be worth exploring adjusting immunotherapy strategy to overcome the adaptive immune suppression.

Our results highlighted that ACC tumors have the lowest median APM score and immune infiltration median (**Figures 3A, B**). The expression of APM genes and IFN-

TABLE 3 | Patients' characteristics.

Nr	Sex	Age	Subtype	Stage	Anti-PD-1 Inhibitor	Line	Previous lines of therapy
P-18	F	60	Solid	Local recurrence, metastatic (bone)	Camrelizumab	1	
P-05	M	51	Solid	Local recurrence, metastatic (bone, mediastinal lymph nodes)	Camrelizumab	2	Paclitaxel,cisplatin
P-30	F	71	Unknown	Metastatic (lung, mediastinum, pleura)	Pembrolizumab	2	Apatinib
P-08	M	28	Mixed	Metastatic (lung)	Camrelizumab	4	Apatinib/Everolimus/Docetaxel, Nedaplatin
P-17	M	29	Mixed	Metastatic (lung, adrenal gland)	Camrelizumab	2	Apatinib

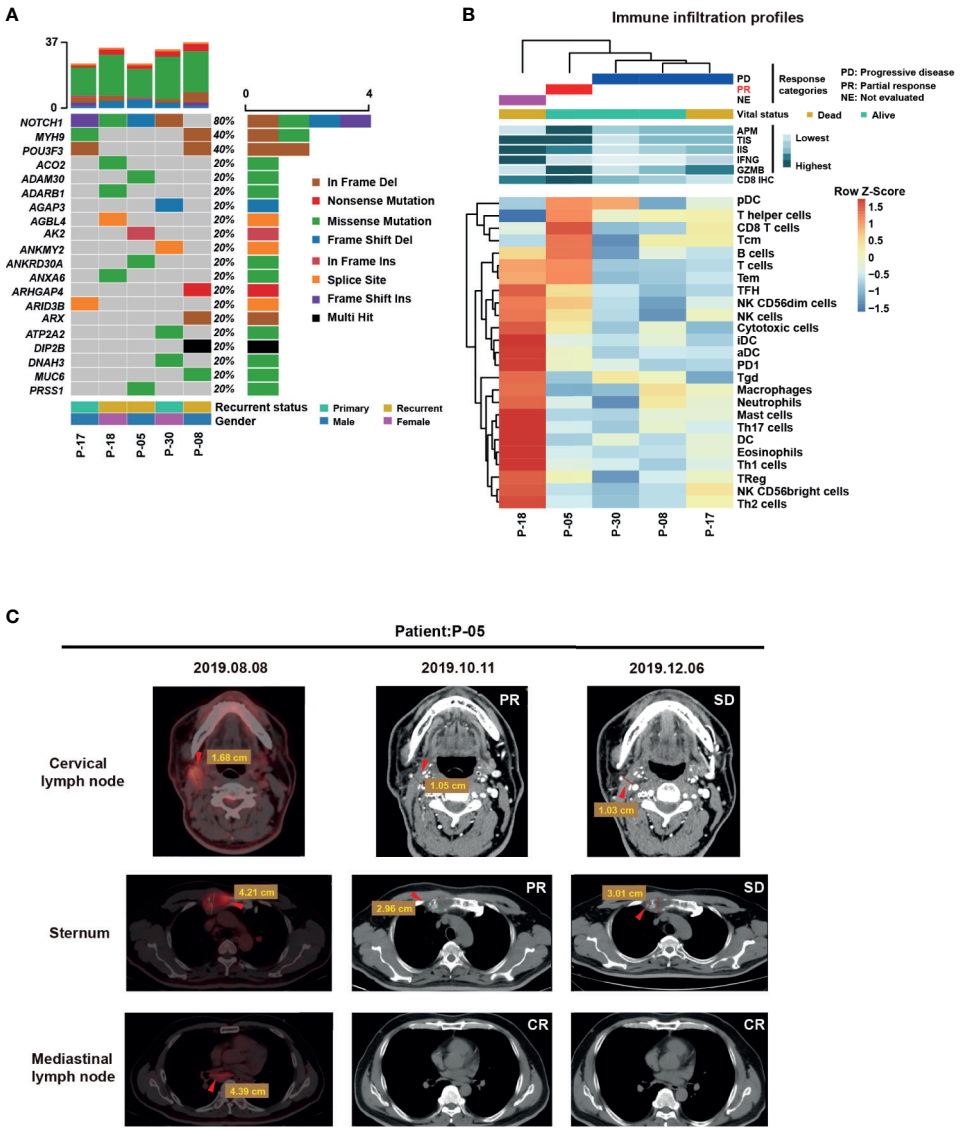

FIGURE 5 | Mutational and Immune Infiltration Profiles in anti-PD1 inhibitor- treated ACC patients. **(A)** Data from combined WES sequencing of samples was analyzed to generate an oncoplot showing distribution of mutations. **(B)** RNA-Seq profiles of five ACC patients were generated and the patients were then treated with the anti-PD1 inhibitor pembrolizumab or camrelizumab. T cell infiltration as well as APM, TIS, and GZMB levels are generally high in a responder (partial response to camrelizumab). **(C)** Detection of recurrent metastatic ACC on positron-emission tomography-computed tomography (PET-CT) with ^{18} F-fluorodeoxyglucose (^{18} F-FDG) or CT. Images represent axial CT scans with volume reduction by RECIST criteria. The 53-year-old man (case No.P-05) in these images underwent immunotherapy and radiation therapy from August to December 2019. PET-CT was performed in August 2019. Axial views on PET (left panel) show disease recurrence with widespread metastases (red arrows). Contrast-enhanced CT was performed after the 4 cycles of immunotherapy in October 2019 and showed remarkable reduction in the volume of three metastases (middle panel, red arrows). Contrast-enhanced CT was performed after the 8 cycles of immunotherapy in December 2019 and showed stable disease (SD) (right panel, red arrows).

related genes was investigated and characterized as a similar expression pattern in primary cases (**Supplementary Figures S2A, S3D**), which is consistent with the previous discovery that IFN-γ activated antigen presentation (64). Preliminary evidence from TME studies suggests that the ACC microenvironment has low immunogenicity, represented by low TIL and DC density (65, 66), which was associated with β-catenin/Wnt and PI3K pathways (67, 68). Moreover, immune tolerance phenotype tumors are also accompanied by low or no PD-L1 expression (69), as observed in our ACC cohort. These observations could be explained to some extent by Theelen et al., who reported that impaired IFN-γ response signal transduction in tumor cells may be related to the devoid of PD-L1 expression (70). Compared with the other two clusters, low PD-L2 expression was observed in cluster I (**Figure 4F**), which may be related to the inactive IFN-γ signaling pathway (**Supplementary Figure S3D**).

Unsupervised clustering of primary cases using immune infiltration levels revealed three clusters of infiltrated tumors; however, this classification did not show prognostic significance (**Supplementary Figures S3A, B**). On the contrary, IIS and TIS are significant immune predictors of locoregional recurrence. We speculated that due to intertumor heterogeneity of immune infiltration in cluster I (**Figure 4D**), principal component analysis (PCA) on the ACC 28 immune cell types showed that a good difference between cluster II and III tumors (**Supplementary Figure S2B**). Mosconi et al. (65) have reported that the majority of ACC cases in their cohort would be classified as immune tolerance type (71). Even though ACC tumors exhibited supressed immune infiltration and T-cell responsiveness, IIS and TIS may still be indicators of disease recurrence. Coincidentally, equivalent results of univariate analysis of locoregional recurrence were obtained through IIS and TIS grouping (**Figures 4B, C**).

In our cohort, primary tumors are predominantly enriched for cluster II or III; however, cluster III patients showed increased propensity to relapse (**Supplementary Figure S3A**). There are similar observations in other tumor types (72–75). Strikingly, MYB fusion along with poor antigen-presenting function and the presence of a highly exhausted T cell, but low levels of inhibitory checkpoints, were observed in cluster I tumors. Although dysfunctional T cells have been exhibited to upregulate inhibitory receptors in multiple studies (76–78), comparable results have not yet observed in ACCs (**Figures 4E, F**). In other words, T-cell dysfunction may not be a distinguished indicator of ACC immunotherapy. Therefore, our data indicate that cluster III patients may respond poorly to T-cell checkpoint inhibitors due to reduced T-cell infiltration. In contrast, due to the relatively high level of inhibitory checkpoint molecules and APM score, cluster II patients could deserve to be further studied to evaluate possible immunotherapeutic strategies.

It should be noted that, although diverse clustering patterns were analyzed in recurrent disease cohort, we did not observe clear associations among APM, IIS, TIS, and clinical outcome (**Supplementary Figures S4A–C**). The intricacy of immune cell populations infiltrating tumors with their synergistic or opposing effects may affect tumors differently depending on their histological and molecular type, their stage, the microenvironment of the organ in which they plant, or the nature of the primary tumor or its metastases (79). Numerous researches have shown that strong lymphocyte infiltration was associated with improved clinical outcome in multiple tumor types (80–83), but in this case, immune evasion occurs perhaps due to the fact that T cell could not migrate to the tumor site.

The optimal immunotherapy for ACC has not yet been fully established. To date, the activity of multiple checkpoint inhibitors in ACC is currently under active investigation (ClinicalTrials.gov identifier: NCT03146650 and NCT04209660). In completed studies, the NISCAHN study showed that single-agent nivolumab had limited efficacy in patients with recurrent/metastatic ACC (45). Schoenfeld et al. presented a randomized phase II study of pembrolizumab with or without hypofractionated radiation; no objective response was observed in this study, but over half of ACCs achieved disease stability (84). Our anti-PD-1 mAb treatment response results indicate that monotherapy may not be an optimal choice for patients with ACC, especially those with poor antigen presentation. In addition, we analyzed the differential genes of patients with partial response (PR) to immunotherapy compared with progressive disease (PD). These genes have been analyzed through Connectivity Map (CMap) to output 10 drugs, of which chorambucil and altretamine are chemotherapy drugs with similar efficacy with PD-1 inhibitors (**Supplementary Figure S3E**). Preliminary studies have shown that tumor immune evasion can occur through high expression of PD-L1 or tumor immune infiltration of PD-1-positive T lymphocytes (85). Although PD-L1 expression was absent in most ACC cases reported here, immune infiltration plays an important role in TME and can be manipulated as a mechanism of immune surveillance. We have observed that patients with relatively lower dewelIIS and TIS recurred faster. It is noteworthy that the responding patient may be due to the existence of a clinically significant abscopal effect by combining radiotherapy (**Supplementary Figure S3F**). Checkpoint inhibitors administered before or concomitant with radiotherapy may be a worthwhile therapeutic strategy to be further explored in ACCs.

Several caveats limit the generalizability of our works. Given the rarity of ACC and the risks associated with tissue collection, we were limited by the absence of matched adjacent normal tissue that could be quantified for various immune markers and mRNA expression, despite retrieving samples collected over decades.

In the current study, we performed a comprehensive evaluation of the genomic characteristics and immune microenvironment of ACC. Collectively, ACC is a cancer type that is slightly immune infiltrated, which may partially explain the poor response to immunotherapy. On the other hand, immune infiltration may facilitate immune surveillance and act as an indicator of primary tumor recurrence. This analysis hints that immune infiltration patterns may act as potential biomarkers of immune therapy and may guide the development of novel drug combination strategies.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Shanghai Ninth People's Hospital (approval number: 2016-74-T31). A single-center database project involving head and neck cancer was approved in 2020 (approval number: SH9H-2020-T58-1). 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

GPZ and DC designed the project. SJD, RRL, NH, YDY, and GDZ contributed to development of methodology. GPZ, SJD, RRL, WJ, LLY, and JL performed the patient treatment and collected the ACC samples and clinical information. SJD, NH, and DC processed and interpreted the data. SJD, DC, MZ, and GPZ wrote and revised the manuscript. YDY and YNY provided administrative, technical, and material support. 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.2021.618367/full#supplementary-material

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PD-L1-Mediated Immunosuppression in Oral Squamous Cell Carcinoma: Relationship With Macrophage Infiltration and Epithelial to Mesenchymal Transition Markers

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To date, immune check-point inhibitors (ICIs), particularly inhibitors of programmed cell death-1 (PD-1) and PD ligand-1 (PD-L1) have become prominent in cancer treatment and also improved life expectancy of cancer patients. As key regulators of PD-1/PD-L1 axis, the recruitment of tumor-associated macrophages (TAMs) enhances aggressive and invasive properties of tumors in immunosuppressive tumor microenvironment (TME) and promotes epithelial-mesenchymal transition (EMT). The aims of the study were first to characterize the critical links among PD-L1, TME and EMT process and, further, to explore the sensitivity of different chemical agents to different PD-L1 expression groups. Bioinformatical analysis revealed that PD-L1 was highly expressed in OSCC and higher PD-L1 expression correlated with worse survival in patients. Notably, PD-L1 was positively correlated with macrophages infiltration and EMT markers gene expression. Moreover, patients in the PD-L1^{high} group were at a significant chance of benefiting from ICI treatment and they also showed higher sensitivity to the chemical drugs (olaparib, paclitaxel, docetaxel, and pazopanib). These findings implicate PD-L1 could serve as a novel target for prognostic and therapeutic approaches in OSCC patients; PD-L1-mediated immune evasion might be attributable to the infiltration of macrophages, resulting EMT progress; Chemical agents in combination with PD-L1 inhibitor could be served as personalized treatment plan for OSCC patients so as to maximize patient benefit.

Keywords: PD-L1, immunosuppressive tumor microenvironment, macrophages, epithelial-mesenchymal transition, chemical drugs

INTRODUCTION

Oral squamous cell carcinoma (OSCC), as one of the 10 most frequent cancers with approximately 300,000 new cases diagnosed annually is perceived as an immunosuppressive cancer (1). Despite advances in therapeutic approaches, including surgical methods and radiotherapy, patients with locally advanced or metastatic OSCC still face the risk of a poor prognosis (2). Therefore, further efforts are still demanded to identify clinically relevant biomarkers, establish effective mechanism-based combinations, and develop effective targeted therapies for OSCC.

Cancer immunotherapy, including immune check-point inhibitors (ICIs), Oncolytic virotherapy (OVT), and chimeric antigen receptor (CAR) T cells has led to major improvements in tumor treatment over the past two decades (3). ICIs expressed on the cell surface, including programmed cell death-1 (PD-1) and PD ligand-1 (PD-L1), play crucial roles in activating negative regulatory pathways and evading immune surveillance (4). Upon activation, ICIs can dampen antitumor immune responses, leading to cancer cells escaping from host immune system (5). The interaction between these ligands can be blocked by ICIs, thereby reactivating the cytocidal immune response. Unprecedented advances in tumor control have been made using therapeutic monoclonal antibodies (mAbs) to block ICIs. Particularly, mAbs targeting PD-1/PD-L1 have brought great clinical benefits in multiple indications, either as monotherapy or in combination regimens (6–8). Pembrolizumab and nivolumab as anti-PD-1 mAbs have shown remarkable anti-tumor activity in the treatment of head and neck squamous cell carcinoma (HNSCC), leading to their regulatory approval (9–11). The PD-1/PD-L1 interaction inhibits antitumor activity of cytotoxic lymphocytes (CTLs) (12), which contributes to multiple Suppressive effects, such as immune escape, tumor proliferation, invasion, angiogenesis, and epithelial-mesenchymal transition (EMT) (13). At present, the links among PD-L1, TME, and EMT process in OSCC are not well understood, and the expectation that immunotherapy in combination with standard-therapy can maximize patient benefit necessitates further research on PD-L1-mediated immunosuppression. The present study was aimed at characterizing the critical links among PD-L1, TME and EMT process in OSCC, and further exploring the sensitivity of ICI treatment and different chemical agents to different PD-L1 expression groups.

METHODS AND MATERIALS

Data Acquisition

All Clinical and sequencing data were obtained from the Cancer Genome Atlas (TCGA) and the Gene Expression omnibus (GEO) research network. Transcriptome data of 150 OSCC tissue samples and 30 normal oral tissue samples were extracted from TCGA. Patients' information on age, tumor-node-metastasis (TNM), stage, survival time and status were organized (**Supplementary Table 1**). The GEO database was used to obtain the OSCC microarray data set GSE30784, and 167 OSCC cancer patient samples were selected as the validation set based on the sample information (14). The EMT related genes were extracted from the Molecular Signature Database (MSigDB) (15).

Bioinformatic Analysis

Differentially expressed genes (DEGs) in different groups were identified using EdgeR package and filtered by | log2 (Fold Change) | > 1 and adjusted P value < 0.05 which was adjusted using the Benjamini-Hochberg (BH) approach (16). Furthermore, the enrichment analysis including Gene ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation was conducted by cluster Profiler package. The PD-L1 expression level in diverse cancer types and correlation between PD-L1 and immune infiltrates were conducted using Tumor Immune Estimation Resource (TIMER) database (17). Gene marker sets of immune cell types were obtained from Bindea et al. (18) and Newman et al. (19). Single-sample Gene Set Enrichment Analysis (ssGSEA) and relative abundance of immune cells were calculated by Gene Set Variation Analysis (GSVA) (20). The classical chemokines and markers of macrophages and EMT signaling pathway were also included (21–24). Tumor Immune Dysfunction and Exclusion (TIDE) algorithm was applied to get individual immunotherapy response (25, 26), and individual chemotherapeutic response was predicted based on Genomics of Drug Sensitivity in Cancer (GDSC) database (27, 28). Drug sensitivity prediction was perform using R package 'pRRophetic' (29).

Statistical Analysis

R statistical language and SPSS 22.0 software were used for statistical analyses. Expression differences among different tumor grade groups were compared using one-way analysis of variance (ANOVA) test. Overall survival (OS) distribution and survival curves were performed by R package survival. The optimal cutoff point for PD-L1 expression level was determined by the 'surv_cutpoint' function of 'survminer' R package and calculated utilizing the maximally selected rank statistics that calculated the most optimal cut-off for continuous variables using log-rank statistics. Independent prognostic factors were evaluated using univariate and multivariate Cox proportional hazards regression analyses, and relationships between variables were calculated using Pearson correlation coefficients. Differences of TIDE scores and chemotherapy responses between groups were analyzed using Wilcoxon rank sum test.

RESULTS

PD-L1 Was Highly Expressed in OSCC and Predicted Poor OS

PD-L1 was differentially expressed in different tumor types and was highly expressed in HNSCC (**Supplementary Figure 1A**). TIMER analysis revealed that PD-L1 had positive correlations with various types of immune cells in HNSCC (**Supplementary Figure 1B**). Similarly, in OSCC, PD-L1 was also highly expressed compared with control group, but it gradually decreased with the progress of

tumor stages ($P < 0.05$, **Figures 1A, B**). Prognostic value of PD-L1 in OSCC patients was further analyzed; Kaplan–Meier analysis of OS revealed that patients with high PD-L1 expression exhibited a shorter survival time ($P < 0.05$, **Figures 1C–E**); The cutoff point for PD-L1 was 3.66. Cox regression analyses revealed that PD-L1 upregulation was significantly associated with a poor OS (**Table 1**).

Functional Enrichment Analysis of DEGs

Differential expression analysis was performed to identify the DEGs between two groups, so as to further study the function of these genes (**Figure 2A**). Among these DEGs, 384 genes were up-regulated in PD-L1^{high} group, and 1090 genes up-regulated in PD-L1^{low} group (**Figure 2A**).

Additionally, tumor-associated macrophages (TAMs) characteristic cytokines such as CXCL10 and CXCL11 were significantly differentially expressed (**Figures 2A, B**). Notably, the GO enrichment analysis indicated 745 immune-related GO terms including lymphocyte activation, activated leukocyte adhesion, and effector cytokine production were significantly enriched in PD-L1^{high} group, while 414 GO terms including digestion, regulation of postsynaptic membrane potential, drug transport, glutamate receptor signaling pathway, regulation of membrane potential were significantly enriched in PD-L1^{low} group (**Figures 2C, D**). KEGG pathway analysis showed that 47 KEGG pathways were characteristic enriched in PD-L1^{high} group, and 23 KEGG pathways were characteristic enriched in PD-L1^{low} group (**Figures 2E, F**).

PD-L1 Was Correlated With Macrophage Infiltration and Macrophage-Derived Chemokines

The results of GSVA revealed that PD-L1 was positively associated with cell proliferation of 12 immune cell types in

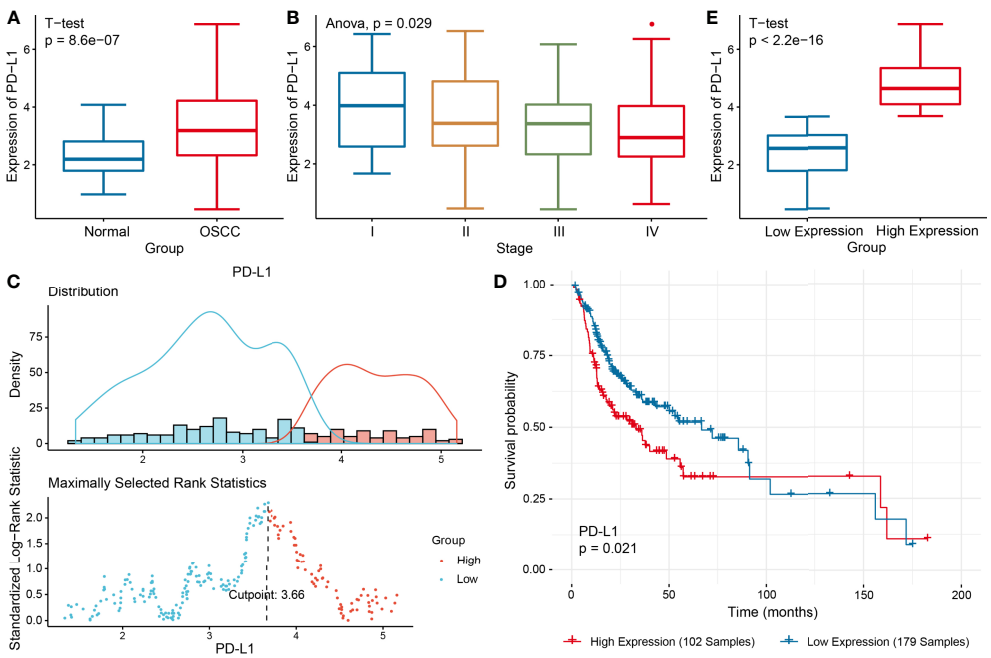

FIGURE 1 | Comprehensive analysis of PD-L1 in OSCC. **(A)** PD-L1 expression in OSCC group and the control group. **(B)** PD-L1 expression in different clinical stages of OSCC. **(C)** The optimal cutoff threshold value **(D)** Kaplan–Meier survival analysis according to the optimal cutoff value. **(E)** PD-L1 expression level in different OSCC groups.

TABLE 1 | Prognostic value of PD-L1 expression in OSCC.

Type	Univariate Cox Analysis			Multivariate Cox Analysis		
	P values	HR	95%CI	P values	HR	95%CI
Age	0.289998	1.205949	0.852479 - 1.705982	0.341135	1.223076	0.807963 - 1.851465
T	0.00025	1.413316	1.174466 - 1.700741	0.031705	1.433184	1.032024 - 1.99028
N	0.000497	1.46845	1.18289 - 1.822947	0.063043	1.320594	0.984993 - 1.770538
Stage	0.000488	1.470667	1.184029 - 1.826695	0.834734	1.053163	0.647391 - 1.713264
Grade	0.053395	1.307115	0.996091- 1.715256	0.135115	1.294857	0.922618 - 1.81728
PD-L1	0.02162	1.502635	1.061568 - 2.126959	0.01391	1.66808	1.109532 - 2.507807

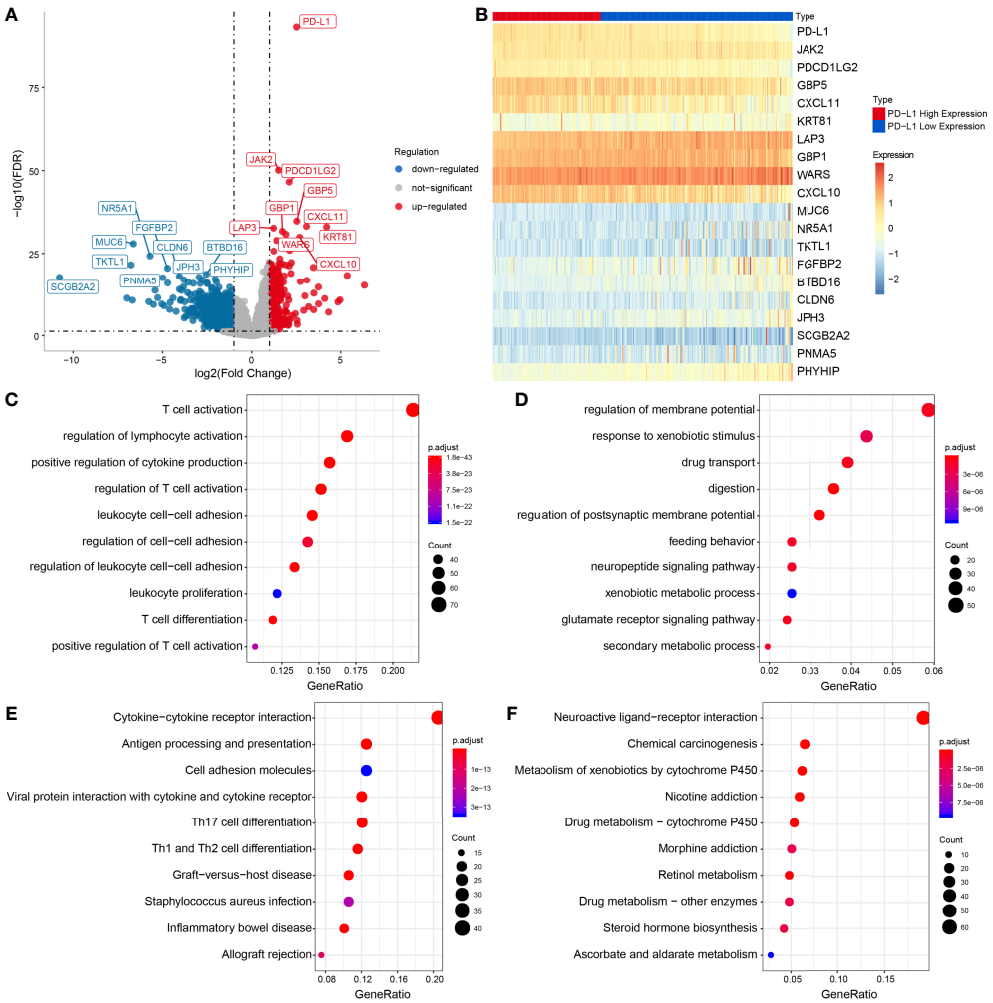

FIGURE 2 | Functional enrichment analysis of DEGs in PD-L1high and PD-L1low groups. **(A)** Volcanic map of DEGs. **(B)** Heat map showing top DEGs. Top 10 enriched GO terms in PD-L1 high **(C)** and PD-L1low group **(D)**. Top 10 significantly enriched KEGG pathways in PD-L1 high **(E)** and PD-L1low group **(F)**.

OSCC (**Supplementary Figure 2** and **Figures 3A, B**), especially with M0 macrophages (cor = 0.42, $P < 0.001$), M1 macrophages (cor = 0.68, $P < 0.001$), and M2 macrophages (cor = 0.53, $P < 0.001$) (**Figures 3C–E**). In addition, the results of ssGSEA in the GEO OSCC validation set showed PD-L1 was strongly positively associated with cell proliferation in several types of immune cell infiltration (**Supplementary Figures 3A–H**), especially with M1 macrophages (cor = 0.63, $P < 0.001$, **Supplementary Figure 3D**) and M2 macrophages (cor = 0.54, $P < 0.001$, **Supplementary Figure 3E**).

To validate the above results, we further analyzed correlations between PD-L1 expression and macrophage markers, which revealed that M1-related chemokines (IL12A, IL-12B, IL-23A, TNF, and IFNG) and M2-related chemokines (TGFBs, IL-10, and IL-13) showed the strongest positive correlations with PD-L1 expression ($P < 0.05$, **Figures 4A–D**). We also observed statistically significant correlations between M1-related chemokines and M2-related chemokines (**Figures 4B, D**).

Associations Between PD-L1 Expression, Macrophage Infiltration and EMT Biomarkers

Since the EMT process has been considered to be particularly relevant to TME, we analyzed the potential association of EMT with immune infiltration, and found significant correlations between EMT biomarkers and various immune cells infiltration (**Figure 5A**), especially with M0 macrophages (cor = 0.568, $P < 0.001$) and M2 macrophages (cor = 0.425, $P < 0.001$). We also evaluated the association between PD-L1 and EMT biomarkers, which showed significant correlations between PD-L1 and vimentin (VIM) (cor = 0.322, $P < 0.001$) (**Figures 5B, C**).

Sensitivity Differences to Immunotherapy/ Chemotherapy Between Groups

TIDE algorithm was employed to assess individual immunotherapy response in different PD-L1 expression groups, and higher TIDE prediction score represented a higher

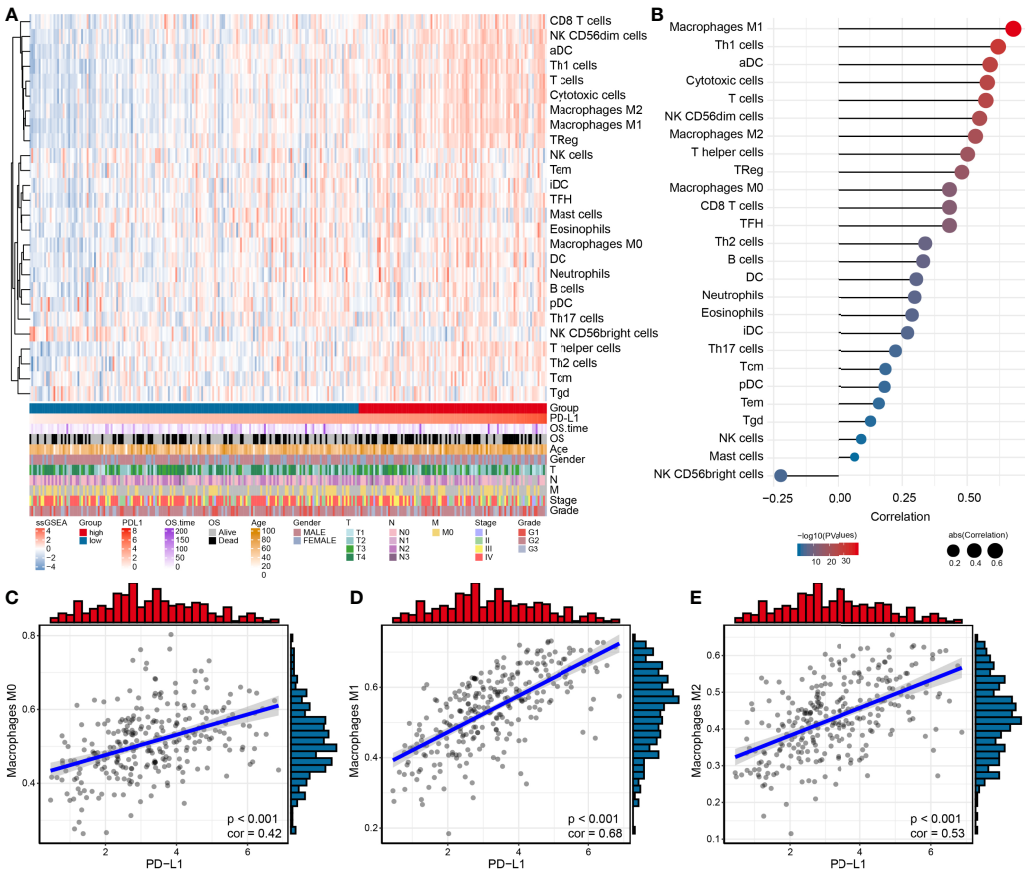

FIGURE 3 | Relationship between immune cell infiltration and PD-L1 expression in OSCC samples. **(A)** Heat map by using the ssGSEA scores from 26 immune cell types. **(B)** Correlation between PD-L1 and immune infiltrates in OSCC samples. Correlation between PD-L1 and M0 **(C)**, M1 **(D)** and M2 **(E)** macrophages infiltration in OSCC samples.

immune evasion potential (**Figure 6A**). The results revealed patients with higher PD-L1 expression had a higher microsatellite instability (MSI) score and T cell dysfunction score (**Figures 6B, C**), but a lower T cell exclusion score (**Figure 6D**), indicating that these patients were less likely to benefit from ICI treatment.

We also took into account the differences in the response of chemotherapy in OSCC patients, and evaluated sensitivity of two patient groups to four chemical drugs (olaparib, docetaxel, paclitaxel, and pazopanib). The IC50 value of each sample in OSCC was estimated, and significant differences were observed between groups, which revealed that PD-L1^{high} group showed higher sensitivity to all drugs (**Figures 6E–H**, $P < 0.001$).

DISCUSSION

PD-L1 overexpression has been demonstrated in many common cancers, inducing T-cell tolerance and promoting immune escape. Blockades targeted on PD-1/PD-L1 have already shown striking effectiveness in clinical applications (6–8). Immune characteristics relevant to PD-L1 in TME and EMT process during cancer progression were depicted in our study. Firstly, PD-L1 expression was highly expressed in OSCC, but it gradually decreased with the progress of tumor stages. As an immunosuppressive cell surface molecule that promotes T cell depletion, PD-L1 upregulation may link with increased cancer aggression and poorer prognosis, as proposed in several previous studies (29, 30). In contrast to this expectation, we observed an decrease in PD-L1 expression as disease progressed; Some previous studies on the association between PD-L1 expression and improved prognosis also Supported our findings (31–34). The most likely explanation for this paradox is that PD-L1 expression can be induced by cytokines, primarily the production of interferon-γ within TME, and therefore, its expression actually reflects the contribution of endogenous anti-tumor immune response, which typically occurred in the early stages of tumor development and progression (35, 36). Moreover, our results also showed that PD-L1 was positively associated with cell proliferation in activated TAMs and EMT process. The DEGs in PD-L1^{high} group were significantly enriched in canonical signaling pathways that related to regulation of lymphocyte activation, suggesting the critical involvement of PD-L1 in regulating TAMs function. Additionally, our results suggested that patients in the PD-L1^{high}

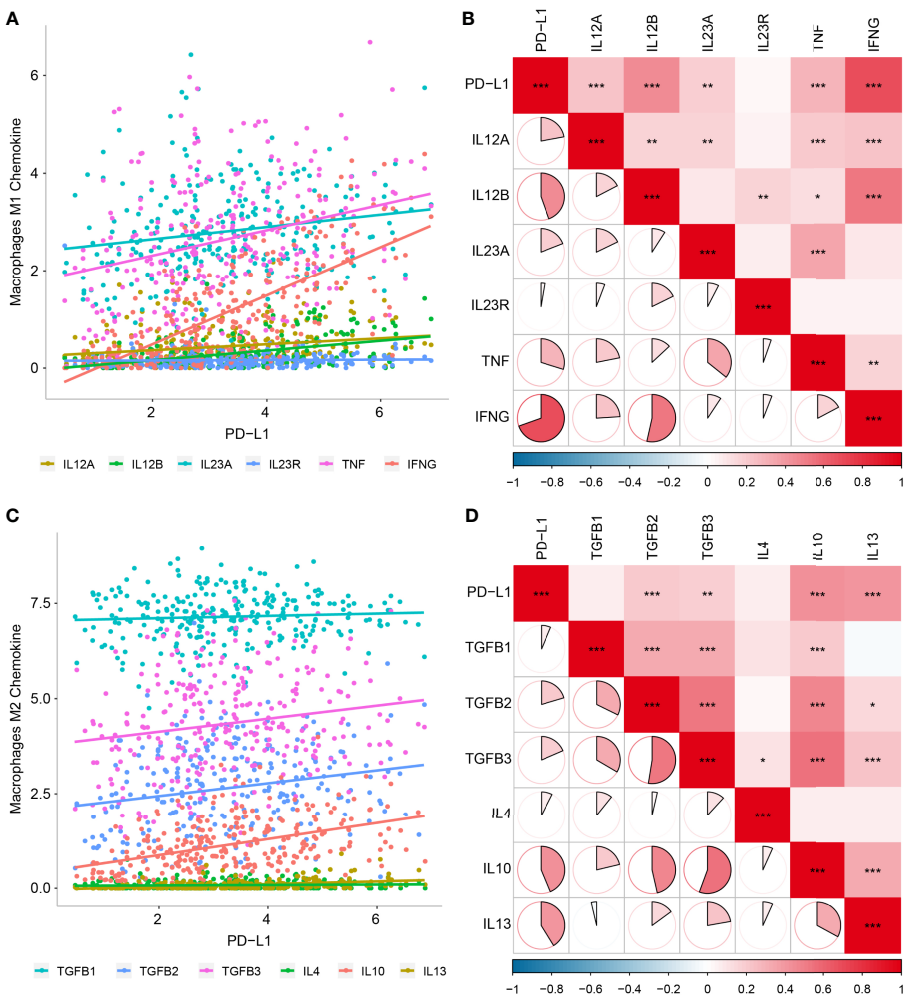

FIGURE 4 | Relationship between PD-L1 and macrophage-derived chemokines in OSCC samples. **(A)** Scatter plot showing correlation between PD-L1 and M1-derived chemokines. **(B)** Correlation between PD-L1 and M1-derived chemokines. **(C)** Scatter plot showing correlation between PD-L1 and M2-derived chemokines. **(D)** Correlation between PD-L1 and M2-derived chemokines. *P < 0.05; **P < 0.01; ***P < 0.001.

group were at a significant chance of benefiting from ICI treatment and they also showed higher sensitivity to the chemical drugs (olaparib, paclitaxel, docetaxel, and pazopanib). This study provided preliminary evidence regarding the tight correlations among PD-L1, TAMs and EMT process, further supporting the notion that clinical efficacy of both chemotherapy and immunotherapy could be greatly improved by utilizing PD-L1 inhibitors in OSCC.

A previous research on PD-L1 regulating the proliferation of macrophages revealed that the phenotype and function of macrophages could be altered by anti-PD-L1 treatment, suggesting a crucial role of PD-L1 in regulating macrophages activation and function (37). Circulating monocytes can be recruited into TME and further polarize into TAMs. Classically, in response to microenvironmental stimuli, TAMs polarize to M1-like phenotype exhibiting proinflammatory and tumor-inhibiting phenotypic effects, while certain cytokines convert TAMs into an M2-like phenotype with anti-inflammatory but tumor-promoting functions (38, 39). Previous studies have shown that M2 macrophages elevated PD-L1 expression in cancer cells and at the same time facilitating immune escape (40, 41). As for M1 macrophages, the PD-L1 expressed on the surface was also reported to result in immune escape of cancer cells, resulting in bidirectional effects of both anti-tumoral and pro-tumoral activities (42): On one hand, M1-like phenotype had the unique ability to promote the activation and recruitment of various immune effectors, performing surveillance tasks (43), and their infiltration indicated good prognosis in some cancers (44). On the other hand, M1 macrophages involved in cancer phenotype maintenance and tumorigenicity regulation *in vivo*. For example, previous studies confirmed the important protumorigenic factor role of M1 macrophages in urethane-induced lung tumorigenesis (45); Higher aggregation level of human leukocyte antigen-DR+ (HLA-DR+) M1-like TAMs was related to poor response to

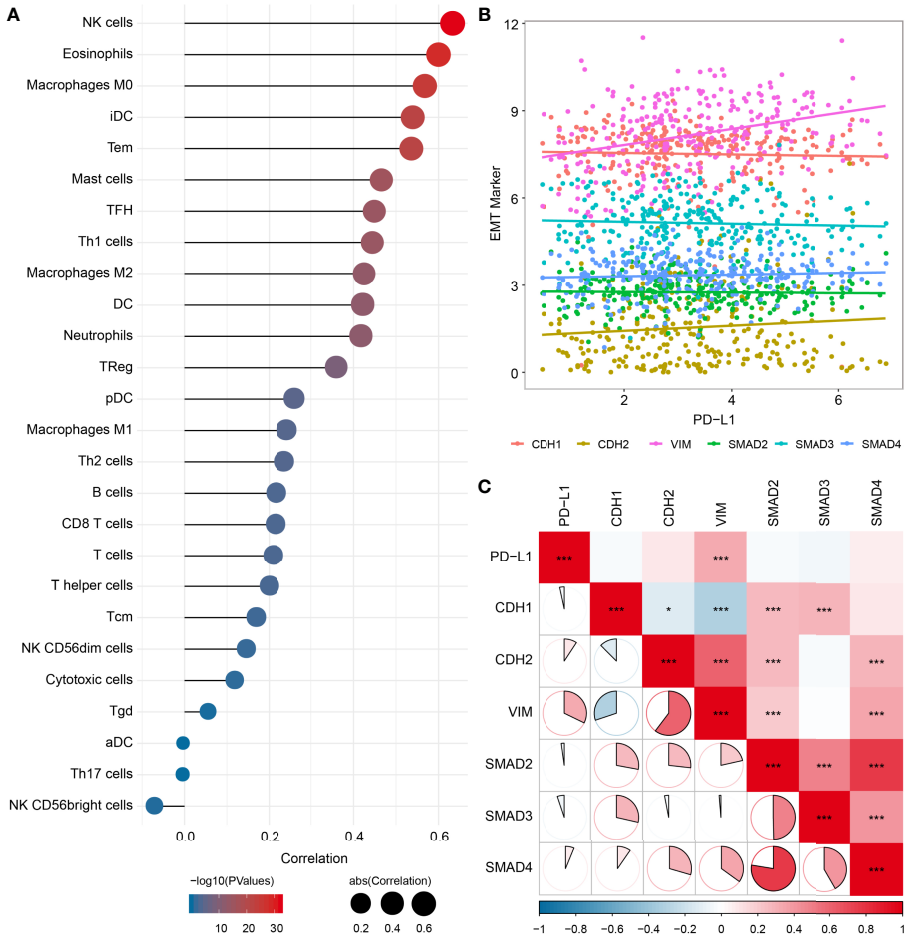

FIGURE 5 | Relationship among PD-L1, immune infiltrates and EMT in OSCC samples. **(A)** Correlation between EMT pathway enrichment scores and immune cell infiltration. **(B)** Scatter plot showing correlation between PD-L1 and EMT-related genes. **(C)** Correlation between PD-L1and EMT-related genes. *P < 0.05; ***P < 0.001.

ionizing radiotherapy in rectal cancers patients (46). In the present study, positive association between PD-L1 expression and TAMs infiltration (both M0, M1 and M2) was observed in OSCC, which was in consistent with previous findings. These findings suggested that TAMs may contribute to pro-tumorigenic effects by promoting PD-L1 expression in OSCC.

EMT is a novel mechanism involved in cancer metastasis, by which epithelial cells acquire both mesenchymal and epithelial phenotypes for cell migration and proliferation. The type III intermediate filament protein VIM that constitutes a key cytoskeletal element of mesenchymal cells, is a canonical marker of EMT process, and EMT process is characterized by marked VIM upregulation (47, 48). It is generally accepted that epithelial cells undergoing EMT are able to survive better under adverse environmental conditions, which enables tumor cells to evade immune destruction (49, 50). In addition, accumulating evidences have suggested strong correlations between EMT and immune evasion by activating multiple ICIs (51, 52); Cancer cells with PD-L1 upregulation displays an EMT phenotype that aids in immune escape. Macrophages and cancer cells were reported to establish a two-way cross-talk: Macrophages facilitated EMT changes in the latter while the latter skewed TAMs polarization into an M2 phenotype (53). Interestingly, recent evidences have indicated that in addition to M2 phenotype, M1-like macrophages also promoted EMT and chemoresistance (54); TAMs generally share both M1- and M2-like phenotypes instead of being strictly classified into above two phenotypes (55), which does not rule out the possibility that these two phenotypes are exchangeable (56, 57).

Immuno-oncology revolutionized cancer treatment. Pembrolizumab and nivolumab as anti-PD-1 mAbs have shown remarkable anti-tumor activity in the treatment of patients with recurrent/metastatic HNSCC. Ferris et al. (10) experienced nivolumab in a population of 347 HNSCC patients to evaluate the efficacy of nivolumab comparable to that of single-agent chemotherapy (CheckMate 141): Both ORR (13.3% *vs* 5.8%) and median OS (7.5 *vs* 5.1 months) were significantly improved in the nivolumab group. The estimated 12-month OS in nivolumab group was 36% *versus* 16.6% in standard-therapy group, while there was little difference in median progression-free survival

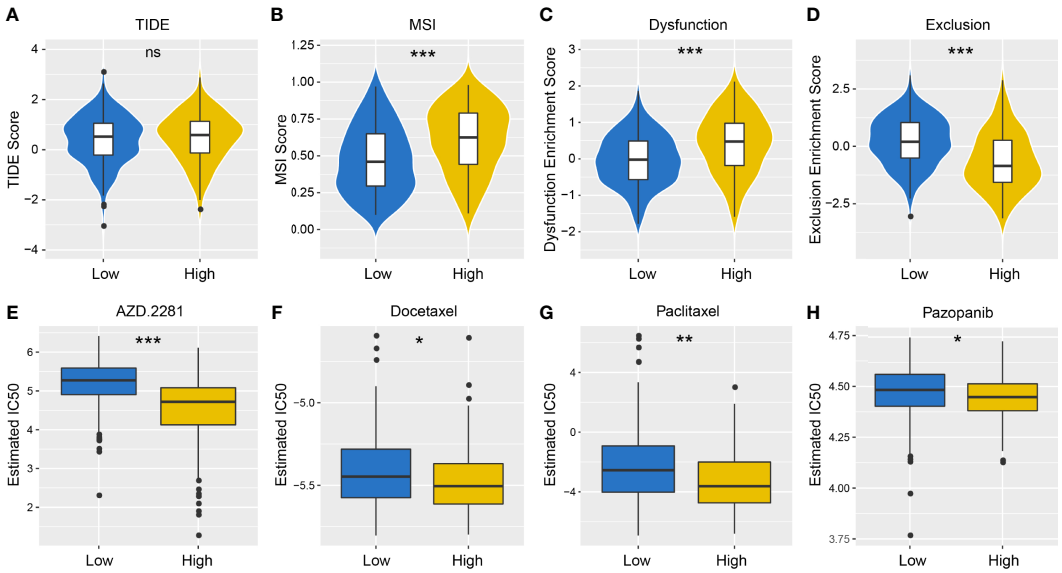

FIGURE 6 | Differences in the sensitivity of immunotherapy and chemotherapy between different PD-L1 expression groups. TIDE **(A)**, MSI **(B)**, T cell Dysfunction **(C)** and T cell Exclusion **(D)** scores in PD-L1^{high} and PD-L1^{low} Groups (NS: not significant; *P < 0.05; **P < 0.01; ***P < 0.001). Sensitivity to olaparib (AZD. 2281) **(E)**, docetaxel **(F)**, paclitaxel **(G)** and pazopanib **(H)** in PD-L1^{high} and PD-L1^{low} Groups (NS: not significant; *P < 0.05; **P < 0.01; ***P < 0.001).

(PFS) of these two groups. Similarly, the KEYNOTE-040 phase III study also compared the clinical efficacy of pembrolizumab *versus* current standard-therapy with a total of 495 patients enrolled: Both ORR (14.6% *vs* 10.1%) and median OS (8.4 *vs* 6.9 months) were significantly improved in the pembrolizumab group (11). These randomized phase III trials indicated that survival benefit could be well conferred by immunotherapy. Thus, it is of great importance to perform immune monitoring on patients, thereby identifying potential biomarkers, accurately stratifying patients and delineating responders and non-responders. Olaparib (AZD. 2281), a competitive inhibitor of poly (ADP-ribose) polymerase-1 (PARP-1), has been used in the clinical treatment of ovarian cancer with BRCA1/2 gene mutations (58, 59). An ongoing phase 1/2 study of olaparib and the PD-L1 inhibitor durvalumab in breast cancer patients with BRCA1/2 mutations demonstrated that 24 of 30 patients who were eligible for trial entry by study design achieved durable and adaptable cancer control at 12 weeks of combination therapy (60). Olaparib in combination with durvalumab exhibited promising anti-tumor efficacy and safety, which was confirmed in numerous clinical studies (61–63). In addition, paclitaxel, including nanoparticle albumin-bound paclitaxel (nab-paclitaxel), is a widely used chemotherapy drug for various cancers; Combination therapy of anti-PD-L1 mAb atezolizumab and nab-paclitaxel as a first-line treatment exhibited significantly improved PFS in patients with PD-L1-positive tumors (64, 65). In this study, we analyzed the sensitivity differences of immunotherapy and chemotherapy between different PD-L1 expression groups with the expectation of screening potential benefit populations and achieving enhanced efficacy. The results revealed that patients in the PD-L1^{high} groups were at a significant chance of benefiting from ICI treatment and they also showed higher sensitivity to the four chemical drugs (olaparib, paclitaxel, docetaxel, and pazopanib. Further research is needed to fully confirm the promising efficacy of these agents in combination with PD-L1 inhibitor in improving personalized treatment for OSCC patients. By screening the subgroups of potential beneficiaries, immunotherapy and chemotherapy can harnessed to maximize the immunostimulatory effects of therapeutic agents.

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

Conceived and designed the study: TW, CT, and RT. Data collection and analysis: TW and RT. Writing and revising the manuscript: TW, CT, XY, QJ, CF, and RT. 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.2021.693881/full#supplementary-material

Supplementary Figure 1 | PD-L1 expression level in various cancers. **(A)** PD-L1 expression level in various cancers was analyzed by box-plot. **(B)** Correlation between PD-L1 and immune infiltrates in HNSCC.

Supplementary Figure 2 | Scatter diagram of the correlation between PD-L1 expression and immune cell infiltration in OSCC.

Supplementary Figure 3 | Relationship between PD-L1 and immune infiltrates in the GEO validation set. **(A)** Heat map of OSCC samples by using the ssGSEA scores from 26 immune cell types. **(B)** PD-L1 significantly associated with Immune cells. Scatter plot showing correlation between PD-L1 and various immune cells, including aDC **(C)**, M1 macrophages **(D)**, M2 macrophages **(E)**, Cytotoxic cells **(F)**, T cells **(G)** and Th1 cells **(H)**.

Supplementary Table 1 | The Clinicopathological data of patients.

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REVIEW
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Defining the Role of Immunotherapy in the Curative Treatment of Locoregionally Advanced Head and Neck Cancer: Promises, Challenges, and Opportunities

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Recent advancements in the development of immunotherapies have raised the hope for patients with locally-advanced HNSCC (LA-HNSCC) to achieve improved oncologic outcomes without the heavy burden of treatment-related morbidity. While there are several ongoing late phase clinical trials that seek to determine whether immunotherapy can be effectively employed in the definitive setting, initial results from concurrent immuno-radiotherapy therapy trials have not shown strong evidence of benefit. Encouragingly, evidence from preclinical studies and early-phase neoadjuvant studies have begun to show potential pathways forward, with therapeutic combinations and sequences that intentionally spare tumor draining lymphatics in order to maximize the synergy between definitive local therapy and immunotherapy. The intent of this review is to summarize the scientific rationale and current clinical evidence for employing immunotherapy for LA-HNSCC as well as the ongoing efforts and challenges to determine how to optimally deliver and sequence immunotherapy alongside traditional therapeutics. In both the preclinical and clinical settings, we will discuss the application of immunotherapies to both surgical and radiotherapeutic management of HNSCC.

Keywords: immunotherapy, head and neck (H&N) cancer, curative treatment, immune oncology (IO), treatment sequences

INTRODUCTION

Squamous cell carcinomas arising from the upper aerodigestive tract, including the pharynx, larynx, and oral cavity, present unique therapeutic challenges in oncology. Representing approximately 3% of new cancer cases and 3% of cancer deaths worldwide (1), head and neck squamous cell carcinomas (HNSCC) arise adjacent to or within anatomy central to ventilation, speech, mastication, salivation, and swallowing. As such, progression of HNSCC causes significant

morbidity, even in the non-metastatic setting. Similarly, curative-intent treatments for HNSCC, which also compromise aerodigestive function, carry a high degree of morbidity. In select cases of early-stage disease, surgical extirpation alone may be sufficient; however, in cases of locally advanced disease, curative-intent treatment often requires multimodal therapy with surgery, radiation therapy, and chemotherapy. The sequelae of curative-intent treatment are manifold and may include permanent alterations in swallowing function, speech, taste, salivation, and dental health. At the same time, even with aggressive therapy, loco-regional and distant recurrences after treatment are all too common and carry a dismal prognosis (2).

While innovations in both surgical technique (3) and radiation therapy (4) have led to improvements in treatment related morbidity without sacrificing treatment efficacy, there remains a critical need for therapies that can deliver cures without incurring undue toxicity (5). Two decades ago, the addition of cisplatin chemotherapy to definitive radiation improved oncologic outcomes, albeit at the cost of significant additional toxicity (6–8). The advent of molecularly targeted therapies in the 1990s raised hopes that cisplatin chemotherapy could be replaced by less toxic targeted systemic therapies, particularly after the EGFR-inhibitor cetuximab added to radiotherapy was shown to improve outcomes over radiation therapy alone (9). Unfortunately, several recent studies have shown inferior outcomes with cetuximab *versus* cisplatin given concurrently with radiation (10, 11) and newer targeted agents are yet to demonstrate comparable efficacy in clinical trials (12). However, the recent development and clinical implementation of novel immunotherapeutic agents - drugs that promise to overcome tumor-immune evasion and stimulate an anti-neoplastic immune response - has once again raised hopes that the efficacy of treatment for HNSCC can be enhanced without increasing treatment-related morbidity.

PRECLINICAL MODELS

Immunotherapies represent a unique class of cancer therapeutics that target host immunity to invigorate systemic anticancer immunity. This therapeutic strategy diverges from traditional therapies which target cancer cells specifically to induce cytotoxicity or inhibit growth. It is precisely for this reason that the field has had to refocus overall therapeutic algorithms. Moreover, because the anti-tumor effects of immunotherapy depend on interactions amongst multiple organ systems (hematologic, lymphatic, vascular), it is critical that we study these therapies in immunocompetent, syngeneic *in vivo* model systems, a requirement that has brought with it new methodological challenges.

Preclinical models of murine HNSCC include *ex vivo* models derived from human disease and primary murine models arising either spontaneously after carcinogen exposure or driven by activated oncogenic signaling networks. The variety of contemporary preclinical models reflects their utility and relevance to address open questions in the field, with each model offering certain advantages and limitations.

Ex Vivo Derived Preclinical Models

Human Derived tumor models have played a critical role in the study of traditional chemotherapies and targeted therapies. However, by virtue of their inherent immunogenicity in animal models, they are limited in their ability to inform immunotherapy research. Immortalization of *ex vivo* cultured HNSCC cells was first reported four decades ago (13). Since then, several groups have rigorously profiled panels of the more commonly employed immortalized HNSCC lines, culminating in the assembly of repositories such as the Cancer Cell Line Encyclopedia (CCLE) (14, 15). Collectively, these efforts inform our understanding of the genetic and molecular drivers of HNSCC and serve as an ideal platform for novel therapeutic testing (16). Additionally, they afford insight into the extrinsic selective pressures imposed by antitumor immunosurveillance that shape tumor heterogeneity and clonal selection (17), a phenomenon predicted by the immunoediting hypothesis (18, 19).

Similar to immortalized cell line models, organoid and related three-dimensional models derive directly from human tissues. As first documented in a pharyngeal mucosa-derived organoid model (20), these models feature the advantage of a more physiologically representative tissue architecture and cellular milieu. Interestingly, divergent response profiles have been observed between three-*versus* two-dimensional patient derived models (21–23) with three-dimensional systems modeling the clinical responses to radiation more closely (24, 25). Three-dimensional tissue culture systems also offer the ability to deconstruct the proximal events in tumorigenesis. This was elegantly demonstrated in a series of studies identifying the central role of cancer-associated fibroblasts in tumorigenesis of the lingual mucosa (26, 27). More recently, in an effort to recapitulate the dynamic tumor-immune microenvironment *ex vivo*, Neal et al. developed the unique air-liquid-interface model for both murine-derived and patient-derived organoids (28). With this unique model system, the authors demonstrated a faithful representation of not only neoplastic cells and the endogenous immune infiltrate, including the complete tumor infiltrating lymphocyte repertoire, but also the native responsiveness to immune checkpoint inhibition (ICI) in organoids from > 100 human biopsies and murine tumors.

Patient-derived xenografts (PDXs) comprise a unique preclinical modelling strategy in which fragments of patient tumors are directly implanted into animals. PDXs are well-suited for targeted drug screens and examination of oncogenic signaling. However, use of this model *in vivo* is necessarily limited to immune-deficient animals or in humanized rodent models as PDXs are inherently immunogenic and are rejected in immune competent animals. Generally, humanized preclinical models are generated by inoculating immune-deficient recipient animals with either (i) human peripheral blood mononuclear cells; (ii) human CD34+ hematopoietic stem cells (HSCs); or, (iii) concurrent human CD34+ HSCs and autologous human fetal liver and thymus tissue transfer. While humanized models offer tremendous opportunity to study PDXs-immune system interactions *in vivo*, they are limited primarily by graft *versus* host disease in recipient animals and by human donor variability.

For a more detailed information regarding contemporary HNSCC PDXs and human preclinical modelling see (29–33).

Primary Murine Preclinical Models

In contrast, syngeneic tumor models, which are derived from inbred mice and can be transplanted into immune-competent animals of the same strain, have afforded tremendous insight into mechanisms of tumor-immune evasion and immunotherapeutic resistance. Syngeneic models include those that have arisen spontaneously or as a consequence of carcinogen exposure. The SCC VII/SF model, which was derived from a spontaneous cancer in C3H/HeJ mice, is among the most widespread, contemporary spontaneous, syngeneic HNSCC models (34). Another versatile and popular model is the panel of murine oral-cavity (MOC) squamous cell lines developed with exposure of 7,12-dimethylbenz(a)anthracene (DMBA)-induced (35, 36). Early therapeutic efforts with these models focused on delivery of systemic immunotherapy with stimulatory cytokine delivery (37, 38). More recently, syngeneic models have served as the mainstay to evaluate advancements in immunotherapy with checkpoint blockade inhibitors and adoptive cell transfer.

Arguably, the ideal preclinical HNSCCC model is one which mimics the oncogenic mutanome of its human disease counterpart, can be transplanted orthotopically, progresses in immune-competent hosts and features both an immune infiltrate and a response to immunotherapy similar to that observed clinically. Lastly, the ideal preclinical model should be dynamic both in its genesis and behavior *in vivo*; and, as such, reflect more accurately the dynamic changes occurring downstream from selective immune pressure, as has been documented in human disease over time (17, 39). Collectively, these properties offer the greatest promise to not only better understand fundamental cancer-immune dynamics but also develop translatable therapeutic strategies and biomarkers of therapy response.

Excitingly, examples of such 'next-generation' preclinical models are emerging. Following from early 4NQO-carcinogen-induced models (40, 41), newer syngeneic, orthotopic murine oral SCC model demonstrate a remarkable homology to the human tobacco-signature mutanome (42, 43). Such models are ideally suited to efforts aimed at understanding tumor-immune interactions and, by extension, developing precision immune-oncology therapies, as described below.

A parallel effort in preclinical HNSCC modeling is to manufacture carcinogenesis by driving specific oncogenic pathways, and, in so doing, generate genetically engineered mouse models (GEMMs). Historically, preclinical GEMMs were derived by driving oncogenic programs downstream from K-ras activation (44–47). However, based upon several studies of common mutational signatures amongst human cancers (48–50), early GEMMs have come under scrutiny for featuring genetic alterations rarely seen in HNSCC and lacking the signature mutanomes now associated with both human papillomavirus (HPV)-positive and HPV-negative HNSCC (49, 50). However, efforts aimed at deconstructing oncogenic networks in HNSCC tumorigenesis are particularly-well suited to the GEMM preclinical platform. GEMMS affords an opportunity to examine the key milieu of true genetic drivers with the promise of exposing targetable molecular vulnerabilities in HNSCC (51). These efforts are especially true with regards to modeling HPV-associated disease in which targeting viral E6/E7 expression in turn activates genome-wide oncogenic changes (52).

PRECLINICAL IMMUNOTHERAPEUTIC STRATEGIES

Cancer-immune dynamics underlie the efficacy of IO therapies. While major advancements in the management of HNSCC have been achieved by examining immune checkpoint inhibition in the clinic (53, 54), innovation in IO therapy design is tempered by an incomplete understanding of cancer-immune interactions, as evidenced by unexpectedly equivocal results in several recent clinical trials (55–58).

The advent of clinically-relevant, robust preclinical models addresses this problem by affording the opportunity to scrutinize not only anti-tumor immunity but also cancer-derived influence over host immunity. A testament to the power of translating preclinical observations from clinically-relevant models is the breadth of emerging clinical trials examining immune-oncology therapies in HNSCC (42, 59). The following subsections highlight only a fraction of the ground-breaking preclinical work that have contributed to the advancement of IO-therapy design and our collective understanding of cancer-immune interactions in HNSCC.

Because the efficacy of immunotherapies depends on their interactions with tumor, hematologic, and lymphatic organ systems, in the preclinical realm, there is considerable interest in understanding how traditional anti-cancer therapies, surgery, radiation, and systemic therapies affect these interactions and modulate the effects of immunotherapies. There is considerable work underway to optimize the interactions between surgery, radiation, and other systemic therapies and immunotherapies in order to maximize synergy and minimize interference.

Radiation Therapy and Immunotherapy

Combinations of radiation therapy and immunotherapies have been increasingly explored both in the preclinical and clinical arenas (42, 60, 61). A question of considerable interest in the field is whether radiation dose and fractionation can be optimized in order to maximize the synergistic effects of immunotherapy and radiation therapy. Specifically, there is increasing interest in determining whether hypofractionation of radiotherapy delivery (delivery of larger daily radiation doses over a shorter time period, typically to a lower total dose) can improve the anti-neoplastic immune response stimulated by modern immunotherapeutic agents. The rationale for this approach is based in the theory that radiation therapy not only kills cancer cells through direct damage to their DNA, but also *via* stimulation of antitumoral immunity (62). However, radiation therapy can also have detrimental effects on the tumor-immune environment, including toxic effects on the local immune cell population and in secondary lymphoid organs (61). Thus, a careful balance between the immunostimulatory and

immunosuppressive effects of radiation therapy must be achieved to optimize synergy between immunotherapies and radiotherapy.

There is growing preclinical evidence across multiple cancer histologies that hypo-fractionated radiation better achieves this balance than conventionally fractionated radiation. In their seminal 2009 paper, Lee et al. demonstrated that the antitumor effects of highly hypo-fractionated radiation (20Gy in 1 fraction) on B16 melanoma tumors was significantly greater in wild type mice than immune-incompetent nude mice (63), suggesting that the immune system mediates a portion of radiation therapy's antineoplastic effects. Further, they found that by dividing the delivered radiation dose over 4 days (20Gy in 5 fractions), there was a loss of tumor control that was similar to the effect of treating wild type mice with an anti-CD8 antibody after single fraction treatment. They found that highly hypo-fractionated radiation significantly increased dendritic cell maturation and promoted priming of antigen-specific T cells, and they hypothesized that hypo-fractionated radiation might have greater synergistic effects with immunotherapy than conventionally fractionated radiation. Subsequently, Grapin et al. investigated the effects of different radiotherapy schedules with equivalent biologically effective doses on the intra-tumoral immune response of mice bearing subcutaneous CT26 colon tumors (64). They found that while conventionally fractionated radiation (36Gy in 18 fractions) induced a myeloid response dominated by myeloid derived suppressor cells (MDSC) and type 2 tumor associated macrophages (TAM 2), highly hypo-fractionated radiation (either 16.4Gy in 1 fraction or 24Gy in 3 fractions) induced a lymphoid response dominated by CD8+ T-cells and regulatory T cells. Delivery of 24Gy in 3 fractions was also found to induce the highest proportion of T cells secreting granzyme B, while conventional fractionation was found to induce the most durable increase in tumoral expression of PD-L1. Similarly, Lan et al. found in mice bearing subcutaneous LL/2 lung tumors or B16F10 melanoma tumors that highly hypo-fractionated radiation to 23Gy in 2 fractions reduced recruitment of MDSCs into the tumor microenvironment and decreased PD-L1 expression compared with more modestly hypo-fractionated radiation to 36Gy in 9 fractions (65). Together, this growing body of preclinical work suggests that hypo-fractionated radiation is more likely to stimulate an antineoplastic immune response *versus* conventionally fractionated radiation although this remains to be demonstrated in randomized human studies.

Surgery and Immunotherapy

There is also mounting preclinical evidence in support of the employment of immunotherapeutic agents in the neoadjuvant setting prior to definitive surgical intervention. Preclinical models suggest that the efficacy of checkpoint inhibitor therapy may be dependent on communication between the primary tumor and its regional draining lymph nodes (66). Tumor draining lymph nodes have long been implicated in the presentation of tumor antigen to and activation of cytotoxic T-cells in response to tumor growth (67), and enhancement of antigen presentation in lymph nodes through expansion and activation of intratumoral dendritic cells has been shown to potentiate the response to checkpoint inhibition in pre-clinical models. More recently, Liu et al. found improved survival in a mouse model of triple negative breast cancer when checkpoint inhibition was initiated prior to rather than after surgical resection of the primary tumor (68). The improvement in efficacy was associated with a significantly greater increase in tumor-specific CD8 T cells in the peripheral blood and organs of mice treated with neoadjuvant *vs*. adjuvant immunotherapy, suggesting a more robust immune response in the setting of an intact primary tumor. Similarly, Fransen et al. found in two murine orthotopic colon cancer models that the antineoplastic response to checkpoint inhibition was dependent on intact communication between the tumor and draining, but not distant, lymphatics (69). Surgically excising the draining lymph nodes associated with flank tumors significantly diminished the effects of subsequently administered anti-PD-1 therapy. No reduction of efficacy was observed when the contralateral non-draining lymph nodes were excised prior to anti-PD-1 administration. Reduced efficacy was also demonstrated when an S1P receptor inhibitor, which blocks T cells egress from lymphoid organs, was administered prior to anti-PD-1 therapy, suggesting that the effects of the checkpoint inhibitor were mediated by activation of T cells within the regional lymphatics. Taken together, this growing body of preclinical work provides strong rationale for clinical study of checkpoint inhibition in the neoadjuvant setting, when communication between the primary tumor and its draining lymphatics has not yet been disrupted either by surgical excision or radiation.

Systemic Therapy and Immunotherapy

Similarly, a growing body of literature now indicate that systemic therapies (chemotherapies and targeted therapies) can potentiate immunotherapy, either by inducing immunogenic tumor cell death with antigen shedding (70–72) or through depletion of immunosuppressive effector populations within the tumor microenvironment (73–76).

Collectively, observations gleaned from parallel preclinical investigations have converged upon certain key mediators that regulate the response to immunotherapy in HNSCC (76, 77). An illustrative example of such a convergence point in HNSCC that has progressed from the preclinical to clinical arena is the myeloid derived suppressive cell (MDSC). MDSCs are notorious for their role in suppressing antigen-specific T cell cytotoxicity and mediating acquired resistance to immunotherapy (78, 79). In preclinical models of HNSCC, MDSCs have specifically been found to regulate the response to PD-1 checkpoint blockade therapy (80, 81). Interestingly, independent groups have found that selective targeting of the PI3K signaling axis can reverse MDSC accumulation by altering tumor-derived cytokine production, ultimately leading to improved PD-1 responses. These key insights suggest that oncogenic signaling in HNSCC is intrinsically linked to maintaining an immunosuppressive tumor immune microenvironment (TIME). In tandem, other investigators, leveraging the power of next generation preclinical models, have innovated to identify oncogenic aberrations upstream from PI3K signaling as an exploitable and precision therapy target to combine with PD-1 blockade in a similar fashion

(42). These advancements in fundamental cancer-immune interactions in HNSCC open the door for paradigm-altering treatment strategies that combine targeted molecular cancer therapies with immunotherapies.

CLINICAL EVIDENCE SUPPORTING USE OF IMMUNOTHERAPY IN LA-HNSCC

Concurrently With Radiation

Concurrent chemoradiation, with conventionally fractionated radiation and cisplatin-based chemotherapy, is the standard of care definitive treatment strategy for inoperable LA-HNSCC (82). While employment of chemoradiation has produced significant improvements in outcomes compared with radiotherapy alone, it also can result in significant treatment-related morbidity and outcomes remain poor for many patients. Efforts to replace or augment cisplatin-based chemoradiation with targeted systemic therapies have to date produced disappointing results in the clinical setting (10, 11, 83, 84). In contrast, by virtue of their demonstrated efficacy in the recurrent and metastatic setting, immunotherapies present a promising alternative (54, 85, 86). A great deal of work in this area is ongoing, and early results from the initial clinical trials have not been uniformly encouraging (57). However, even negative studies yield important insights into how best to employ immunotherapy with definitive radiotherapy.

Table 1 highlights an illustrative listing of recent efforts to interrogate the efficacy of combining IO and radiotherapy. Among these initial efforts, Powell et al. conducted a phase IB study evaluating the safety and efficacy of pembrolizumab when given during and after definitive radiation therapy with concurrent weekly cisplatin (87). The study enrolled 59 patients with locally advanced (Stage III-IVB, AJCC 7th) HNSCC regardless of HPV status with the majority (67.8%) presenting with oropharyngeal primaries. Safety was the primary endpoint of the study with efficacy assessed by the rate of complete response as defined by imaging or pathologic criteria. The therapy was overall well tolerated with only 5 patients (8.8%) requiring discontinuation of pembrolizumab due to toxicity and all but one patient receiving the full planned dose of radiotherapy without any treatment delays exceeding five days. The complete response rate (CR) was 85.3% and 78.3% among HPV-positive and HPV-negative patients, respectively, which met the pre-specified response in the HPV-negative but not HPV-positive cohorts. Progression free and overall survival outcomes were encouraging, with 2-year overall survival (OS) and progression-free survival (PFS) of 86.5% and 72.6%, respectively, for the HPV-negative cohort and 97.1% and 92.8%, respectively, for the HPV-positive cohort. This study concluded that pembrolizumab given concurrently with cisplatin based chemoradiation was safe and did not limit delivery of definitive chemoradiation.

However, the promising efficacy results from the Powell study have not been confirmed by the more recently reported phase III trial, JAVELIN head and neck 100 (57). JAVELIN was a randomized, placebo-controlled, double blind trial in which

TABLE 1 | Illustrative listing of clinical trials examining combination immunotherapy and definitive radiation for LA-HNSCC.

Trial	Ref	Phase	Study Design	Study Eligibility	Primary Outcome	Status	Estimated Completion
NCT02586207	Powell JCO 2020	IB	Pembro (200mg d-7) → CRT → RAA + Pembro (200mg x5)	LA-HNSCC (Stage III-IVB)	Safety/Tolerability	Active, Not Reruiting	Sep-23
NCT02952586	Lee Lancet 2021	III	Avelumab (10mg/kg x1, d-7) → Avelumab (10mg/kg q2wk) + CRT → RAA + Avelumab (10mg/kg q2wk)	LA-HNSCC	PFS	Discontinued	Aug-20
NCT03383094	n/a	III	Pembro (200mg q3wk x20) + RT	LA-HNSCC	PFS	Recruiting	Jun-24
NCT03258554	n/a	II/III	Durva (1500mg wk-2 x7) + RT	LA-HNSCC	DLT/PFS/OS	Recruiting	Dec-25
NCT02707588	Bourhis AnnOnc 2020	II	Pembro (200mg q3wk) + RT	LA-HNSCC	LRC	Active, Not Reruiting	Dec-21
NCT03952585	n/a	II/III	Reduced-Dose RT + Cisplatin *vs* Nivo (d-7 q2wk x6)	LA-HNSCC	PFS/QOL	Recruiting	Feb-25

patients with locally advanced HNSCC received chemoradiation with bolus cisplatin chemotherapy and either concurrent and adjuvant avelumab or placebo. The trial enrolled 697 patients and the primary endpoint was PFS. At interim analysis tolerability was similar in both arms. However, the trial was closed early after it was determined that PFS would not be improved in the experimental avelumab arm [hazard ratio (HR), 1.21; 95% confidence interval (CI) 0.93-1.57; 1-sided p = 0.920]. Similarly, OS was not improved in the avelumab arm [HR, 1.31; 95% confidence interval (CI) 0.93-1.85; 1-sided p = 0.94]. Subset analysis did not find any subgroup with improved outcomes on the avelumab arm, though there was a non-significant trend toward improved PFS in the patients with PD-L1 high tumors [HR 0.59, 95% confidence interval (CI) 0.28-1.22]. The failure of this highly anticipated trial to meet its primary endpoint raises a number of important questions. Given the demonstrated efficacy in the recurrent and metastatic setting, what could explain the failure of checkpoint inhibitors to improve outcomes in the upfront concurrent/adjuvant setting? The results of the study could not be easily explained by poor tolerability or increased toxicity with the study drug, as no significant differences in safety outcomes were observed in the investigational and placebo arms. Another potential explanation is that the efficacy of avelumab was restricted to patients with PD-L1 high tumors. While this may be the case, this potential subgroup effect in PD-L1 high expressing patients was not found to be statistically significant upon exploratory analysis in this study.

Alternatively, suboptimal IO treatment sequencing could be part of the reason underlying the negative results of the JAVELIN head and neck 100 study. The optimal timing of ICI relative to chemoradiation is unknown, and several trials conducted for non-HNSCC have shown benefit when employing ICIs in the adjuvant rather than concurrent setting. For example, the PACIFIC trial, a randomized, controlled phase III trial in unresectable locally advanced non-small-cell lung cancer (NSCLC), compared durvalumab consolidation therapy against placebo in patients with no disease progression after at least two cycles of platinum-based chemoradiotherapy, finding a significant benefit in progression-free and overall survival in the ICI group (PFS 16.8 months *versus* 5.6 month; HR 0.52, p<0.001; OS at 24 months 66.3% *versus* 55.6%, p=0.005) (88, 89). Similarly, the CheckMate 577 trial, a large, randomized, placebo-controlled phase III study evaluating ICI in the adjuvant setting in patients with esophageal or gastroesophageal junction cancer after neoadjuvant chemoradiation and surgery, found significant improvements in disease-free survival with ICI (22.4 months *versus* 11 months; HR 0.69, p<0.001) (90). These favorable results along with those from the CheckMate 141 (53) and KEYNOTE-048 trials (54) – both evaluating adjuvant ICI *versus* standard risk-adjusted chemoradiotherapy within the recurrent and metastatic HNSCC population – suggest that immune-oncology treatment sequencing may influence the tumor response to combination therapy.

A final, intriguing hypothesis is that the antineoplastic effects of avelumab were antagonized by the non-investigational components of the therapeutic regimen. One potential suspect is the cisplatin chemotherapy, given its known hematologic toxicity; however, this hypothesis is inconsistent with the results of the recent KEYNOTE-048 study – a large, randomized phase III trial in recurrent and metastatic HNSCC which found that ICI combined with platinum-based chemotherapy (including cisplatin) improved overall survival in the total study population (54). An alternative suspect is the radiation itself. As discussed above, conventionally fractionated radiation has been found to alter the tumor immune environment in ways that may antagonize immune surveillance and promote tumor-immune escape. Further, definitive intent radiation therapy entails elective radiation of the draining lymphatics of head and neck cancers, which may reduce the efficacy of immunotherapy by a similar mechanism to surgical lymphadenectomy.

A series of clinical studies that may shed additional light on these questions include those that assess whether checkpoint inhibitors can replace standard of care systemic agents in the setting of definitive intent radiation. Amongst these studies are the KEYCHAIN trial (NCT03383094) (91), the GORTEC 2015-01 'PembroRad' trial (NCT02707588) (58), NRG HN004 (NCT03258554) (83) and NRG HN005 (NCT03952585). The KEYCHAIN trial is a prospective, multi-institutional, open-label, randomized phase II trial investigating whether concurrent and adjuvant pembrolizumab can improve PFS over standard concurrent cisplatin in patients with HPV-associated locally advanced HNSCC (Stage III-IVB, AJCC 8th) undergoing definitive intent radiation therapy. Both PembroRad and HN004 are prospective, randomized trials investigating whether checkpoint inhibitors (pembrolizumab and durvalumab, respectively) can improve outcomes over concurrent cetuximab when combined with definitive intent radiation therapy for cisplatin-ineligible patients. NRG HN005 is a prospective phase II/III trial interrogating progression-free survival and quality of life with reduced-dose definitive radiation with cisplatin or nivolumab compared to standard of care definitive chemoradiation. Of note, the PembroRad and HN005 trials omit adjuvant IO while the HN004 and KEYCHAIN trials include adjuvant IO to 3 months and 12 months, respectively. KEYCHAIN, HN004 and HN005 are actively enrolling at this time. PembroRad has completed enrollment and reported preliminary results at the ESMO 2020 annual conference (58). The trial enrolled 133 patients between 2016 and 2017 with a near-even split of p16-positive (46%) and p16-negative patients. Locoregional control at 15 months was 59% and 60% with cetuximab-RT and pembrolizumab-RT, respectively. There was no significant difference in two-year PFS. Notably, acute toxicity was lower in the pembrolizumab-RT arm compared to the cetuximab-RT arm, with at least one grade 3 or greater acute adverse event in 74% and 92% of patients, respectively. While the toxicity data reported in this trial is encouraging, the failure of pembrolizumab to improve PFS over cetuximab is disappointing, especially in light of the recent trials demonstrating the inferiority of cetuximab when compared with cisplatin. If KEYCHAIN and HN004 similarly fail to meet their efficacy endpoints, it may suggest a need to fundamentally rethink the timing, dose and fractionation, or nodal volume coverage of definitive-intent radiation therapy when delivered with checkpoint inhibitors.

While there is little clinical experience with elimination of elective nodal irradiation in the definitive setting, efforts have been made to study the effect of altered fractionation and dose. The current standard of care for definitive radiation therapy in LAHNSCC is to deliver 66-72Gy in approximately 2Gy daily fractions over 6-7 weeks to areas of gross disease, with modestly lower doses (44-63Gy) delivered to areas of subclinical disease, including elective nodal volumes, over the same time period. Compared to the burgeoning preclinical data, there is relatively little clinical evidence that highly hypo-fractionated radiation better stimulates a tumor immune response than conventionally fractionated radiation in patients with HNSCC. However, there is clinical evidence that modestly hypo-fractionated radiation can improve outcomes in certain clinical scenarios. For example, in early stage glottic larynx cancer, a prospective, randomized trial found that delivery of definitive radiation monotherapy in 2.5Gy fractions resulted in superior local control than delivery of a higher biologically effective dose (based on linear-quadratic modeling) in 2Gy fractions (92). At the time, the improved efficacy of the hypo-fractionated treatment regimen was attributed to the shorter overall treatment time needed to deliver the complete course of therapy, which may reduce the opportunity for cancer cells to repopulate during treatment. The results of this trial have led to the widespread clinical adoption of modestly hypo-fractionated radiation in early stage glottic larynx cancer. Outside of the glottic larynx, definitive radiation therapy continues to be standardly delivered in fractions of 2Gy or less; however, recently a retrospective case series of patients with oropharynx, hypopharynx, or larynx cancers treated at Princess Margaret from 2005-2017 has suggested that modestly hypo-fractionated radiation may be effective more broadly in the definitive setting (93). In this study, patients treated either with hypo-fractionated radiation monotherapy (60Gy in 25 fractions over 5 weeks), moderately accelerated radiation monotherapy (70Gy in 35 fractions over 6 weeks), or conventional radiation with concurrent chemotherapy, were assessed for locoregional control and distant control at 3 years post treatment. They found locoregional control and distant control to be similar for patients with HPV+ tumors with AJCC 7th edition stage T1-T3N0-N2c disease across the three treatment schedules as well as for patients with HPV- tumors with stage T1-2N0 disease. Patients with more advanced disease demonstrated more clear benefit from concurrent chemoradiation. This study was published during the COVID-19 pandemic with the recommendation that highly impacted treatment facilities adopt the hypo-fractionated approach to conserve healthcare resources and limit patient exposure to the virus.

In the setting of recurrent and metastatic HNSCC, multiple retrospective series have reported good local control and acceptable toxicity with highly hypo-fractionated radiation therapy delivered using an SBRT technique (94–98). These studies have examined heterogeneous patient populations and employed varied dose-fractionation schedules ranging from 13-18Gy in a single fraction (96) to 35-50Gy in 4-6 fractions (97), making comparisons across dose-fractionation schedules challenging. Nevertheless, early evidence of the safety and efficacy of SBRT in this setting have made it an intriguing technique to pair with systemic immunotherapies. Recently, investigators at Memorial Sloan Kettering Cancer Center set out to assess specifically whether employment of SBRT in the setting of recurrent and metastatic HNSCC could stimulate a systemic antineoplastic immune response beyond that generated by immune checkpoint blockade monotherapy (99). The rationale for the study was based on the theory that radiation therapy, and in particular highly hypo-fractionated radiation therapy, can improve the presentation of tumor neoantigens to the immune system, thereby stimulating a more robust antitumor immune response, even in unirradiated lesions (100). This study enrolled 62 patients with recurrent or metastatic HNSCC, and all patients were required to have at least two cancer lesions, one of which could be irradiated, and one of which could be monitored for response by RECIST criteria. 30 patients were randomized to receive nivolumab monotherapy, while 32 received nivolumab plus SBRT to one metastatic site to a dose of 27Gy in 3 fractions. The primary outcome was the overall response rate in the unirradiated lesions. Unfortunately, the study was not able to meet its primary endpoint. There was no statistically significant difference in overall response rate between the two arms (34.5% for nivolumab monotherapy *vs*. 29% for nivolumab plus SBRT, p=0.86). Similarly, there was no statistically significant difference in overall survival, progression-free survival, response duration, or grade 3-5 toxicity between the two arms. The authors concluded that they could find no evidence of synergistic effect between SBRT and immunotherapy in unselected patients with metastatic HNSCC.

Lastly, efforts are underway to determine whether select patients with favorable HNSCC can safely be treated definitively with doses of radiation that are far lower than the current standard of care. The recently reported 30 ROC Trial was a single institution study that investigated the feasibility of using hypoxia imaging to identify patients that could be effectively treated to a dose of 30Gy in 15 fractions rather than the standard 70Gy in 35 fractions (101). In this study, 19 patients with T1-2, N1-2b p16+ cancers of the oropharynx or unknown primary underwent surgical resection of the primary followed by ^{18}F-MISO PET to assess oxygenation status of their unresected nodal disease. Patients without pre-treatment hypoxia were assigned to receive chemoradiation to the post-op bed, gross nodal disease, and elective cervical lymphatics to a dose of 30Gy in 15 fractions with two doses of bolus cisplatin or carboplatin/5-FU. Patients with evidence of tumor hypoxia were started with standard of care chemoradiation but could be reassigned to low-dose radiation if gross disease became normoxic within 10 days of starting therapy. All patients who underwent low-dose radiation underwent a selective neck dissection 3-4 months after completing radiotherapy to assess for pathologic response. Ultimately, 15 of 19 patients enrolled were assigned to receive a low dose of radiation. Eleven of 15 had a pathologic complete response on completion neck dissection. Two-year locoregional control was 94.4% (95% CI 84.4-100%) and two-year overall survival was 94.7% (95% CI 85.2-100%). While the results of this study are early, uncontrolled, and applicable only to a highly-select population of patients, they are encouraging in that

they represent a willingness to study the employment of novel and less intensive radiation strategies in HNSCC. Whether 30Gy is sufficiently low to reduce the immunosuppressive effects of radiation remains unknown, and additional work will be required to determine whether such a treatment strategy is more compatible with immunotherapy than is conventional radiation.

In summary, despite encouraging evidence of tolerability and safety, and proven efficacy in the recurrent and metastatic setting, clinical trials of checkpoint inhibition have not yet demonstrated efficacy when combined with definitive-intent radiation therapy. The reasons for this remain unclear, however, antagonistic activity from the radiation itself is an intriguing hypothesis. Based upon preclinical principles, theoretical approaches to reducing antagonism between radiation and checkpoint inhibition could potentially include altering the relative timing of delivery of radiation and immunotherapy, hypofractionating radiation therapy to reduce potentially immunosuppressive effects on the tumor immune microenvironment or reducing the volume of elective radiation coverage to promote communication between the primary tumor and the draining lymphatics and promote early antitumor immunity. Clinical evidence for any of these strategies, beyond basic safety and tolerability, remains lacking, however, and careful work will be required to ensure that the proven benefits of conventional radiation therapy are not lost in the effort to increase its synergy with immunotherapy.

Neoadjuvant

Local therapy, either surgical or radiotherapeutic, is the standard of care strategy for the initial management of non-metastatic SCC for the majority of sites in the head and neck. However, the propensity of these tumors to recur both locoregionally and distantly despite aggressive local therapy, as well as the significant morbidity associated with definitive treatment of locoregionally advanced disease, has made neoadjuvant or induction systemic therapy an attractive treatment approach. The efficacy of induction chemotherapy prior to definitive chemoradiation has been assessed in multiple phase III clinical trials (102). However, with the exception of nasopharyngeal carcinoma (103), improvements in overall survival have not been consistently observed (8, 104–107). Similarly, neoadjuvant chemotherapy prior to definitive surgery has not been shown consistently to improve survival over upfront surgery (55, 94).

The development of checkpoint inhibitors and other novel immunotherapeutic agents has reignited interest in neoadjuvant strategies for locoregionally advanced head and neck cancers. As with chemotherapy, the goals of neoadjuvant immunotherapy include upfront treatment of potential distant sites of microscopic metastatic disease and downstaging of locoregional disease to decrease treatment-associated morbidity while increasing efficacy.

Currently, multiple clinical studies are ongoing to assess the efficacy of immunotherapy in the neoadjuvant setting for HNSCC, and the promising results of several trials have been reported. Early neoadjuvant immunotherapy studies employed cytokine-based therapies combined with immunomodulatory chemotherapy (108, 109). Their results suggested these regimens were tolerable and did not adversely impact subsequent surgery. Tímár et al. found that neoadjuvant immunotherapy altered the ratio of CD4+:CD8+ T cells within the surgically excised tumors (109). Wolf et al. found a potential correlation between the degree of tumor lymphocyte infiltration after neoadjuvant immunotherapy and survival (108). More recent studies have employed checkpoint inhibitors in the neoadjuvant setting. The CIAO trial, the first study to examine ICI for oropharyngeal squamous cell carcinoma (OPC) in the neoadjuvant setting, compared durvalumab *versus* combination durvalumab and tremelimumab in stage II-IVA OPC, finding that safety endpoints were met and that combination therapy did not increase CD8+ T lymphocyte tumor infiltration above durvalumab alone (110). At the ESMO annual conference in 2017, Ferris et al. presented the early results of the CheckMate 358 study of nivolumab in the pre-operative setting for patients with HNSCC (111). Twenty-nine patients with previously untreated, resectable HNSCC of the oral cavity, pharynx, or larynx with at least T1 primary disease and at least N1 nodal disease received nivolumab 250mg on days 1 and 15 and underwent surgery on day 29+/-7. The primary endpoints of the study were safety and a delay >4 weeks for planned surgery. At the time of data lock, four grade 3-4 treatment related adverse events had been reported, and there were no protocol defined surgery delays. CT-defined tumor responses were observed prior to surgery in 11/23 evaluated patients. More recently, Uppaluri et al. published the results of a multicenter phase II study investigating the safety of and pathologic response to pembrolizumab in the neoadjuvant setting (112). Thirty-six patients with stage III-IVb (AJCC 7th ed), HPV-unrelated SCC of the oral cavity, larynx, hypopharynx or oropharynx were administered a single dose of pembrolizumab (200mg) 2-3 weeks prior to surgical resection. Patients with high-risk pathology at surgery, defined as extranodal extension or positive margins, received postoperative chemoradiation and adjuvant pembrolizumab (200mg) every 3 weeks for 6 doses. Low and intermediate risk patients could receive post-operative radiation if indicated but did not receive adjuvant immunotherapy. The co-primary endpoints of the study were the percentage of patients with at least 50% pathologic tumor response at time of surgery and 1-year relapse rate in patients with high-risk pathology compared with a historical control. There were no reported grade 3-4 adverse events associated with treatment and no unexpected surgical delays. Twenty-two percent of patients achieved at least a 50% pathologic tumor response and an additional 22% achieved a 10-50% response. Baseline PD-L1, immune infiltrate, and interferon-gamma activity were associated with achieving a pathologic tumor response. In patients with high-risk pathologic features, the 1-year relapse rate was 16.7%, which was numerically lower than the historical comparator rate of 35%, though not statistically significant. There were no relapses at 1 year in the low-intermediate risk group. Schoenfeld et al. have also recently published results of a phase II study of neoadjuvant checkpoint inhibition prior to surgical resection for HNSCC (113). In this

TABLE 2 | Illustrative listing of clinical trials examining combination immunotherapy in the Neoadjuvant setting for LA-HNSCC.

Trial	Ref	Phase	Study Design	Study Eligibility	Primary Outcome	Status	Estimated Completion
NCT02274155		I	Anti-OX40 x3 (0.4mg/kg 1-3 weeks pre-op) → S → RAA	LA-HNSCC	Safety/Feasibility	Active, Not Reruiting	Oct-22
NCT02488759 (CheckMate 358)	Ferris 2017 ESMO	I/II	Nivo x2 (240mg D1 & D15) → S D30 → RAA	Multiple Cohort (including HPV+ HNSCC)	Objective Response Rate	Active, Not Reruiting	Aug-22
NCT03003637		I/II	Nivo x2 +/- Ipi x1 (240mg and 1mg/kg Wk 1 & 3 pre-op) → S → RAA	LA-HNSCC	Pathologic Treatment Response, Delay to Surgery	Completed	Feb-21
NCT03247712		I/II	Nivo x3 + Xrt x3 (240mg and 8Gy GTV+3mm x3) → S → RAA	LA-HNSCC	Delay to Surgery	Recruiting	Dec-26
NCT02296684	Uppaluri CCR 2020	II	Pembrolizumab x1 (200mg 2-3 weeks pre-op) → S → RAA +/- Pembro x6	HPV- LA-HNSCC; any site	Major Pathologic Treatment Response	Active, Not Reruiting	Dec-21
NCT02919683	Schoenfeld JamaOnc 2020	II	Nivo x2 (3mg/kg Wk1 & Wk 3) or Nivo + Ipi (3mg/kg & 1mg/kg Wk1) → S → RAA	LA-HNSCC; oral cavity	Volumetric Treatment Response	Active, Not Reruiting	Apr-24
NCT03021993		II	Nivo x3 or 4 (3mg/kg D1, 15, 29 +/- 43 for response or stable disease) → S → RAA	LA-HNSCC; oral cavity	Objective Response Rate	Active, Not Reruiting	Dec-21
NCT03721757		II	Nivo x1 (240mg 1-2 weeks pre-op) → S → RAA +/- Nivo x6 (480mg q4wk)	LA-HNSCC; oral cavity	Disease Free Survival	Active, Not Reruiting	Nov-23
NCT02827838		II	Durval x2 (D1 & D14 pre-op) → S → RAA	HNSCC (any stage, surgical candidates)	Immune Biomarkers	Recruiting	May-21
NCT03708224		II	Atezo (840mg total from D1-D15 pre-op) +/- Tiragolumab or Tocilizumab → S → RAA +/- Atezo x12 (1200mg q3 weeks)	LA-HNSCC	CD3 T Cell Tumor Infiltration, R0 Resection Rate	Recruiting	Nov-25
NCT04080804		II	Nivo x1 +/- anti-LAG3 x1 (480mg and 160mg) or Nivo x2 +/- Ipi x1 (240mg and 1mg/kg)	LA-HNSCC	Adverse Events	Recruiting	May-25
NCT03247712	Leidner JITC 2021	Ib	Nivo x3 (240mg q2 wk pre-op) +/- SBRT (GTV; 40Gy x5 fxs or 24Gy x3 fxs or 8Gy x3) → S → RAA +/- Nivo x3	LA-HNSCC	pCR, mPR & clinical to pathologic downstaging	Active, Not Reruiting	Dec-26
NCT03765918 (MK-3475-689)		III	Pembro x2 (200mg D1 & D21) → S → RAA +/- Pembro x6	Multiple Cohort (including LA-HNSCC)	Major Pathologic Treatment Response	Recruiting	Jul-26
NCT03700905 (IMSTAR-HN)		III	Nivo x1 (3mg/kg within 2 weeks pre-op) → S → RAA +/- Nivo x3 +/- Ipi x1 (3mg/kg and 1mg/kg q2 weeks)	LA-HNSCC	Disease Free Survival	Active, Not Reruiting	May-24

single-center study at the Dana Farber Cancer Institute, twenty-nine patients with at least T2 or node positive SCC of the oral cavity were randomized to receive either two cycles of nivolumab (14 patients) or two cycles of nivolumab and 1 cycle of ipilimumab (15 patients) prior to undergoing surgical resection. Adjuvant radiation or chemoradiation was given according to standard of care based on pathological findings. The coprimary endpoints were safety, including surgical delays, and volumetric response. At 14.2 months median follow up, two grade 3-4 events were observed in the nivolumab arm and five grade 3-4 events in the ipilimumab-nivolumab arm. There was one death that was not thought to be related to the study drugs. There were no unplanned surgical delays. A volumetric response, based on re-staging imaging obtained a median of 14 days after treatment initiation, was observed in 50% of patients in the nivolumab arm *vs.* 53% in the ipilimumab-nivolumab arm. Responses met RECIST criteria in 13% and 38% of patients in each arm, respectively. Clinical to pathological downstaging at the time of surgery occurred in 69% and 53% of patients in each arm, respectively. Intriguingly, post-immunotherapy, pre-surgery PET/CT restaging imaging demonstrate a high rate of increased FDG avidity in cervical lymph nodes that later proved to be pathologically negative, suggesting that immune response could potentially confound interpretation of post-immunotherapy FDG-PET imaging.

At this time, the preponderance of evidence from published studies of checkpoint inhibitors in the neoadjuvant setting suggests that these agents are tolerable, with low rates of severe adverse events, and do not lead to unexpected surgical delays. While clinically meaningful efficacy endpoints remain to be assessed, the clinical and pathological responses observed to date have been encouraging and correlative histopathological and radiological analysis has provided important information for selection of patients for late phase clinical trials. Currently, there are several additional early phase trials underway to further assess the safety and feasibility of checkpoint inhibitors and other immunotherapeutic agents in this setting (**Table 2**). These trials, which feature checkpoint inhibitors delivered as monotherapy or in combination with agonistic immunomodulators or radiation, will provide key insights into the treatment sequences that maximize responses. Of note, there are two open phase III trials examining neoadjuvant immunotherapy in HNSCC. The MK-3475-689 trial (NCT03765918) will assign 704 patients with locoregionally advanced HNSCC to receive upfront surgical resection or neoadjuvant pembrolizumab for two 21-day cycles prior to surgical resection. Patients randomized to the pembrolizumab arm will also receive adjuvant therapy for fifteen 21-day cycles. All patients will receive standard of care adjuvant radiation or chemoradiation based on pathological assessment. The co-primary endpoints of the study will include the proportion of patients with a major pathological response (defined as less than or equal to 10% invasive SCC in the primary specimen) and event free survival for up to 5 years. Similarly, the IMSTAR-HN (NCT03700905) will deliver neoadjuvant immunotherapy prior to surgery with risk adapted adjuvant therapy and maintenance immunotherapy; however, in this study, the investigational drug will be nivolumab with a sub-cohort also receiving ipilimumab in the maintenance phase. The primary endpoint of IMSTAR-HN will be DFS.

CONCLUSIONS/FUTURE DIRECTIONS

Although immunotherapy is now a mainstay of treatment for recurrent and metastatic HNSCC, the addition of immunotherapy to standard therapies in the curative setting has yet to improve outcomes for patients with locally-advanced disease. The effects of ICI on clinically relevant outcomes in the neoadjuvant setting are still largely unknown, and the negative results of the recent Javelin HN 100 trial inspire several thought-provoking questions about how to optimally combine checkpoint inhibitors with chemoradiation. Excitingly, ongoing work in the preclinical arena is promising and raises the intriguing hypothesis that the efficacy of ICI may be improved by treatment strategies that spare communication between a target tumor and its draining lymphatics. As we look ahead, it will be critical to re-evaluate not only the timing for delivering immunotherapies in relation to standard therapies but also the importance of maintaining the integrity of the tumor-immune-lymphatic axis during immunotherapy. However, as exciting as these hypotheses are, careful work will be required to ensure that the proven benefits of standard therapies are not compromised in the effort to maximize the efficacy of immunotherapy.

AUTHOR CONTRIBUTIONS

RS-K and ABS contributed equally to the manuscript, including concept, sourcing literature and writing. WS contributed to sections involving curative-intent immunoradiotherapy trials. AS and LM contributed to concept and editing. EC contributed to all aspects of the manuscript from concept to editing. All authors contributed to the article and approved the submitted version.

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Tumor-Infiltrating Immune-Related Long Non-Coding RNAs Indicate Prognoses and Response to PD-1 Blockade in Head and Neck Squamous Cell Carcinoma

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Long non-coding RNAs (lncRNAs) in immune cells play critical roles in tumor cell–immune cell interactions. This study aimed to characterize the landscape of tumor-infiltrating immune-related lncRNAs (Ti-lncRNAs) and reveal their correlations with prognoses and immunotherapy response in head and neck squamous cell carcinoma (HNSCC). We developed a computational model to identify Ti-lncRNAs in HNSCC and analyzed their associations with clinicopathological features, molecular alterations, and immunotherapy response. A signature of nine Ti-lncRNAs demonstrated an independent prognostic factor for both overall survival and disease-free survival among the cohorts from Fudan University Shanghai Cancer Center, The Cancer Genome Atlas, GSE41613, and GSE42743. The Ti-lncRNA signature scores in immune cells showed significant associations with *TP53* mutation, *CDKN2A* mutation, and hypoxia. Inferior signature scores were enriched in patients with high levels of PDCD1 and CTLA4 and high expanded immune gene signature (IGS) scores, who displayed good response to PD-1 blockade in HNSCC. Consistently, superior clinical response emerged in melanoma patients with low signature scores undergoing anti-PD-1 therapy. Moreover, the Ti-lncRNA signature was a prognostic factor independent of PDCD1, CTLA4, and the expanded IGS score. In conclusion, tumor-infiltrating immune profiling identified a prognostic Ti-lncRNA signature indicative of clinical response to PD-1 blockade in HNSCC.

Keywords: HNSCC, Ti-lncRNA, PD-1 blockade, CTLA4, prognosis

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) originates from epithelial cells at sites of oral cavity, pharynx, and larynx, which is the sixth most common cancer worldwide, with 890,000 new cases and 450,000 deaths in 2018 (1, 2). Several common risk factors for HNSCC have been uncovered, such as smoking, alcohol abuse, consumption of areca catechu, human papillomavirus

(HPV) infection, and exposure to environmental pollutants (3, 4). Surgery, radiation, and systemic therapy are the principal modalities for locally confined HNSCC. A majority of the patients with recurrent or metastatic HNSCC are considered for systemic therapy, especially immunotherapy, except for some patients cured by local management (5, 6).

In general, the tumor microenvironment (TME) of HNSCC is highly infiltrated by immune cells with regard to tumor biology, which mediate immune surveillance or evasion through various mechanisms (3). In advanced-staged HNSCC, it is demonstrated that the cytotoxic activities of T cells are repressed due to the upregulation of immunosuppressive factors such as PD-1 and CTLA4 in TME, leading to persistent efforts of reactivating T cells to treat this malignancy (7–10). Until now, immune checkpoint inhibitors have significantly updated the therapeutic modalities of HNSCC. The Food and Drug Administration approved the use of the immune checkpoint inhibitors pembrolizumab and nivolumab for the treatment of cisplatin-refractory recurrent or metastatic HNSCC and pembrolizumab as a first-line therapy for unresectable or metastatic disease in 2016 and 2019, respectively (9–11). However, it is noted that only a subset of patients are expected to respond to immune checkpoint inhibitors and that reliable predictive biomarkers are needed.

Therefore, it is necessary to identify molecular biomarkers that can be used to predict the disease progression, survival status, and response to immunotherapy of HNSCC. The search for such biomarkers has focused on the molecular abnormalities of tumor-infiltrating immune cells. In recent years, long non-coding RNAs (lncRNAs) in immune cells have demonstrated to play critical roles in tumor cell–immune cell interactions (12, 13). In the present study, we initially characterized the lncRNA landscape of immune cells specifically altered in HNSCC and then aimed to identify a prognostic lncRNA signature that is useful for the prediction of immunotherapy response through integrated analyses of tumor-infiltrating immune-related lncRNAs (Ti-lncRNAs) and clinicopathological features.

MATERIALS AND METHODS

Transcriptional Data of Immune Cells and Tumor Cell Lines

The transcriptional profiles of 115 purified cell lines of 19 immune cell types based on the Affymetrix HG-U133_Plus 2.0 platform were obtained from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo), including GSE13906 (14), GSE23371 (15), GSE25320 (16), GSE27291 (17, 18), GSE27838 (19), GSE28490 (20), GSE28698 (21), GSE28726 (22), GSE37750 (23), GSE39889 (24), GSE42058 (25), GSE49910 (26), GSE51540 (27), GSE59237 (28), GSE6863 (29), and GSE8059 (30). We obtained the transcriptional profiles of HNSCC cell lines based on the Affymetrix HG-U133_Plus 2.0 platform from the Cancer Cell Line Encyclopedia (CCLE) project (https://portals.broadinstitute.org/ccle) and collected 34 cell lines that matched the tumor type 'HNSC' from the cell annotation files of The Cancer Genome Atlas (TCGA).

Microarray Data Processing

All raw data (.cel files) of the 115 immune cells and the 34 'HNSC' cell line microarray data profiled by the Affymetrix HG-U133_Plus 2.0 platform were downloaded and processed together using robust multi-array average (RMA) normalization with the R 'affy' packages. RMA normalization for the patient datasets GSE41613 and GSE42743 (31) was performed separately as well. The Affymetrix Human Genome U133 Plus 2.0 Array probes were re-annotated into unique Ensembl gene IDs using custom library file downloaded from the Brainarray database (HGU133Plus2_Hs_GENCODEG, version 24; http://mbni.org/customcdf/24.0.0/gencodeg.download/HGU133Plus2_Hs_GENCODEG_24.0.0.zip). In total, 54,675 probes were mapped to 21,311 ensemble genes, including 3,599 genes that were annotated as lncRNAs in the GENCODE annotation file (version 32, http://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_32/gencode.v32.annotation.gtf.gz). The 3,477 lncRNAs that existed in both the microarray and TCGA profiles were selected for subsequent analysis.

Transcriptional Profiles of HNSCC Patients

HNSCC patients with transcriptional profiles were obtained from the GEO database, the TCGA database (https://portal.gdc.cancer.gov), and The cBioPortal for Cancer Genomics (32) (http://www.cbioportal.org/) according to the following selection criteria: 1) with detailed information of stage, age, gender, and overall survival (OS) time and status; 2) profiled with the Affymetrix HG-U133_Plus 2.0 or Illumina HiSeq platform; and 3) sample size large than 50. In total, 671 HNSCC patients were enrolled, including two microarray profiles, GSE41613 (n = 97) and GSE42743 (n = 74), and TCGA dataset (n = 500). TCGA dataset was used as the training dataset for discovering a lncRNA signature; the other two microarray datasets were used as independent test datasets for validating the lncRNA signature. Detailed clinical information of the three patient sets is shown in **Table 1**.

Development of a Ti-lncRNA Signature

We established a novel Ti-lncRNA by integrative lncRNA profiling analyses on purified immune cells, HNSCC cell lines, and cancer bulk tissues as follows (**Figure 1**): 1) the top 10% expressed lncRNAs in each immune cell (average expression value) were obtained for the 19 immune cell types; 2) the immune cell specificity of lncRNA was calculated with the tissue specificity index (TSI) using the following formula:

$$\mathrm{TSI}_{\mathrm{lnc}} = \frac{\sum_{i=1}^{N}(1 - x_{\mathrm{lnc},i})}{N - 1}$$

where N denotes the total number of immune cell types and $x_{\mathrm{lnc},i}$ is the expression intensity of immune cells normalized by the maximal expression of any immune cell types for lncRNAs. A higher TSI value represents a higher cell specificity of the

TABLE 1 | Clinical characteristics of patients with head and neck squamous cell carcinoma (HNSCC) enrolled in this study.

Variables	TCGA (training)		TCGA (test)		FUSCC		GSE41613		GSE42743	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%
Age (years)										
≤60	142	47.3	101	50.5	41	51.25	50	51.5	40	54.1
>60	158	52.7	99	49.5	39	48.75	47	48.5	34	45.9
Gender										
Male	216	72.0	151	75.5	70	87.5	66	68.0	58	78.4
Female	84	28.0	49	24.5	10	12.5	31	32.0	16	21.6
TNM stage										
I–II	67	22.3	47	23.5	25	31.25	41	42.3	30	40.5
III–IV	227	75.7	145	72.5	51	63.75	56	57.7	44	59.5
Unknown	6	2.0	8	4.0	4	5.0	0	0.0	0	0.0
Survival status										
Alive	169	56.3	112	56.0	55	68.75	46	47.4	32	43.2
Dead	130	43.3	87	43.5	25	31.25	51	52.6	42	56.8
Unknown	1	0.3	1	0.5	0	0.0	0	0.0	0	0.0

TCGA, The Cancer Genomics Atlas; FUSCC, Fudan University Shanghai Cancer Center; TNM, tumor node metastasis.

lncRNA. TSI ranges from 0 to 1; 3) differential expression analyses were further performed for the top expressed lncRNAs with high TSI values (>0.1) between immune cells and the HNSCC cell line profiles. The lncRNAs with a false discovery rate (FDR) <0.05 upregulated in immune cells were recognized as Ti-lncRNAs; 4) the OS-related Ti-lncRNAs were selected using univariate Cox regression analyses. Finally, nine lncRNAs with a univariate *p*-value <0.01 were selected for the construction of a prognostic risk model using the linear combination of the expression values of the prognostic Ti-lncRNAs, weighted by their estimated regression coefficients from multivariate Cox regression analyses.

HNSCC Patients from FUSCC

A total of 80 HNSCC patients were enrolled in this study, who received surgical therapy at Fudan University Shanghai Cancer Center (FUSCC) from 2011 to 2019. The 80 samples obtained from these patients, whose diagnoses were confirmed by

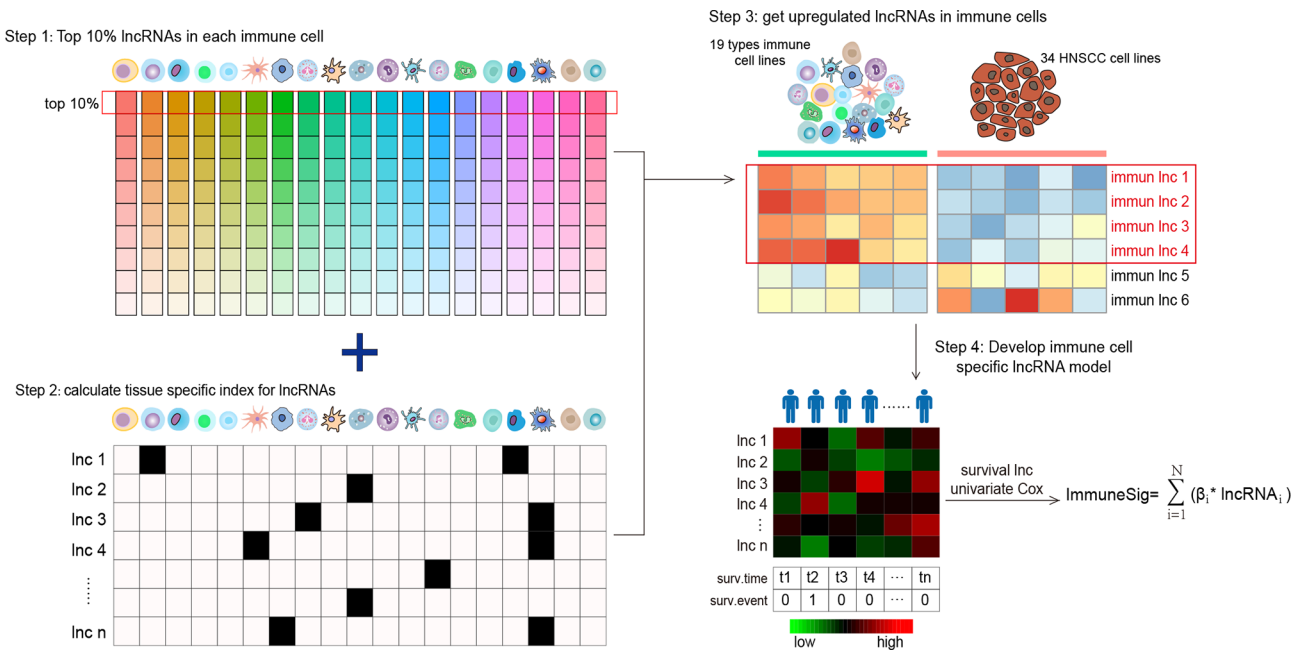

FIGURE 1 | Flow graph for characterizing the lncRNA landscape of HNSCC-infiltrating immune cells and for building a prognostic signature. Step 1: all lncRNAs were ranked according to their mean expression levels in each immune cell type, and the top 10% expressed lncRNAs were chosen as candidate lncRNAs. Step 2: the TSI scores of a total of 958 lncRNAs were calculated to reflect their expression specificity with respect to the 19 immune cell types. Step 3: lncRNAs with high TSI values (> 0.1) were further analyzed for differential expression between the 19 types of immune cells and the 34 HNSCC cell lines. Step 4: the lncRNAs upregulated in immune cells were screened out and compared with HNSCC cells. lncRNA, long non-coding RNA; HNSCC, head and neck squamous cell carcinoma; TSI, tissue specificity index.

pathological experts, were subjected to RNA extraction and further lncRNA expression analyses. The clinical features of the 80 patients are described in **Table 1**. Each patient provided written informed consent for his/her specimens and information to be used for research and stored in the hospital database. This study was approved by the Medical Ethics Committee of the FUSCC. All procedures performed in our study were in accordance with the ethical standards of our institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

RNA Extraction and Expression Analyses of lncRNAs

We extracted total RNA from the HNSCC samples using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Total RNA was reverse transcribed to cDNA using the TAKARA PrimeScriptTM RT Master Mix (Perfect Real Time). As previously described (33), for real-time quantitative PCR, the TAKARA TB green was used and the following lncRNAs were employed: ENSG00000265148, ENSG00000281358, ENSG00000262089, ENSG00000240889, ENSG00000253230, ENSG00000261888, ENSG00000235304, ENSG00000226806, and ENSG00000260244. The assays were performed in triplicate for each sample, and the mean value was used for the calculation of the lncRNA expression levels. The relative lncRNA expression levels were determined by the comparative CT ($2^{-\Delta CT}$) method. The lncRNA expression levels were given as ratios to the β-actin messenger RNA (mRNA) level. The primer sequences for each lncRNA are attached in **Supplementary Table S1**.

Hypoxia, Cancer-Associated Fibroblast, and Tumor-Associated Macrophage

The Nurmik mRNA-based cancer-associated fibroblast (CAF) gene signature was used to quantify the CAFs in HNSCC according to previous studies (34, 35). We quantified tumor hypoxia in HNSCC by applying the mRNA-based hypoxia signature from Buffa et al. (36) The signature genes of hypoxia and CAF are shown in **Supplementary Table S1**. A summary score of hypoxia or CAF is defined in each sample as the median of the absolute expression values of the genes in the signature, as described in previous studies (35, 37). The xCell tool (http://xcell.ucsf.edu/) (38) was used to infer the enrichment score of the M2 cell type by using the transcriptome data of TCGA cohort, and M2 was considered as a tumor-associated macrophage (TAM) in our study.

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was performed using the GSEA software, version 4.1.0, which was obtained from the Broad Institute (http://www.broad.mit.edu/gsea), as previously described (39, 40). Enrichment Map was used for the visualization of the GSEA results. The normalized enrichment score (NES) and *p*-value were used to sort the pathways enriched in each phenotype after gene set permutations were performed 1,000 times for each analysis.

Statistical Analysis

Continuous variables were expressed as the mean value ± standard deviation (SD), and categorical data were summarized with frequencies and percentages. Independent *t*-test was used to compare the continuous variables between two groups. χ^2 and Fisher's exact tests were used for categorical variables. The expression levels of the nine lncRNAs as a signature in each patient were integrated into a risk score: $-0.724 \times$ ENSG00000265148 + $(-1.047) \times$ ENSG00000281358 + $(-0.159) \times$ ENSG00000262089 + $(-0.887) \times$ ENSG00000240889 + $2.137 \times$ ENSG00000253230 + $(-0.656) \times$ ENSG00000261888 + $(-0.556) \times$ ENSG00000235304 + $(-1.257) \times$ ENSG00000226806 + $(-0.195) \times$ ENSG00000260244. To analyze the associations between the lncRNA signature and the clinicopathological parameters, patients were divided into two subgroups (low risk score and high risk score groups) according to the median value of the risk score. Nonparametric receiver operating characteristic (ROC) analyses were performed to calculate the area under the curve (AUC) for the signature that would be predictive of the survival status. Univariate and multivariate logistic regression analyses were performed to determine the risk factors for the signature. The Kaplan–Meier method was used to construct survival curves, and the survival difference was determined by the log-rank test. A *p*-value <0.05 was considered significant. Univariate and multivariate Cox regression methods were utilized to conduct survival analyses. Data preparation and statistical analyses were performed using the SPSS for Windows (version 22.0; IBM Corp., Armonk, NY, USA), the R software (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria), and GraphPad Prism (version 6.01; GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Identification of Ti-lncRNAs Specifically Altered in Immune Cells of HNSCC

The landscape of lncRNAs was initially characterized in all human immune cells, and a differential expression pattern was observed in 19 immune cell types (**Supplementary Figure S1**). As shown in **Figure 1**, to capture representative lncRNAs in different immune cell types, we firstly ranked all lncRNAs according to the mean expression levels of each immune cell, and the top 10% expressed lncRNAs were chosen as candidate lncRNAs. A total of 958 lncRNAs were selected for the next procedure (**Supplementary Table S2**). Then, we calculated the TSI scores for the 958 immune-related lncRNAs to reflect their expression specificity with respect to the 19 immune cell types. The top 872 expressed lncRNAs with high TSI values (>0.1) were further analyzed for differential expression between the 19 types of immune cells and the 34 HNSCC cell lines. As a result, 492 lncRNAs were identified as Ti-lncRNAs, which were significantly upregulated with FDR < 0.05 in immune cells compared with HNSCC cells (**Supplementary Table S2**).

We further sought to identify Ti-lncRNAs associated with HNSCC survival outcomes using univariate Cox regression analyses in the training cohort of TCGA. Patients from TCGA were randomly split into 60% (n = 300) and 40% (n = 200) as a training cohort and a test cohort, respectively. There were nine Ti-lncRNAs (ENSG00000265148, ENSG00000281358, ENSG00000262089, ENSG00000240889, ENSG00000253230, ENSG00000261888, ENSG00000235304, ENSG00000226806, and ENSG00000260244) with different expression specificities in the 19 immune cell types (**Figure 2A** and **Supplementary Table S3**), which were remarkably correlated with OS in the training cohort (**Figure 2B**).

The Ti-lncRNA Signature as an Independent Prognostic Factor for HNSCC

We developed a Ti-lncRNA signature score using a linear combination of the expression values of the nine Ti-LncRNAs, which were weighted by their estimated regression coefficients from multivariate Cox regression analysis. The Ti-lncRNA signature was identified as a prognostic indicator associated with OS in the training cohort of TCGA (log-rank $p < 0.001$) (**Figures 2C, D**). As shown in **Figure 2C**, a majority of the immune signaling pathways (16/17, 94.11%) were enriched in patients with low Ti-lncRNA signature scores, while the neutrophil signaling pathway (1/17, 5.89%) was enriched in patients with high risk scores. We confirmed the significant correlation of a high Ti-lncRNA score with a decreased OS time (log-rank $p = 0.033$) (**Figure 2E**) in the test cohort of TCGA. We performed GSEA using the RNA sequencing data of the whole TCGA cohort to investigate the associations of the signature with the tumor signaling pathways. Among all predefined pathway gene sets, the biological pathways Myc targets v2 (NES = 1.86, $p = 0.010$), interferon alpha response (NES = 2.07, $p = 0.020$), TGF-β signaling (NES = 1.68, $p = 0.009$), and glycolysis (NES = 2.07, $p = 0.043$) were enriched in the phenotype with a high risk score (**Figure 2F**).

We further validated the prognostic effect of the Ti-lncRNA signature in the FUSCC cohort (80 patients) and the public cohorts (TCGA, 498 patients; GSE41613, 97 patients; and GSE42743, 74 patients). The Ti-lncRNA signature demonstrated a significant association with the OS of patients among all four cohorts (TCGA: log-rank $p < 0.001$; FUSCC: log-rank $p = 0.020$; GSE41613: log-rank $p = 0.006$; GSE42743: log-rank $p = 0.040$) (**Figure 3**), and its predictive scores for decease status were proven to be relatively high at 3 years (TCGA: AUC = 0.671; FUSCC: AUC = 0.671) and at 5 years (TCGA: AUC = 0.639; FUSCC: AUC = 0.619) (**Figures 3B, D**, respectively). Additionally, we analyzed the correlations of the Ti-lncRNA signature with recurrence of HNSCC in 80 patients from FUSCC and in 374 patients from TCGA. A high signature score indicated

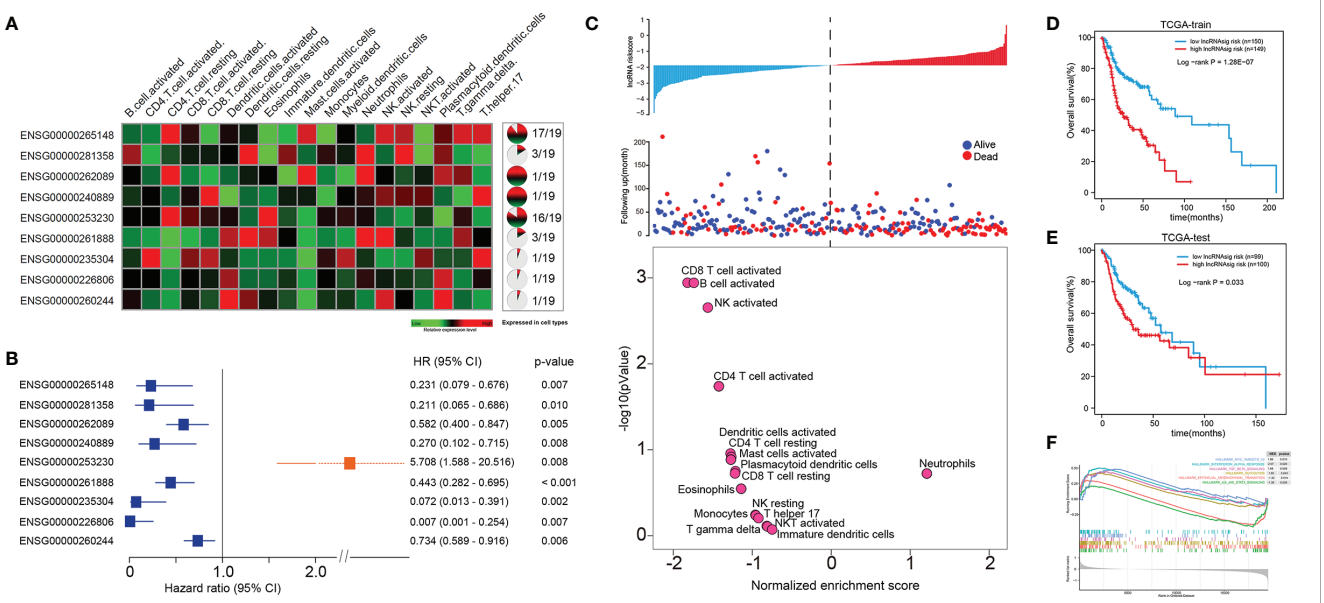

FIGURE 2 | A Ti-lncRNA signature consisting of nine lncRNAs was identified as a prognostic indicator associated with OS in TCGA cohort. **(A)** Expression heatmap (*left*) and expression specificity proportion (*right*) of the nine Ti-lncRNAs in the 19 immune cell types. **(B)** Forest plot of the hazards ratios (HRs) with 95% confidence interval **(CI)** to show the associations of the Ti-lncRNAs with OS in the training cohort of TCGA. **(C)** A Ti-lncRNA signature was developed using a linear combination of the expression values of the nine Ti-LncRNAs, weighted by their estimated regression coefficients from multivariate Cox regression analysis, and volcano plots for the enrichment of immune cell types for patients with high scores and low scores calculated based on the NES from the GSEA. **(D)** Differences in OS between patients with low signature risk scores and those with high risk scores was analyzed in the training cohort of TCGA using the Kaplan–Meier method to construct an OS curve. **(E)** The Ti-lncRNA signature was analyzed for its association with survival in the test cohort of TCGA. **(F)** Biological signaling pathways enriched in patients with high Ti-lncRNA scores. lncRNA, long non-coding RNA; OS, overall survival; TCGA, The Cancer Genome Atlas; Ti-lncRNA, tumor-infiltrating immune-related lncRNA; NES, normalized enrichment score; GSEA, gene set enrichment analysis.

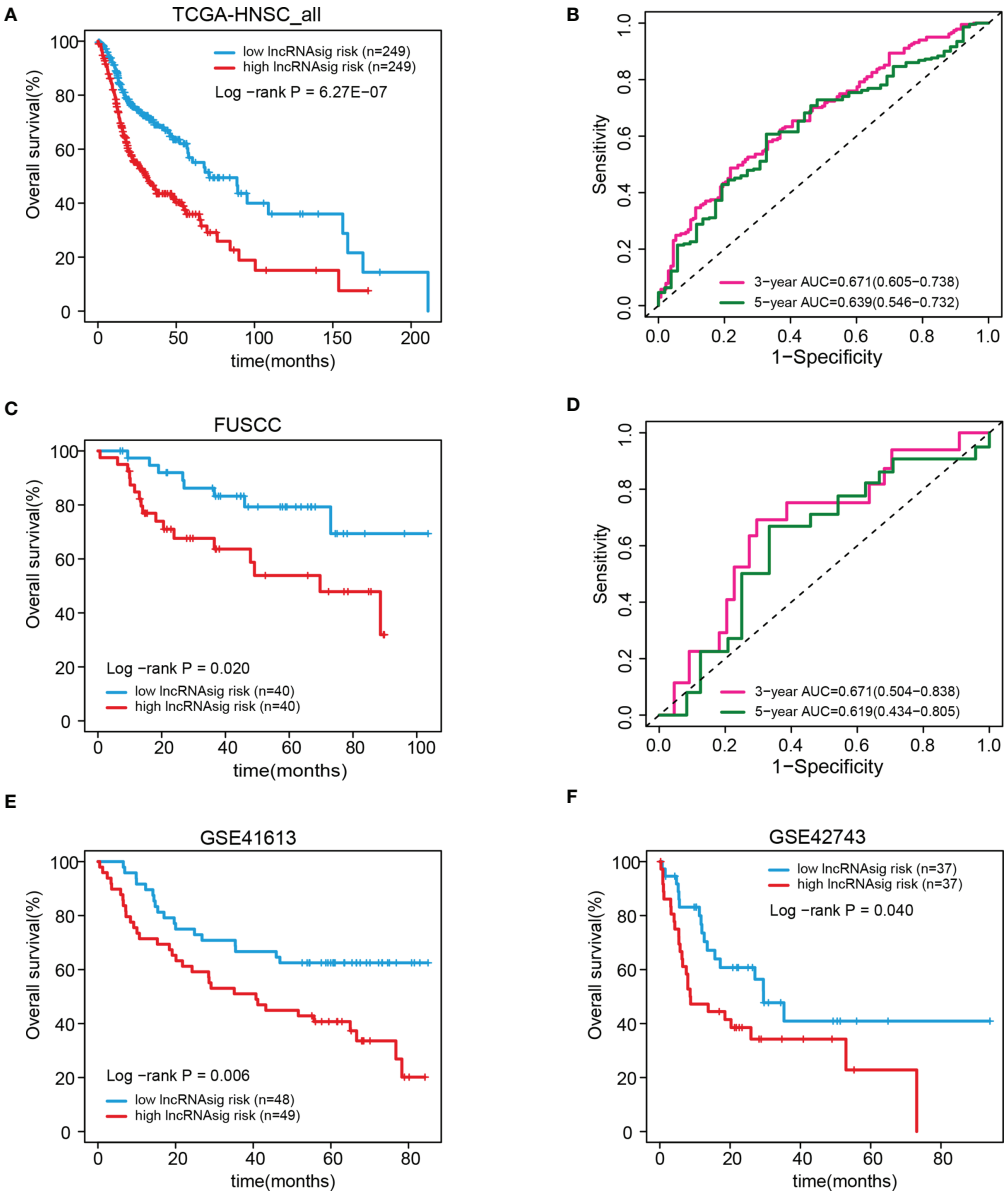

FIGURE 3 | Validation of the prognostic effect of the Ti-lncRNA signature in patients from TCGA, FUSCC, GSE41613, and GSE42743. **(A–F)** Differences in OS and the predictive effect of the signature risk score were analyzed in the whole TCGA cohort **(A, B)**, the FUSCC cohort **(C, D)**, and the GSE41613 and GSE42743 cohorts **(E, F)**. lncRNA, long non-coding RNA; Ti-lncRNA, tumor-infiltrating immune-related lncRNA; TCGA, The Cancer Genome Atlas; FUSCC, Fudan University Shanghai Cancer Center; OS, overall survival.

a shortened disease-free survival (DFS) in both cohorts (TCGA: log-rank p = 0.0015; FUSCC: log-rank p = 0.002), with a relatively high predictive effect for recurrence (FUSCC: AUC = 0.64; TCGA: AUC = 0.66) (**Figure 4**).

Moreover, univariate and multivariate Cox regression analyses were performed to verify the independent effect of the Ti-lncRNA signature on the prognoses of patients. As shown in **Table 2**, after adjusting for age, gender, and tumor node metastasis (TNM) stage, a high signature score was repeatedly recognized as a risk factor for shortened OS time in the FUSCC cohort (multivariate: HR = 2.495, 95%CI = 1.058–5.881, p = 0.037), TCGA cohort (HR = 2.214, 95%CI = 1.669–2.939, p < 0.001), the GSE41613 cohort (HR = 1.768, 95%CI = 0.980–3.192, p = 0.059), and the GSE42743 cohort (HR = 1.911, 95%CI = 1.003–3.641, p = 0.049). The Ti-lncRNA signature was an independent risk factor for recurrence in the FUSCC cohort (p = 0.010, HR = 3.005, 95%CI = 1.307–6.910) and TCGA cohort (p = 0.001, HR = 1.835, 95%CI = 1.299–2.593), as well as in the multivariate Cox regression analyses after adjusting for age, gender, and TNM stage (**Table 3**).

The Ti-lncRNA signature was analyzed for its associations with the prognostic outcomes of 32 malignancies using the pan-

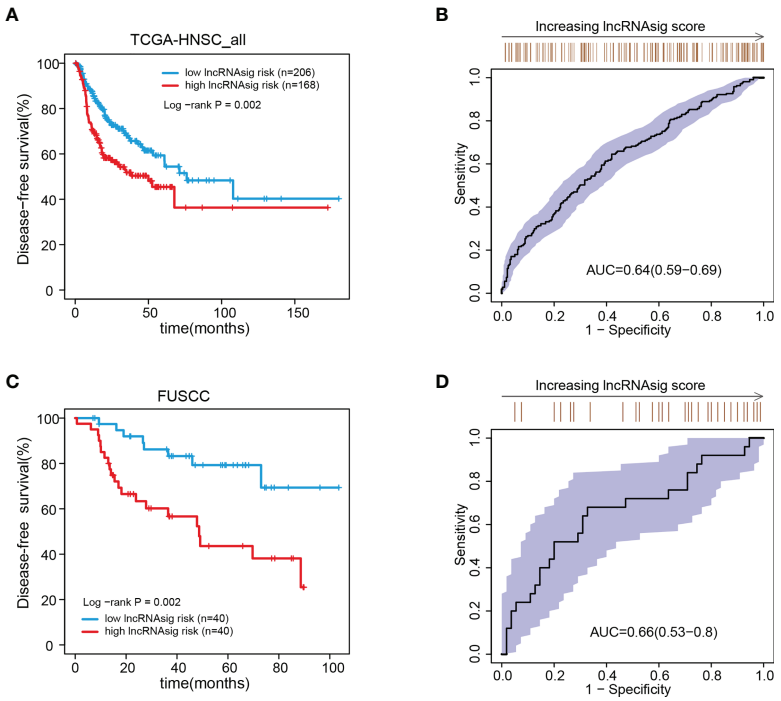

FIGURE 4 | Associations of the Ti-lncRNA signature with DFS in patients from TCGA and FUSCC. **(A–D)** Differences in DFS and the predictive effect of the signature risk score were analyzed in TCGA cohort **(A, B)** and in the FUSCC cohort **(C, D)**. *lncRNA*, long non-coding RNA; Ti-*lncRNA*, tumor-infiltrating immune-related lncRNA; *DFS*, disease-free survival; *TCGA*, The Cancer Genome Atlas; *FUSCC*, Fudan University Shanghai Cancer Center.

TABLE 2 | Univariate and multivariate Cox regression analyses of overall survival in the datasets.

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI of HR	*p*-value	HR	95%CI of HR	*p*-value
TCGA						
lncRNAsig (high/low)	1.996	1.513–2.633	**<0.001**	2.214	1.669–2.939	**<0.001**
Age	1.021	1.008–1.033	**<0.001**	1.024	1.011–1.037	**<0.001**
Gender (male/female)	0.751	0.563–1.000	0.050	0.798	0.594–1.073	0.136
TNM stage (I/II *vs*. III/IV)	1.216	0.877–1.685	0.242	1.259	0.906–1.748	0.170
GSE41613						
lncRNAsig (high/low)	2.185	1.230–3.882	**0.008**	1.76812	0.980–3.192	**0.059**
Age group[a]	0.924	0.689–1.239	0.598	0.9365	0.685–1.281	0.681
Gender (male/female)	1.123	0.621–2.029	0.702	1.11925	0.618–2.029	0.710
TNM stage (I/II *vs*. III/IV)	3.829	1.959–7.485	**<0.001**	3.36525	1.699–6.666	**<0.001**
GSE42743						
lncRNAsig (high/low)	1.892	1.019–3.512	**0.043**	1.91053	1.003–3.641	**0.049**
Age	1.004	0.980–1.028	0.764	1.013	0.988–1.038	0.305
Gender (male/female)	0.676	0.337–1.355	0.270	0.56938	0.281–1.154	0.118
TNM stage (I/II *vs*. III/IV)	2.917	1.210–7.034	**0.017**	3.09155	1.259–7.590	**0.014**
FUSCC						
lncRNAsig (high/low)	2.636	1.132–6.137	**0.025**	2.4945	1.058–5.881	**0.037**
Age	1.016	0.971–1.063	0.505	1.0137	0.967–1.063	0.574
Gender (male/female)	0.751	0.256–2.205	0.602	0.8999	0.301–2.695	0.851
TNM stage (I/II *vs*. III/IV)	2.276	0.777–6.667	0.134	2.3862	0.801–7.107	0.118

HR, hazard ratio; CI, confidence interval; lncRNAsig, long non-coding RNA signature; TCGA, The Cancer Genomics Atlas; TNM, tumor node metastasis; FUSCC, Fudan University Shanghai Cancer Center.

[a]*Age groups: 19–39 years, 40–49 years, 50–59 years, and 60–68 years.*

Bold type indicates statistical significance.

TABLE 3 | Univariate and multivariate Cox regression analyses of disease-free survival in the datasets.

Variable	Univariate analysis			Multivariate analysis		
	HR	95%CI of HR	*p*-value	HR	95%CI of HR	*p*-value
TCGA						
lncRNAsig (high/low)	1.707	1.221–2.385	**<0.001**	1.835	1.299–2.593	**0.001**
Age	1.012	0.996–1.028	**<0.001**	1.017	1.000–1.034	0.051
Gender (male/female)	0.959	0.654–1.406	0.829	0.967	0.649–1.442	0.871
TNM stage (I/II *vs*. III/IV)	1.369	0.884–2.119	0.159	1.428	0.918–2.222	0.114
FUSCC						
lncRNAsig (high/low)	3.336	1.472–7.562	**0.004**	3.005	1.307–6.910	**0.010**
Age	1.015	0.974–1.059	0.480	1.011	0.967–1.057	0.625
Gender (male/female)	0.669	0.254–1.764	0.417	0.782	0.290–2.108	0.627
TNM stage (I/II *vs*. III/IV)	2.613	0.902–7.563	0.077	2.698	0.918–7.928	0.071

HR, hazard ratio; CI, confidence interval; lncRNAsig, long non-coding RNA signature; TCGA, The Cancer Genomics Atlas; TNM, tumor node metastasis; FUSCC, Fudan University Shanghai Cancer Center.
Bold type indicates statistical significance.

cancer TCGA data as well, of which 18 malignancies demonstrated increased disease risk patients with high signature risk scores compared to those with low risk scores, especially including kidney renal papillary cell carcinoma (KIRP), lung adenocarcinoma (LUAD), and pancreatic adenocarcinoma (PAAD) (**Supplementary Figure S4**).

Correlations of the Ti-lncRNA Signature With Clinicopathological Features, Genetic Mutations, Hypoxia, CAF, and TAM in TME

Using the cohort data from TCGA, we analyzed the correlations of the Ti-lncRNA signature with the clinicopathological features, genetic mutations, hypoxia, CAF, and TAM in TME. The Ti-lncRNA signature showed no statistical associations with gender, alcohol history, HPV status, TNM stage, and histological grade, except for age. In the analyses of the factors affecting the Ti-lncRNA signature, hypoxia ($p < 0.001$) and *TP53* ($p < 0.001$) and *CDKN2A* ($p = 0.048$) mutations showed significant associations with a high signature score, while *SYNE1* mutation ($p = 0.003$) occurred more frequently in patients with low risk scores than in those with high risk scores (**Figure 5** and **Supplementary Table S3**). CAF and TAM failed to have an impact on the Ti-lncRNA signature. After adjusting for age, sex, alcohol history, hypoxia, and *TP53*, *CDKN2A*, and *SYNE1* mutations, the multivariate logistic regression analysis showed that hypoxia ($p < 0.001$) and *TP53* mutation ($p = 0.001$) were independent risk factors for patients with a high signature score, whereas *SYNE1* mutation was an independent protective factor ($p = 0.004$) (**Supplementary Table S3**).

The Ti-lncRNA Signature and Immunotherapy Response

To investigate the predictive effect of the Ti-lncRNA signature on blockade therapy of immune checkpoints, we initially analyzed the relationship between the signature score and the expression of immune checkpoints in HNSCC. As shown in **Figures 5**, **6A**, the signature score showed a negative correlation with the expressions of PDCD1 and CTLA4 (**Supplementary Table S3**). We also utilized an expanded IGS consisting of 18 genes that can be predictive of the response to anti-PD-1 therapy for HNSCC in order to verify the value of the Ti-lncRNA signature on the evaluation of immunotherapy response (41). Consistently, HNSCC patients with low signature scores demonstrated increased expended IGS scores, suggesting an effective response to anti-PD-1 therapy (**Figures 5**, **6A** and **Supplementary Table S3**). Moreover, using available data of melanoma patients undergoing immunotherapy, it was found that anti-PD-1 therapy tended to achieve good responses in patients with low Ti-lncRNA signature scores (**Figure 6B**).

Integrating the Ti-lncRNA signature and immune checkpoints to analyze their impacts on the prognoses of patients, we found that a high signature score was significantly associated with a reduced OS independent of the level of PDCD1 or CTLA4 or the expanded IGS score (**Figure 6C**). Patients with the combination of a high signature risk score and a low level of PDCD1 or CTLA4 or expanded IGS score had the worst survival prognosis, while those with a low risk score and a high level of the immune factor had extended survival time.

DISCUSSION

In addition to the existing FDA-approved PD-1 inhibitors, nivolumab and pembrolizumab, emerging agents targeting PD-1 and CTLA4 are under ongoing clinical trials for HNSCC patients (3). So far, limited knowledge on tumor-infiltrating immune cells has confined the ability to effectively predict the effects of immunotherapy. Although some studies have reported that lncRNAs in immune cells play critical roles in tumor cell–immune cell interactions (12, 13), the roles of lncRNAs remain unclear in tumor-infiltrating immune cells of HNSCC. Due to the temporal and spatial specificity of the expressions of lncRNAs in human cells, tissues, and organs, alterations in their expressions can be well predictive of the state of cells and their response to stimuli (12, 42, 43). The present study aimed to explore the characteristics of lncRNA expressions in HNSCC-infiltrating immune cells in order to screen out a lncRNA signature that can clinically reflect survival prognoses and effectively predict response to therapy targeting immune checkpoints for HNSCC.

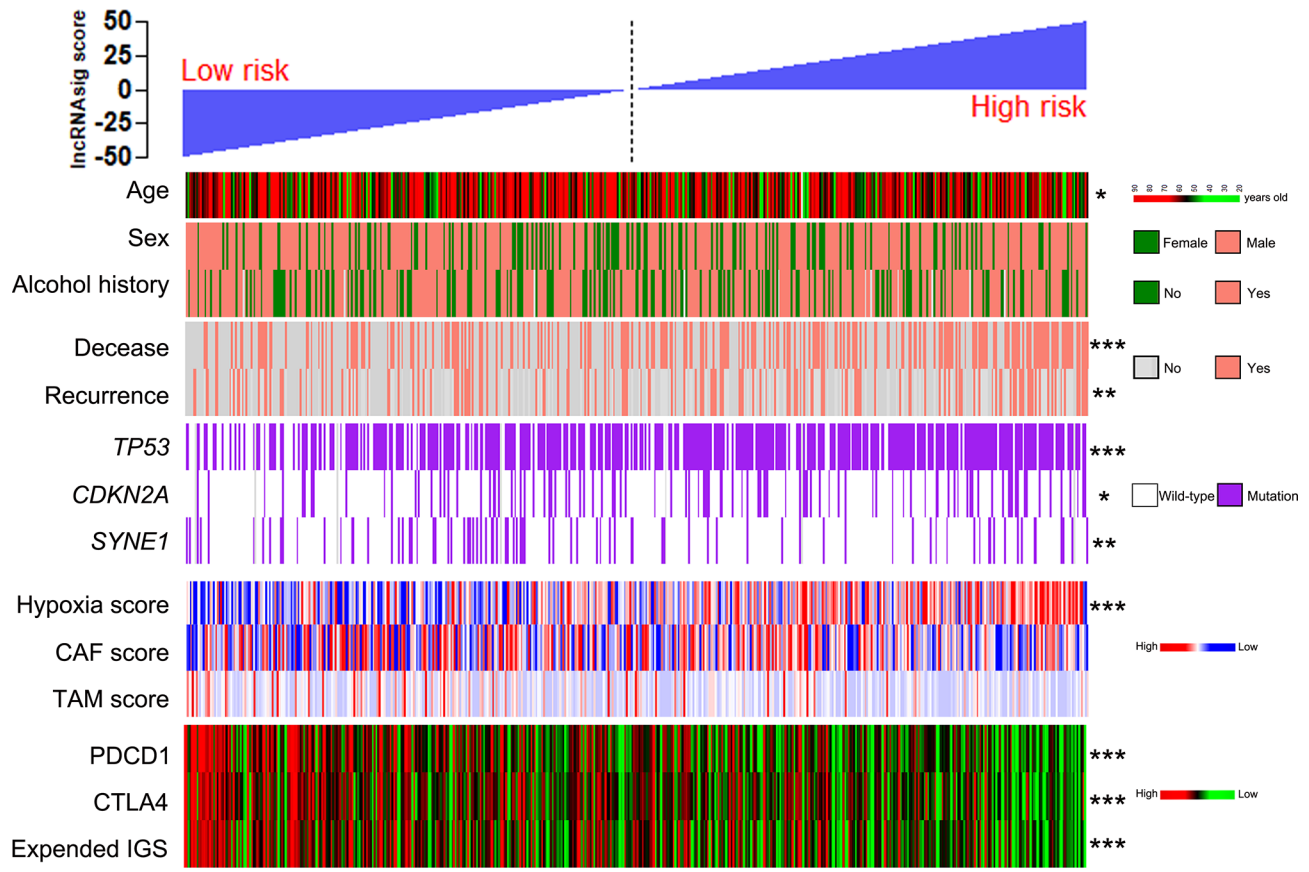

FIGURE 5 | Patients from TCGA cohort with high and low Ti-lncRNA scores were ranked by clinicopathological factors, genetic mutations, the hypoxia, CAF, and TAM scores, PDCD1 and CTLA4 expressions, and the expanded IGS score. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. lncRNA, long non-coding RNA; Ti-lncRNA, tumor-infiltrating immune-related lncRNA; TCGA, The Cancer Genome Atlas; CAF, cancer-associated fibroblast; TAM, tumor-associated macrophage; IGS, immune gene signature.

Therefore, to our knowledge, this is the first time a novel model was built for the lncRNA expression patterns of tumor-infiltrating immune cells in HNSCC. Specific expressions of lncRNAs were initially evaluated in 19 types of immune cells, and then the top 10% lncRNAs were selected in each immune cell type. Each lncRNA was further analyzed for its cell and tissue specificity index. Comparison analyses of the lncRNAs between 19 immune cell types and HNSCC cell lines identified the upregulated lncRNAs in immune cells, which were then validated in HNSCC patients for their prognostic associations in order to develop a prognostic lncRNA model of immune cells for HNSCC. As a result, a signature of nine lncRNAs was discovered in the training cohort of TCGA and validated in the test cohort.

The Ti-lncRNA signature was demonstrated to be a prognostic predictor for HNSCC patients among the TCGA, FUSCC, GSE41613, and GSE42743 cohorts. A high signature score was an independent risk factor for both death and recurrence in HNSCC patients. In addition, the signature was confirmed to be associated with the prognoses of patients for multiple types of cancers in the pan-cancer analyses, suggesting its efficacy as a prognostic factor for cancer. The significant correlations of the Ti-lncRNA signature with poor prognoses may be attributed to the alterations of the expressions of Ti-lncRNAs associated with immunosuppressive TME, which could either affect the activated state of tumor-infiltrating immune cells or receive tumor–stromal crosstalk signaling from aggressive cancer cells.

It is well known that the intricate interaction between tumor cells and stromal cells within the TME contributes to immune evasion and immunotherapy resistance (44–46). In our study, a high signature score was found to be positively correlated with mutations in *TP53* and *CDKN2A*, the most frequently altered tumor suppressor genes in HNSCC. In addition to its significant associations with shortened survival time and resistance to radiotherapy and chemotherapy (47), *TP53* mutation has been recently reported by Zhang et al. to be indicative of poor response to immunotherapy in HNSCC (48), which supports the signature as a potential predictor for prognoses and immunotherapy response. Immunosuppressive TME is characterized by enriched CAFs, TAMs, T regulatory cells, myeloid-derived suppressor cells, and hypoxia, leading to tumor progression and reduced responses to pembrolizumab and nivolumab (49). Therefore, we explored the correlations of the signature score with the CAF, TAM, and hypoxia

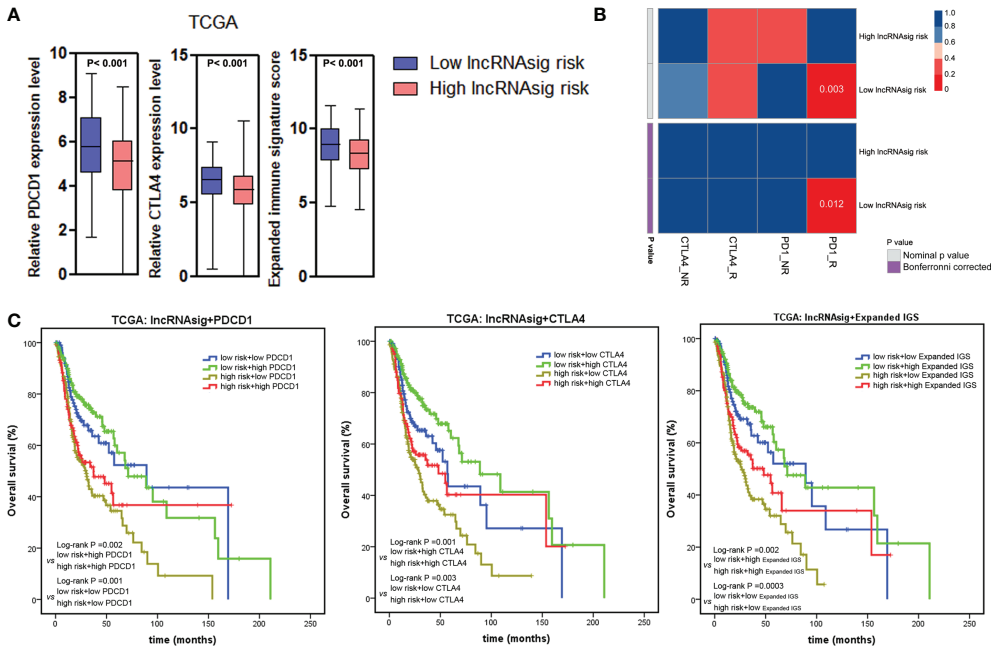

FIGURE 6 | Ti-lncRNA signature and immunotherapy response. **(A)** Associations of the signature risk score with the expression of PDCD1 and CTLA4 and the expanded IGS score. **(B)** Sub-map analyses of the Ti-lncRNA signature in melanoma patients undergoing blockade therapy of PD-1 and CTLA4. **(C)** Kaplan–Meier survival curves of OS among four patient groups stratified by the Ti-lncRNA signature, PDCD1 and CTLA-4 expressions, and the expanded IGS scores. lncRNA, long non-coding RNA; Ti-lncRNA, tumor-infiltrating immune-related lncRNA; TCGA, The Cancer Genome Atlas; CAF, cancer-associated fibroblast; TAM, tumor-associated macrophage; IGS, immune gene signature; OS, overall survival.

scores as well. Patients with high signature scores tended to stay at high hypoxia status. There was no statistical association of the signature score with the CAF and TAM scores in our study. It is interesting to find that *TP53* mutation in tumor cells and hypoxia within the TME can affect the signature score independently. A series of studies have revealed that *TP53* mutation confers an immunosuppressive phenotype in multiple tumors (50–52). Hypoxia also plays a pivotal role in immunosuppressive effect in a variety of ways, such as reducing the activities of cytotoxic T cells and natural killer (NK) cells, increasing the release of immunosuppressive cytokines, and inducing the expressions of immune checkpoint inhibitors (53, 54). These outcomes may suggest that tumor cells harboring *TP53* mutations alter the lncRNA expression patterns through crosstalk with immune cells and that hypoxia significantly induces the expressions of the signature lncRNAs in immune cells.

The signature score has been confirmed to be negatively correlated with the expressions of PDCD1 and CTLA4, suggesting that the signature score can provide evidence for determining blockade therapy of immune checkpoints. Ayers et al. described the expanded IGS consisting of 18 genes, and a high expanded IGS was associated with the clinical response to PD-1 blockade for HNSCC patients in a previous study (41). The correlation of a low signature score with a high expanded IGS further verified its predictive effect for anti-PD-1 response. Consistent with the above results, patients with low signature scores showed clinical response to anti-PD-1 therapy in melanoma. Thus, our study reveals that a low signature score is indicative of response to blockade of immune checkpoints, while a high score means increased risk of therapeutic resistance. This finding can be explained by the following aspects: on one hand, a majority of the activated immune cell signaling pathways were enriched in patients with low signature scores, as described in the context; on the other hand, PDCD1 and CTLA4 exhibited high expressions in patients with low signature scores.

Finally, we have to stress that some limitations exist in the present study. Because our clinical trials of anti-PD-1 therapy are ongoing, it is not available for us to evaluate the predictive effect of the Ti-lncRNA signature on therapeutic response to immunotherapy. Although the signature is a potent indicator of immunotherapy response, the molecular mechanisms of the nine lncRNAs in immunosuppressive TME remain unclear. Therefore, the next step is to validate the predictive value of this signature in our patients undergoing immunotherapy; an RNA scope will be performed to determine the localization and distribution of the nine Ti-lncRNAs in immune cells. Moreover, the Ti-lncRNAs specifically localized in cytotoxic T lymphocytes will be selected with priority according to the results of the RNA scope, which will be further investigated for their biological functions in the transformation between immunoactivation and immunosuppression and the crosstalk between tumor cells and immune cells.

In summary, the Ti-lncRNA signature was identified as a prognostic factor independent of the TNM stage, PDCD1,

CTLA4, and the expanded IGS, which may become a potential clinical indicator of therapeutic response to anti-PD-1/PD-L1 and anti-CTLA4 therapies.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Fudan University Shanghai Cancer Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YW, QJ, CD, and BM designed the study. BM, HJ, YL, and TL were responsible for performing experiments. BM and HJ performed data analyses. WX and XW contributed to the collection of surgical samples and clinical information and the data preparation. BM and CD wrote the manuscript. YW and QJ revised the paper. 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.2021.692079/full#supplementary-material

Supplementary Figure 1 | Heatmap of differential expression pattern observed in 19 immune cell types.

Supplementary Figure 2 | The immune cell specificity of the nine lncRNAs.

Supplementary Figure 3 | The Ti-lncRNA signature's associations with prognostic outcomes of 32 malignancies. **(A)** Hazards ratios (HR) with 95% confidence interval (CI) of the signature were calculated in the analyses of OS in 32 malignancies by using the pan-cancer TCGA data; **(B-D)** OS difference of the signature risk score was analyzed in kidney renal papillary cell carcinoma (KIRP) **(B)**, lung adenocarcinoma (LUAD) **(C)** and pancreatic adenocarcinoma (PAAD) **(D)**.

Supplementary Table 1 | Primer sequence of the Ti-lncRNA signature genes, and hypoxia and CAF signature.

Supplementary Table 2 | LncRNA landscape of HNSCC-infiltrating immune cells.

Supplementary Table 3 | Tissue specificity index (TSI) scores of the nine lncRNAs included in our signature and associations of the Ti-lncRNA signature with clinicopathological and molecular factors.

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