Expression of PD-L1 and CD4+ tumor-infiltrating lymphocytes predict survival in head and neck squamous cell carcinoma

ZE-MING FU[1], DE-JUN ZHANG[1], YING-YUAN GUO[1], SHUANG HAN[1], FANG GUO[1], JIE BAI[1], YI-NING WAN[1], GUO-FANG GUAN[1], KE-WEI SUN[2] and NA YANG[2]

[1]Department of Otolaryngology-Head and Neck Surgery, The Second Hospital of Jilin University; [2]Department of Otolaryngology-Head and Neck Surgery, Changchun Center Hospital, Changchun, Jilin 130041, P.R. China

Received August 28, 2021; Accepted December 10, 2021

DOI: 10.3892/mco.2022.2492

Abstract. The clinical efficacy of immune checkpoint blockade has been recently demonstrated in a variety of cancer types. The aim of the present study was to characterize the expression profile of tumor-infiltrating lymphocytes (TILs) and programmed death-ligand 1 (PD-L1) in head and neck squamous carcinoma (HNSCC). A total of 63 patients with HNSCC were enrolled in the present study. $CD3^+$ and $CD4^+$ TILs and the expression of PD-L1 were detected by immunohistochemistry. PD-L1 mRNA levels were evaluated by reverse transcription-quantitative PCR analysis. The association of TILs and PD-L1 with patient clinicopathological characteristics was also assessed. $CD3^+$ and $CD4^+$ TILs were detected in 100% of the samples. $CD3^+$ was the predominant subset of TILs. PD-L1 was expressed in 53 of 61 (86%) patients when a score of ≥1 on tumor cells was considered positive and in 28 patients (45.2%) when a score of >5 on tumor cells was considered positive. PD-L1 mRNA levels were determined to be significantly correlated with PD-L1 protein expression. Survival analysis demonstrated that high $CD4^+$ TILs were associated with improved overall survival (OS) and disease-free survival (DFS), and furthermore, the association of high PD-L1 expression with unfavorable OS and DFS was statistically significant. Multivariate analysis identified $CD4^+$ TILs and PD-L1 as prognostic markers for HNSCC. The results of the present study suggested that increased $CD4^+$ TILs in HNSCC may be associated with improved outcomes, while high expression of PD-L1 may indicate unfavorable OS and DFS; thus, these factors may serve as predictors of the response to immune checkpoint therapy.

Correspondence to: Dr Guo-Fang Guan, Department of Otolaryngology-Head and Neck Surgery, The Second Hospital of Jilin University, 218 Ziqiang Street, Changchun, Jilin 130041, P.R. China
E-mail: guangf@jlu.edu.cn

Key words: tumor-infiltrating lymphocytes, PD-L1, head and neck squamous cell carcinoma, prognostic value

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant tumor type worldwide and the most common malignancy originating in the mucosal epithelium of the oral cavity, pharynx, larynx and hypopharynx (1). Smoking and excessive alcohol consumption have been associated with the tumorigenesis of HNSCC (2). Human papillomavirus has been associated with tumors arising in the oropharynx (3). Various cases are usually diagnosed at a later stage, which is associated with high mortality and morbidity rates (2). Although multimodality approaches, such as EGFR monoclonal antibody in combination with chemotherapy, are used for more advanced-stage disease, 30-40% of patients develop distant metastases within 5 years (3); therefore, understanding the molecular characteristics and immunological profile of HNSCC may help overcome certain obstacles associated with targeted therapies and prove beneficial for the patients.

The tumor microenvironment significantly affects tumor aggressiveness and response to treatment (4). Tumor-infiltrating lymphocytes (TILs) are an important histopathological characteristic of HNSCC (5). Tumor infiltration by T lymphocytes is a highly informative prognostic factor for predicting the clinical outcome of the disease. In several studies, TILs of different types and locations in primary tumors are of prognostic value for overall survival (OS) and disease-free survival (DFS) (6,7). However, different subsets of lymphocytes have different functions. $CD4^+$ T-helper cells stimulate cytotoxic $CD8^+$ T cells to enhance the antitumor immune response (8), while $CD4^+$ regulatory T cells (Tregs) are considered to serve as suppressors of antitumor immune response (9). $CD3^+$ T cells have been considered as an important T-cell marker for the classification of malignant lymphomas and leukemias (10). Although TILs have been extensively investigated, the prognostic significance of specific TIL subgroups, such as $CD3^+$ or $CD4^+$, is different due to the complexity of the composition and function of TILs, and has been inconsistent across different studies. The role of TILs in immune surveillance in HNSCC remains to be clarified and previous studies have reported conflicting results.

The immune checkpoint molecules programmed death 1 (PD-1) and programmed death 1 ligand 1 (PD-L1) have been evaluated in a number of cancer types (11,12). PD-1 is mainly

expressed on the surface of T and B cells during activation. PD-1 signaling is mediated through binding to its two ligands, PD-L1 and PD-L2, which are mainly expressed by cancer cells (13). As an inhibitory immune checkpoint molecule, PD-1 mediates the immune escape of tumor cells (14). PD-L1 is also referred to as cluster of differentiation 274, which is a protein encoded by the CD274 gene (15). There is increasing evidence that tumor cells express various levels of PD-L1 in numerous types of cancer, including HNSCC. However, the currently available results remain controversial. The clinical prognostic significance of PD-L1 expression in HNSCC tissues varies across different studies, which may be due to different sample sources and intratumor heterogeneity, different antibodies and staining protocols, and inconsistent cut-off values, among others (16). In three recent randomized phase III trials, the PD-1 targeting antibodies nivolumab and pembrolizumab, which have been used to treat patients with advanced HNSCC, exhibited superior efficacy and lower toxicity compared with traditional chemotherapy and radiotherapy alone (17). Pembrolizumab combined with chemotherapy was determined to be superior to cisplatin and 5-fluorouracil (18). Furthermore, pembrolizumab monotherapy was more effective as a first-line treatment in patients with HNSCC whose tumors express PD-L1 compared with those with non-PD-L1-expressing tumors (17). However, a large proportion of patients do not respond to treatment, while certain initial responders eventually develop resistance; thus, further research is required.

The aim of the present study was to investigate the frequency of TILs expressing CD3 and CD4 and the expression of PD-L1 in tumor cells using formalin-fixed paraffin-embedded (FFPE) samples from patients with HNSCC. Furthermore, the association of TILs and PD-L1 with clinical characteristics and prognosis was assessed in order to evaluate the predictive value of TILs and PD-L1 expression.

Materials and methods

Patient cohort. A total of 63 FFPE tissue specimens were collected from patients with pathologically confirmed HNSCC at the Second Hospital of Jilin University (Changchun, China) between May 2008 and July 2019. Patients who had previously received conventional radiotherapy and/or chemotherapy prior to surgery were excluded. All tumor specimens were obtained from primary resections. The study protocol was approved by the Medical Ethics Committee of Jilin University (Changchun, China; no. 2021-157). Approximately half of the patients provided written informed consent for the study. For the other half of the patients, a telephone interview was conducted and verbal consent was obtained.

The clinical characteristics of the patients are summarized in Table I. The majority of the patients were male [45 (71.4%)] and the median age was 64.6 years (range, 40-76.8 years). Approximately half of the patients had advanced disease with T stage 3/4 (52.4%), N stage 2/3 (54%) and clinical stage III/IV (55.6%). All studied tumour specimens were assessed on HE-stained slides using standard diagnostic criteria.

Immunohistochemistry (IHC). $CD3^+$ and $CD4^+$ TILs were determined by IHC. Archival FFPE HNSCC tumor blocks were cut into 4-μm sections, deparaffinized with xylene and rehydrated through a decreasing ethanol gradient. Boiling in 10 mM citrate buffer, pH 6.0 (Novocastra; Leica Microsystems, Ltd.) in a microwave was used for epitope retrieval. After endogenous peroxidase blocking (0.3% H_2O_2; Thermo Fischer Scientific, Inc.) at room temperature for 30 min, the sections were incubated with primary antibodies against CD3 (1:200 dilution; clone A0452; cat. no. A045229-2; Dako; Agilent Technologies, Inc.) and CD4 (1:500 dilution; clone 4B12; cat. no. NCL-L-CD4-368; Novocastra; Leica Microsystems, Ltd.) at 4˚C overnight. The CD4 antibody was used to stain all $CD4^+$-expressing cells, including Tregs. Secondary detection was performed using the Level-2 Ultra Streptavidin (horseradish peroxidase) system (Dako; Agilent Technologies, Inc.) at room temperature, which included anti-rabbit for CD3 or anti-mouse for CD4 for 30 min. Streptavidin was applied to all sections for 30 min at room temperature. Color detection was performed by using 3,3'-diaminobenzidine for 5 min at room temperature. The slides were counterstained with hematoxylin and mounted with coverslips.

PD-L1 was detected by using the mouse monoclonal anti-human PD-L1 antibody (1:100 dilution; CD274, clone UMAB229; cat. no. UM800121; OriGene Technologies, Inc.) at 4˚C overnight. Accel Retrieval Solution (GBI Labs) was used for antigen retrieval. Human tonsillar samples known to be positive for CD3, CD4 and PD-L1 expression served as positive controls. Sections without primary antibodies were used as negative controls.

Evaluation of TILs and PD-L1 expression. A semi-quantitative method was utilized to assess the CD3 and CD4 expression of TILs inside the infiltrated tumor tissues. As described previously (6,19), three of the most densely stained fields were randomly selected, with necrotic areas excluded. The proportion of cells with positive staining (range, 0-100%) and staining intensity (0, negative; 1, weak; 2, moderate; and 3, strong) were assessed and recorded. The final score was calculated by multiplying the percentage of positive cells with the average staining intensity.

The abundance and location of PD-L1 expression within the tumor were also determined semi-quantitatively. PD-L1 expression was membranous, with variable cytoplasmic staining. A four-level score was used to quantify the proportion of total cells stained and the staining intensity on the membrane: 0 (0-<5% positive cells), 1 (5-10% positive cells), 2 (10-20% positive cells), 3 (20-50% positive cells) and 4 (>50% positive cells). The staining intensity was determined as 0, 1, 2 or 3 (20). The final score was calculated by multiplying the proportion of stained cells (scored as 0, 1-4) by the staining intensity (scored as 0, 1-3) (21). A Leica light microscope linked with a camera was used for capturing and analysing images (magnification, x200). All evaluations were performed in a blinded manner regarding the clinical outcome of the patients.

RNA extraction and quantification of PD-L1 expression by reverse transcription-quantitative (RT-q)PCR. A total of 60 available FFPE surgical samples from the same cohort of patients with HNSCC and 10 adjacent normal tissue samples were collected. Based on HE-stained tissue sections, only samples containing >60% tumor cells were considered. The

Table I. Patient characteristics.

Characteristic	Value
Age, years [median (range)]	64.6 (40-76.8)
Sex	
Male	45 (71.4)
Female	18 (28.6)
T classification	
T1	11 (17.5)
T2	19 (30.1)
T3/4	33 (52.4)
N classification	
N0	13 (20.6)
N1	16 (25.4)
N2/3	34 (54)
Clinical stage	
I	3 (0.05)
II	25 (39.7)
III/IV	35 (55.6)
PD-L1 expression score	
High, >5	28 (45.2)
Low, ≤5	34 (56.8)
CD3 expression score	
High, >26.8	30 (50)
Low, ≤26.8	30 (50)
CD4 expression score	
High, >17.3	29 (48.3)
Low, ≤17.3	31 (51.7)

Values are expressed as n (%) unless otherwise specified. PD-L1, programmed death ligand 1.

expression of PD-L1 at the mRNA level was measured by using RT-qPCR. A total of 5-10 8-μm sections were obtained from each sample and total RNA was extracted from the samples using the Recover All Total Nucleic Acid Isolation kit for FFPE (Thermo Fisher Scientific, Inc.). In brief, the sections were deparaffinized with xylene and rehydrated through a decreasing ethanol gradient. After washing with 100% ethanol, the air-dried pellets were incubated with 200 μl digestion buffer and 4 μl of protease K (included in the kit) in heat blocks for 15 min at 50˚C and then 15 min at 80˚C. After adding 100% ethanol, the mixture was loaded onto a filter cartridge, centrifuged, the flow-through was discarded and the filter cartridge was washed twice. Subsequently, 60 μl DNase was added, followed by incubation at room temperature for 30 min. After two additional washes, the RNA was eluted with nuclease-free water. According to the manufacturer's protocol, RT of RNA was performed with Super Script III reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). Primer 3 (v.0.4.0; https://bioinfo.ut.ee/primer3-0.4.0/) was used to design the primers. The primers for PD-L1 were as follows: Forward, 5'-GTGGCATCCAAGATACAAACT CAA-3' and reverse, 5'-TCCTTCCTCTTGTCACGCTCA-3'. The primers for GAPDH were as follows: Forward, 5'-GTC TCCTCTGACTTCAACAGCG-3' and reverse, 5'-ACCACC CTGTTGCTGTAGCCAA-3'. Amplification was performed using SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the ABI PRISM 7900 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The 10-μl reaction volume contained 50 ng cDNA (either from FFPE tumor samples or normal adjacent tissue samples), 200 nmol/l of each primer and 5 μl of SYBR Green Master Mix (Thermo Fisher Scientific, Inc.). The thermocycling conditions included initial denaturation at 94˚C for 2 min; 40 cycles of denaturation at 94˚C for 15 sec; and annealing and extension at 60˚C for 1 min. Relative quantification of PD-L1 mRNA levels was performed by using the $2^{-\Delta\Delta Cq}$ method (22). As calculated from the duplicate reactions for each tested sample, the mean Cq value was used. The relative fold change of mRNA expression, in which the mean of the ΔCq values of the target amplicon was normalized to the endogenous gene GAPDH, compared with normal tissue specimens. Each experiment was performed three times.

Statistical analysis. Fisher's exact test was used to assess differences between categorical variables. The associations between the expression of each detected biomarker and various clinicopathological parameters were assessed. The Kaplan-Meier method was used to calculate OS and DFS and the log-rank test was used for comparisons. The Cox proportional hazard model was used to perform univariate and multivariate analyses. Factors with prognostic significance in the univariate model were further analyzed in a multivariate Cox proportional hazards regression model. $P<0.05$ was considered to indicate statistically significant differences. Pearson's correlation coefficient analysis was used to determine the correlation between PD-L1 expression and $CD3^+$ or $CD4^+$ TILs. Analyses were performed using the R 4.1.2 package (R Foundation for Statistical Computing).

Results

TILs in HNSCC. The expression of CD3 and CD4 on TILs was evaluated in the tumor parenchyma of patients with HNSCC. Of the 63 tumor samples, 61 were available for CD3 and CD4 assessment. A total of two samples were excluded due to the small sample size or poor tissue morphology after staining. CD3 and CD4 were expressed in 100% of the samples. With a range of 9.0-61.3 for CD3 and 2.3-42.3 for CD4, the median score of CD3 and CD4 expression was 26.8 and 17.3, respectively. As a dichotomous variable, patients were divided into CD3 expression high and low groups. According to the median, high was defined as a score >26.8 and low was defined as a score ≤26.8. Using a similar method, patients were assigned to CD4 high (>17.3) and low (≤17.3) groups (Table I). IHC analysis revealed that the expression of CD3 was abundant in tumors, while the expression of CD4 was relatively low.

Evaluation of PD-L1 expression by IHC. Of the 61 available tumors, PD-L1 was expressed in 53 (86.0%, score ≥1). According to the median score, PD-L1 expression was divided into low and high groups, with 28 samples (45.2%) exhibiting high PD-L1 expression (score >5) and 34 samples exhibiting

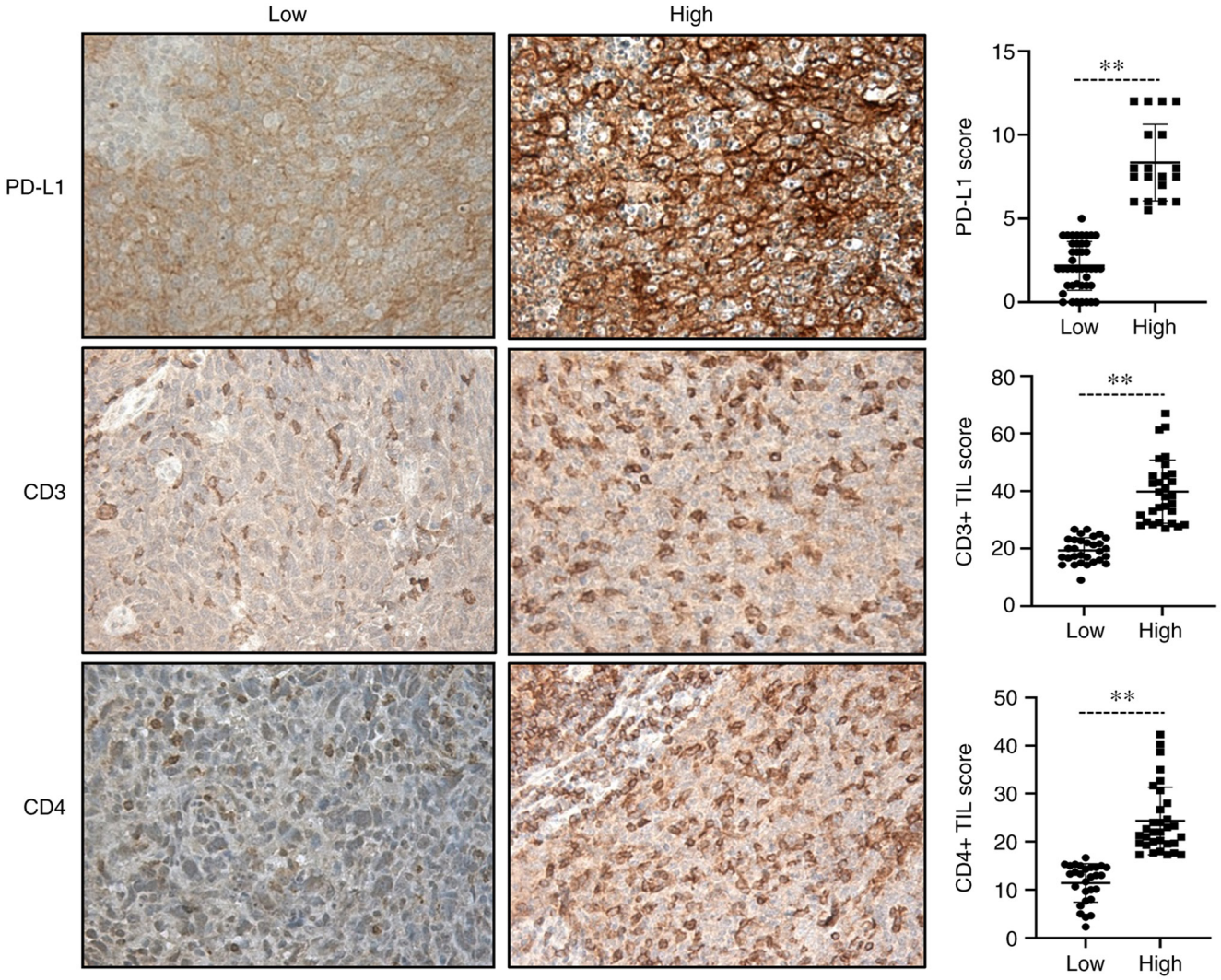

Figure 1. Immunohistochemistry for tumor-infiltrating lymphocytes and PD-L1 staining in head and neck squamous cell carcinoma. Representative images and quantification of PD-L1, $CD3^+$ and $CD4^+$ cells (magnification, x200). **P<0.001. PD-L1, programmed death ligand 1.

low expression (score ≤5; Table I). In addition, PD-L1 expression was observed in 53 samples in which TILs were present. Representative results on CD3, CD4 and PD-L1 immunostaining and quantification in tumor cells are provided in Fig. 1.

Evaluation of PD-L1 expression by RT-qPCR. The mRNA expression of PD-L1 was evaluated by RT-qPCR in 60 available patient samples. By comparing with 10 normal tissue samples, the mean expression level of PD-L1 in HNSCC was 2.07-fold higher compared with that in normal tissues (range, -3.69- to 6.84-fold). PD-L1 mRNA expression was detected in 49 of 60 patients (81.7%; Fig. 2A), highlighting the overexpression of PD-L1 gene in tumor cells. Furthermore, PD-L1 mRNA levels were indicated to be significantly correlated with PD-L1 protein levels (R=0.461; P<0.001; Fig. 2B). However, there was no significant association between PD-L1 mRNA levels and patient clinicopathological characteristics.

Association of TILs and PD-L1 expression with clinicopathological characteristics. The clinical value of TILs and PD-L1 protein expression was further evaluated. With a mean follow-up of 72 months (range, 3-151 months), constructed Kaplan-Meier curves revealed that patients with high CD4 expression were associated with improved OS (P=0.004) and DFS (P=0.004), which was confirmed by univariate Cox regression analysis [HR=0.31, 95% confidence interval (CI): 1.13-0.72, P=0.004 for OS; and HR=0.30, 95% CI: 0.13-0.71, P=0.006 for DFS; Fig. 3A and B; Table II].

As a categorical variable, PD-L1 was also used to divide the patients into low and high expression groups. Kaplan-Meier survival analysis demonstrated that high PD-L1 expression was significantly associated with unfavorable OS and DFS (P=0.024 and P=0.025, respectively; Fig. 3C and D), compared with low PD-L1 expression. Univariate Cox regression analysis revealed significant differences between the high and low expression groups, [HR=2.47, 95% confidence interval (CI): 1.12-5.46, P=0.024 for OS; and HR=2.57, 95% CI: 1.17-5.66, P=0.015 for DFS (Table II).

Furthermore, multivariate analysis using the Cox proportional hazards model indicated that CD4 and PD-L1 were significantly associated with OS and DFS after adjusting for age (for OS, CD4: HR=0.35, 95% CI: 0.15-0.86 and P=0.006; and PD-L1: HR=2.29, 95% CI: 0.89-5.86, P=0.025; for DFS,

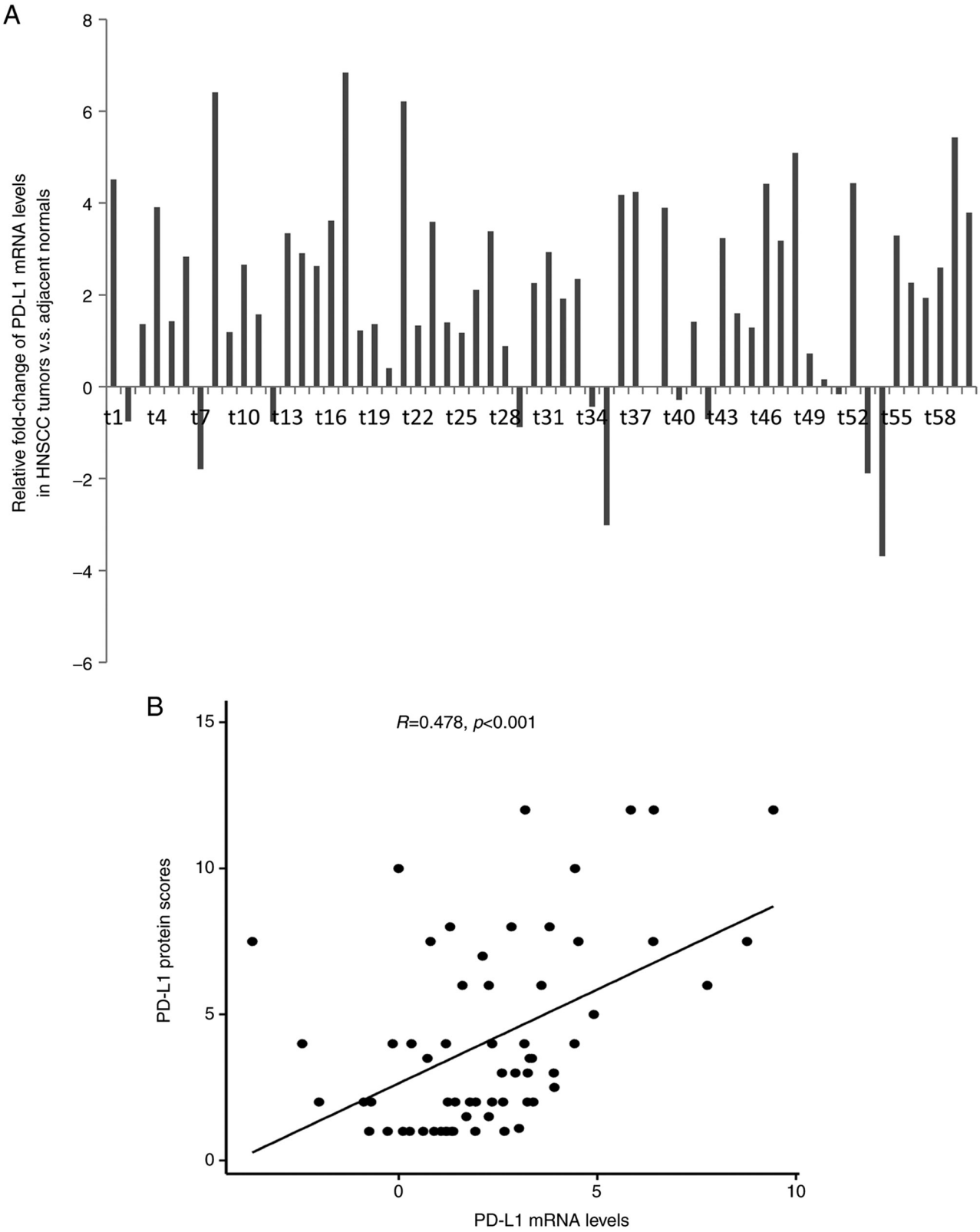

Figure 2. Evaluation of PD-L1 mRNA expression in patients with HNSCC. (A) Bar plot representing the relative fold change of PD-L1 mRNA levels in HNSCC compared with adjacent normal tissues. (B) Correlation analysis of PD-L1 protein levels with PD-L1 mRNA levels (P<0.001). R, Spearman correlation coefficient; HNSCC, head and neck squamous cell carcinoma; PD-L1, programmed death ligand 1.

CD4: HR=0.33, 95% CI: 0.14-0.80 and P=0.009; and PD-L1: HR=2.24, 95% CI: 0.90-5.57 and P=0.051; Table II). However, CD3 expression was not indicated to be significantly associated with disease outcome. Furthermore, no significant associations of patient sex and tumor stage with CD4 or PD-L1 expression were observed.

To elucidate the correlation between PD-L1 expression and $CD3^+$ or $CD4^+$ TILs, a Pearson's correlation coefficient

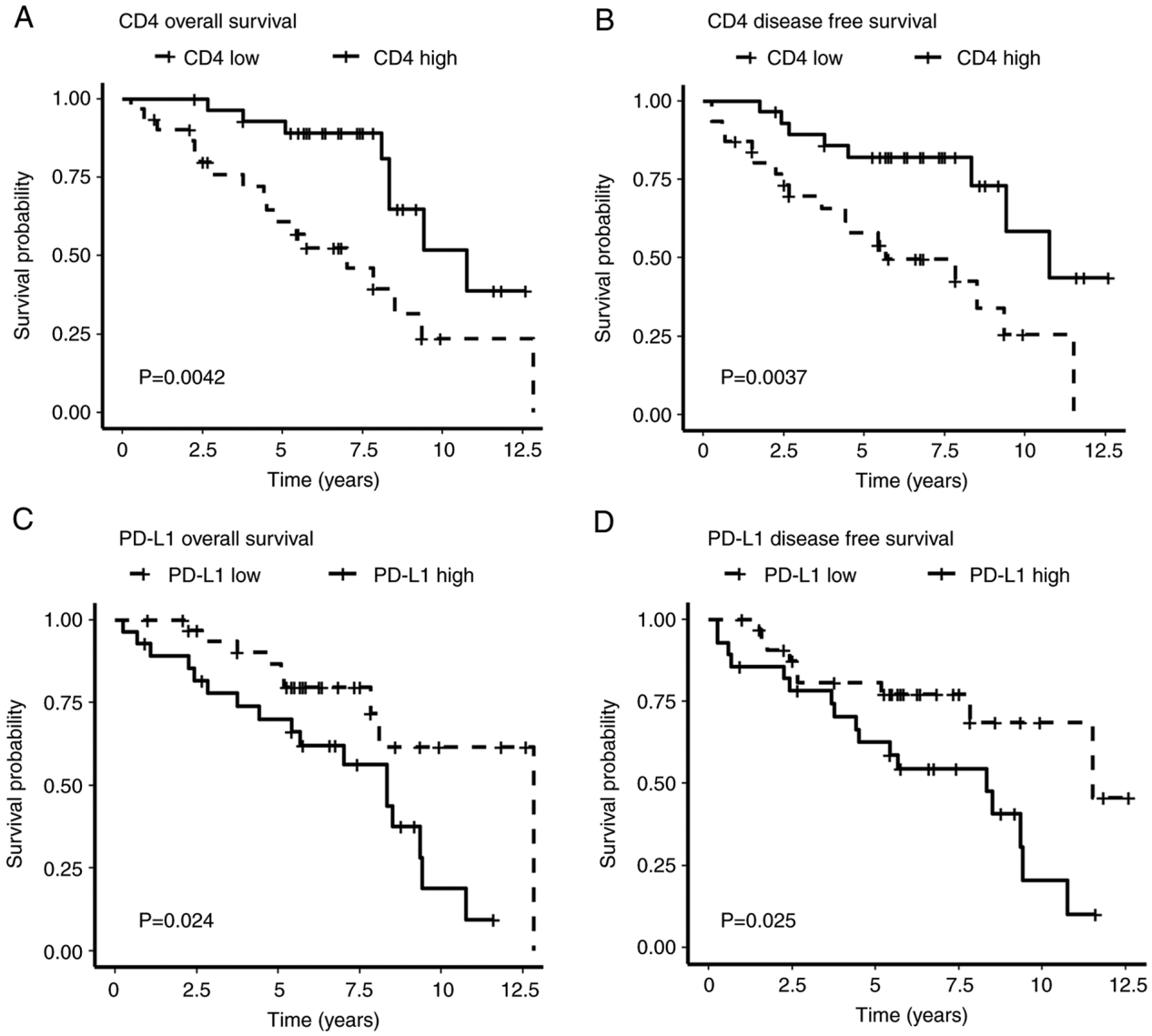

Figure 3. Prognostic significance of $CD4^+$ TILs and PD-L1 protein expression in patients with head and neck squamous cell carcinoma. (A and B) Kaplan-Meier curves indicated that the number of $CD4^+$ TILs was associated with (A) OS (P=0.0042) and (B) DFS (P=0.0037). (C and D) Association of PD-L1 expression with (C) OS and (D) DFS. The P-value was calculated by using the log-rank test. PD-L1, programmed death ligand 1; OS, overall survival; DFS, disease-free survival; TIL, tumor-infiltrating lymphocyte.

analysis was performed. The scatter plot demonstrated that the expression of $CD4^+$ TILs had a negative, if insignificant, correlation with the PD-L1 score (R=-0.22, P=0.08). No significant correlation between $CD3^+$ TILs and PD-L1 expression was obtained (Fig. S1). Overall, these results indicated that $CD4^+$ TILs and PD-L1 have a role as independent prognostic factors for patients with HNSCC.

Discussion

The infiltration of the tumor parenchyma by a large number of TILs has been associated with clinical outcomes in various types of cancer. Tumor immune checkpoint-targeted therapies have achieved responses in multiple cancer types (23), including HNSCC (16). However, the clinical response rates vary widely and the reasons for this have yet to be fully elucidated. In the present study, the distribution of TILs and the expression of PD-L1 were quantitatively analyzed in a population of patients with HNSCC. Both PD-L1 protein and mRNA levels were analyzed. The clinical significance of these immune parameters and the clinical characteristics of the patients were assessed. It was demonstrated that $CD3^+$ and $CD4^+$ TILs were present in 100% of the samples. Patients whose tumors were infiltrated by high numbers of $CD4^+$ T cells exhibited significantly improved OS and DFS compared with patients exhibiting poor tumor infiltration. In addition, when a score of >1 of tumor cells was considered positive, PD-L1 expression was detected in 86% of the samples and when a score of >5 of cells was considered positive, PD-L1 expression was observed in 45% of the samples. Of note, PD-L1 mRNA levels were indicated to be significantly associated with PD-L1 protein levels (R=0.461; P<0.001). It is worth noting that high PD-L1 expression was significantly associated with unfavorable OS and DFS. These results were independent of clinicopathological characteristics and were of predictive value for disease outcome.

Table II. Univariate and multivariate analyses of factors associated with survival in patients with head and neck squamous cell carcinoma.

	Overall survival				Disease-free survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (> vs. ≤64.6 years)	2.62 (1.09-6.27)	0.03	1.45 (0.56-3.71)	0.003	2.29 (0.98-5.27)	0.05	1.32 (0.53-3.27)	0.004
Sex (female vs. male)	0.67 (0.28-1.56)	0.357			0.83 (0.36-1.91)	0.667		
T classification (T1/2 vs. T3/4)	0.76 (0.20-2.93)	0.698			0.66 (0.17-2.53)	0.548		
N classification (N0/1 vs. N2/3)	1.94 (0.86-4.37)	0.11			1.71 (0.76-3.87)	0.193		
Clinical stage (I/II vs. III/IV)	1.65 (0.73-3.71)	0.229			1.65 (0.74-3.69)	0.223		
CD3 expression (score > vs. ≤26.8)	0.59 (0.27-1.29)	0.183			0.56 (0.27-1.26)	0.175		
CD4 expression (score > vs. ≤17.3)	0.31 (1.13-0.72)	0.004	0.35 (0.15-0.86)	0.006	0.30 (0.13-0.71)	0.006	0.33 (0.14-0.80)	0.009
PD-L1 expression (score > vs. ≤5)	2.47 (1.12-5.46)	0.024	2.29 (0.89-5.86)	0.025	2.57 (1.17-5.66)	0.015	2.24 (0.90-5.57)	0.051

HR, hazard ratio; PD-L1, programmed death ligand 1.

There is growing interest in evaluating TILs in solid tumors. It has been demonstrated that the immune attack or immune escape of tumor cells induces the immune response in the tumor microenvironment and serves an important role in the response to cancer therapy (1). However, as different subsets of lymphocytes have different functions in the tumor microenvironment, there is currently no standardized method for evaluating TILs. Previous studies analyzed the peritumoral stroma and tumor core area separately, and evaluated the numbers and percentage of TILs in solid tumors with different results. In many cancers, $CD3^+$ or $CD8^+$ TILs is positively correlated with favorable clinical outcomes (6,24,25), whereas others suggest that $CD4^+$ or $CD8^+$ TILs is associated with better prognosis (26) or has no directly correlation with patient survival, independent of HPV status (27). Therefore, the focus of the present study was to assess the numbers of $CD3^+$ and $CD4^+$ TILs in the tumor parenchyma and determine their association with the clinical variables of the patients.

It has been indicated that $CD3^+$ T cells are the major subtype of TILs in gastric cancer (28). In laryngeal squamous cell carcinoma, a high density of $CD3^+$ cells in the tumor core area was associated with a lower risk of metastasis (29). $CD3^+$ and $CD8^+$ T cells were observed to be associated with improved disease outcome in patients with head and neck cancer treated with chemoradiotherapy (30). The results of the present study consistently demonstrated that $CD3^+$ cells were the predominant TIL subtype in the present HNSCC cohort, although the results did not exhibit any clinical significance, possibly due to the small sample size.

The complexity of the $CD4^+$ T cell response has led to ambiguous results. $CD4^+$ T cells are able to promote antitumor immunity through Th1 and Th2 cells (31). Other subtypes, such as Th17 cells, are mainly characterized by the production of high cytokine IL-17A levels, which has been linked to antitumor immunity in mouse models (32). However, $CD4^+$ forkhead box (Fox)$p3^+$ Tregs contribute to the inhibition of autoimmunity and prevent excessive immune response to pathogens (33). $CD4^+$ TIL infiltration has been evaluated in several studies on HNSCCs. RT-qPCR analysis indicated that CD4 mRNA expression levels were significantly correlated with the $CD4^+$ cell infiltration score in cancer epithelium and cancer stroma (8). Higher $CD4^+$ TIL numbers in patients with HNSCC are associated with improved OS and relapse-free survival (26,34). This phenomenon was also observed in the present study. The present results indicated that patients whose tumors were infiltrated by high numbers of $CD4^+$ T cells had superior OS and DFS compared with patients exhibiting lower tumor infiltration. By contrast, another study demonstrated that neither CD4 nor Foxp3 expression intratumoral or in the stroma area showed significance for the clinical outcome (6).

Immunotherapy by using PD-1 and PD-L1 immune checkpoint blockade has been used in different cancer clinical trials with certain success. With regard to HNSCC, Schneider *et al* (35) reported that PD-L1 expression was present in 36% of primary carcinomas and the presence of lymph node metastasis further indicated that PD-L1 expression may be associated with reduced OS and DFS. However, in a meta-analysis of 3,105 patients from 23 studies, no significant difference between patients with PD-L1-positive and -negative HNSCC was obtained in terms of OS and

DFS (36), while a recently reported randomized phase III clinical study (KEYNOTE-048) (18) demonstrated that pembrolizumab (anti-PD-L1) plus platinum treatment is suitable for PD-L1-positive recurrent or metastatic HNSCC. The results of the present study supported this evidence, as it was demonstrated that >80% of tumors have a PD-L1 score ≥1 and indicated that increased PD-L1 expression may be associated with unfavorable OS and DFS in HNSCC.

PD-1/PD-L1 immune checkpoint therapy has revolutionized the traditional cytotoxic anticancer treatments in recent years and has achieved a long-lasting therapeutic response in various types of cancer (37). However, these therapies still pose significant clinical challenges for patients with HNSCC. In order to further understand the mechanism of the PD-1/PD-L1 pathway, the associations between PD-L1, CD3 and CD4 were investigated, but there were limitations in detecting PD-1 expression due to the availability of samples; hence, a new cohort of patients may be required for further study.

In conclusion, the data of the present study demonstrated that the numbers of $CD4^+$ TILs as detected by IHC were associated with the clinical outcome of patients with HNSCC. PD-L1 is commonly expressed in HNSCC at the protein and mRNA levels. In particular, high expression of PD-L1 on IHC examination may reflect the deterioration of OS and DFS and indicate the opportunity for immune checkpoint blocking therapy in patients with HNSCC.

Acknowledgements

Not applicable.

Funding

The present study was funded by the Health Special Project of Jilin Province (grant no. 2020SCZT002).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

GFG designed the study. ZMF and GFG are responsible for confirming the integrity and authenticity of the data and the accuracy of the data analysis. ZMF, DJZ, YYG, KWS and NY collected and analyzed the patient data. SH, FG and YNW evaluated and interpreted the clinicopathological data. DJZ, YYG and JB were responsible for immunohistochemically staining and evaluated the results. FG and YNW performed the statistical analysis and interpreted the results. ZMF and DJZ wrote the manuscript. ZMF and GFG revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study protocol was approved by the Medical Ethics Committee of Jilin University (no. 2021-157) and all patients consented to participate in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Argiris A, Karamouzis MV, Raben D and Ferris RL: Head and neck cancer. Lancet 371: 1695-1709, 2008.
2. Johnson DE, Burtness B, Leemans CR, Lui VWY, Bauman JE and Grandis JR: Head and neck squamous cell carcinoma. Nat Rev Dis Primers 6: 92, 2020.
3. Stein AP, Saha S, Kraninger JL, Swick AD, Yu M, Lambert PF and Kimple RJ: Prevalence of human papillomavirus in oropharyngeal cancer: A systematic review. Cancer J 21: 138-146, 2015.
4. Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, Gong Z, Zhang S, Zhou J, Cao K, *et al*: Role of tumor microenvironment in tumorigenesis. J Cancer 8: 761-773, 2017.
5. de Ruiter EJ, Ooft ML, Devriese LA and Willems SM: The prognostic role of tumor infiltrating T-lymphocytes in squamous cell carcinoma of the head and neck: A systematic review and meta-analysis. Oncoimmunology 6: e1356148, 2017.
6. Balermpas P, Michel Y, Wagenblast J, Seitz O, Weiss C, Rödel F, Rödel C and Fokas E: Tumour-infiltrating lymphocytes predict response to definitive chemoradiotherapy in head and neck cancer. Br J Cancer 110: 501-509, 2014.
7. Hendry S, Salgado R, Gevaert T, Russell PA, John T, Thapa B, Christie M, van de Vijver K, Estrada MV, Gonzalez-Ericsson PI, *et al*: Assessing tumor-infiltrating lymphocytes in solid tumors: A practical review for pathologists and proposal for a standardized method from the International Immunooncology Biomarkers Working Group: Part 1: Assessing the host immune response, TILs in invasive breast carcinoma and ductal carcinoma in situ, metastatic tumor deposits and areas for further research. Adv Anat Pathol 24: 235-251, 2017.
8. Sakaguchi S, Yamaguchi T, Nomura T and Ono M: Regulatory T cells and immune tolerance. Cell 133: 775-787, 2008.
9. Nishimura T, Iwakabe K, Sekimoto M, Ohmi Y, Yahata T, Nakui M, Sato T, Habu S, Tashiro H, Sato M and Ohta A: Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. J Exp Med 190: 617-627, 1999.
10. Neisig A, Vangsted A, Zeuthen J and Geisler C: Assembly of the T-cell antigen receptor. Participation of the CD3 omega chain. J Immunol 151: 870-879, 1993.
11. Zhang JY, Yan YY, Li JJ, Adhikari R and Fu LW: PD-1/PD-L1 based combinational cancer therapy: Icing on the cake. Front Pharmacol 11: 722, 2020.
12. Rahimi Kalateh Shah Mohammad G, Ghahremanloo A, Soltani A, Fathi E and Hashemy SI: Cytokines as potential combination agents with PD-1/PD-L1 blockade for cancer treatment. J Cell Physiol 235: 5449-5460, 2020.
13. Wang X, Yang X, Zhang C, Wang Y, Cheng T, Duan L, Tong Z, Tan S, Zhang H, Saw PE, *et al*: Tumor cell-intrinsic PD-1 receptor is a tumor suppressor and mediates resistance to PD-1 blockade therapy. Proc Natl Acad Sci USA 117: 6640-6650, 2020.
14. Sharpe AH and Pauken KE: The diverse functions of the PD1 inhibitory pathway. Nat Rev Immunol 18: 153-167, 2018.
15. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, *et al*: Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. Nat Med 8: 793-800, 2002.
16. Qiao XW, Jiang J, Pang X, Huang MC, Tang YJ, Liang XH and Tang YL: The evolving landscape of PD-1/PD-L1 pathway in head and neck cancer. Front Immunol 11: 1721, 2020.
17. Cramer JD, Burtness B and Ferris RL: Immunotherapy for head and neck cancer: Recent advances and future directions. Oral Oncol 99: 104460, 2019.

18. Burtness B, Zhang Y, Harrington KJ and Rischin D: Further clinical interpretation and implications of KEYNOTE-048 findings-Authors' reply. Lancet 396: 379-380, 2020.
19. Larbcharoensub N, Mahaprom K, Jiarpinitnun C, Trachu N, Tubthong N, Pattaranutaporn P, Sirachainan E and Ngamphaiboon N: Characterization of PD-L1 and PD-1 expression and $CD8^+$ tumor-infiltrating lymphocyte in epstein-barr virus-associated nasopharyngeal carcinoma. Am J Clin Oncol 41: 1204-1210, 2018.
20. Badoual C, Hans S, Merillon N, Van Ryswick C, Ravel P, Benhamouda N, Levionnois E, Nizard M, Si-Mohamed A, Besnier N, *et al*: PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. Cancer Res 73: 128-138, 2013.
21. Cho YA, Yoon HJ, Lee JI, Hong SP and Hong SD: Relationship between the expressions of PD-L1 and tumor-infiltrating lymphocytes in oral squamous cell carcinoma. Oral Oncol 47: 1148-1153, 2011.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
23. Upadhaya S, Neftelino ST, Hodge JP, Oliva C, Campbell JR and Yu JX: Combinations take centre stage in PD1/PDL1 inhibitor clinical trials. Nat Rev Drug Discov 20: 168-169, 2021.
24. Maibach F, Sadozai H, Seyed Jafari SM, Hunger RE and Schenk M: Tumor-infiltrating lymphocytes and their prognostic value in cutaneous melanoma. Front Immunol 11: 2105, 2020.
25. Canning M, Guo G, Yu M, Myint C, Groves MW, Byrd JK and Cui Y: Heterogeneity of the head and neck squamous cell carcinoma immune landscape and its impact on immunotherapy. Front Cell Dev Biol 7: 52, 2019.
26. Spector ME, Bellile E, Amlani L, Zarins K, Smith J, Brenner JC, Rozek L, Nguyen A, Thomas D, McHugh JB, *et al*: Prognostic value of tumor-infiltrating lymphocytes in head and neck squamous cell carcinoma. JAMA Otolaryngol Head Neck Surg 145: 1012-1019, 2019.
27. van Kempen PM, Noorlag R, Swartz JE, Bovenschen N, Braunius WW, Vermeulen JF, Van Cann EM, Grolman W and Willems SM: Oropharyngeal squamous cell carcinomas differentially express granzyme inhibitors. Cancer Immunol Immunother 65: 575-585, 2016.
28. Zhang D, He W, Wu C, Tan Y, He Y, Xu B, Chen L, Li Q and Jiang J: Scoring system for tumor-infiltrating lymphocytes and its prognostic value for gastric cancer. Front Immunol 10: 71, 2019.
29. Höing B, Kanaan O, Altenhoff P, Petri R, Thangavelu K, Schlüter A, Lang S, Bankfalvi A and Brandau S: Stromal versus tumoral inflammation differentially contribute to metastasis and poor survival in laryngeal squamous cell carcinoma. Oncotarget 9: 8415-8426, 2018.
30. Barnes TA and Amir E: HYPE or HOPE: The prognostic value of infiltrating immune cells in cancer. Br J Cancer 117: 451-460, 2017.
31. Schüler T, Qin Z, Ibe S, Noben-Trauth N and Blankenstein T: T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in interleukin 4-deficient mice. J Exp Med 189: 803-810, 1999.
32. Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, Hwu P, Restifo NP, Overwijk WW and Dong C: T helper 17 cells promote cytotoxic T cell activation in tumor immunity. Immunity 31: 787-798, 2009.
33. Quezada SA, Peggs KS, Curran MA and Allison JP: CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. J Clin Invest 116: 1935-1945, 2006.
34. Nguyen N, Bellile E, Thomas D, McHugh J, Rozek L, Virani S, Peterson L, Carey TE, Walline H, Moyer J, *et al*: Tumor infiltrating lymphocytes and survival in patients with head and neck squamous cell carcinoma. Head Neck 38: 1074-1084, 2016.
35. Schneider S, Kadletz L, Wiebringhaus R, Kenner L, Selzer E, Füreder T, Rajky O, Berghoff AS, Preusser M and Heiduschka G: PD-1 and PD-L1 expression in HNSCC primary cancer and related lymph node metastasis-impact on clinical outcome. Histopathology 73: 573-584, 2018.
36. Yang WF, Wong MCM, Thomson PJ, Li KY and Su YX: The prognostic role of PD-L1 expression for survival in head and neck squamous cell carcinoma: A systematic review and meta-analysis. Oral Oncol 86: 81-90, 2018.
37. Marin-Acevedo JA, Kimbrough EO and Lou Y: Next generation of immune checkpoint inhibitors and beyond. J Hematol Oncol 14: 45, 2021.