Association of PD-L1 expression in oral squamous cell carcinoma with smoking, sex, and p53 expression

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Objectives. The aim of this study was to investigate the association between oral cavity squamous cell carcinoma (OSCC) and PD-L1 expression, and smoking, and p53 expression.

Study Design. Histologic review of archival slides of patients with OSCC, obtained from the Sydney Head and Neck Cancer Institute database from 1995 to 2015, was undertaken with tissue microarray construction and immunohistochemistry to identify PD-L1 and p53 expression.

Results. Of the 255 patients identified, PD-L1 expression was observed in 70 (27.5%) and more commonly in females (odds ratio [OR] = 2.19; $P = .005$). PD-L1 expression of 1% or greater was associated with p53 expression ($P = .019$) and associated with absence of smoking ($P = .06$). PD-L1 expression, at 1%, was not significantly associated with overall survival ($P = .482$), disease-specific survival ($P = .864$), and disease-free survival ($P = .731$).

Conclusions. Our data demonstrate that PD-L1 expression of 1% or greater is more frequent in OSCC in females, nonsmokers, and in patients with p53-positive OSCC. These findings have important implications for immune therapy for OSCC. (Oral Surg Oral Med Oral Pathol Oral Radiol 2019;128:631−638)

Greater than 300,000 new oral squamous cell carcinomas (OSCCs) are diagnosed worldwide annually.[1] Approximately 30% of these patients die as a result of either distant metastases or locoregional recurrence.[1-4] The mainstay of treatment is surgery and adjuvant radiotherapy with or without concurrent chemotherapy. There are almost no effective second-line therapies for recurrent or metastatic disease.[4]

Immune checkpoints are increasingly being considered as targets for pharmacologic blockade to recruit the patients' immune system to aid in tumor elimination.[5] One such checkpoint is provided by the programmed cell death receptor, PD-1, and its ligand, PD-L1.[6] The PD-1 checkpoint, first described by Ishida et al., is a negative regulator of the immune system.[7,8] PD-1 is expressed on the surface of T cells, B cells, and natural killer cells and plays an important role in limiting the response to self-antigens by binding to its ligand PD-L1.[7,8] Several cancers express PD-L1 and, thus, escape the normal immune surveillance mechanisms.[9] Several anti-PD-1 and PD-L1 agents that block the PD1/PD-L1 axis have recently shown sustained response in patients with advanced melanoma, non−small cell lung cancer (NSCLC), and urothelial carcinoma.[10] There is increasing evidence of PD-L1 expression in OSCC and that PD-L1 expression can potentially predict response to immune check point inhibitors in OSCC.[11,12]

Smoking tobacco, which is a well-known carcinogen, is the main risk factor for OSCC.[13] A higher incidence of PD-L1 expression has been demonstrated in NSCLC in smokers.[14] This is possibly mediated by a high mutation burden associated with exposure to the carcinogen.[15] Immunohistochemical expression of p53 has been considered as a surrogate marker of the tumor mutation burden and has been demonstrated to be adversely associated with response to therapy and outcome in several malignancies.[16,17] The association between OSCC and PD-L1 expression, smoking status, and p53 expression has not been explored. Thus, the primary aim of this study was to compare the incidence of PD-L1 expression in OSCC according to smoking status and p53 overexpression. The secondary aim of this study was to evaluate the effect of PD-L1 expression, smoking, and p53 expression on prognosis.

MATERIALS AND METHODS

Records of patients with OSCC treated with curative intent between 1995 and 2015 were obtained from the

Statement of Clinical Relevance

PD-L1 is a key immune checkpoint in oral squamous cell carcinoma (OSCC) and we examine the association between OSCC and PD-L1, smoking, and p53 to predict response to treatment. This study showed that PD-L1 expression is more common in females, nonsmokers, and p53-positive patients.

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Received for publication Jan 3, 2019; returned for revision Jun 19, 2019; accepted for publication Jul 16, 2019.

2212-4403/$-see front matter
https://doi.org/10.1016/j.oooo.2019.07.008
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Sydney Head and Neck Cancer Institute database, Sydney, Australia. The study included 255 patients after the exclusion of patients for whom information regarding smoking was not available, patients for whom archival slides and blocks were either unavailable or unsuitable for histologic analysis, and those with no clinical follow-up or perioperative mortality. Ethics approval was obtained from the relevant institutional human research ethics committee (HREC 14/RPA/301).

Clinical and follow-up data

Patient demographic characteristics, smoking history, postoperative adjuvant therapy, locoregional failure, distant metastases, and survival data were obtained from the Sydney Head and Neck Cancer Institute database. With regard to those patients with incomplete smoking data, their files were retrieved, and smoking status was determined retrospectively.

Histopathologic review

The archival slides were reviewed and scored for pathologic features, including tumor size, depth of invasion, pattern of invasion, and bone involvement. Tumors were staged according to the pTNM staging provided in the 7th edition of the *American Joint Committee on Cancer Cancer Staging Manual.*[18]

Tissue microarray construction

Tissue microarrays were constructed by using two cores of the tumor from whole formalin-fixed, paraffin-embedded blocks from each patient. Each core measured 1 mm in diameter and was taken from the invasive front of each tumor, selecting the highly cellular areas and avoiding excessive keratin deposition, necrosis, or haemorrhage.[19]

Immunohistochemistry

Immunohistochemistry (IHC) was performed by utilizing a Dako autostainer/PT-Link with high pH target retrieval buffer (K8005; Dako, Glostrup, Denmark), according to the manufacturer's instructions. The primary antibody against PD-L1 (E1 L3 N-XP-Rb mAb; CST#13684) was incubated for 45 minutes at room temperature at a 1:500 dilution and visualized by using the MACH3 Rabbit HRP polymer detection system (M3 R531; Biocare, Irvine, CA) and DAB chromogen kit (BDB2004; Biocare, Irvine, CA), according to the manufacturer's instructions. Tonsil tissue was used as external control for PD-L1. The majority of crypt epithelial cells showed strong membranous staining (positive control), the majority of macrophages showed weak to moderate membranous staining (weak positive control), and the vast majority of lymphocytes showed complete lack of staining (negative control).[20] Colon tissue was used as an external control for p53. Scattered epithelial cells in the basal part of the crypts showed strong nuclear staining (positive control). Luminal epithelial cells were negative (negative control). Negative isotype staining was performed on a proportion of cases (Rabbit Monoclonal Negative Control; 16 minutes incubation at 36°C) and did not demonstrate any background/unexpected staining.

Membranous PD-L1 staining of at least 1% of tumor cells in the entire tissue microarray core was considered as positive according to initial industry-sponsored phase Ib clinical trials.[21] The percentage of tumor cells showing membranous staining in each core was recorded (Figures 1A to 1D). An average of the number of cells in the two cores from the same patient was used in case of heterogeneity of expression. IHC for p53 (BP53.12, 1:800 dilution; Invitrogen Inc., Carlsbad, CA) was performed, according to the manufacturer's instructions, and recorded as positive or negative, with no heterogeneity in staining of p53 observed, per standard clinical criteria (Figure 2).

Statistical analysis

Statistical analysis was performed by using SPSS version 22 (SPSS Inc., Chicago, IL) and EcStat, a locally developed program. The χ^2 test was utilized to analyze categorical data. Three-way association was used to assess smoking, p53, and PD-L1 expression. A P value of less than .05 was considered statistically significant. Disease-free survival (DFS) was calculated from the date of surgery to the date of recurrence. For disease-specific survival (DSS), patients were excluded if they had died as a result of any cause other than OSCC. Overall survival (OS) was calculated from the date of surgery to the date of last follow-up or date of death. Survival was calculated by using the Kaplan-Meier method, and differences were analyzed by using the log-rank test.

RESULTS

The cohort included 145 males and 110 females (1.3:1; median age of years). A total of 232 patients had a neck dissection, and of these, 69 had a bilateral neck dissection. Postoperative adjuvant therapy was administered to 126 patients, including radiotherapy alone to 101 patients and chemoradiotherapy to 25 patients. The median follow-up was 19.2 months (0.2−162 months), and 2.9% of patients were lost to follow-up. Local recurrence was observed in 26 patients (10.2%), regional recurrence in 30 patients (11.8%), and distant metastasis in 11 patients (4.3%). At the last follow-up, 42 patients had died, with 26 deaths (10.2%) attributed to OSCC. Table I summarizes the relevant clinicopathologic characteristics of the cohort.

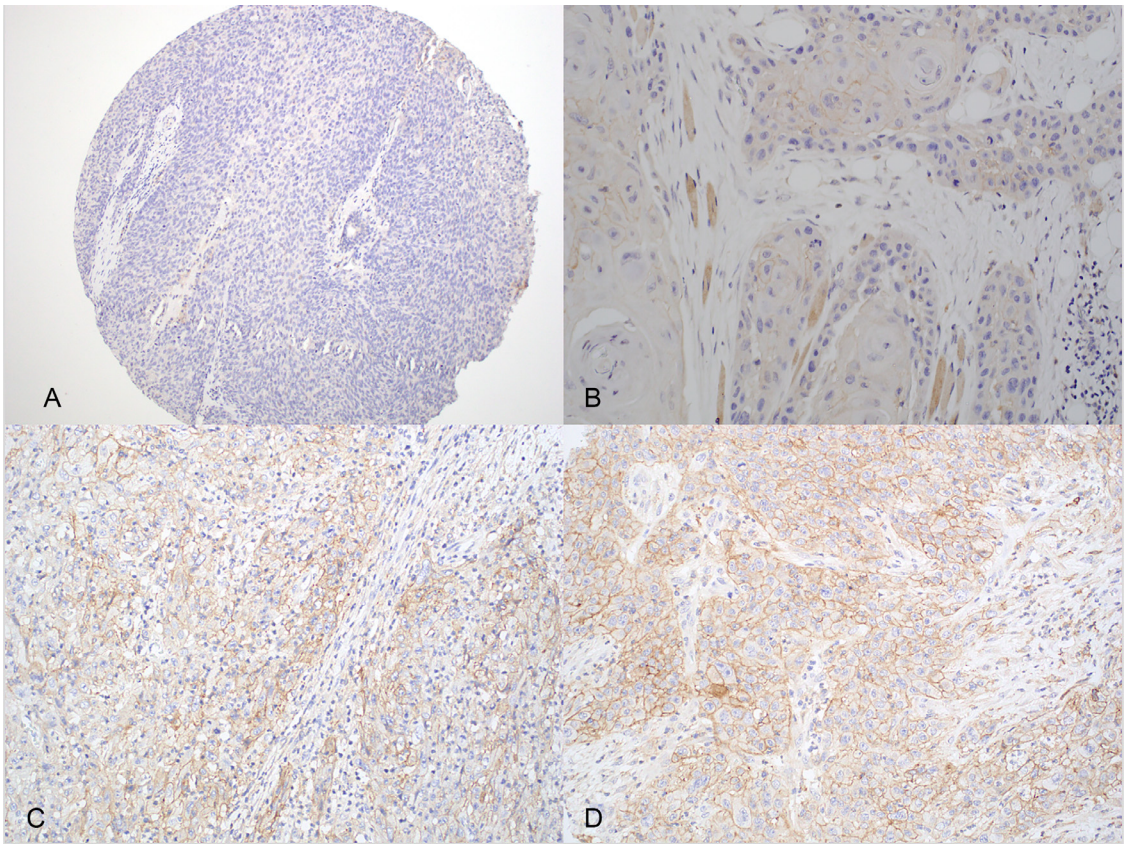

Fig. 1. Membranous PD-L1 expression in the tumor cells. **(A)** No PD-L1 expression (0%) at × 40 magnification. **(B)** PD-L1 expression in less than 25% of the tumor cells at × 200 magnification. **(C)** PD-L1 expression in 25% to 50% of the tumor cells at × 200 magnification. **(D)** PD-L1 expression in greater than 50% of the tumor cells at × 200 magnification.

PD-L1 expression

PD-L1 expression at a threshold of 1 % or greater was observed in 70 cases (27.5%) and was absent in 185 cases (72.5%). PD-L1 expression in 1% to 24% of the tumor cells was seen in 28 cases (11.0%); in 25% to 50% of the tumor cells in 30 cases (11.8%), and in 50% or greater of the cells in 12 cases (4.7%).

PD-L1 expression of 1% or greater was more frequent in females compared with males (36.4% in females vs 20.7% in males; odds ratio [OR] = 2.19; $P = .005$).

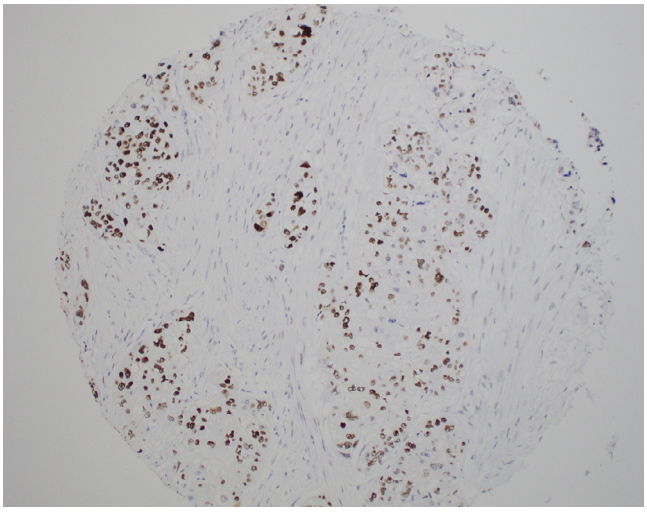

Fig. 2. p53 expression in tumor cells. Strong nuclear staining with p53 malignant cells. The adjacent stromal and inflammatory cells show complete lack of staining at × 100 magnification.

Table I. Demographic characteristics of patient cohort

Demographic characteristics	*Subgroup*	*PD-L1 ≥ 1 n = 70*	*PD-L1 % < 1 n = 185*	*P value*
Sex	Female	40 (36.3%)	70 (63.7%)	.005
	Male	30 (20.7%)	115 (79.3%)	
Age group	<45 years	4 (22.2%)	14 (77.8%)	.610
	≥45 years	66 (27.8%)	171 (72.2%)	
Smoking status	Smoker	45 (24.2%)	141 (75.8%)	.060
	Nonsmoker	25 (36.2%)	44 (63.8%)	
Margin*	Clear	11 (36.7%)	19 (63.3%)	.220
	Close/Involved	58 (26.0%)	165 (74.0%)	
Differentiation†	Well/Moderate	56 (28.5%)	140 (71.5%)	.760
	Poor	14 (26.4%)	39 (73.6%)	
Thickness‡	≤5 mm	11 (25.0%)	33 (75.0%)	.690
	>5 mm	57 (27.9%)	147 (72.1%)	
Tumor size§	≤3 cm	41 (27.9%)	106 (72.1%)	.990
	>3 cm	28 (28.0%)	72 (72.0%)	
Perineural invasion	N	45 (29.4%)	108 (70.6%)	.390
	Y	25 (24.5%)	77 (75.5%)	
Lymphovascular invasion	N	56 (28.4%)	141 (71.6%)	.520
	Y	14 (24.1%)	44 (75.9%)	
Bone invasion	N	52 (29.5%)	124 (70.5%)	.260
	Y	18 (22.8%)	61 (77.2%)	
Extracapsular spread	N	16 (29.1%)	39 (70.9%)	.480
	Y	14 (23.3%)	46 (76.7%)	
T stage	T1 T2	43 (30.3%)	99 (69.7%)	.260
	T3 T4	27 (23.9%)	86 (76.1%)	
N stage	N0	40 (28.6%)	100 (71.4%)	.660
	N+	30 (26.1%)	85 (73.9%)	
p53	Negative	23 (20.2%)	91 (79.8%)	.019
	Positive	47 (33.3%)	94 (66.7%)	
Radiotherapy	N	31 (24.0%)	98 (76.0%)	.220
	Y	39 (31.0%)	87 (69.0%)	

*Margin: Information not available for 2 patients.
†Differentiation: Information not available for 6 patients.
‡Thickness: Information not available for 7 patients.
§Tumor size: Information not available for 8 patients.

PD-L1 expression of 1% or greater was not associated with tumor thickness (≤5 mm or >5 mm), T stage (T1/T2 or T3/T4), perineural invasion, lymphovascular invasion, bone involvement, lymph node involvement (N0/N+), primary tumor size (≤3 cm or >3 cm), extranodal extension, or adjuvant radiotherapy.

Smoking

In this cohort, 186 patients (72.9%) were smokers. The association between PD-L1 expression and smoking was evaluated at thresholds of PD-L1 expression in 1% or greater of the tumor cells and in 50% or greater of the tumor cells. PD-L1 expression at a threshold of 1% or greater was present in 24.2% of smokers compared with 36.2% of nonsmokers ($P = .060$). Interestingly, an association was found between PD-L1 expression threshold of 50% or greater and being a nonsmoker ($P = .027$). PD-L1 expression threshold of 50% or greater was present in 5.9% of smokers compared with 14.5% of nonsmokers

p53 status

Nuclear p53 expression was seen in 141 cases (55.3%) in this cohort. The association between PD-L1 and p53 expression was also evaluated at thresholds of PD-L1 expression in 1% or greater of the tumor cells and in 50% or greater of the tumor cells. PD-L1 expression was associated with the presence p53 expression at both the thresholds of PD-L1 expression in 1% or greater ($P = .019$) and at 50% or greater ($P = .003$). PD-L1 expression at a threshold of 1% or greater was present in 33.3% of p53-positive cases compared with 20.2% of p53-negative cases. PD-L1 expression at a threshold of 50% or greater was present in 12.8% of p53-positive cases compared with 2.6% of p53-negative cases.

There was no association between p53 and smoking status (53.2% smokers vs 60.9% nonsmokers; $P = .280$) (Table II). No association between p53 expression and PD-L1 expression was observed in the nonsmoking group ($P = .153$) or in the smoking group ($P = .083$)

Survival

A survival advantage was not observed in those with PD-L1 expression of 1% or greater in terms of OS ($P = .482$), DSS ($P = .864$), or DFS ($P = .731$). Similarly, PD-L1 expression threshold of 50% or greater was not associated with OS ($P = .975$), DSS ($P = .911$), or DFS ($P = .819$).

Among the patients who smoked, there were no significant differences in survival between PD-L1 expression of 1% or greater and PD-L1 expression of 1% or greater (OS: $P = .420$; DSS: $P = .727$; DFS: $P = .960$), as shown in Figures 3A, 3B, and 3C. Among the patients whose tumors were p53 positive, there was no difference in survival between PD-L1 expression of 1% or greater and PD-L1 expression of 1% or less (OS: $P = .647$; DSS: $P = .825$; and DFS: $P = .802$), as shown in Figures 4A, 4B, and 4C. Similarly, there were no differences in survival between smokers and nonsmokers in either the PD-L1-positive group or the PD-L1-negative group, as shown in Figures 5A, 5B, and 5C.

DISCUSSION

The present study, which included 255 patients with a complete smoking history, is the first and largest to examine the association between OSCC and PD-L1 expression, smoking, and p53 expression. In this cohort, 27.5% of patients demonstrated PD-L1 expression, and PD-L1 expression was more common in females than in males. There was a correlation between PD-L1 and p53 expression at both the 1% and 50% thresholds. However, nonsmokers were more likely to show PD-L1 expression at a threshold of 1% or greater ($P = .060$), which was more significant at threshold of 50% or greater ($P = .027$). In this cohort, PD-L1 expression, irrespective of the threshold of expression, was not associated with DSS or OS.

Tobacco smoke is a complex mixture of more than 60 carcinogens and has been linked with multiple cancers in humans, particularly cancers of the oral cavity and the lungs.[22] Foy et al. demonstrated overexpression of PD-L1 in OSCC in nonsmokers and nondrinkers in their cohort of 44 patients.[23] Nonsmokers were more likely to demonstrate PD-L1 expression at a threshold of 1% or greater in our cohort, which included 69 self-reported nonsmokers and 186 smokers. Interestingly, Rizvi et al, showed that smokers with NSCLC respond better to immune checkpoint inhibitors in terms of objective response (56% smokers vs 17% nonsmokers; $P = .03$), durable clinical benefits (77% smokers vs 22% nonsmokers; $P = < .01$), and longer DFS ($P < .01$) when utilizing a molecular signature for smoking, but

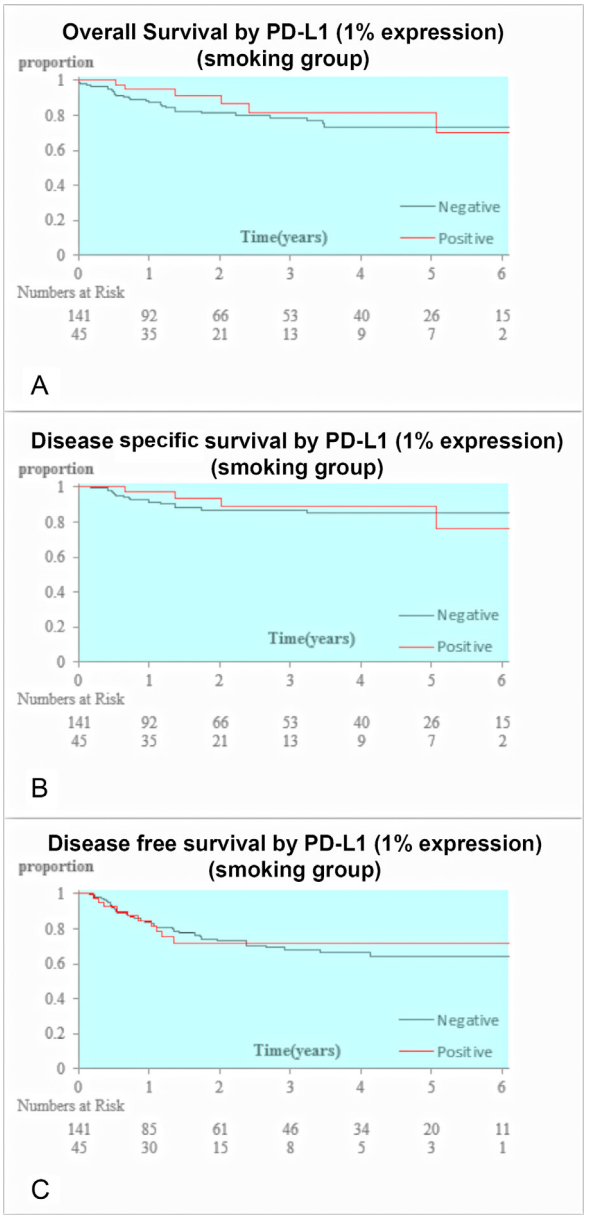

Fig. 3. Survival curves for the smoking group, subdivided on the basis of PD-L1-negative (<1% expression) and positive (≥1% expression) groups. **(A)** Overall survival ($P = .420$). **(B)** Disease-specific survival ($P = .727$). **(C)** Disease-free survival ($P = .960$).

Table II. Three-way analysis of PD-L1 expression of 1% or greater against p53 expression and smoking status

PD-L1 ≥ 1%	*p53-negative*		*p53-positive*		*Total*
	Nonsmoker	*Smoker*	*Nonsmoker*	*Smoker*	
Positive	7 (25.9%)	16 (18.4%)	18 (42.9%)	29 (29.3%)	70
Total	**27**	**87**	**42**	**99**	**255**

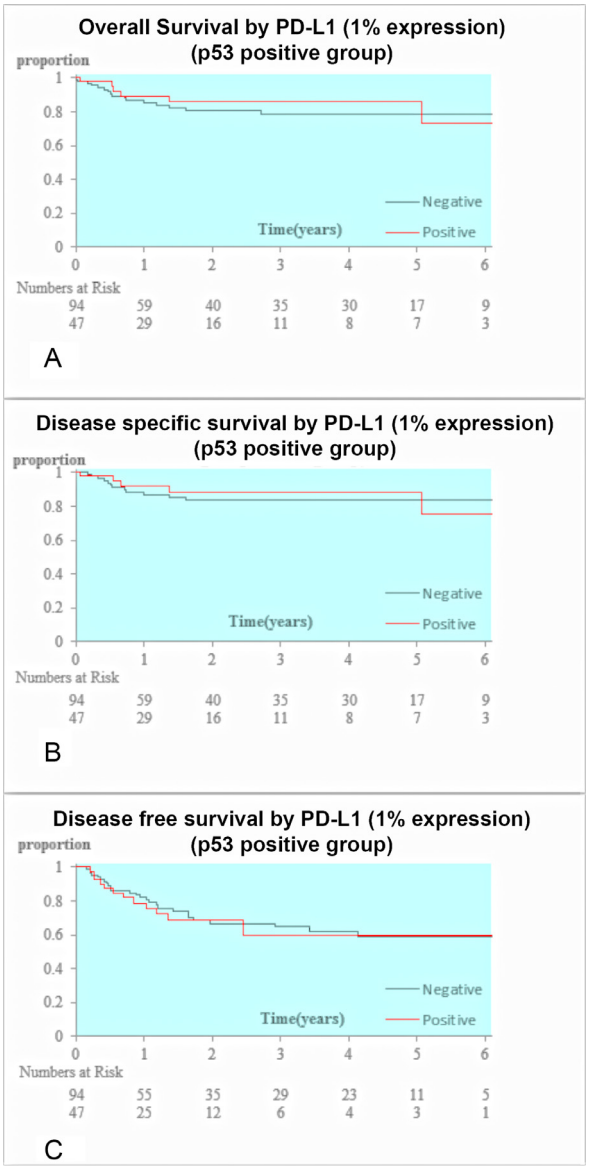

Fig. 4. Survival curves for p53 positive group, subdivided on the basis of PD-L1-negative (<1% expression) and positive (≥1% expression) groups. **(A)** Overall survival ($P = .647$). **(B)** Disease-specific survival ($P = .825$). **(C)** Disease-free survival ($P = .802$).

not with a self-reported smoking history.[24] These findings raise questions regarding the reliability of a self-reported smoking history, as has been used in the current cohort, especially because there was no association between a self-reported smoking history and survival in the current cohort. Unfortunately, there are no reliable methods of exploring smoking habits besides a self-reported smoking history in OSCC. Alexandrov et al., using next-generation sequencing data, reported that the smoking signature four is detected in a lower number of oral cancers compared with NSCLC.[25]

We sought to identify an additional cost-effective marker for PD-L1 expression that could be utilized clinically. p53 plays a critical role in cell cycle regulation, DNA damage repair, and apoptosis and is one of the most commonly mutated genes in cancer.[16,26,27] Cortez et al. recently demonstrated that p53 regulates PD-L1 expression in NSCLC.[28] However, only a few studies have examined the correlation between immunohistochemical expression of p53 and PD-L.[16] PD-L1 expression was seen in a larger proportion of tumor cells that demonstrated nuclear p53 expression in our cohort. Unlike immunostaining for PD-L1, immunostaining for p53 is widely available, does not show temporal and spatial heterogeneity as observed in PD-L1, and is not fraught with interobserver variability.

Cortez et al. showed that patients with NSCLC with high PD-L1 and low p53 levels had a worse survival rate compared with patients with low PD-L1 and high p53.[28] Similar analyses were limited by the number of patients available in each category, and neither PD-L1 expression nor p53 expression was associated with survival in this cohort. Currently, there are conflicting data regarding the clinicopathologic correlation and/or prognostic relevance of PD-L1 expression in OSCC.[29] Thus, Satgunaseelan et al., Lin et al., Oliveira-Costa et al., Straub et al., and Cho et al. have demonstrated the variable association between PD-L1 expression in tumor cells and prognosis and survival.[11,29-32] Mattox et al. showed that there is a significant positive correlation between PD-L1 expression and CD4+ and CD8+ tumor infiltrating lymphocytes in tongue OSCC tumors.[33] Although this may reflect the variety of antibody clones, staining platforms, evaluation methods, and thresholds, it may also indicate that PD-L1 expression does not provide reliable information for the prognosis of OSCC in an immunotherapy-naïve cohort. It is well established that tumors with PD-L1 expression are more likely to respond to immunotherapy compared with those that do not demonstrate PD-L1.[24] However, the CheckMate 141 clinical trial, evaluated the difference in OS in patients showing PD-L1 expression of 1% or greater and those showing PD-L1 expression of less than 1%. The IHC 28-8 PharmaDx platform was used for PD-L1 evaluation. In comparison with conventional chemotherapy, the CheckMate 141 trial reported a 45% reduction in risk of death with immunotherapy among patients with PD-L1 expression in 1% or greater of the tumor cells and 27% reduction in risk of death in patients with PD-L1 expression in less than 1% of the tumor cells.[34] Thus, it is well established that immunotherapy leads to better survival outcomes compared with conventional chemotherapy. However,

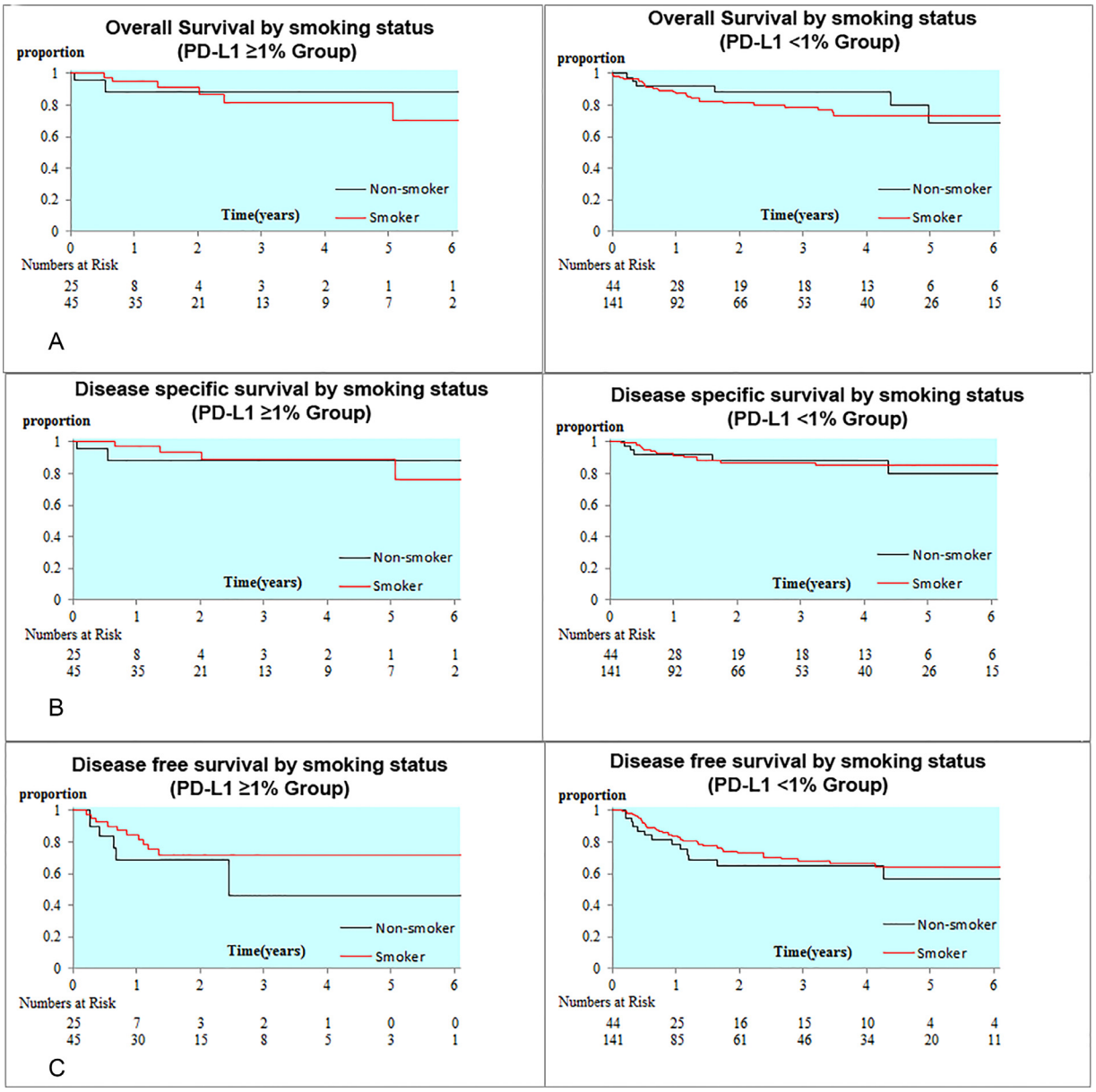

Fig. 5. Survival curves by smoking status, subdivided on the basis of PD-L1-negative (<1% expression) and positive (≥1% expression) groups. **(A)** Overall survival (PD-L1 positive: $P = .821$; PD-L1-negative: $P = .444$). **(B)** Disease-specific survival (PD-L1 positive: $P = .484$; PD-L1-negative: $P = .817$). **(C)** Disease-free survival (PD-L1 positive: $P = .252$; PD-L1-negative: $P = .405$).

the methods to identify patients who are most likely to benefit from single-agent immunotherapy or those who may require combination therapies remain to be defined in OSCC. As such, utilizing multiple factors, including patient factors, such as female sex, nonsmoking status, and immunohistochemical tumor characteristics, such as PD-L1 and p53 expression in tumor cells, could help better predict response to immunotherapy and assist with designing clinical trials with optimal benefits and reduced costs for patients.

CONCLUSIONS

Our data demonstrate that patients with OSCC who are females and nonsmokers are more likely to show PD-L1 expression. Also, OSCC with nuclear p53 expression are more likely to show PD-L1 expression in a higher proportion of the cells. These factors may be useful in predicting response to immune checkpoint inhibitors in OSCC. However, larger, multi-institutional studies with cohorts of patients with OSCC treated with immunotherapy are essential to validate these findings.

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