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Prognostic Value of Programmed Death Ligand-1 and Human Papilloma Virus Expression in Head and Neck Squamous Cell Carcinoma

ABSTRACT

Background: Human papilloma virus infection and programmed death-1/programmed death ligand-1 pathway play a role in the development of immune tolerance against the tumor. The aim of this study was to analyze the prevalence and prognostic value of human papilloma virus and programmed death ligand-1 expression in head and neck squamous cell carcinoma.

Methods: The study included 73 cases with oropharyngeal and oral cavity squamous cell carcinoma. The immunohistochemistry method was used to determine p16 and programmed death ligand-1 expression. Membranous staining in tumor cells of ≥5% was considered programmed death ligand-1 positive. Human papilloma virus status, programmed death ligand-1 expression, and prognostic associations were statistically analyzed.

Results: Median follow-up was 37.2 months (2-105 months). Of the total 73 patients, 61.6% (45/73) were p16 positive and 29% (21/73) were positive for programmed death ligand-1 expression by immunohistochemistry. There was no significant relationship between p16 and programmed death ligand-1 expressions ($P = .62$). Programmed death ligand-1 expression did not correlate with disease-free survival and overall survival ($P = .62$, $P = .92$, respectively). In regression analysis, the advanced stage ($P = .011$) was associated with poor overall survival, whereas p16 and programmed death ligand-1 independently did not affect overall survival ($P > .05$).

Conclusion: There was no correlation between tumor cell programmed death ligand-1 and human papilloma virus expression in oral cavity squamous cell carcinoma and oropharyngeal squamous cell carcinoma patients, and programmed death ligand-1 was not a prognostic biomarker associated with survival. The predictive and prognostic role of programmed death ligand-1 should be supported by multicenter prospective studies with larger patient populations.

Keywords: Immune system, human papilloma virus, prognosis, programmed death ligand-1, head and neck squamous cell carcinoma

Ağah Yeniçeri[1]
İpek Işık Gönül[2]
Ceren Bilkan Öge[2]
Mehmet Düzlü[3]

[1]Department of Otorhinolaryngology, University of Health Science, Ankara City Hospital, Ankara, Turkey
[2]Department of Pathology, Gazi University School of Medicine, Ankara, Turkey
[3]Department of Otorhinolaryngology, Gazi University School of Medicine, Ankara, Turkey

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Corresponding author:
Ağah Yeniçeri
Email: *agahyeniceri@gmail.com*
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INTRODUCION

Head and neck cancers are the seventh most common cancer among all cancers.[1] Histologically, ≥90% of head and neck cancers consist of squamous cell carcinomas. Smoking and alcohol consumption are known to be the most important risk factors.[2] Oncogenic human papilloma virus (HPV) infection has become known to be an important risk factor for head and neck squamous cell carcinomas (HNSCC) in recent years.[2,3] HPV-associated HNSCC is most commonly seen in the oropharynx, and HPV-associated oropharyngeal squamous cell carcinoma (HPV-OPSCC) is considered a separate subtype of HNSCC.[3] Furthermore, HPV-OPSCC accounts for approximately 25% of all HNSCC cases.[4] Although the incidence of smoking-related HNSCC has decreased, the overall incidence is still increasing, mainly due to the increase in HPV-OPSCC.[3] Compared to other etiological causes, HPV-associated HNSCCs show different biological features. It is seen at a younger age, responds better to radiotherapy and chemotherapy, and has been reported to have better survival rates.[2,4]

Cancer and the immune system are interrelated, as the basis of malignancy development is potentially immunogenic. The aggressive nature of tumor tissues can be determined by

their ability to evade the immune system. One of the mechanisms used to achieve this is the immune checkpoint interaction and up-regulation such as programmed death-1 (PD-1)/programmed death ligand-1 (PD-L1).[5]

Programmed death ligand-1 is a surface protein expressed in cells such as cancer cells, myeloid cells, and parenchymal cells.[6] When the PD-1 receptor, which is from the CD28 receptor family and found on tumor-infiltrating lymphocytes, interacts with its ligand PD-L1, the PD-1/PD-L1 pathway is activated, causing impaired cytotoxic CD8 T cell proliferation and function, tumor progression, and the development of immune tolerance to infections.[7,8] It is effective in both persistence of HPV infection and resistance to the immune system in the existence of malignancy.[5] It has been reported that immunotherapeutic agents such as PD-L1 and PD-1 antibodies, which have opened new doors in clinical oncology, will reverse the anergic state and increase antitumor immunity by inhibiting the PD-1/PD-L1 immune checkpoint.[9,10] Some clinical studies have reported promising response rates of these monoclonal antibodies in diverse malignancies such as lung, renal cell carcinoma, and melanoma.[11,12] Initially, in 2016, nivolumab and pembrolizumab were confirmed by the U.S. Food and Drug Administration for use in patients with HNSCC.[13,14] Some authors have come to the conclusion that PD-L1-positive patients have a richer response to immunotherapeutics such as pembrolizumab and nivolumab than PD-L1-negative patients.[15]

The PD-L1/PD-1 pathway reduces the effect of T cells on HPV by suppressing the immune system. This shows the relation between HPV status and PD-L1 expression levels and the prognostic importance of PD-L1 levels, especially in HNSCC.[5,8] Although there is consensus among authors about HPV-HNSCC, PD-L1 is a biomarker that has uncertainties and its clinical significance and prognostic value should be investigated. For this reason, the aim of this article was to examine HPV status and PD-L1 expression levels, the relationship of these biomarkers with clinicopathological features, and their effects on prognosis in patients with HNSCC.

MATERIAL AND METHODS

Patients

The study population included 73 patients, comprising 13 diagnosed with oropharynx and 60 with oral cavity squamous cell carcinoma, who presented at Gazi University Faculty of Medicine, Department of Otorhinolaryngology between January 1, 2010, and December 31, 2019. Written informed consent was obtained from the patients/patient who agreed to take part in the study. The study was approved by Gazi University Ethics Committee (Number: 91610558-604.01.02). The patients consisted of in the study were 18 years of age and older, had not got any treatment such as surgery and chemoradiotherapy, had sufficient tissue material in the tissue archive of the Pathology department, and attended regular follow-up appointments. The patients who had previously been treated, had recurrent tumors, did not have sufficient tissue material, or did not have regular clinical follow-up were excluded from the study. The clinicopathological characteristics of the patients, such as demographic information, cigarette/alcohol consumption, clinical follow-up information, survival status, imaging data, TNM (the extent of the tumor (T), extent of spread to the lymph nodes (N), presence of metastasis (M)) staging, and treatment method, were obtained from the hospital database. The staging was determined by the eighth edition of the AJCC (American Joint Committee On Cancer) TNM staging guideline.[16]

Tissue sections of 4 µm thickness were prepared from 10% formalin-fixed paraffin-embedded (FFPE) tissue blocks obtained from diagnostic biopsy or surgical material in the tissue archive of the Pathology department. Immunohistochemistry (IHC) staining was applied to determine p16 and PD-L1 expression. All the stained preparations were evaluated by an experienced pathologist unaware of the patient's information.

Expression of p16 by Immunohistochemistry

Sections of 4 µm thickness of FFPE tissues were taken into positively charged lamps. Mouse monoclonal anti-p16 antibody clone E6H4 (The CINtec® E6H4 p16 clone) was used as the primary antibody and staining was applied with an automatic IHC stainer (Ventana Benchmark XT). Commercial ready-to-use kits (Ultraview universal Diaminobenzidine (DAB) detection kit) of biotinylated binding (secondary) antibody, streptavidin–biotin complex, and 3,3′-diaminobenzidine used as chromogen were used. Cervical tissue containing high-grade cervical intraepithelial neoplasia was used as a positive control.

Expression of Programmed Death Ligand-1 by Immunohistochemistry

Rabbit monoclonal anti-PD-L1 (SP142) clone was used as the primary antibody for PD-L1. Staining was performed on an automatic IHC stainer (Ventana Benchmark XT) using the biotin-free indirect method with the OptiView DAB Detection Kit. Tissue sections of 4 µm were placed in the Ventana device. PD-L1 was incubated in EDTA buffer (pH: 8.0) for 64 minutes for antigen retrieval and in the device for 32 minutes for primary antibody incubation. The OptiView DAB Detection kit was used to provide the image with coloration. Counterstaining with Hematoxylin I was completed. The slides were then washed in tap water and kept in alcohol for 2 minutes and in xylol for 2 minutes, respectively. Normal tonsil tissue was used as a positive control. Several cutoff values were determined for PD-L1 which is membranous in expression 5% staining was selected as the cutoff value providing optimal assessment, and therefore ≥5% membranous staining in tumor cells (TCs) was defined as PD-L1 positive.

Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences version 22 software (IBM SPSS Corp.; Armonk, NY, USA). The compliance of numerical data with normal distribution was evaluated by the Shapiro–Wilk test and histogram. The Fisher exact test and Pearson chi-square test were used to compare

MAIN POINTS

- Programmed death ligand-1 (PD-L1) did not differ significantly according to human papilloma virus (HPV) status.
- The HPV-positive/PD-L1-positive group showed the best survival.
- Immune cell and tumor cell PD-L1 expressions were not adequate prognostic biomarkers for survival.

categorical data. Kaplan–Meier (with 95% confidence interval)-log-rank (Mantel–Cox) test was used for survival analysis. The effect of independent variables on survival was evaluated with Cox regression analysis. A value of $P < .05$ was considered statistically significant.

RESULTS

Of the patient population, 82% (60/73) had oral cavity cancer and 18% (13/73) had oropharyngeal cancer. The most frequently involved subsites in the oral cavity and oropharynx were the tongue (23.3%, 17/60) and tonsils (9.6%, 7/13), respectively. The median age of the patients was 63.89 (27-93 years, range). In the study, 32 patients were female (43.8%) and 41 patients were male (56.2%). There was a history of smoking in 26 patients (35.6%) and using alcohol in 13 patients (17.8%). Median follow-up was 37.24 months (2-105 months, range). At the time of diagnosis, 29 patients (39.7%) had advanced stage (stage III–IV) disease. As a treatment, surgery was applied to 26 patients (35.6%) and surgery + radiotherapy and/or chemotherapy was applied to 36 patients (49.3%) **(**Table 1**)**.

In a total of 45 patients (61.6%), p16 was positive in 36 patients (60%) diagnosed with oral cavity squamous cell carcinoma (OCSCC) and 9 patients (69%) diagnosed with OPSCC. No statistically significant correlation was determined between oral cavity or oropharyngeal cancer and p16 expression ($P = .53$). Of the clinicopathological features, there was a statistically significant relation was determined between gender and p16 expression ($P = .038$) **(**Table 1**)**.

PD-L1 was negative in 52 patients (71%) and positive in 21 patients (29%). PD-L1 was positive in 20 patients (33.3%) with OCSCC and in 1 patient (7.7%) with OPSCC. PD-L1 expression was higher in the oral cavity cancer patients, but this difference was not significant ($P = .064$). There was only a significant correlation between gender and PD-L1 expression ($P = .048$). Of the patients with PD-L1 positivity, 12 (26.7%) were HPV positive and 9 (32%) HPV negative. No significant relationship was determined between p16 and PD-L1 expressions ($P = .62$) **(**Table 1**)**. In the evaluation of PD-L1 expression with different cutoff values (≥1%, ≥10%, and ≥20%), no significant relationship was determined between PD-L1 and p16 ($P = .61$, $P = .27$, and $P = .36$, respectively).

During follow-up, 14 patients (19%) developed locoregional recurrence and 21 patients (29%) died. At the time of diagnosis, 23 patients (31.5%) had neck metastases and 3 (4%) had distant metastases. Overall survival (OS) and disease-free survival (DFS) rates in the p16-positive patients were significantly better than those of the p16-negative patients ($P = .039$, $P = .039$). No significant difference was determined between p16 and disease-specific survival (DSS) ($P = .139$). No significant correlation was found between PD-L1 expression and OS, DFS, and DSS (respectively, $P = .92$, $P = .62$, and $P = .26$) **(**Figure 1**)**. The 3-year OS was 73% in PD-L1-negative patients and 70% in positive patients. In the PD-L1 analysis with different cutoff values (≥1%, ≥10%, and ≥20%), no significant relationship was detected between OS and DFS and PD-L1. Immune cell (IC) PD-L1 expression levels were evaluated in the tumor microenvironment and IC PD-L1 was negative in 28 patients (38.4%) and positive in 45 patients (61.6%). No significant difference was detected between IC PD-L1 and DFS and OS ($P = .39$, $P = .43$, respectively).

The survival of the p16-positive and p16-negative patient groups was analyzed according to PD-L1 expression. In the p16-positive group, survival was better in those expressing PD-L1, but no significant correlation was seen between DFS and OS ($P = .46$, $P = .33$, respectively). There was no significant difference in OS and DFS according to PD-L1 in the p16-negative group ($P = .32$, $P = .136$, respectively) **(**Figure 2**)**.

Of the clinicopathological features, there was a statistically significant correlation between stage ($P < .0001$), distant metastasis ($P < .0001$), locoregional recurrence ($P = .002$), and neck metastasis ($P = .004$) and survival. The prognostic value of independent factors affecting OS was determined by Cox regression analysis. In univariate analysis, only advanced stage (HR = 0.198; 95% confidence interval, 0.057-0.687: $P = .011$) was an independent factor related with poor prognosis for OS. PD-L1 and p16 did not independently affect OS **(**Table 2**)**.

DISCUSSION

It is now known that malignancy and the immune system are related to each other.[17,18] Immune cell and cancer cell interactions in the tumor microenvironment creates an immunosuppressive environment that supports tumor development by protecting the tumor from immune attack. From this perspective, patients with advanced tumors have a predominant immune tolerance to the tumor.[17] It is known that viral infections and ICs in the tumor microenvironment are interrelated and that viruses affect PD-L1 expression, thereby helping TCs escape from immune checkpoints.[18] Among these viruses, HPV, which has been proven to be connected with OPSCC in particular, renders tumors more immunogenic.[6,19] It co-evolves by targeting PD-L1 and aids tumor development by producing immunosuppression against tumor progression.[18] This information shows that evaluating PD-L1 expression and HPV status together can give more accurate results. The results of the study yielded p16 expression rates and survival results similar to findings in the literature.[4,20] A relevant relationship was determined between gender and p16 expression ($P = .038$). Females showed a higher rate of p16 positivity. Human papilloma virus-positive patients were mostly at the early stage (stage I–II) (62.2%). In p16-positive patients (45 patients, 61.6%), both DFS and OS were significantly better than in p16-negative patients ($P = .039$, $P = .039$).

Expression status of PD-L1 and HPV in HNSCC have been the subject of research by many authors.[6,14,19-21] Balermpas et al[19] declared that HPV and PD-L1 positivity were associated in 221 patients, and there was a positive prognostic effect for PD-L1 overexpression, and Hong et al[6] supported this result with a study of 214 patients. Yang et al[21] declared that PD-L1 was expressed in 52% of laryngeal squamous cell carcinoma, and PD-L1 and HPV were independent protective factors affecting OS. Kim et al[20] detected 68% PD-L1 positivity in 133 OPSCC patients, found no correlation between p16 and PD-L1, DFS, OS, and clinicopathological features, and stated that PD-L1 and HPV expression were not associated with prognosis. Another author reported that p16 and PD-L1 status have a greater prognostic impact when evaluated together rather than independently.[22] In the current study, there was a relatively lower PD-L1 positivity of 29% compared to the literature. This result can be attributed to the interobserver variability of IHC and the cut-off value of PD-L1. It was seen that

Table 1. Clinicopathological Features of Patients and Their Relationship with p16 and PD-L1 Expressions

Characteristics	No. of Patients (%)	p16 Positive (%)	p16 Negative (%)	*P*	PD-L1 Negative (%)	PD-L1 Positive (%)	*P*
Age (years)							
<65	37 (50.7%)	24 (53.3%)	13 (46.4%)	.56	28 (53.8%)	9 (43%)	.4
≥65	36 (49.3%)	21 (46.7%)	15 (53.6%)		24 (46.2%)	12 (57%)	
Gender							
Female	32 (43.8%)	24 (53.3%)	8 (28.6%)	.038*	19 (36.5%)	13 (62%)	.048*
Male	41 (56.2%)	21 (46.7%)	20 (71.4%)		33 (63.5%)	8 (38%)	
Smoking							
No	47 (64.4%)	30 (66.7%)	17 (60.7%)	.6	31 (59.6%)	16 (76%)	.18
Yes	26 (35.6%)	15 (33.3%)	11 (39.3%)		21 (40.4%)	5 (24%)	
Alcohol							
No	60 (82.2%)	38 (84.4%)	22 (78.6%)	.52	42 (81%)	18 (86%)	.61
Yes	13 (17.8%)	7 (15.6%)	6 (21.4%)		10 (19%)	3 (14%)	
Site of tumor							
Oral cavity	60 (82%)	36 (80%)	24 (85.7%)	.53	40 (77%)	20 (95%)	.064
Oropharynx	13 (18%)	9 (20%)	4 (14.3%)		12 (23%)	1 (5%)	
T stage							
T1	23 (31.5%)	17 (37.8%)	6 (21.4%)	.44	19 (36.5%)	4 (19%)	.41
T2	30 (41.1%)	16 (35.6%)	14 (50%)		20 (38.5%)	10 (47.6%)	
T3	11 (15.1%)	6 (13.3%)	5 (17.9%)		8 (15.4%)	3 (14.3%)	
T4	9 (12.3%)	6 (13.3%)	3 (10.7%)		5(9.6%)	4 (19%)	
N stage							
N0	51 (69.9%)	30 (66.7%)	21 (75%)	.65	39 (75%)	12 (57%)	.18
N1	13 (17.8%)	10 (22.2%)	3 (10.7%)		8 (15.4%)	5 (24%)	
N2	7 (9.6%)	4 (8.9%)	3 (10.7%)		3 (5.8%)	4 (19%)	
N3	2 (2.7%)	1 (2.2%)	1 (3.6%)		2 (3.8%)	0	
AJCC TNM stage							
I	24 (32.9%)	18 (40%)	6 (21.4%)	.17	21 (40.4%)	3 (14.3%)	.074
II	20 (27.4%)	10 (22.2%)	10 (35.7%)		15 (28.8%)	5 (23.8%)	
III	14 (19.2%)	10 (22.2%)	4 (14.3%)		8 (15.4%)	6 (28.6%)	
IV	15 (20.5%)	7 (15.6%)	8 (28.4%)		8 (15.4%)	7 (33.3%)	
HPV status							
Positive	45 (61.6%)				33 (73.3%)	12 (26.7%)	.62
Negative	28 (38.4%)				19 (68%)	9 (32%)	
PD-L1 status							
Positive	21 (29%)	12 (26.7%)	9 (32%)	.62			
Negative	52 (71%)	33 (73.3%)	19 (68%)				
Treatment							
Surgery	26 (35.6%)	14 (31.1%)	12 (42.9%)	.6	18 (34.6%)	8 (38%)	.21
Chemoradiation therapy	9 (12.3%)	7 (15.6%)	2 (7.1%)		9 (17.3%)	0	
Surgery+ radiotherapy ± chemo	36 (49.3%)	23 (51.1%)	13 (46.4%)		24 (46%)	12 (57%)	
Non	2 (2.7%)	1 (2.2%)	1 (3.6%)		1 (2%)	1 (5%)	

*$P < .05$.

PD-L1 did not affect DFS and OS, and PD-L1 did not differ significantly according to HPV status ($P = .62$). In the p16-positive and p16-negative patient subgroups, PD-L1 was not associated with DFS and OS, but the p16-positive/PD-L1-positive group indicated the best survival, similar to the literature.[6,20,22] In univariate analysis, PD-L1 and p16 were not independent prognostic factors affecting OS. The differing opinions among authors are evidence that PD-L1 expression and its prognostic value need further investigation.

The different results of analyses of PD-L1 in the literature may be due to the lack of a standardized clone used during PD-L1 staining, the use of different cutoff values of PD-L1, and the varying results of immune and TC expression levels when scoring PD-L1.

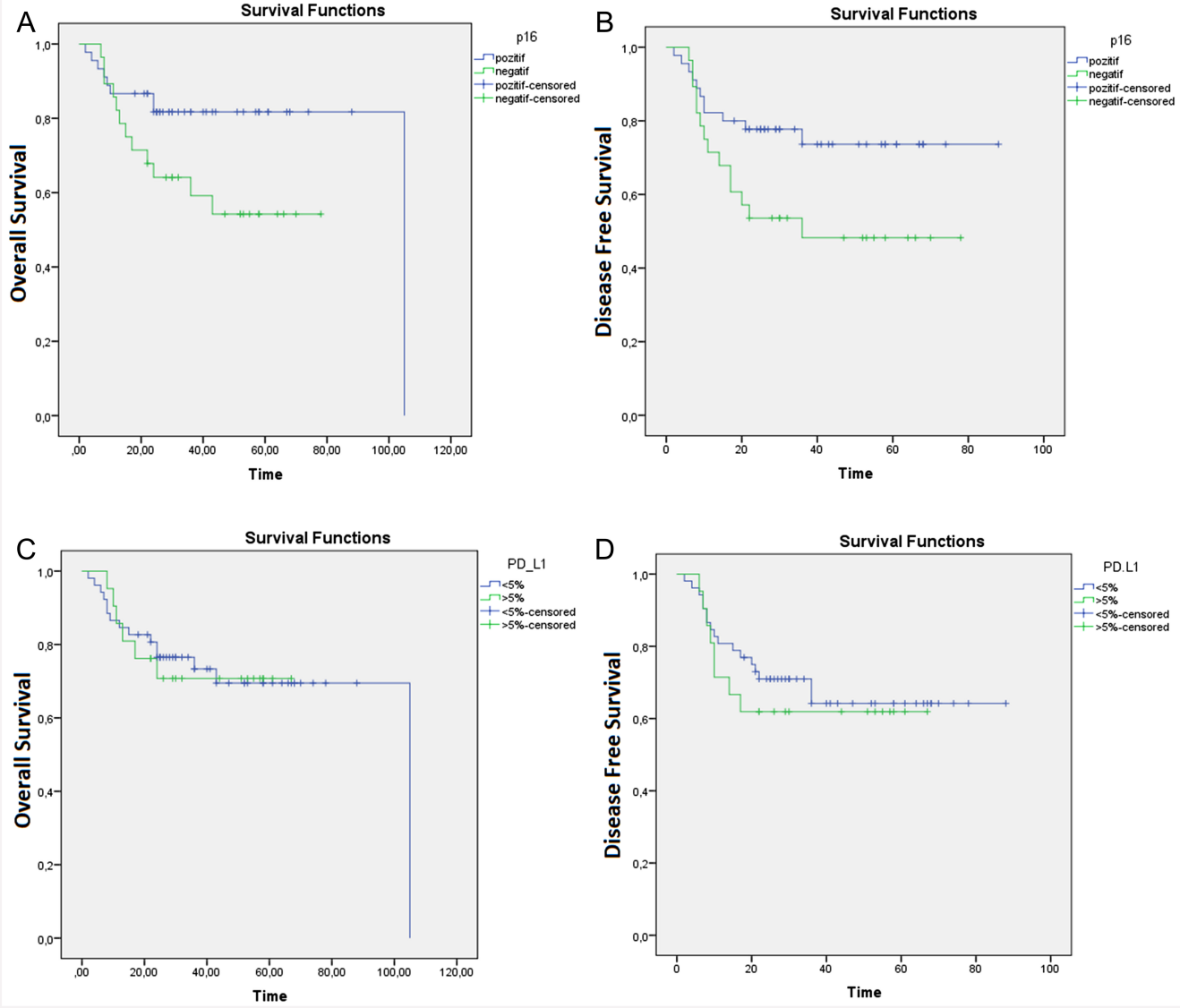

Figure 1. A-D. Kaplan-Meier analysis of Disease-free survival (DFS) and Overall survival (OS) according to Programmed Death Ligand-1 (PD-L1) and p16 expression in patients with head and neck squamous cell carcinoma. (A-B) OS and DFS relationship with p16 status (C-D) OS and DFS relationship with PD-L1 expression.

Many authors have used different cutoff values when scoring PD-L1 expression.[13,14,20-22] Scognamiglio et al[23] reported that different cutoff values would change the frequency of PD-L1-positive tumors, and this would have implications for investigating the predictive or prognostic role of PD-L1. In our study although the PD-L1 expression levels changed when analyses were performed with different cutoff values (≥1%, ≥10%, and ≥20%), these results did not affect OS and DFS ($P > .05$). No significant difference was determined between IC membranous PD-L1 levels and DFS and OS ($P = .39$, $P = .43$, respectively). In contrast, some studies of HNSCC patients have found an association between IC PD-L1 levels and OS and DFS, indicating that IC PD-L1 is a more appropriate independent prognostic biomarker than TC affecting OS.[22,24]

Immunotherapy treatment containing immune checkpoint inhibitors such as anti-PD-L1 and anti-PD-1 has been shown to be effective in many tumors including HNSCC.[25-27] In recurrent HNSSC (CheckMate 141), nivolumab has been shown to result in longer survival than standard treatments, independent of p16 and PD-L1.[14] On the contrary, the KEYNOTE-055 and KEYNOTE-012 studies reported higher response rates to pembrolizumab in PD-L1-positive HNSCC patients.[13,27] Therefore, PD-L1 levels in immunotherapy candidates may have an effective predictive role that needs further investigation.

Our study had some limitations, primarily the small number of patients included. Second, PD-L1 expression may be higher than reported, as the SP142 clone has been shown to have weaker

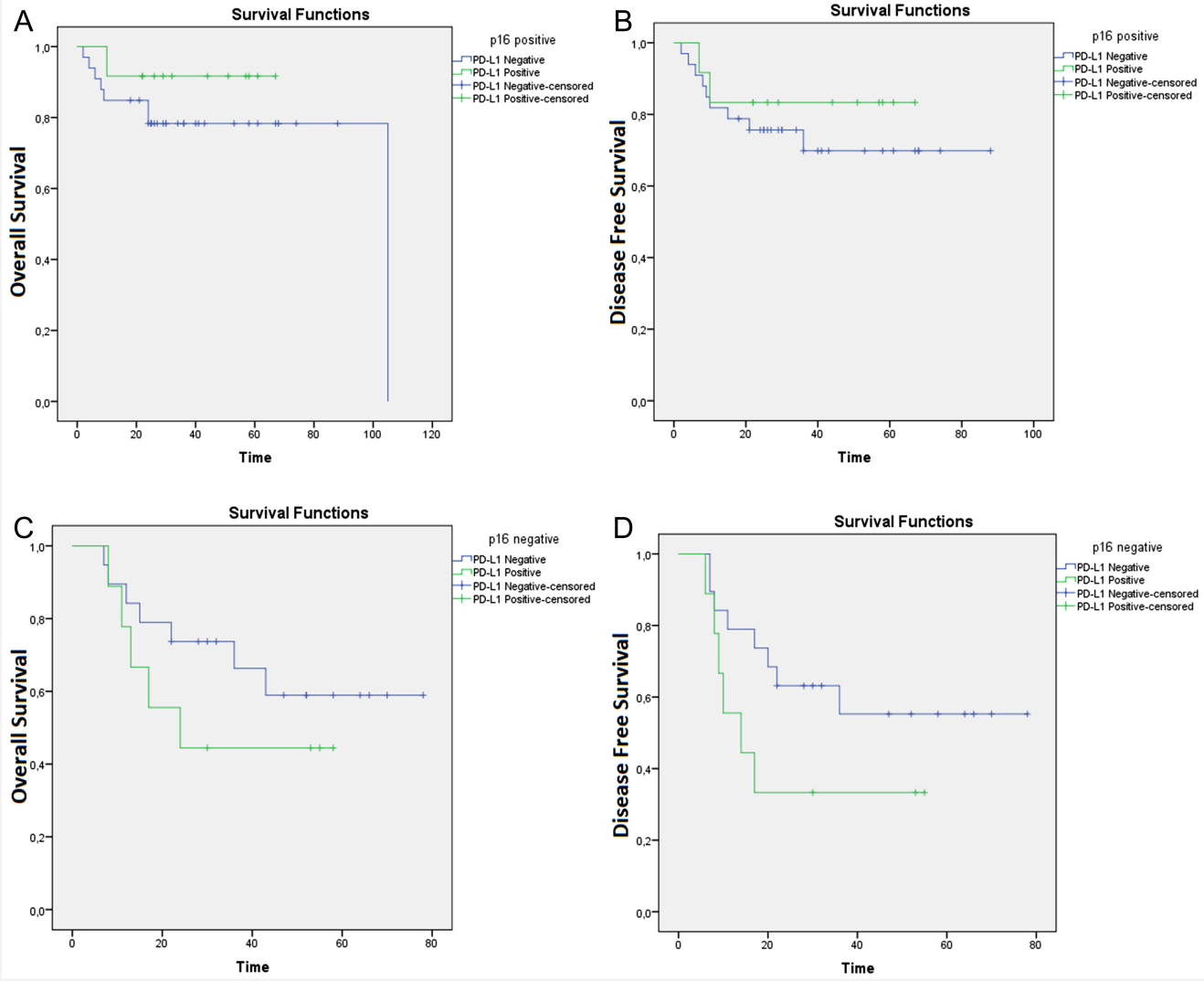

Figure 2. Kaplan-Meier analysis of Disease-free survival (DFS) and Overall survival (OS) according to Programmed Death Ligand-1 (PD-L1) expression in p16 positive and p16 negative patient groups. (A-B) OS and DFS according to PD-L1 expression in p16 positive patient group (C-D) OS and DFS according to PD-L1 expression in p16 negative patient group

Table 2. Univariate Cox Regression Analysis of Independent Factors Affecting Survival

	Univariate Analysis	
Characteristics	**HR (95% Confidence Interval)**	***P***
Age (<65 vs. ≥65)	0.597 (0.219-1.623)	.312
Gender (male vs. female)	1.337 (0.364-4.911)	.662
Smoking (yes vs. no)	2.36 (0.881-6.325)	.088
Alcohol (yes vs. no)	0.705 (0.201-2.473)	.585
Site of tumor (oc vs. op)	1.029 (0.266-3.985)	.967
p16 (negative vs. positive)	0.42 (0.144-1.231)	.114
PD-L1 (negative vs.positive)	0.634 (0.206-1.954)	.428
Neck metastasis (yes vs. no)	0.519 (0.138-1.946)	.33
Distant organs metastasis (yes vs. no)	1.628 (0.290-9.126)	.58
Stage (I–II: early stage vs. III–IV: advanced stage)	0.198 (0.057-0.687)	.011*

*$P < .05$.

staining results in several publications.[28,29] Third, the PD-L1 expression in tumor tissues included in our study may show dynamic variability over time, which may not indicate the real PD-L1 expression level at the time of diagnosis and its relationship with survival.

CONCLUSION

In conclusion, this study demonstrated p16 and PD-L1 levels in OCSCC and OPSCC patients. No correlation was found between HPV status and TC PD-L1 expression, and PD-L1 was not an independent prognostic biomarker associated with survival. With increasing cancer immunology research, the predictive and prognostic role of PD-L1 should be supported by multicenter studies with larger patient populations.

Ethics Committee Approval: Ethics committee approval was received for this study from Gazi University Assessment and Evaluation Ethics Sub-Working Group (Approval number: 91610558-604.01.02).

Informed Consent: Written informed consent was obtained from the patients who agreed to take part in the study.

Peer-Review: Externally peer-reviewed.

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Declaration of Interests: The authors have no conflict of interest to declare.

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