

A Correlative Analysis of PD-L1, PD-1, PD-L2, EGFR, HER2, and HER3 Expression in Oropharyngeal Squamous Cell Carcinoma

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Abstract

We explored potential associations of the PD-1/PD-L1/PD-L2 pathway with clinical characteristics, outcome, and expression of EGFR, HER2, HER3 in oropharyngeal squamous cell carcinoma (OPSCC) using an institutional database. Protein expression was assessed by IHC on tissue microarray sections (EGFR, HER2, HER3) or whole tissue sections (PD-1/PD-L1/PD-L2). Expression of EGFR, HER2, HER3, PD-L1, and PD-L2 was quantified on tumor cells. Maximum density of PD-1 positive lymphocytes was measured on a scale of 0 to 4 within the tumor mass and peritumoral stroma. Associations between biomarkers and patient outcomes were tested using descriptive and inferential statistics, logistic regression, and Cox proportional hazards models. We analyzed tissue samples from 97 OPSCC cases: median age 59 years, p16+ (71%), male (83.5%), never smokers (18%), stage 3 to 4 disease (77%). Twenty-five percent of cases were PD-L1 positive. The

proportion of PD-L1+ tumors was higher in p16+ (29%) than p16– OPSCC (11%, $P = 0.047$). There was no correlation between PD-L1, PD-L2, PD-1, EGFR, HER2, or HER3 expression. Positive PD-L1 status correlated with advanced nodal disease on multivariate analysis (OR 5.53; 95% CI, 1.06–28.77; $P = 0.042$). Negative PD-L2 expression was associated with worse survival (HR 3.99; 95% CI, 1.37–11.58; $P = 0.011$) in p16– OPSCC. Lower density of PD-1 positive lymphocytes in peritumoral stroma was associated with significantly increased risk of death on multivariate analysis (HR 3.17; 95% CI, 1.03–9.78; $P = 0.045$) after controlling for prognostic factors such as stage and p16 status. PD-L1 expression on tumor cells correlates with p16 status and advanced nodal status in OPSCC. PD-1 positive lymphocytes in peritumoral stroma serve as an independent prognostic factor for overall survival. *Mol Cancer Ther*; 17(3); 710–6. ©2018 AACR.

Introduction

Until recently, the standard of care for the treatment of metastatic or recurrent oropharyngeal squamous cell carcinoma (OPSCC) was chemotherapy, with cetuximab as the only targeted treatment option (1). However, the care of OPSCC patients with recurrent or metastatic disease has been revolutionized with the development of immunotherapy. Immune checkpoint inhibitors have proven to be an effective treatment strategy for a wide range of cancers. Blockade of the programmed cell death-1 (PD-1) pathway has shown promising results (15%–40% response rate) in several cancers and PD-1 targeted therapeutics have received

FDA approval for the treatment of melanoma, bladder cancer, renal cell carcinoma, non-small cell lung cancer, and squamous cell carcinoma of the head and neck (SCCHN; refs. 2–4). The PD-1 inhibitors pembrolizumab and nivolumab have been approved by the FDA for patients that progress after platinum-based chemotherapy in SCCHN (5).

Biomarkers that can predict benefit of immunotherapy treatment are still under investigation. The most commonly used is PD-L1. Despite a seemingly higher likelihood of response to immunotherapy in a number of PD-L1 positive tumors, the adequate cut-off for PD-L1 positivity remains under debate (6). The correlation between the PD-1/PD-L1 pathway with clinical characteristics and outcomes in OPSCC has not been fully elucidated, with conflicting reports in the literature (7, 8). In addition, PD-L2 is emerging as a potential biomarker for immunotherapy as well (9). Furthermore, the relationship between PD-1/PD-L1/PD-L2 and other biologically relevant alterations, including those in the EGFR pathway (EGFR, HER2, HER3), is an understudied yet important area due to existing evidence of interactions between these pathways, especially in the tumor immune microenvironment (10, 11). Our group has previously described HER3 and its ligand as poor prognostic markers in OPSCC (12). We sought to examine the relationships of PD-1/PD-L1/PD-L2 biomarkers with clinical characteristics, outcome, and biologically relevant alterations of the EGFR family of receptors (EGFR, HER2, and HER3) using our annotated OPSCC database.

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Materials and Methods

A total of 97 formalin-fixed paraffin-embedded (FFPE) tissue specimens were obtained from patients diagnosed with OPSCC at the Winship Cancer Institute of Emory University from 1994 to 2008. The study was conducted under an Institutional Review Board-approved protocol at Emory University and clinical characteristics were decoded in compliance with the Health Insurance Portability and Accountability Act (HIPAA). No treatments for OPSCC were administered to patients before tissue biopsy or surgery. Information regarding clinical characteristics was retrieved as documented by the treating physicians.

All immunostains were examined and scored by a head and neck pathologist (CCG). FFPE tissue sections were used for IHC, as described previously (13). The slides were incubated with primary antibodies for PD-L1 (Cell Signaling Technology, dilution 1:20), PD-L2 (Cell Signaling Technology, dilution 1:50), PD-1 (Abcam, dilution 1:100), EGFR (Biogenex, dilution 1:200), HER2 (Cell Signaling Technology, dilution 1:200), or HER3 (Cell Signaling Technology, dilution 1:100). Staining of the antibodies was observed by 3,3'-diaminobenzidine tetrahydrochloride peroxidase substrate solution (Vector Laboratories). Cell surfaces were counterstained using Hematoxylin QS (Vector Laboratories). Mouse IgG served as a negative control, and normal epithelial tissues with known positive immunoreactions to selected antibodies were used as positive controls. For EGFR, HER2, HER3, PD-L1, and PD-L2 the intensity of staining on tumor cells was measured using a numerical scale (0, no expression; 1+, weak expression; 2+, moderate expression; and 3+, strong expression). The overall score for these stains was taken as the product of the intensity score and the percentage of tumor cells with any staining.

The maximum density of PD-1 positive lymphocytes was quantified within the tumor mass and in the stroma immediately adjacent to the infiltrating tumor (i.e., peritumoral stroma within a single 400× microscopic field of the tumor front). In both the tumor mass and the peritumoral stroma, the slides were examined for the area of maximum density of PD-1 positive lymphocytes and this area of maximum density was given a score of 0 to 4 (0, no PD-1 positive lymphocytes; 1, rare PD-1 positive lymphocytes [1–4/400× high power field (HPF)]; 2, moderate PD-1 positive lymphocytes [5–19/400× HPF]; 3, high PD-1 positive lymphocytes [20–49/400× HPF]; 4, very high PD-1 positive lymphocytes [≥50/400× HPF]). When scoring the peritumoral stroma, germinal centers were avoided.

Statistical analysis was conducted using SAS Version 9.3, and SAS macros or software developed at the Biostatistics and Bioinformatics Shared Resource at Winship Cancer Institute (14). The significance level was set at 0.05. Overall survival (OS) was defined as months from date of diagnosis to death or last follow up, and disease-free survival (DFS) was defined as months from date of diagnosis to progression or last follow up scan. Descriptive statistics for each variable were reported. The univariate association of each covariate with each biomarker was assessed using the chi-square test for categorical covariates and ANOVA for numerical covariates. The association with OS or DFS was assessed using Cox proportional hazards models and the association with advanced nodal status, defined as N2 disease or above, was carried out by logistic regression model. The multivariable model was fit by a backward variable selection method applying an $\alpha = 0.20$ removal criteria. KM plots were produced to compare survival curves along with the log-rank *P*-value.

Results

Demographic and clinical information for the 97 OPSCC patients included is shown in Table 1. The median age of the patients was 59 years, 83.5% of patients were male, 81.4% were Caucasian, and 17.7% were never-smokers. Among these oropharyngeal tumors, 71.1% were p16 positive. The majority of tumors were early T stage (T1/2 = 86.2%) compared to late T stage (T3/4 = 13.8%; *P* < 0.05). The majority of patients had advanced nodal disease (N2–N3, 66.7%) and had locally advanced disease (stage 3–4, 77.4%).

The results of the IHC stains are also shown in Table 1. Based on the methods described above, 25% of tumors were positive (score of 1–130) for PD-L1, 61% PD-L2 positive, and 64.5% had at least a high maximum lymphocyte density within the tumor (scaled value of 2–4). Within the peritumoral stroma, 88.2% had at least a high density of PD-1 positive lymphocytes. Finally, 47.4%, 26%, and 50.5% of carcinomas were positive for EGFR, HER2 and HER3, respectively. Representative samples of tumor PD-L1, PD-1, and peritumoral stroma PD-1 staining are shown in Supplementary Fig. S1.

We first sought to establish if the expression of the biomarkers studied correlated with p16 status (Table 2). There was a higher proportion of PD-L1 positivity in p16 positive than in p16 negative OPSCC tumors (29% vs. 11%, *P* = 0.047). PD-L2 and

Table 1. Patient characteristics and tumor staining

Variable	Level	N (%) = 97
Age	Median	59.00
p16	Negative	28 (28.9)
	Positive	69 (71.1)
Gender	Male	81 (83.5)
	Female	16 (16.5)
Race	AA	12 (12.4)
	White	79 (81.4)
	Other/unknown	6 (6.2)
Smoking	Never	17 (17.7)
	Former	41 (42.7)
	Current	38 (39.6)
Differentiation	WD	5 (5.2)
	MD	40 (41.2)
	NK	52 (53.6)
Tumor stage	1	39 (41.5)
	2	42 (44.7)
	3–4	13 (13.8)
		13 (13.8)
Node metastasis	N0–N1	32 (33.3)
	N2–N3	64 (66.7)
Overall stage	1–2	19 (22.6)
	3–4	65 (77.4)
		65 (77.4)
EGFR average overall	≤ Median (0.8)	50 (52.6)
	>Median (0.8)	45 (47.4)
HER2 average overall	0	71 (74.0)
	0.025–0.3	25 (26.0)
HER3 average overall	≤Median (0.1625)	47 (49.5)
	>Median (0.1625)	48 (50.5)
PD-L1	0	71 (74.7)
	1–130	24 (25.3)
PD-L2	0	33 (38.8)
	1–130	52 (61.2)
PD-1 positive lymphocytes in tumor	0–1	33 (35.5)
	2–4	60 (64.5)
		60 (64.5)
PD-1 positive lymphocytes in peritumoral stroma	0–1	11 (11.8)
	2–4	82 (88.2)

AA, African-American; WD, well-differentiated; MD, moderately differentiated; NK, non-keratinized.

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Table 2. Univariate association with p16

Covariate	Statistics	Level	p16		P-value ^a
			Negative N = 28	Positive N = 69	
PD-L1	N (Row %)	0	24 (34)	45 (65)	0.047
	N (Row %)	1-130	3 (13)	20 (87)	
PD-L2	N (Row %)	0	11 (35)	20 (65)	0.243
	N (Row %)	1-130	12 (24)	39 (76)	
PD-1 positive lymphocytes in tumor	N (Row %)	0-1	12 (36)	21 (64)	0.316
	N (Row %)	2-4	15 (26)	42 (74)	
PD-1 positive lymphocytes in peritumoral stroma	N (Row %)	0-1	2 (18)	9 (82)	0.494
	N (Row %)	2-4	25 (32)	54 (68)	
EGFR average overall	N (Row %)	≤ Median (0.8)	9 (19)	38 (81)	0.028
	N (Row %)	> Median (0.8)	18 (40)	27 (60)	
HER2 average overall	N (Row %)	0	18 (26)	51 (74)	0.496
	N (Row %)	0.025-0.3	8 (33)	16 (67)	
HER3 average overall	N (Row %)	≤ Median (0.1625)	15 (33)	31 (67)	0.354
	N (Row %)	> Median (0.1625)	11 (24)	35 (76)	

^aThe P-value is calculated by chi-square test or Fisher's exact, where appropriate.

PD-1 lymphocyte density, however, whether examined in the tumor or peritumoral stroma, did not correlate with p16 status. OPSCC tumors that were EGFR positive were associated with p16 tumor negativity ($P = 0.028$). There was no correlation between PD-1, PD-L1, PD-L2, HER2, HER3, and EGFR expression (Table 3).

Subsequently, we examined whether PD-L1, PD-L2 tumor expression, and PD-1 positive lymphocyte density in our OPSCC population correlated with advanced nodal status, defined as N2 disease or above. On multivariate analysis, tumors that were PD-L1 positive were significantly associated with advanced nodal

status (OR 5.53; 95% CI, 1.06–28.77; $P = 0.042$). Conversely, PD-1 positive lymphocyte density did not correlate with nodal status. PD-L2 positive tumors trended towards more advanced nodal disease (OR 3.12; 95% CI, 0.71–10.99; $P = 0.054$), but this did not reach statistical significance (Table 4). Interestingly, patients with a high density of PD-1 positive lymphocytes in the peritumoral stroma had improved survival compared with patients that had lower PD-1 positive lymphocyte density in the peritumoral stroma in a multivariable analysis controlling for other prognostic factors such as age, p16 status, and stage (HR 3.17; 95% CI, 1.03–9.78; $P = 0.045$). The improved survival

Table 3. Univariate association of EGFR, HER2, HER3 and PD-1, PD-L1, PD-L2

Covariate	Statistics	Level	PD-1 positive lymphocytes in tumor		P-value ^a
			0-1 N = 33	2-4 N = 57	
EGFR average overall	N (Row %)	≤ Median (0.8)	17 (39.53)	26 (60.47)	0.5
	N (Row %)	> Median (0.8)	14 (32.56)	29 (67.44)	
HER2 average overall	N (Row %)	0	23 (33.82)	45 (66.18)	0.505
	N (Row %)	0.025-0.3	8 (42.11)	11 (57.89)	
HER3 average overall	N (Row %)	≤ Median (0.1625)	19 (44.19)	24 (55.81)	0.116
	N (Row %)	> Median (0.1625)	12 (27.91)	31 (72.09)	
PD-1 positive lymphocytes in peritumoral stroma					
Covariate	Statistics	Level	0-1 N = 11	2-4 N = 79	P-value ^a
EGFR average overall	N (Row %)	≤ Median (0.8)	6 (13.95)	37 (86.05)	0.747
	N (Row %)	> Median (0.8)	5 (11.63)	38 (88.37)	
HER2 average overall	N (Row %)	0	8 (11.76)	60 (88.24)	0.699
	N (Row %)	0.025-0.3	3 (15.79)	16 (84.21)	
HER3 average overall	N (Row %)	≤ Median (0.1625)	6 (13.95)	37 (86.05)	0.747
	N (Row %)	> Median (0.1625)	5 (11.63)	38 (88.37)	
PD-L1					
Covariate	Statistics	Level	0 N = 69	1-130 N = 23	P-value ^a
EGFR average overall	N (Row %)	≤ Median (0.8)	38 (84.44)	7 (15.56)	0.061
	N (Row %)	> Median (0.8)	29 (67.44)	14 (32.56)	
HER2 average overall	N (Row %)	0	51 (73.91)	18 (26.09)	0.770
	N (Row %)	0.025-0.3	16 (80)	4 (20)	
HER3 average overall	N (Row %)	≤ Median (0.1625)	34 (77.27)	10 (22.73)	0.622
	N (Row %)	> Median (0.1625)	32 (72.73)	12 (27.27)	
PD-L2					
Covariate	Statistics	Level	0 N = 31	1-130 N = 51	P-value ^a
EGFR average overall	N (Row %)	≤ Median (0.8)	15 (41.67)	21 (58.33)	0.686
	N (Row %)	> Median (0.8)	16 (37.21)	27 (62.79)	
HER2 average overall	N (Row %)	0	22 (36.07)	39 (63.93)	0.520
	N (Row %)	0.025-0.3	8 (44.44)	10 (55.56)	
HER3 average overall	N (Row %)	≤ Median (0.1625)	13 (36.11)	23 (63.89)	0.755
	N (Row %)	> Median (0.1625)	17 (39.53)	26 (60.47)	

^aThe P-value is calculated by chi-square test or Fisher's exact, where appropriate.

Table 4. Multivariate analysis of advanced nodal metastasis

Covariate	Level	Node metastasis = N2-N3	
		OR (95% CI)	OR P-value
PD-L1	1-130	5.53 (1.06-28.77)	0.042
	0	—	—
PD-L2	1-130	3.12 (0.71-10.99)	0.054
	0	—	—
PD-1 positive lymphocytes in tumor	0-1	0.87 (0.31-2.48)	0.795
	2-4	—	—
PD-1 positive lymphocytes in peritumoral stroma	0-1	2.44 (0.43-13.91)	0.316
	2-4	—	—

seen in this population was observed regardless of p16 status (Fig. 1A). Tumor expression of PD-L1, EGFR, HER2, and HER3, and PD-1 positive lymphocyte density within the tumor were not associated with overall survival (Table 5; Fig. 1B and C). In addition, PD-L2 overall was not associated with survival, however, for p16- OPSCC tumors, those that did not express PD-L2 had worse survival outcomes (HR 5.29; 95% CI, 1.64-17.03; $P = 0.005$; Fig. 1D).

Discussion

As immunotherapy has ushered in a new era for the treatment of SCCHN and PD-1 inhibitors are now standard of care in the treatment of recurrent or metastatic SCCHN, further investigation of immune-related biomarkers and their interaction with other cancer-related pathways is warranted. In our study, we examined potential correlations between EGFR, HER2, HER3, PD-1, PD-L1, and PD-L2 expression and their association with clinical characteristics and outcomes in patients with OPSCC.

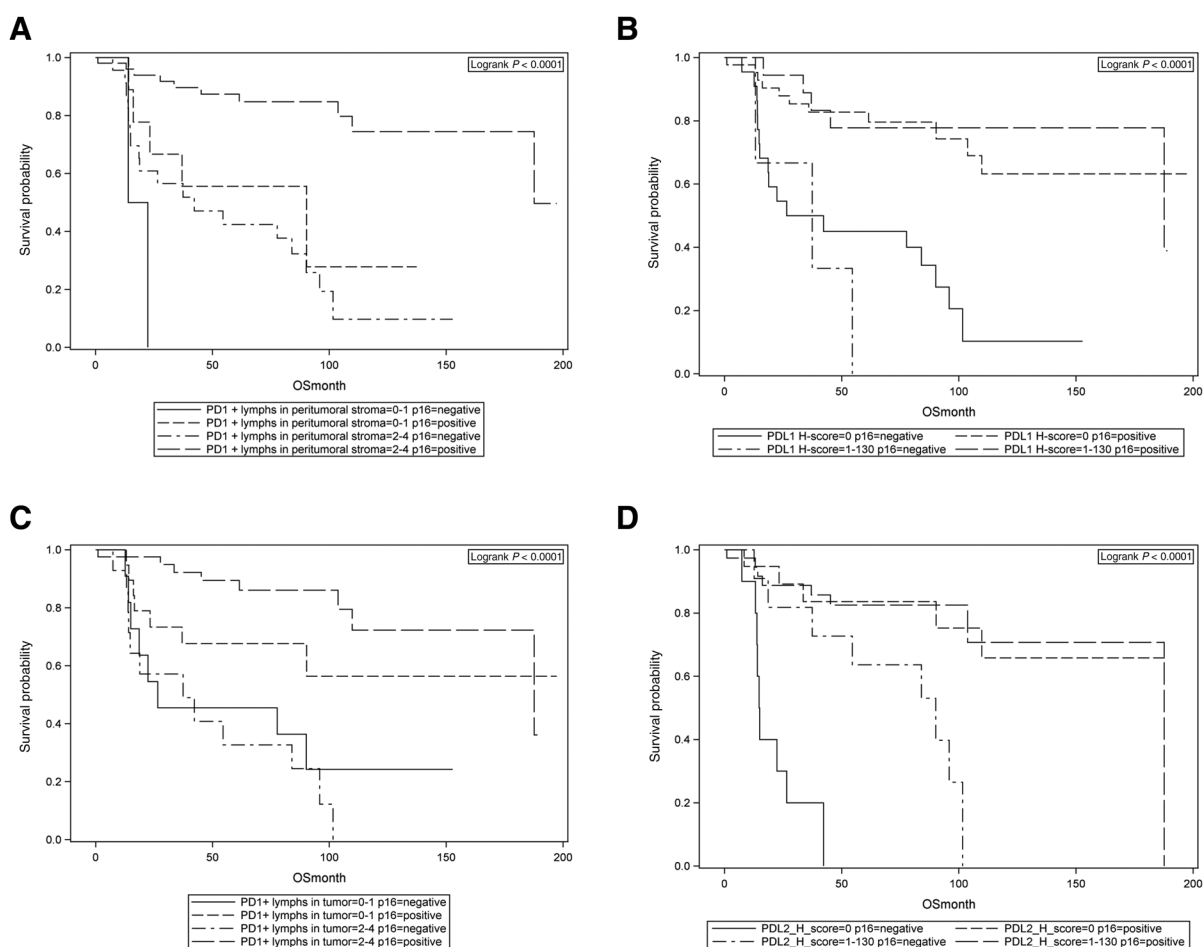
Our study demonstrated that the density of PD-1 positive lymphocytes in peritumoral stroma correlates with increased mortality. This highlights the potential role the tumor microenvironment may play in OPSCC. However, given that the PD-L1/PD-1 pathway acts as a "brake" of the immune system, it was expected that increased PD-1 signaling on tumor infiltrating lymphocytes (TIL) and/or the peritumoral stroma would be associated with worse outcomes (15). Previous studies have demonstrated the activity of the PD-L1/PD-1 pathway in HPV-driven OPSCC, theorizing the potential role immune resistance may have in these tumors (16). Badoual and colleagues examined the role of PD-1 positive TILs as a prognostic biomarker in

HPV-associated OPSCC. Not only did HPV-associated OPSCC have higher levels of TILs, but the increased infiltration of TILs around the tumor correlated with better prognosis for this group of patients. A possible explanation of these findings is that PD-1 status could be a marker of immune activation during HPV-driven OPSCC tumorigenesis, leading to the observed improved outcomes (8). Other studies have also demonstrated that the density of TILs in SCCHN may predict outcome (17, 18). However, evidence is mounting that there is heterogeneity within the tumor microenvironment and more complete characterization of TILs is lacking (19). This problem is noted in other tumor types as well. Increased PD-1 TIL signaling in the tumor microenvironment has been shown to be a positive prognostic marker in a variety of tumor types, including ovarian and pancreatic tumors, whereas high PD-1 TIL expression was associated with worse outcomes in other histologies such as renal cell carcinoma (20-22). Our study demonstrates a correlation between PD-1 staining on TILs within the tumor microenvironment, specifically the peritumoral region, and better survival in both p16 positive and negative cohorts. Interestingly, PD-L1 expression was not associated with survival. There was a correlation between higher PD-L1 expression and increased nodal status (P -value = 0.042). In addition, PD-L2 is currently being examined as potentially playing an important role in the PD-1 pathway. Although less studied than PD-L1, PD-L2 has been shown to play an important role in immunosuppression and T-cell function (23). Therefore, it holds interest as a potential biomarker and target for immunotherapy treatment. In fact, Yearley and colleagues examined PD-L2 expression across tumor types, and found that HNSCC had the highest level of tumor staining, at greater than 50% of HNSCC tested. This correlates with our data where we found 61% of tumors were PD-L2

Table 5. Multivariate survival analysis

Covariate	Level	N	Median survival (m) (95% CI)	OS month	
				HR (95% CI)	HR P-value
EGFR average overall	≤ Median (0.8)	47	109.8 (90.3, NA)	0.77 (0.35-1.69)	0.516
	>Median (0.8)	43	90.2 (37.4-187.6)	—	—
HER3 average overall	≤Median (0.1625)	46	103.7 (84-187.6)	0.89 (0.39-2.03)	0.778
	>Median (0.1625)	44	109.8 (22.4, NA)	—	—
HER2 average overall	0	68	103.7 (90.2-187.6)	0.70 (0.29-1.65)	0.414
	0.025-0.3	23	42.3 (18.6, NA)	—	—
PD-L1	0	67	101.6 (84, NA)	1.06 (0.61-3.01)	0.911
	1-130	23	187.6 (45.2-187.6)	—	—
PD-L2	0	30	109.8	1.44 (0.61-3.43)-	0.406
	1-130	50	103.7	—	—
PD-1 positive lymphocytes in tumor	0-1	33	90.2 (23.2, NA)	1.11 (0.51-2.42)	0.797
	2-4	55	109.8 (95.8-187.6)	—	—
PD-1 positive lymphocytes in peritumoral stroma	0-1	11	37 (14.2, NA)	3.17 (1.03-9.78)	0.045
	2-4	77	187.6 (90.2, NA)	—	—

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**Figure 1.**

A, Overall survival based on PD-1 in peritumoral stroma. **B**, Overall survival based on PD-L1 in tumor. **C**, Overall survival based on PD-1 in tumor. **D**, Overall survival based on PD-L2 in tumor.

positive. In addition, they found that PD-L2 was an independent predictor of PFS and OS for patients undergoing treatment with pembrolizumab (9). Our study showed that PD-L2 is a poor prognostic marker for p16 negative HNSCC patients, and highlights that this group may get superior benefit from checkpoint blockade.

The EGFR, HER2, and HER3 pathways have been proven to play important roles in the tumorigenesis of SCCHN (24). Prior work from our group demonstrated that high heregulin mRNA and high HER3 protein levels were associated with worse prognosis (12). The importance of these pathways has led to the development of novel therapeutics for the treatment of this patient population, such as the anti-EGFR antibody cetuximab, now FDA-approved for SCCHN in both the definitive and metastatic setting (1, 25). Of additional interest is that several studies have demonstrated an association of the EGFR pathway with the immune system in SCCHN. This has been less well defined for HER2 and HER3 (26, 27). We did not see a correlation between EGFR, HER2, and HER3 expression analyzed by IHC and immune markers or overall outcomes in our study. Dynamic expression of these markers is expected during the course of therapy and therefore analyses such as ours

may not reflect an accurate picture of the interaction between these pathways. PD-1 and PD-L1 expression have been shown to be upregulated in relation to treatment with concurrent chemotherapy and radiation in HPV positive SCCHN patients and other tumors (28, 29). Further insight into the dynamic interplay of the EGFR pathway and PD-1/PD-L1 is needed and may help guide the design of future clinical trials using these combinations.

Despite the unique results of our study, there are important limitations that should be noted. Given the retrospective nature of our analysis and that patients were treated with curative modalities, we were unable to assess the predictive value of the biomarkers studied in regards to immunotherapy. Furthermore, biopsies may not provide enough information regarding the topography of the entire tumor to relay a more accurate status for PD-L2/PD-1/PD-L1 and TILs. In the biomarker analysis of the KEYNOTE-012 study, PD-L1 expression correlated with response and PFS in patients treated with pembrolizumab in the metastatic setting, although the sample size was too small to make any firm conclusions (30, 31). Likewise, in the Checkmate-141 phase III trial examining nivolumab in comparison to investigator's choice chemotherapy in recurrent or metastatic SCCHN post-platinum

exposure, a trend for improved survival in patients receiving nivolumab was noted in the PD-L1 positive (PD-L1 \geq 1%) group (32). These results cannot, however, be considered conclusive given the nature of the analysis (2). Further exploration of relevant biomarkers is underway in these landmark studies. More work is needed to better define the population that will benefit from these new drugs.

As the future of SCCHN treatment will rely on different combinations of immunotherapeutic agents with or without cytotoxic or targeted systemic therapy, understanding the interactions between anti-cancer agents, tumor biology, and the microenvironment is of utmost importance. Our results add to existing evidence suggesting that the tumor immune microenvironment plays a role in OPSCC and ought to be a major focus of future research.

Disclosure of Potential Conflicts of Interest

C.E. Steuer is a consultant/advisory board member of Merck and EMD Serono. M.R. Patel has received speakers bureau honoraria from Intuitive Surgical. Nabil F. Saba is a consultant/advisory board member of BMS, Merck, Pfizer, and Lilly. No potential conflicts of interest were disclosed by the other authors.

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