PD-L1/PD1 Expression, Composition of Tumor-Associated Immune Infiltrate, and HPV Status in Conjunctival Squamous Cell Carcinoma

Priyadharsini Nagarajan,[1] Christian El-Hadad,[2] Stephen K. Gruschkus,[3] Jing Ning,[3] Courtney W. Hudgens,[1] Oded Sagiv,[2] Neil Gross,[4] Michael T. Tetzlaff,[1,5] and Bita Esmaeli[2]

[1]Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States
[2]Orbital Oncology and Ophthalmic Plastic Surgery, Department of Plastic Surgery, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States
[3]Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States
[4]Department of Head and Neck Surgery, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States
[5]Department of Translational and Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States

Correspondence: Bita Esmaeli, Orbital Oncology and Ophthalmic Plastic Surgery, Department of Plastic Surgery, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 1488, Houston, TX 77030, USA; besmaeli@mdanderson.org.

Submitted: February 15, 2019
Accepted: April 29, 2019

Citation: Nagarajan P, El-Hadad C, Gruschkus SK, et al. PD-L1/PD1 expression, composition of tumor-associated immune infiltrate, and HPV status in conjunctival squamous cell carcinoma. *Invest Ophthalmol Vis Sci*. 2019;60:2388–2398. https://doi.org/10.1167/iovs.19-26894

Purpose. Conjunctival squamous cell carcinoma (SCC), a type of ocular surface neoplasia, is primarily treated by surgical resection and topical immuno- or chemotherapy. Metastatic disease may be treated with systemic chemo- or immunotherapy, albeit with variable response. The purpose of this study was to determine whether immune checkpoint blockade might be considered in the management of conjunctival SCC.

Methods. In this retrospective study, we evaluated tumor programmed death-ligand 1 (PD-L1) expression, high-risk human papillomavirus (HPV) status, and immunohistochemical expression of cluster of differentiation 3 (CD3), cluster of differentiation 8 (CD8), and programmed death 1 (PD1) in tumor-associated immune infiltrate in a series of 31 conjunctival SCCs.

Results. PD-L1 expression in $\geq$1% of tumor cells was noted in 14 conjunctival SCCs (47%) and was more prevalent in invasive than in situ SCC and among tumors with higher American Joint Committee on Cancer (AJCC) T category ($\geq$T3 versus $\leq$T2). The density of CD3-positive T cells was higher in primary than recurrent tumors and higher in invasive than in situ tumors. Density of CD3-positive and CD8-positive T cells was higher in higher AJCC stage tumors. Density of CD8-positive T cells was higher in HPV-positive than HPV-negative tumors. PD-L1 expression correlated with a higher density of CD3-, CD8-, and PD1-positive cells in the tumor-associated immune infiltrate but not with HPV status.

Conclusions. Our findings demonstrate that PD-L1 is expressed in almost half of conjunctival SCCs. The density of tumor-associated immune cells correlated with invasive SCC, stage, and HPV status in conjunctival SCC. Our findings support further studies to establish the potential application of immune checkpoint blockade in the management of conjunctival SCC.

Keywords: ocular surface squamous neoplasia, conjunctiva, squamous cell carcinoma, oncology

Ocular surface squamous neoplasia encompasses a spectrum of disease that includes conjunctival premalignant dysplasia, carcinoma in situ, and invasive conjunctival squamous cell carcinoma (SCC).[1] The annual incidence of ocular surface squamous neoplasia ranges from 0.02 to 3.5 cases per 1 million population depending on the geographic location and its attendant degree of ultraviolet B light exposure.[2–8] Incidence is higher close to the equator and decreases with increasing latitude.[9] Additional predisposing factors include smoking; human papillomavirus (HPV) infection; immunosuppression, including human immunodeficiency virus (HIV)/AIDS, hematolymphoid malignancies, and transplant; chronic inflammatory states such as allergic conjunctivitis and cicatricial pemphigoid; male sex; and Fitzpatrick type I and II skin.[7,10–16]

Surgery and adjuvant cryotherapy have historically been considered standard for local management of conjunctival SCC; however, topical and/or intralesional chemotherapy and immunotherapy with interferon have recently gained popularity.[17–24] In the United States, the reported 1-year local recurrence rates after definitive therapy with any of the above-mentioned modalities range from 3% to 10%,[25,26] although the rates are much higher among immunocompromised patients.[27] The rate of regional lymph node metastasis is 0% to 16%,[27] but systemic dissemination is rare.[24,28,29] Patients with metastatic disease may require systemic therapy with cytotoxic agents and/or EGFR inhibitors and radiation thera-

py.[24,30,31] Patients with locally advanced and/or recurrent conjunctival SCC with orbital invasion may require orbital exenteration, a radical and debilitating surgery. Invasive conjunctival SCC with orbital invasion is endemic in parts of Africa and leads to significant morbidity.[12,32,33] Thus, new treatments are needed to effectively prevent recurrence and preserve ocular function.

Conjunctival SCC shares several features with cutaneous SCC, including demographic characteristics (predominance in males and individuals with Fitzpatrick type I and II skin), etiopathogenic factors (high cumulative ultraviolet B light exposure, immunosuppression, and chronic inflammatory states), management strategies (surgical resection, cryo-, photodynamic, topical, systemic, and radiation therapies), and genomic alterations (gains in 3q22.3–3q28 and 5p and losses in 9p, 13q, 17p, and 18q).[34] Moreover, the presence of primary cutaneous SCC or basal cell carcinoma is correlated with increased risk for developing conjunctival SCC.[8]

Management of advanced cutaneous SCC has not been standardized; however, since the advent of immune checkpoint therapy, studies have evaluated the role of tumor-infiltrating lymphocytes[35,36] and programmed death-ligand 1 (PD-L1) expression in tumor cells in cutaneous SCC[37,38] and have established the efficacy of programmed death 1 (PD1) blockade in the management of advanced cutaneous SCC.[39,40] To explore whether use of PD1/PD-L1-based immunotherapy might also be feasible in conjunctival SCC, we evaluated, in a series of 31 conjunctival SCCs, the expression of PD-L1 within tumor cells; the composition and density of tumor-associated immune infiltrates; relationships between PD-L1 expression, immune infiltrates, and clinicopathologic features, including HPV status; and relationships between various patient and disease features and prognosis.

Methods

Selection of Cases and Collation of Clinicopathologic Data

This study was approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center and adhered to the tenets of the Declaration of Helsinki. We identified 42 patients treated by the ophthalmic plastic surgeon (BE), between July 2009 and April 2017, of which, we were able to procure tissue blocks with residual tumor from the archived surgical specimens for 31 patients. For each patient, we collected demographic features (age at initial diagnosis and at presentation to our institution, sex, and ethnicity), risk factors for conjunctival SCC (cutaneous SCC, hematolymphoid malignancy, HIV positivity, and transplant), primary tumor features (laterality, anatomic site, disease status at presentation [primary or recurrent], in situ versus invasive disease, and size/diameter of invasive carcinoma), extraconjunctival extension, American Joint Committee on Cancer (AJCC) stage (Supplementary Table S1), local recurrence (anatomic site and date), metastases (regional or distant and date), and vital status at last follow-up and cause of death. Types of surgery and adjuvant therapy were also recorded.

Immunohistochemistry and In Situ Hybridization

Immunohistochemical studies were performed on 4-µm-thick tissue sections using a Leica Bond III autostainer, using the following antibodies: cluster of differentiation 3 (CD3) (catalog no. A0452; dilutions 1:100; Dako, Carpinteria, CA, USA), cluster of differentiation 8 (CD8) (catalog no. MS457s; dilutions 1:25; Life Sciences Technology, Waltham, MA, USA), PD1 (catalog no. ab137132; dilutions 1:250; Abcam, Burlingame, CA, USA), and PD-L1 (catalog no. 13684S; dilutions 1:100; Cell Signaling Technology, Danvers, MA, USA). RNA in situ hybridization (ISH) to identify high-risk HPV (types 16, 18, 31, 33, 35, 45, 52, and 58) was performed using the RNAscope 2.5 LS assay.

Image Analysis

Slides immunostained for PD-L1 or subjected to HPV ISH were evaluated in a blinded manner by two pathologists (MTT and PN). PD-L1 expression was visually estimated as the percentage of tumor cells that displayed complete or partial membranous staining. Tumors with PD-L1 staining in $\geq$1% of tumor cells were considered PD-L1 positive, on the basis of previous studies of cutaneous SCC.[37,38,41] Of the 31 cases evaluated, 1 did not contain tumor cells for analysis on the PD-L1-immunostained slide. HPV status was determined by visual evaluation for the presence of punctate nuclear signals within tumor nuclei at 100× magnification and was scored as positive or negative.

Automated image analysis for quantification of lymphocytes expressing CD3, CD8, and PD1 was performed as described previously.[42] Briefly, the immunostained slides were scanned at 200× magnification by using the Aperio Scanscope AT Turbo instrument (Leica Biosystems, Buffalo Grove, IL, USA). Using the Aperio ImageScope image analysis software, we used 3 to 5 squares, each measuring 0.25 mm^2, to designate regions of interest (ROI) for analysis. First, a square was drawn in the region of tumor-stroma interface with the highest density of immune infiltrate, and then 2 to 4 additional squares (depending on the size of tumor available for evaluation) were drawn in peritumoral and intratumoral regions with progressively decreasing density of immune infiltrate. To ensure optimal image analysis of tumor-associated immune infiltrate, the ROIs were first selected on CD3-immunostained slides, and then the corresponding ROIs were designated on CD8- and PD1-immunostained slides. Positive cells were counted within each square, tabulated either as raw number or as the percentage of the nucleated cells in the designated regions, and reported as overall (average of all designated squares) and "hotspot" (highest-density square) per mm^2 for each sample.

Statistical Analysis

The clinical and histopathologic characteristics were summarized using descriptive statistics. Correlations between immunohistochemical, demographic, and clinicopathologic factors were assessed using the Wilcoxon rank sum, Fisher's exact, and Pearson's correlation tests. Recurrence-free survival (RFS) was defined as the time from surgery to disease recurrence or death from any cause, whichever occurred first. Metastasis-free survival (MFS) was defined as the time from surgery to diagnosis of metastasis or death from any cause, whichever occurred first. Disease-free survival (DFS) was defined as the time from surgery to death. Patients not experiencing the event of interest were censored at their date of last follow-up for each survival outcome, and the survival curves were estimated by the Kaplan-Meier method.[43] Cox regression modeling using Firth's penalized likelihood approach was used to evaluate correlations between demographic, clinicopathologic, and immunohistochemical features and survival outcomes. All statistical tests used a significance level of 5% and were performed using SAS 9.3 for Windows (SAS Institute Inc., Cary, NC, USA).

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Table 1. Demographic, Clinicopathologic, and Immunohistochemical Parameters in Patients With Conjunctival SCC and Correlation With Tumor HPV Status and Proportion of Tumor Cells Expressing PD-L1

		Correlation With:	
Clinicopathologic Parameters	**Value**	**HPV Status, *P* Value***	**PD-L1 Expression, *P* Value***
Age at presentation, y		0.40	0.72
Mean (SD)	61 (10)		
Median (min, max)	62 (36, 81)		
Sex, *n*		0.68	0.73
Male	16 (52%)		
Female	15 (49%)		
Ethnicity, *n*		0.16	0.22
White	28 (90%)		
African American	2 (7%)		
Hispanic	1 (3%)		
Tumor site, *n*†		1.00†	1.00†
Bulbar conjunctiva	18 (65%)		
Palpebral conjunctiva	11 (39%)		
Fornix	2 (7%)		
Tarsal conjunctiva	5 (18%)		
Caruncle	6 (21%)		
Cornea	4 (14%)		
Limbus	2 (7%)		
Eyelid	1 (3%)		
Laterality, *n*		0.15	0.67
Right	8 (26%)		
Left	23 (74%)		
Risk factors for conjunctival SCC, *n*		1.00	1.00
Absent	25 (81%)		
Present	6 (19%)		
Cutaneous SCC only	3 (10%)		
Lymphoma	2 (6%)		
Transplant (renal) + cutaneous SCC	1 (3%)		
SCC type, *n*		0.64	**0.04**
In situ	8 (26%)		
Invasive	23 (74%)		
Size of invasive carcinoma, mm		30	0.52
Mean (SD)	15 (5)		
Median (min, max)	15 (8, 25)		
Tumor type at presentation, *n*		0.64	1.00
Primary	24 (77%)		
Recurrent	7 (23%)		
Extraconjunctival extension, *n*		**0.05**	0.67
Absent	24 (77%)		
Present	7 (23%)		
Orbit	6 (19%)		
Subconjunctival/scleral	2 (7%)		
Bone	1 (3%)		
AJCC 8th edition TNM grouping, *n*		**0.05**‡	**0.01**‡
TisN0M0	8 (26%)		
T1N0M0	0		
T2N0M0	6 (20%)		
T3N0M0	10 (32%)		
T3N1M0	1 (3%)		
T4aN0M0	5 (16%)		
T4bN1M0	1 (3%)		
Surgery type, *n*		–	–
Wide local excision	22 (71%)		
Orbital exenteration	9 (29%)		

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Table 1. Continued

Clinicopathologic Parameters	Value	Correlation With: HPV Status, *P* Value*	Correlation With: PD-L1 Expression, *P* Value*
Adjuvant therapy, *n*		–	–
No	13 (42%)		
Yes	18 (58%)		
Topical chemotherapy	11 (36%)		
Systemic chemotherapy	1 (3%)		
Cryotherapy	4 (13%)		
Immunotherapy	8 (26%)		
Immunohistochemical Markers			
HPV status in tumor cells, *n*		–	1.00
Negative	23 (74%)		
Positive	8 (26%)		
PD-L1 expression in tumor cells, *n*§		0.87	–
<1%	16 (53%)		
1%–10%	9 (30%)		
>10%	5 (17%)		
Overall CD3-positive cells/mm^2			
Mean (SD)	1641 (1051)	||	||
Median (min, max)	1504 (130, 4509)		
Hotspot CD3-positive cells/mm^2			
Mean (SD)	2990 (1777)	||	||
Median (min, max)	2705 (252, 6203)		
Overall CD8-positive cells/mm^2			
Mean (SD)	1121 (1033)	||	||
Median (min, max)	928 (39, 4831)		
Hotspot CD8-positive cells/mm^2			
Mean (SD)	1855 (1321)	||	||
Median (min, max)	1672 (60, 5188)		
Overall PD1-positive cells/mm^2			
Mean (SD)	143 (140)	||	||
Median (min, max)	104 (0, 648)		
Hotspot PD1-positive cells/mm^2			
Mean (SD)	267 (242)	||	||
Median (min, max)	195 (0, 920)		
Outcome			
Recurrence after curative therapy, *n*			
No	26 (84%)	–	–
Yes	5 (16%)		
Metastasis, *n*			
None	29 (94%)		
Regional	2 (6%)	1.00	0.21
Distant	0		
Disease-free at last follow-up, *n*			
Yes	28 (90%)	–	–
No	3 (10%)		
Vital status at last follow-up, *n*			
Dead	4 (13%)	–	–
Alive	27 (87%)		
Cause of death, *n*			
Metastatic conjunctival SCC	1 (25%)	–	–
Other	3 (75%)		

For en dash marks, see Supplementary Table 2 or not available. SD, standard deviation; TNM, tumor, nodes, and metastasis categories as defined by AJCC.

* *P* values based on Wilcoxon rank sum test for continuous variables and Fisher's exact test for categoric variables. Statistically significant *P* values are bolded.

† Anatomic site based on 1 vs. ≥2 sites involved by carcinoma; some patients had multiple sites of involvement.

‡ AJCC 8th edition T categories: Tis+T1+T2 vs. T3+T4.

§ PD-L1 status not available in one case because of inadequate tumor for analysis.

|| See Table 2.

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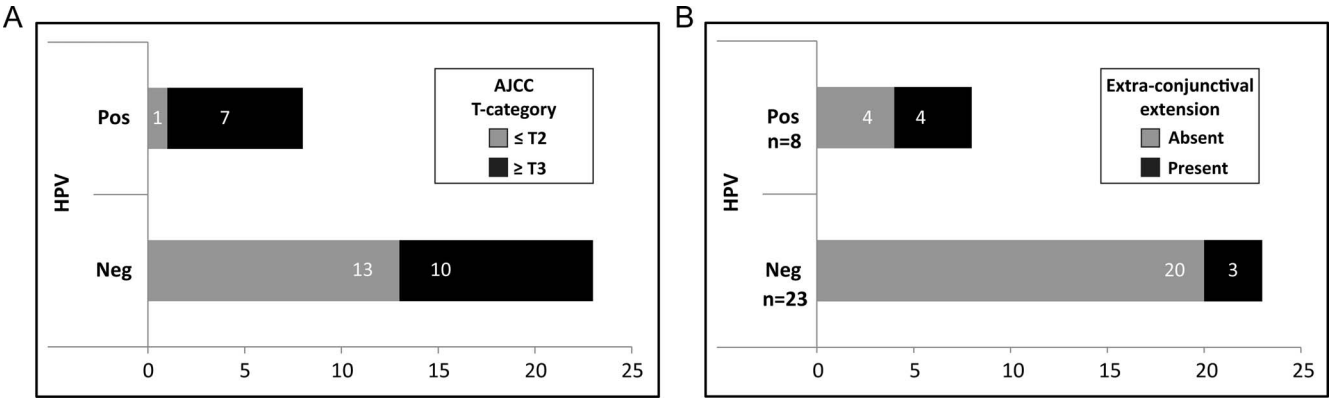

Figure 1. Correlation between HPV status and (**A**) AJCC T category and (**B**) extraconjunctival extension status in conjunctival SCC.

Results

Demographic and clinicopathologic characteristics of the cohort are summarized in Table 1. Twenty-eight of the 31 patients in our cohort (90%) were white; and the cohort included 16 men and 15 women, with a mean age at presentation of 61.3 years. Six patients had at least one risk factor for conjunctival SCC, and cutaneous SCC was the most common. The left eye was involved in 23 patients (74%), and 17 patients (54%) had contiguous involvement of multiple parts of the ocular surface epithelium. Seven patients (23%) presented with recurrent disease. Twenty-three patients (74%) had invasive SCC and eight (26%) had in situ tumor. There was a slight predominance of AJCC T category T3 or higher tumors ($n = 17$; 55%) (Supplementary Table S1). Orbital exenteration was necessary for local disease control in 9 patients (29%), and 18 patients (58%) underwent adjuvant therapy, most commonly topical chemotherapy (11 patients; 36%). Five patients (16%) experienced local recurrence after curative surgery, and two patients developed regional metastases. Of this group, one patient refused treatment and died with disease 13.3 months after diagnosis of regional metastases; the other patient was alive without disease at 31.9 months after diagnosis of regional metastases. Four patients (13%) died, and one of which was due to conjunctival SCC (described above). Twenty-eight patients (90%) were disease free at their last follow-up.

The majority of the conjunctival SCCs in our cohort were HPV negative ($n = 23$; 74%) (Table 1). Most of the HPV-negative primary tumors were AJCC T category ≤T2 (13/23, 57%), whereas most of the HPV-positive primary tumors were AJCC ≥T3 (7/8, 88%; $P < 0.05$) (Fig. 1A). The HPV-positive tumors exhibited a higher incidence of extraconjunctival extension than the HPV-negative tumors did (50% vs. 13%, $P = 0.05$) (Fig. 1B).

Fourteen of 30 tumors (47%) were PD-L1 positive (staining in ≥1% of tumor cells); of which in 5 tumors, more

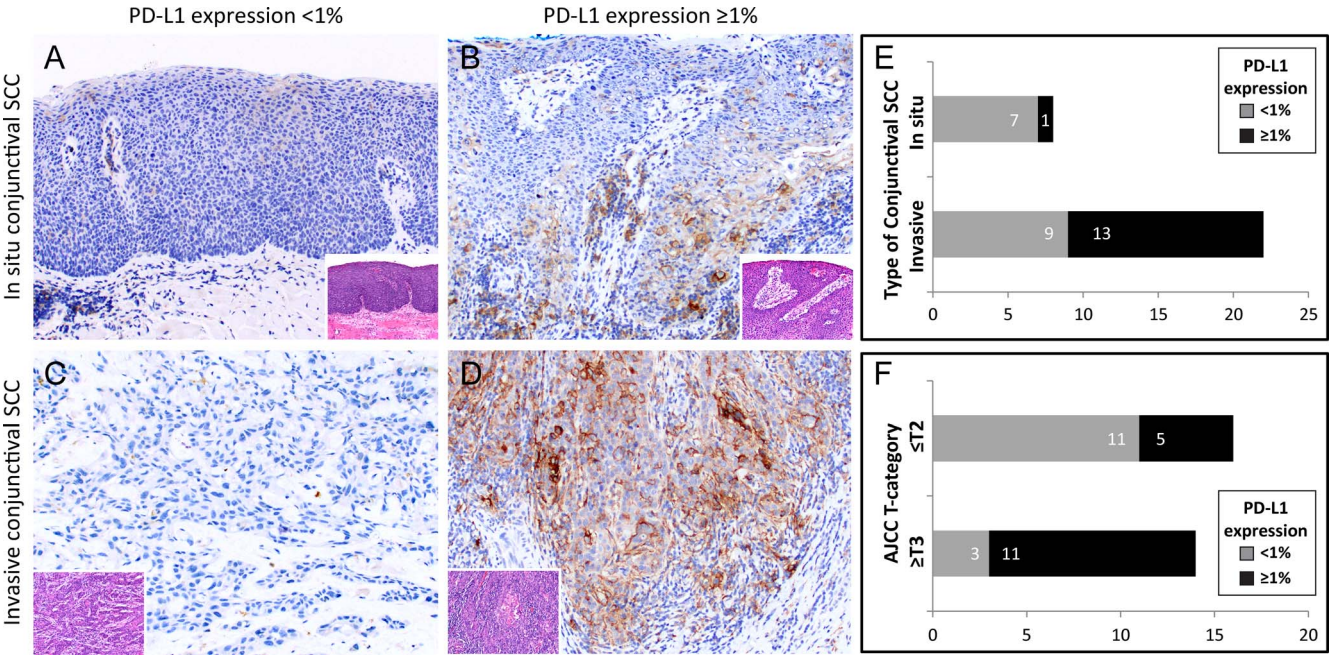

Figure 2. PD-L1 expression in conjunctival SCC and correlation between PD-L1 expression and tumor type and AJCC T category of conjunctival SCC. (**A–D**) Representative micrographs showing PD-L1 expression in (**A**, **B**) in situ and (**C**, **D**) invasive conjunctival SCCs with (**A**, **C**) <1% and (**B**, **D**) ≥1% of tumor cells staining positive for PD-L1 (magnification ×200). Inset: corresponding field in hematoxylin-eosin-stained section (magnification ×200). (**E**, **F**) Correlation between PD-L1 staining and (**E**) tumor type and (**F**) AJCC T category. PD-L1 status not available in one case because of inadequate tumor for analysis.

Table 2. Correlation Between Tumor HPV Status and PD-L1 Expression

HPV Status	Negative		Positive		
PD-L1	***n***	**%**	***n***	**%**	***P* Value***
<1%	12	54.5	4	50	1.00
≥1%	10	45.5	4	50%	
<1%	12	54.5	4	50	0.87
1%–10%	6	27.3	3	37.5	
>10%	4	18.2	1	12.5	

* *P* values based on Fisher's exact test.

than 10% of tumor cells expressed PD-L1 (Table 1). PD-L1 positivity was more prevalent among invasive SCCs ($n = 13$ of 22, 59%) than among in situ tumors ($n = 1$ of 8, 13%) ($P = 0.04$) (Figs. 2A–E) and more prevalent among tumors with higher (≥T3) AJCC T category (11 of 14, 79%, including the 2 tumors that gave rise to regional nodal metastasis) than among tumors with lower (≤T2) AJCC T category (5 of 16, 31%) ($P = 0.01$) (Fig. 2F). No correlation was noted between HPV status and tumor PD-L1 expression, irrespective of the percentage of tumor cells expressing PD-L1 (≥1% vs. 1%–10% and ≥10%) (Table 2; Fig. 3).

When the spatial distribution of CD3-, CD8- and PD1-positive immune infiltrate in and around the tumors were evaluated, we did not identify any obvious differences with respect to the HPV status or the pattern/percentage of PD-L1 expression in the primary (Fig. 3) or metastatic (Supplementary Fig. S1) tumors. However, the following correlations were

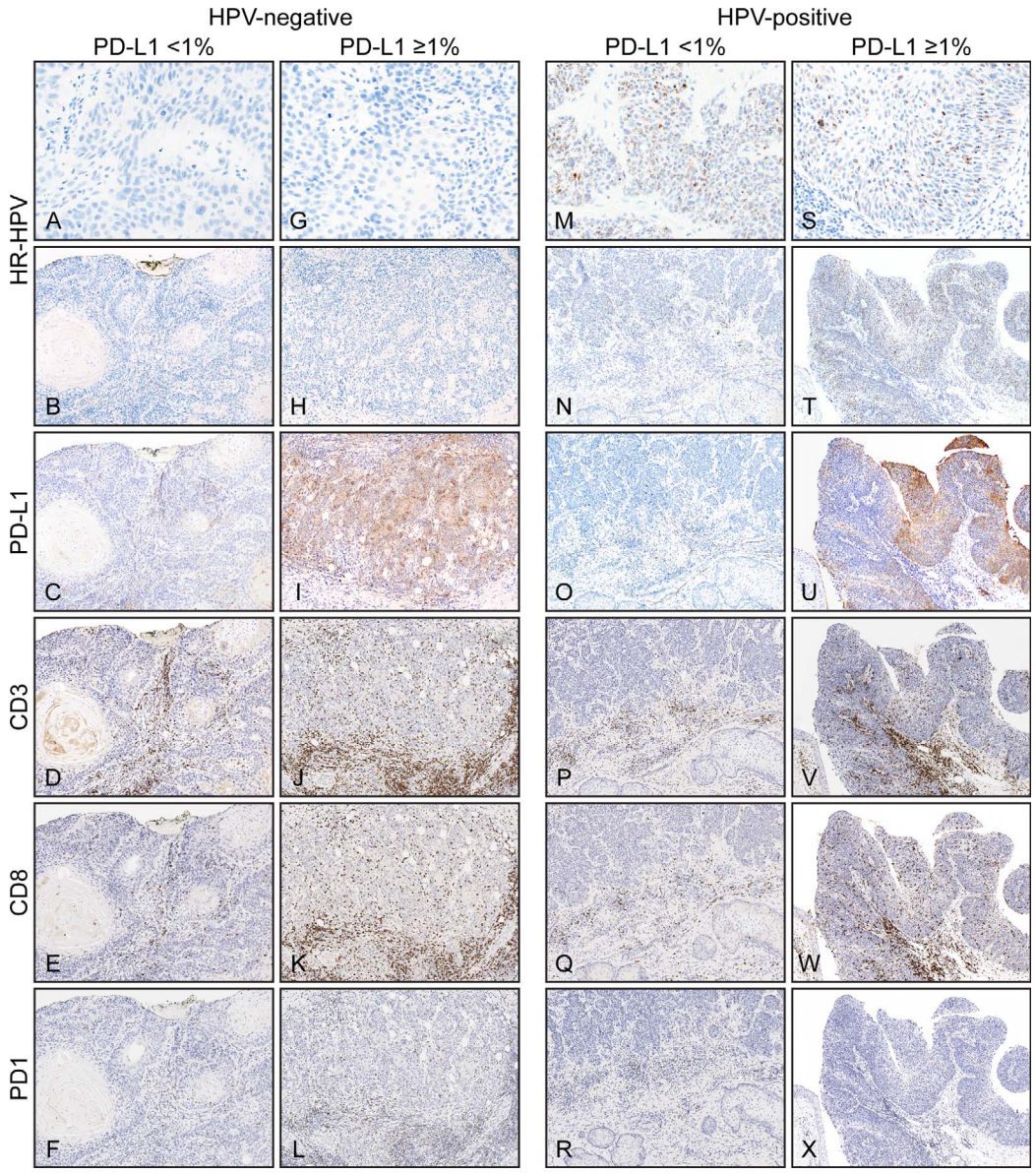

Figure 3. Expression of immune markers in primary conjunctival squamous cell carcinoma, with respect to HPV status: negative (**A–L**) versus positive (**M–X**) and PD-L1 expression in <1% (**A–F**, **M–R**) vs. ≥1% (**G–L**, **S–X**) of tumor cells. High-risk HPV: **A**, **G**, **M**, **S** (400× magnification); **B**, **H**, **N**, **T** (100× magnification); PD-L1: **C**, **I**, **O**, **U** (100× magnification); CD3: **D**, **J**, **P**, **V** (100× magnification); CD8: **E**, **K**, **Q**, **W** (100× magnification); PD1: **F**, **L**, **R**, **X** (100× magnification).

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Table 3. Correlation Between Clinicopathologic Characteristics, HPV and PD-L1 Status, and Immune Infiltrates

	P Value					
	% CD3-Positive Cells		% CD8-Positive Cells		% PD1-Positive Cells	
Variable	Overall	Hotspot	Overall	Hotspot	Overall	Hotspot
Mean (SD)	19.3 (9.5)	31.5 (14.0)	13.4 (9.2)	20.1 (11.3)	1.7 (1.3)	2.8 (2.1)
Median (min, max)	19.3 (1.1, 39.2)	30.3 (4.2, 57.5)	14.1 (0.8, 35.5)	19.2 (1.1, 41.7)	1.5 (0.0, 4.8)	2.6 (0.0, 7.7)
Age*	0.41	0.73	0.90	0.99	0.29	0.37
Sex†	0.13	0.20	0.29	0.24	0.19	0.23
Ethnicity†	0.48	0.44	1.00	0.82	0.07	0.07
Risk factors†	0.24	0.36	0.24	0.50	0.90	0.82
Laterality†	0.95	0.95	0.87	0.80	0.70	0.54
Primary vs. recurrent†	0.09	**0.04**	0.14	0.10	0.55	0.59
In situ vs. invasive†	**0.03**	0.08	0.06	0.08	0.10	0.06
Invasive tumor size*	0.52	0.29	0.33	0.37	0.51	0.29
AJCC 8th edition stage†	**0.03**	**0.05**	**0.03**	0.06	0.26	0.21
Extraconjunctival extension†	0.38	0.41	0.18	0.33	0.91	0.69
Anatomic site†‡	0.92	0.82	0.46	0.58	0.58	0.61
HPV status†	0.33	0.13	**0.04**	0.06	0.94	0.98
PD-L1 expression in tumor cells (<1% vs ≥1%)†	**0.0001**	**0.0003**	**0.0002**	**0.001**	**<0.0001**	**0.0002**

Statistically significant *P* values are bolded, and those approaching statistical significance are underlined.
* *P* values based on Pearson correlation.
† *P* values based on Wilcoxon rank-sum test.
‡ Anatomic site based on 1 vs. ≥2 sites involved by carcinoma.

observed between primary tumor features and tumor-associated immune infiltrate (Table 3; Supplementary Table S2; Fig. 4): (1) patients with recurrent tumors had lower hotspot CD3 expression than patients presenting with primary tumors ($P = 0.04$); (2) patients with invasive SCC had higher overall CD3 expression than patients with in situ tumors ($P = 0.03$); (3) AJCC T category ≥T3 was correlated with higher CD3 expression (overall, $P = 0.03$; hotspot, $P = 0.05$) and overall CD8 expression ($P = 0.03$), compared to T category ≤T2; (4) HPV-positive tumors had higher overall CD8 expression than HPV-negative tumors ($P = 0.04$); and (5) PD-L1 expression in ≥1% of tumor cells correlated with higher CD3 (overall, $P = 0.0001$; hotspot, $P = 0.0003$), CD8 (overall, $P = 0.0002$; hotspot, $P = 0.001$), and PD1 (overall, $P < 0.0001$; hotspot, $P = 0.0002$) expression in tumor-associated immune infiltrate.

Univariate Cox regression and Kaplan-Meier analyses did not reveal significant correlations between patient, tumor, or immune features and RFS, MFS, or DFS (Supplementary Table S2). However, the correlation between hotspot CD3 expression and DFS approached significance ($P = 0.09$) by univariate analysis. Also, MFS and DFS appeared to be slightly lower in patients with PD-L1-positive SCCs than PD-L1-negative tumors, although this was not statistically significant.

Discussion

To the best of our knowledge, this is one of the first studies to survey the prevalence of PD-L1 expression in conjunctival SCC and to evaluate the prognostic significance of tumor cell expression of PD-L1 and the density and composition of the tumor-associated immune infiltrates in conjunctival SCC. We and others have previously evaluated the expression of these markers in the context of response to immune checkpoint therapy in other malignancies, such as cutaneous malignancies such as Merkel cell carcinoma[42] and sebaceous carcinoma.[44] Many studies evaluating the PD-L1/PD1 axis often use only PD-L1 and PD1[45,46] or PD-L1 alone.[47] We chose to include CD3 and CD8 as well because CD3- and CD8-positive immune infiltrate density correlated with outcome in cutaneous malignancies such as Merkel cell carcinoma.[42] Inclusion of CD4 or FoxP3 did not proffer additional advantage because there was no significant association with clinicopathologic parameters or outcome.[44,48] Therefore, we chose to evaluate the expression of only CD3, CD8, PD-L1, and PD1 in this exploratory study. This small panel of immune markers was also advantageous because, in some cases, only limited amount of tumor was available for evaluation.

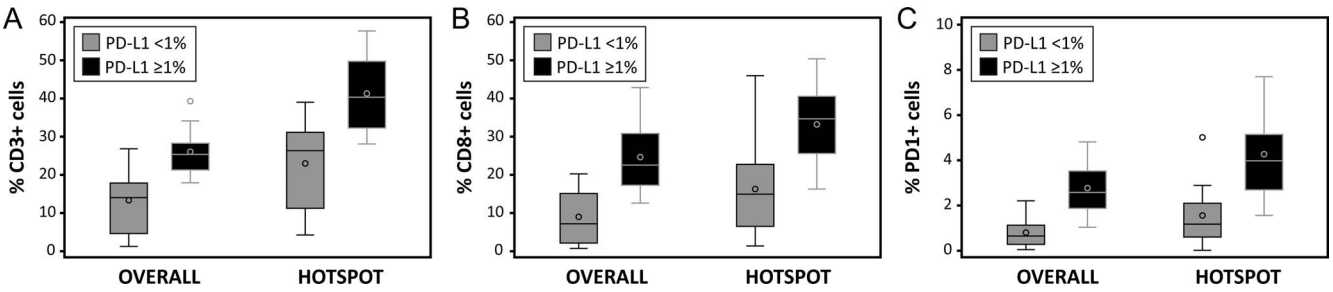

Figure 4. Relationships between immune infiltrate status and proportion of tumor cells expressing PD-L1 in conjunctival SCC. Shown are distributions of percentages of (**A**) CD3-positive, (**B**) CD8-positive, and (**C**) PD1-positive cells in the tumor-associated immune infiltrate according to PD-L1 expression.

In this study, we found that the tumor cells in almost half of conjunctival SCCs (47%) expressed PD-L1. The frequency of PD-L1 positivity in tumor cells was higher among invasive tumors and in tumors with higher AJCC T category (≥T3) and increased with increasing density of CD3-positive, CD8-positive, and PD1-positive cells in the tumor-associated immune infiltrate. Furthermore, we found that the density of CD3-positive cells in the inflammatory infiltrate was lower in patients presenting with recurrent tumors and the relative proportion of CD8-positive T lymphocytes was higher among HPV-positive tumors. PD-L1 expression did not correlate with outcome in our cohort, although there appeared to be a trend toward slightly lower MFS and DFS in patients with PD-L1-positive tumors.

We did not observe an independent association between the presence of risk factors such as cutaneous SCC or immunosuppression (Table 1; Supplementary Table S2) and expression of immune markers in our cohort. However, when immunosuppression was considered separately, the majority of patients with immunosuppression had lower PD-L1 expression, as well as lower overall numbers of immune cells positive for CD3, CD8, and PD1 (Supplementary Table S3). This finding, although statistically insignificant, suggests that the composition and density of tumor-associated immune infiltrate are likely influenced by multiple elements, which include systemic factors such as the patient's general immune competence, HIV status, and age-related immune senescence, as well as local factors such as HPV infection, chronic inflammatory diseases and ultraviolet light-induced immune dysregulation.[11,49,50] Therefore, studies with larger cohorts will be needed to evaluate if and how systemic and/or local immune dysregulation may affect the expression of immune markers and whether they may be modified to enhance antitumor effects against conjunctival SCC.

Our demonstration of PD-L1 expression in almost half of conjunctival SCC tumor samples is similar to the reported frequencies of PD-L1 expression in primary oropharyngeal mucosal SCC of head and neck (39.2%–87%)[48,51–53] and cutaneous SCC (25%–65%).[38,54–56] Our finding that the frequency of PD-L1 positivity in neoplastic cells increased with increasing conjunctival SCC AJCC T category is also similar to trends previously noted in cutaneous SCC. In a study of 45 cases of primary cutaneous SCC, the frequency of PD-L1 expression was reported to be higher in tumors with high-risk features for metastasis (50%–70%) than in low-risk tumors (20%–26%).[37,38] The reported frequency of PD-L1 expression in samples of metastases from patients with cutaneous SCC ranges from 39.5% to 100%.[37,38,52] In a study of 83 patients with head and neck cutaneous SCC, Amoils et al.[56] reported that the percentage of tumor cells positive for PD-L1 was higher in metastatic tumor samples than in primary tumor samples.

The demonstration of PD-L1 expression in conjunctival SCC raises the possibility of applying immune checkpoint blockade with agents targeting the PD1/PD-L1 axis—particularly among patients with locally advanced conjunctival SCC or those with orbital invasion, who are currently treated with radical disfiguring and disabling surgery, and/or among patients with metastatic disease, who tend to die of disease as there are currently few effective treatments.[24] Recent studies have demonstrated that inhibition of the PD1/PD-L1 axis is often effective in the management of advanced cutaneous SCC.[39] Similar, but inconsistent trends have also been noted in SCCs of other mucosal and cutaneous origins, and the inhibition of PD1/PD-L1 axis has been successful in the management of these malignancies.[39,57–60]

Immunohistochemical expression of PD-L1 on tumor cells and/or tumor-infiltrating immune cells has been shown to correlate with favorable response to PD1/PD-L1 inhibitor therapy in several malignancies, including melanoma[61] and nonsquamous non-small-cell lung carcinoma.[62] However, in other cancers, such as renal cell carcinoma and pulmonary SCCs, response to inhibition of the PD1/PD-L1 axis did not appear to correlate with PD-L1 expression on neoplastic cells.[63–65] Thus, in the majority of the previously evaluated malignancies, PD-L1 expression on tumor cells may be predictive of response to PD1/PD-L1 inhibitor therapy, particularly when supplemented by characteristics of immune infiltrate and other previously established biomarkers specific to that cancer as well as other tumor-related factors, such as tumor mutational burden.[66,67] However, PD-L1 expression status may not be independently predictive of response to PD1/PD-L1 inhibitor therapy.[68] For example, in the CheckMate 141 trial, patients with advanced, recurrent, or metastatic mucosal SCC of oral, oropharyngeal, hypopharyngeal, or laryngeal origin (that had progressed within 6 months from the last dose of platinum-based chemotherapy) were randomized to receive nivolumab monotherapy or investigator's choice single-agent chemotherapeutic agent.[69] Nivolumab therapy increased overall survival (OS) at 18, 24, and 30 months, irrespective of PD-L1 expression (<1% vs. ≥1%) and HPV status.[70]

Our finding that PD-L1 expression correlated with increased density of CD3-positive, CD8-positive, and PD1-positive cells in the tumor-associated immune infiltrate is similar to previously reported findings in melanoma[71] and head and neck cutaneous SCC.[52] Although it is unusual that HPV-negative tumors predominated in our cohort, the relative proportion of CD8-positive T lymphocytes was higher among HPV-positive conjunctival SCC. This may be secondary to T-cell activation in response to viral antigens. There was no correlation between HPV status and PD-L1 expression in our cohort. Similar results have been noted in oropharyngeal SCC, where majority of the tumors expressed PD-L1, irrespective of HPV status.[72] Therefore, it remains to be seen if PD-L1 expression may correlate with response to immunotherapy in conjunctival SCC.

Although we did not find any correlation between PD-L1 expression and tumor recurrence, the density of CD3-positive T cells was significantly lower in patients presenting with recurrent tumors in our cohort. Whether this reflects effects of altered microenvironment and/or evasion of immune surveillance is unclear. Kamiya et al.[73] found that a higher intensity of PD-L1 staining, but not the proportion of tumor cells with PD-L1 staining, correlated with nodal metastasis in cutaneous SCC. In contrast, Garcia-Pedrero et al.[74] found that PD-L1 expression in more than 25% of tumor cells correlated with lymph node metastasis in head and neck cutaneous SCC.

Several studies in other types of cutaneous and mucosal SCC have documented conflicting correlations between PD-L1 expression and clinical outcome. In head and neck cutaneous SCC, PD-L1 expression was reported to correlate with longer DFS.[52] In oral mucosal SCC, one study suggested that PD-L1 expression may be an indicator of poor outcome,[75] but a recent meta-analysis did not support this notion in oral SCC.[76] In an analysis of 133 cases of oropharyngeal SCC, PD-L1 expression in tumor cells did not correlate with OS, irrespective of HPV status.[72] Similarly, there was no clear association between PD-L1 positivity in tumor cells and OS in HPV-positive oropharyngeal carcinomas, whereas higher density of CD8-positive tumor-infiltrating lymphocytes correlated with improved OS.[77–79] On the other hand, PD-L1 expression correlated with increased risk for regional lymph node metastasis among head and neck mucosal SCCs, including those of oropharyngeal origin.[80]

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There are important limitations to the current study. There might be a bias for the referral of patients with high-risk and/or recurrent conjunctival SCC to our institution. Of these, patients were selected on the basis of availability of tumor samples for further analysis. Patients lacking adequate tumor samples and patients treated exclusively with topical or intralesional chemotherapy (who had no surgical specimen available for analysis) were excluded. In view of this selection bias, it is not surprising that PD-L1 expression did not predict clinical outcomes in our cohort. Some of the specimens included in our study were subjected to intraoperative frozen section evaluation; it is unclear if and how this might have affected the detection of PD-L1 expression. The percentage of CD3-positive, CD8-positive, and PD1-positive cells compared to all nucleated cells in the ROIs may have been underestimated in large tumors. Additionally, the size of our study cohort was small ($n = 31$) and there was a predominance of high-risk HPV-negative tumors in the cohort. Also, the length of follow-up (less than 2 years in some patients) may not be sufficient for long-term outcome analyses. Therefore, additional studies will be needed to corroborate our findings. Nevertheless, this initial survey of conjunctival SCC reveals that PD-L1 is expressed in almost half of tumors and correlates with increased density of CD3-positive, CD8-positive, and PD1-positive immune infiltrate, justifying the possible initiation of clinical trials to determine the efficacy of PD1/PD-L1 blockade in patients with locally advanced, recurrent, or metastatic conjunctival SCC.

Acknowledgments

Supported by the National Cancer Institute under award number P30CA016672, which supports the MD Anderson Cancer Center Clinical Trials Support Resource.

Disclosure: **P. Nagarajan**, None; **C. El-Hadad**, None; **S.K. Gruschkus**, None; **J. Ning**, None; **C.W. Hudgens**, None; **O. Sagiv**, None; **N. Gross**, None; **M.T. Tetzlaff**, None; **B. Esmaeli**, None

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