Original Study

PD-L1 and p16 Expression in Penile Squamous Cell Carcinoma From an Endemic Region

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Abstract

Penile carcinoma is a rare malignancy with higher incidence in developing countries. To understand its biology may help the development of new therapeutic strategies. A cohort of 40 patients was analyzed. Programmed death-ligand 1 (PD-L1) and p16 expression were performed. PD-L1 appears to be associated with p16, larger tumors, and worse clinical outcomes and may provide clinical data to evaluate anti-PD-L1 immune therapies.

Background: Penile squamous cell carcinoma (PSCC) is a rare malignancy with higher incidence in developing countries. Treatment options include surgery, radiation therapy, and systemic chemotherapy. However, effective treatments for advanced disease are lacking. To understand the biology underlying PSCC may help the development of new therapeutic strategies. The objective of this study was to **e**valuate immunohistochemical expression of programmed death-ligand 1 (PD-L1) and p16 in PSCC and its association with clinicopathologic features and outcomes. **Patients and Methods:** A cohort of 40 patients with PSCC from an academic institution in Brazil was analyzed. Clinicopathologic features and outcomes were retrospectively collected. PD-L1 and p16 immunohistochemical expression were performed in formalin-fixed paraffin-embedded specimens. PD-L1 was positive with any staining in more than 1% of tumor, and p16 was positive in more than 10%. Associations were performed using the Mann-Whitney and Fisher exact test. Kaplan-Meier curves were used to estimate survival rates with log-rank. **Results:** Of 35 patients, 5 were excluded, 4 owing to a lack of data and 1 owing to no tumor available; 18 (51.4%) patients were PD-L1-positive (PD-L1$^+$). PD-L1$^+$ was associated with larger tumors ($P = .027$). There was an association between PD-L1$^+$ and p16 expression ($P = .002$). PD-L1$^+$ was more frequent in grade II and III disease than grade I (77.8% vs. 22.2%) and was expressed in all patients with grade III disease. Lymph node involvement was associated with PD-L1 expression (69.2% PD-L1$^+$ vs. 30.8% PD-L1-negative). The 5-year mortality was 37.1%. **Conclusion:** PD-L1 expression appears to be associated with p16 expression, larger tumors, and worse clinical outcomes in PSCC and may provide clinical data for new studies to evaluate anti-PD-L1 immune therapies.

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Introduction

Penile squamous cell carcinoma (PSCC) is a rare and aggressive malignancy, which carries a major psychological impact.[1] In the United States, 2080 men were diagnosed with PSCC in the year of 2018, representing less than 0.4% of male malignancies, and 410 patients died from the disease. In contrast, PSCC is relatively common in developing countries affecting low income and non-circumcised males.[2] In Brazil, this tumor represents 2% of all men's cancer and is more frequent in the North and Northeast regions.[3] In this endemic area, two-thirds of patients are diagnosed with advanced disease (T2 or higher), resulting in a higher risk of local or systemic metastasis.

Worldwide, PSCC has a recurrence rate of about 29%, and lymph node metastases are found in 28% to 39%, depending upon the extent and grade of the tumor.[3] In a large series, stage I cases have an estimated 10-year survival of 89%.[4] In contrast, only 21% of patients with metastatic PSCC are alive at 2 years.[4,5] Effective therapeutic options for advanced penile cancers are lacking,

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partially owing to the poor understanding of the biology and genomic alterations that underlie the tumor development and progression.

Human papilloma virus (HPV) infection has been associated with the development of penile cancer in approximately 30% to 80% of the cases. Subtypes 6 and 11 are most often related to genital warts with low dysplasia. Subtypes 16, 18, 31, and 33 are associated with in situ carcinoma or invasive disease, with 16 being the most prevalent subtype and also detected in metastases of penile carcinoma.[6] p16 overexpression has been widely used as a surrogate marker for HPV infection in head and neck squamous cell carcinoma as well as uterine cervical cancers and penile cancers.[7,8] The role of HPV infection as a prognostic factor in penile cancer remains unclear in developing countries.[9]

Programmed cell death protein 1 (PD-1) is a co-inxhibitory receptor present on CD8-positive cytotoxic T cells and interacts with its ligand programmed death-ligand 1 (PD-L1) on tumor cell membranes, resulting in suppression of T-cell activation and proliferation and dampening of the host anti-tumor immune response.[10] Inhibiting the PD-1/PD-L1 immune checkpoint pathway should augment tumor cell killing by cytotoxic T cells.

Several studies have shown that PD-L1-positive (PD-L1$^+$) expression is associated with adverse clinicopathologic features and worse outcome in other malignancies, like renal-cell carcinoma and bladder cancer.[11,12] Furthermore, PD-L1 expression may be a biomarker to predict oncologic outcome and treatment response.[13,14]

In this study, we sought to characterize the immunohistochemical (IHC) expression of PD-L1 and p16 in PSCC from an endemic region and evaluate its association with clinicopathologic features and outcome.

Patients and Methods

A cohort of 40 patients treated between 1998 and 2016 at São Lucas Hospital of PUCRS were retrospectively selected. This is an academic hospital in the south of Brazil that is a referral center for PSCC treatment. Five of 40 patients were excluded from the study, 4 owing to lack of clinical data and 1 because no tumor specimens were available.

Medical records were retrospectively reviewed using standard templates. Clinicopathologic characteristics, such as age, size, histologic subtype, presence of lymph node invasion, inguinal lymphadenectomy surgery, pathologic staging, surgical margins, vascular invasion, perineural invasion, and postoperative follow-up, including death, were retrieved. Clinical outcomes were also collected. Five-year survival was defined as the date from diagnosis until 60 months after it. To define the survival rate, patients who did not have updated records of consultations at the institution were contacted by telephone for health information and/or date of death. The last follow-up information was obtained in October 2017.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) specimens were obtained from the pathology department. Specimens were analyzed by the HSL/PUCRS Pathology Department. Slides from a representative area of the tumor were prepared, and the IHC analysis for PD-L1 and p16 was performed.

PD-L1 expression was retrospectively evaluated by immunohistochemistry using the monoclonal anti-PD-L1 (colne ZR3, Zeta Corporation) on the DAKO autostainer system. The IHC expression of PD-L1 was evaluated by percentage of membrane and/or cytoplasm stained and was considered positive with any staining in more than 1% of tumor cell membrane.

Immunohistochemistry for p16 was also performed using the DAKO autostainer system using anti-p16 (clone G75-405, Zeta Corporation). Expression for p16 was defined as negative if positivity was lower than 10%.

Statistical Analysis

Quantitative data were described by median and minimum and maximum values. Categorical data were presented by counts and percentages. Comparisons between quantitative data were performed by the Mann-Whitney *U* test and categorical data by the Fisher exact test. Additionally, Kaplan-Meier curves were obtained to estimate the survival rates with statistical significance by the log-rank test. Association strength measures based on the hazard ratio (HR) and their respective confidence intervals (CIs) were estimated using Cox proportional hazards regression. The level of significance adopted in the study was alpha = 0.05. Data was processed and analyzed with IBM-SPSS software version 22.0.

Results

Patient Characteristics

Patient characteristics are summarized in Table 1. Briefly, the mean age was 64.6 years (SD, 12.4 years). The mean size of the primary lesions was 4.0 cm, ranging from 1.5 cm to 16 cm. Of the

Table 1 Patient Characteristics

Characteristic	n	%
Patients	35	100
Race		
White	33	94.2
Black	2	5.8
Subtype		
Usual	31	88.5
Verrucoso	04	11.5
Local		
Foreskin	23	65.7
Glans	33	94.2
Corpus spongiosum	17	48.5
Corpus cavernosum	9	25.7
Urethra	19	54.3
Grade		
I	13	37.2
II	19	54.3
III	03	8.6
Penectomy		
Partial	22	62.8
Total	13	37.2
Linfadenectomy	15	42.8

35 patients, 13 (37.1%) died during the study period. The mean follow-up of the patients was 46.87 months, ranging from 1.12 to 209.54 months.

PD-L1 Expression

Of the 35 primary penile PSCC tumors examined, 18 (51.4%) were considered PD-L1$^+$ (Figure 1). Percentage of tumor cell membrane staining ranged from 1% to 80%. Clinicopathologic characteristics of PD-L1$^+$ and PD-L1-negative (PD-L1$^-$) primary PSCC are presented in Table 2. PD-L1$^+$ was predominant in lesions affecting the glans (94.4%) and urethra (72.2%). Regarding tumor grade, 22.2% were grade I, 61.1% were grade II, and 16.6% were grade III. PD-L1 was expressed in 69% of the patients with lymph node involvement.

PD-L1$^+$ patients presented with larger tumors, with a median size of 4.25 cm ($P = .027$). There was statistical correlation between PD-L1$^+$ and p16 expression ($P = .002$). Patients with PD-L1$^+$ had a trend to present with high-grade tumors, grade II and III in 77.8% of the lesions versus 22.2% grade I. PD-L1 was expressed in all patients with grade III PSCC ($P = .061$). There was a 2-fold relationship in lymph node involvement of patients who expressed PD-L1 (69.2% of patients with lymph node involvement had PD-L1$^+$, and only 30.8% had lymph node involvement with PD-L1$^-$).

p16 Expression

IHC expression of p16 was considered positive in 13 (37.1%) patients (Figure 3). Tumor was predominating on the glans in 100% of the patients, on the urethra in 69.2%, and on the foreskin in 61.5%. Considering tumor grade, 23.1% were grade I, 61.5% were grade II, and 15.4% were grade III. p16 was expressed in 38.5% of patients with lymph node involvement. Clinicopathologic characteristics of p16 expression on PSCC are presented in Table 3.

Survival Outcomes

No statistically significant differences on survival at 5 years was observed according to PD-L1 expression. The 5-year survival in PD-L1$^+$ was 42.6% versus 59.2% in PD-L1$^-$. The HR was 2.13 with 95% CI ranging from 0.67 to 7.71 ($P = .199$) (Figure 2).

Similarly, no statistically significant differences on survival at 5 years was observed according to p16 expression. The 5-year survival in p16-positive (p16$^+$) was 28.2% versus 58.1% in p16$^-$. The HR was 2.23 with 95% CI ranging from 0.72 to 6.94 ($P = .167$) (Figure 4).

Figure 1 Programmed Death-Ligand 1-Positive Membranous Staining, 200×

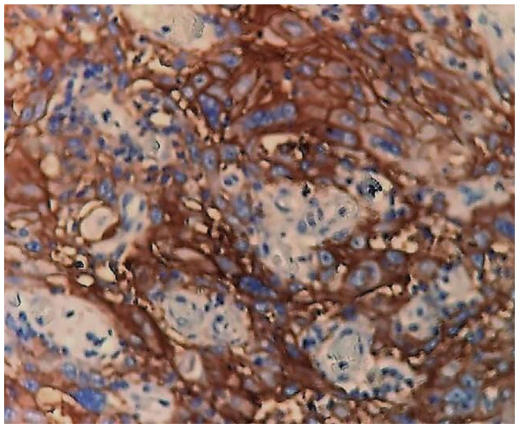


Table 2 Clinicopathologic Characteristics According to PD-L1 Expression in PSCC

	PD-L1$^-$ N = 17, n (%)	PD-L1$^+$ N = 18, n (%)	*P*
Size, cm			
Median	2.6	4.25	.027
Range	1.5-5.5	1.7-16.0	
Subtype			
Usual	14 (82.4)	17 (94.4)	.338
Verrucous	3 (17.6)	1 (5.6)	
Grade			
I	9 (52.9)	4 (22.2)	.061
II	8 (47.1)	11 (61.1)	
III	0	3 (16.7)	
Clinical stage			
Localized	14 (82.4)	13 (72.2)	.691
Advanced	3 (17.6)	3 (27.8)	
Perineural invasion	2 (11.8)	6 (33.3)	.228
Vascular invasion	1 (5.9)	4 (22.2)	.338
Lymph node-positive	4 (23.5)	9 (50.0)	.164
p16$^+$	3 (17.6)	10 (55.5)	.035

Abbreviations: PD-L1 = programmed death-ligand 1; PSCC = penile squamous cell carcinoma.

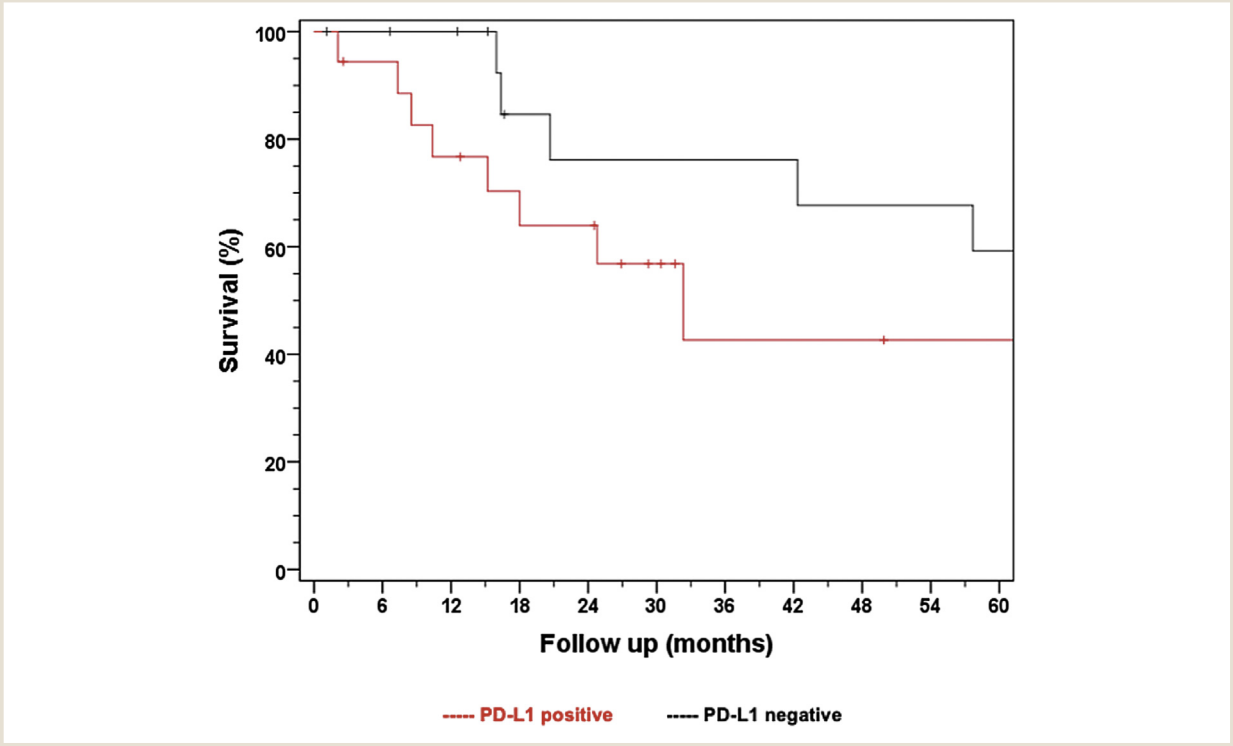
Discussion

Advanced PSCC is a rare and fatal malignancy with limited treatment options, including surgery, radiation therapy, and systemic chemotherapy. However, available therapies have not resulted in meaningful clinical benefit.[15] A challenge in developing novel systemic strategies for advanced penile cancer is the limited understanding of the oncogenic drivers of disease.

Important differences in terms of risk factors have been identified across distinct populations. In developed countries, PSCC is more common in regions with a higher prevalence of HPV (HPV-16 and HPV-18). Conversely, PSCC is associated with poor hygiene habits, tobacco use, and possibly sexual behavior, such as sex with animals, which is common in rural areas in Brazil. Understanding the diversity of PSCC biology and possible correlation with clinicopathologic characterization may help to define the role of HPV infection in the oncogenesis of PSCC, especially in a population from a developing country.

Recently, PD-L1 immunotherapy has been approved to be used in other advanced genitourinary tumors such as renal cell carcinoma and urothelial carcinoma.[13,16] Immunotherapy has been studied in few trials enrolling patients with PSCC (NCT02837042 and NCT02834013). Encouraging data has been published in a case

Figure 2 Overall Survival and PD-L1 Expression



Abbreviation: PD-L1 = programmed death-ligand 1.

Table 3 Clinicopathologic Characteristics According to p16 Expression in PSCC

	$p16^-$ N = 22, n (%)	$p16^+$ N = 13, n (%)	*P*
Size, cm			
Median	3.2	3.5	.682
Range	1.5-10.0	1.7-16.0	
Subtype			
Usual	19 (86.4)	12 (92.3)	.522
Verrucous	3 (13.6)	1 (7.7)	
Grade			
I	10 (45.5)	3 (23.1)	.297
II	11 (50.0)	8 (61.5)	
III	1 (4.5)	2 (15.4)	
Clinical stage			
Localized	16 (72.7)	11 (84.6)	.680
Advanced	6 (27.3)	2 (15.4)	
Perineural invasion	4 (18.2)	4 (30.8)	.433
Vascular invasion	3 (13.6)	2 (15.4)	.626
Lymph node-positive	6 (27.2)	7 (53.8)	.552
$PD\text{-}L1^+$	8 (36.4)	10 (76.9)	.035

Abbreviations: PD-L1 = programmed death-ligand 1; PSCC = penile squamous cell carcinoma.

report of a patient with chemoradiation-refractory advanced PSCC using nivolumab, presenting partial response with reduction of lymph node metastasis.[17] It has been reported that the response to anti-PD-1 therapy is correlated with PD-L1 tumor expression.[18]

Recently, Udager and colleagues were among the first to report the PD-L1 expression in a cohort of 37 patients with PSCC. The PD-L1 expression was positive in 62.2% of the PSCC. Despite the small size of the sample, they found that PD-L1 expression was mainly found in the usual type histology and was associated with

Figure 3 p16-Positive Nuclear Staining, 400×

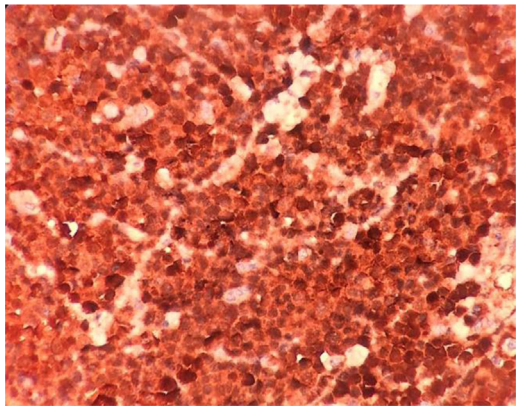
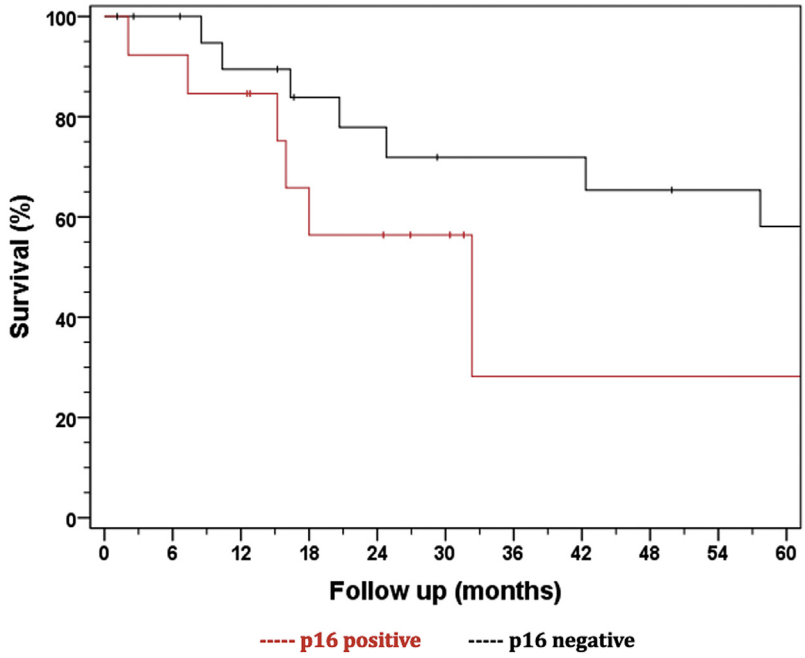


Figure 4 Overall Survival and p16 Expression



regional lymph node metastasis and decreased cancer-specific survival.[19] They also found that, among the warty and verrucous type histology, both with typically better outcomes, none expressed PD-L1. Similarly, our study has shown a non-statically significant association between higher tumor grade and PD-L1 expression. Interestingly, in their study, 9 patients with matched primary and metastatic PSSC had a strong correlation of PD-L1 expression.

In a retrospective multi-center study, Deng and colleagues evaluated the expression of PD-L1 in both tumor cells and tumor-infiltrating lymphocytes in a cohort of 116 patients. They found that 53.4% of the patients had positive expression of PD-L1 and that higher PD-L1 expression was associated with shorter cancer-specific survival and increased CD8+ tumor-infiltrating lymphocytes.[20]

In addition, a retrospective cohort evaluating 213 patients found that 48% were considered PD-L1$^+$ using the same cutoff for positivity (more than 1% of tumor cell staining). PD-L1$^+$ was significantly correlated with tumor grade of differentiation. In this study, they also analyzed the pattern of expression of PD-L1; 62% had a marginal expression, and 38% had a diffuse pattern. The diffuse expression was associated with lymph node metastases and poor survival. Of the PD-L1$^+$ patients, 82% were negative for HPV infection.[21] It is important to note that all patients came from developed countries.

In our study, approximately 51.4% of the patients with PSCC were PD-L1$^+$. Predominant histology was the usual subtype. PD-L1$^+$ tumors presented statistical significance with risk factors for disease of greater clinicopathological aggressiveness and were larger than those with PD-L1$^-$. We also found that patients with PSCC who were PD-L1$^+$ had a statistical correlation with p16 expression. p16 expression on this study is in contrast to those previously published by Ottenhof and colleagues.[21] There was a trend toward PD-L1 expression and high-grade tumors as found in previous studies.[19,21] Although it did not reach statistical significance, there was a 2-fold relationship in lymph node involvement in the PD-L1$^+$ group (69.2% vs. 30.8%). No differences were identified between the PD-L1 groups and the presence of vascular invasion and perineural invasion, both known as risk factors of aggressiveness in PSCC. No statistical significance in the analysis of 60-month survival of patients with PD-L1 expression was detected.

Two recent publications have outlined the genomic landscape of PSCC,[13,22] providing a rationale for targeted agents working in this disease. Key molecular alterations in PSCC are loss of heterozygosity at *CDKN2A*, alterations of *TP53*, and high levels of *EGFR* overexpression, suggesting that this signaling pathway may play an important role in PSCC carcinogenesis and tumor progression. Molecular studies from endemic countries to assess whether the biology is distinct, particularly in light of HPV status, are warranted.

Our study has several limitations. First, PSCC is a very rare disease, and we evaluated a small cohort of patients. In addition, the retrospective nature of analysis may affect the results. Furthermore, differences in choice of antibody, scoring method, and cutoff values may impact the IHC analysis and outcomes. In addition, considering the small group size for patients with PD-L1$^+$ and a small number of deaths, a multivariate analysis may not properly adjust the association of PD-L1 expression and clinical outcomes. Moreover, the relatively short follow-up period may influence the correlation of PD-L1 expression and survival.

According to previous studies evaluating the response to anti-PD-L1 immune therapy in different tumors,[13,22-24] the positivity in PD-L1 expression by tumor cells may be related to higher treatment response rates. There appears to be a possibility that some PSCC are susceptible to immune therapies involving anti-PD-L1.

In conclusion, PD-L1 expression appears to be associated with p16 expression, larger tumors, higher proliferative rate, and worse clinical outcomes in PSCC from developing countries. It provides a rational and opens new avenues for the investigation of PD-1/PD-L1 inhibitors in advanced disease.

Clinical Practice Points

- PSCC is a rare malignancy with higher incidence in developing countries. Treatment options include surgery, radiation therapy, and systemic chemotherapy. However, effective treatments for advanced disease are lacking. Understanding the biology underlying PSCC may help the development of new therapeutic strategies.
- A cohort of 40 patients with PSCC from an academic institution in Brazil was analyzed. Clinicopathologic features and outcomes were retrospectively collected.
- PD-L1 and p16 IHC expression were performed in FFPE specimens. PD-L1$^+$ was associated with larger tumors (P = .027). There was an association between PD-L1$^+$ and p16 expression (P = .002). PD-L1$^+$ was more frequent in grade II and III disease than grade I disease (77.8% vs. 22.2%) and was expressed in all patients with grade III disease.
- Lymph node involvement was associated with PD-L1 expression (69.2% PD-L1$^+$ vs. 30.8% PD-L1$^-$). The 5-year mortality was 37.1%.
- PD-L1 expression appears to be associated with p16 expression, larger tumors, and worse clinical outcomes in PSCC and may provide clinical data for new studies to evaluate anti-PD-L1 immune therapies.

Disclosure

The authors have stated that they have no conflicts of interest.

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