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ORIGINAL PAPER

Programmed death-ligand expression and lymph node involvement in penile squamous cell carcinoma

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Summary ***Purpose: Our objective was to investigate the association between programmed death-ligand (PD-L1) immunoexpression measured as a combined positive score and clinical outcomes in penile SqCC.***
Materials and methods: We retrospectively reviewed all penile SqCC cases diagnosed in our institution between 2018 and 2023. PD-L1 immunohistochemistry was performed as a qualitative assay. Immunoexpression in both tumor and immune cells equal or superior to 1 was considered positive.
Results: A total of 34 patients with conventional penile SqCC were included. Eleven cases were HPV-associated (32.4%). Twelve cases were PD-L1 CPS < 1 and twenty-two were PD-L1 CPS ≥ 1. Nine cases (32.4%) were PD-L1 CPS ≥ 1 and p16 positive, but this did not translate in worse clinicopathological features. Larger tumors (3.0 cm in PD-L1 CPS ≥ 1 vs 2.5 cm in PD-L1 CPS < 1; p = 0.662), vascular invasion (36.4% in PD-L1 CPS ≥ 1 vs. 25.0% in PD-L1 CPS < 1; p = 0.705) and perineural invasion (40.9% in PD-L1 CPS≥1 vs. 16.7% in PD-L1 CPS < 1; p = 0.252) were associated with PD-L1 expression. Among the high-risk features, only lymph node involvement had statistical significance, with 14 out of 22 PD-L1 CPS ≥ 1 patients (63.6%) having lymph node metastases when lymphadenectomy was performed (p = 0.031). With a median follow-up of 16 months (IQR 27.5), PD-L1 CPS ≥ 1 patients had worse overall survival (53.4 months vs 75.9 months), but no statistical significance could be inferred (p = 0.188).
Conclusions: It is noteworthy the clinical significance of lymph node involvement in PD-L1 CPS ≥ 1 cases and a trend towards worse overall survival in this group of patients.

KEY WORDS: PD-L1; Penile carcinoma; Lymph node involvement; Prognostic biomarker.

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INTRODUCTION

Penile cancer (PC) is a rare form of cancer in Western nations, with *squamous cell carcinoma* (SqCC) being the most common type, accounting for around 95% of cases (1) The global incidence of PC varies due to differences in socioeconomic and religious factors, representing less than 1% of all malignancies in Western Europe (2). Regions with high rates of human papillomavirus (HPV) infection are most affected by penile cancer, with approximately one third to half of cases attributed to HPV-related causes (3).
The management of penile cancer presents a significant challenge for clinicians. This malignancy is highly aggressive, and there is a lack of well-defined strategies for patients whose metastatic penile carcinoma progresses or recurs after front-line cisplatin-based chemotherapy (4).
The ability of tumor cells to evade the immune system is one of the many characteristics of tumor cells recognized as a hallmark of cancer (5). The *programmed cell death 1 and its ligand* (PD-1/PD-L1) pathway is one of the primary immune checkpoint targets that has been extensively studied in clinical research in recent years (6).
PD-L1 acts as a co-stimulatory ligand, which, upon binding with its receptor PD-1, functions as a negative regulator of T-cell-mediated antitumor immunity (7). Although PD-L1 expression is typically induced in cells of the macrophage lineage and T cells, abnormal PD-L1 expression has been detected in various types of cancer. This has led to the hypothesis that PD-L1 expression in either tumor cells or *tumor-infiltrating immune cells* (TIICs) might facilitate tumor progression by inhibiting the antitumor immune response (7).
Currently, several clinical studies evaluating PD-1/PD-L1 inhibitors have been conducted in several different tumor types including melanoma, breast cancer, non-small-cell lung cancer, and head and neck cancer. PD-L1 expression has also been extensively studied in a number of urological malignancies such as bladder, kidney, and prostate cancer. However, there have been relatively few studies on PD-L1 expression in penile squamous cell carcinoma (8-10). Given the aforementioned difficulty in treating recurrent and/or metastatic penile SQCC, the targeting of PD-L1 may offer a novel therapeutic avenue for those patients who exhibit PD-L1 expression upon recurrence or progression after first-line chemotherapy.
The aim of our study is to investigate the association between PD-L1 immunoexpression as a combined positive score and clinical outcomes in penile squamous cell carcinoma.

METHODS

Patients and samples

We retrospectively reviewed all penile SqCC cases treated in our institution between 2018 and 2023. Tumors were classified according to the 5th edition of the *World Health Organization Urinary and Male genital tumors* 2004 TNM classification. Exclusion factors included a non-squamous cell carcinoma diagnosis and cases without available

material for additional immunohistochemistry studies and without clinical follow-up. Baseline patient and disease characteristics were assessed for the selected patients. All penile specimens were reviewed by a pathologist specialized in genitourinary pathology.
All research was performed in accordance with relevant national and international regulations and informed consent to use the pathology material was obtained from all participants. The research was performed in accordance with the Declaration of Helsinki.

Immunohistochemistry

PD-L1 expression was assessed by *immunohistochemistry* (IHC) in formalin-fixed, *paraffin-embedded* (FFPE) tumor samples at our institution. PD-L1 IHC 22C3 pharmDx assay was performed using a monoclonal mouse anti-PD-L1 clone 22C3 and a validated protocol for Ventana BenchMark Ultra platform. PD-L1 protein expression was determined by using a *Combined Positive Score* (CPS), counting the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100 (e.g. if 1 staining cell was found out of 100 viable cells the score was 1/100 x 100 = 1). A minimum of 100 viable tumor cells was present in each stained slide for adequate PD-L1 evaluation. PD-L1 staining was evaluated as membranous tumor cell staining and membranous/cytoplasmic staining of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma. PD-L1 CPS ≥ 1 cases were considered when CPS ≥ 1.
Also, in all cases before 2021 IHC for P16 was performed on Ventana® Benchmark ULTRA equipment with the Ventana® optview DAB IHC detection kit (ref 760-700). CC1 was used for *antigen retrieval* (AR) and anti-P16 (Roche Ventana® - clone E6H4) was used. Positive cases were considered if a strong block positivity for p16 was observed.

Statistical analysis

Continuous data were described by median and *interquartile range* (IQR). Categorical data were presented by counts and percentages. Comparisons between continuous data were performed by the Mann-Whitney U test and categorical data by Pearson's chi-squared or Fisher's exact test, accordingly. Kaplan-Meier curves were obtained to estimate the survival rates with statistical significance evaluated by the log-rank test. A $p < 0.05$ was defined as statistically significant. Data was processed and analysed with IBM-SPSS software version 22.0.

Results

Patient characteristics

A total of 34 patients were eligible for analysis in this study. The median age at diagnosis was 67 years (IQR 20). The median size of the tumor was 3.0 cm (IQR 3.3). Among the patients, 11 were classified as HPV-associated (p16 positive). Twenty-two cases were considered to be PD-L1 CPS ≥ 1.
The study uniformly recorded cases across all grades. Regarding pathological stages, no pT1b tumors were observed, but all other stages were represented. A partial penectomy was performed in the majority of the cases (70.6%) and none of the patients had adjuvant treatments. Clinicopathological and demographic characteristics of the cohort are presented in Table 1.

Association of PD-L1 expression with clinicopathological features

Larger tumors (3.0 cm in PD-L1 CPS ≥ 1 *vs* 2.5 cm in PD-L1 CPS < 1; p = 0.662), vascular invasion (36,4% in PD-L1 CPS ≥ 1 *vs.* 25,0% in PD-L1 CPS < 1; p = 0.705) and perineural invasion (40.9% in PD-L1 CPS ≥ 1 *vs.* 16,7% in PD-L1 CPS < 1; p = 0.252) were associated with PD-L1 expression, but with no statistical significance.
Twenty-three patients underwent lymphadenectomy, with lymph node involvement detected in 17 of them. Among those with lymph node involvement, 16 patients were found to be PD-L1 CPS ≥ 1.
Among the high-risk features, only lymph node involvement had statistical significance, with 14 out of 22 PD-L1 CPS ≥ 1 patients (63.6%) having lymph node metastases when lymphadenectomy was performed (p = 0.031).
These associations are presented in Table 2.
Association of PD-L1 expression with survival outcomes: With a median follow-up of 16 months (IQR 27.5), PD-L1 CPS ≥ 1 patients had worse overall survival (53.4

Table 1.
Clinical and pathological characteristics.

Characteristic	Total sample (n = 34)
Median age at surgery, yr	67 (IQR 20)
High-risk HPV status, n (%)	
Negative	23 (67.6%)
Positive	11 (32.4%)
PD-L1 status, n (%)	
CPS < 1	12 (35.3%)
CPS ≥ 1	22 (64.7%)
Median tumor size, cm	3 (IQR 3.3)
Tumor grade, n (%)	
1	11 (32.4%)
2	14 (41.2%)
3	8 (23.5%)
4	1 (2.9%)
Vascular invasion, n (%)	
No	23 (67.6%)
Yes	11 (32.4%)
Perineural invasion, n (%)	
No	23 (67.6%)
Yes	11 (32.4%)
pT stage, n (%)	
1a	9 (26.5%)
2	11 (32.4%)
3	14 (41.2%)
Lymph node involvement, n (%)	
No	17 (50.0%)
Yes	17 (50.0%)
Treatment, n (%)	
Glansectomy	6 (17.6%)
Partial penectomy	24 (70.6%)
Total penectomy	4 (11.8%)

Table 2.
Association of PD-L1 expression with clinicopathological features.

	PD-L1 in tumor cells, n (%)		
	CPS < 1	CPS ≥ 1	
Tumor size (median, cm)	2.5 (IQR 2.5)	3.0 (IQR 3.4)	p = 0.662
p16 immuno-expression, n (%)			p = 0.252
Negative	10 (83.3%)	13 (59.1%)	
Positive	2 (16.7%)	9 (32.4%)	
Histological grade, n (%)			p = 0.360
Grade 1	5 (41.7%)	6 (27.3%)	
Grade 2	6 (50.0%)	8 (36.4%)	
Grade 3	1 (8.3%)	7 (31.8%)	
Grade 4	0 (0.0%)	1 (4.5%)	
Vascular invasion, n (%)			p = 0.705
No	9 (75.0%)	14 (63.6%)	
Yes	3 (25.0%)	8 (36.4%)	
Perineural invasion, n (%)			p = 0.252
No	10 (83.3%)	13 (59.1%)	
Yes	2 (16.7%)	9 (40.9%)	
pT stage, n (%)			p = 0.989
pT1a	3 (25.0%)	6 (27.3%)	
pT2	4 (33.3%)	7 (31.8%)	
pT3	5 (41.7%)	9 (40.9%)	
Lymph node involvement, n (%)			p = 0.031
No	9 (75.0%)	8 (50.0%)	
Yes	3 (25.0%)	14 (63.6%)	

months vs 75.9 months), but statistical significance could not be inferred (p = 0.188). The survival curves are presented in Figure 1.

Discussion

The rarity of penile cancer in Western countries poses challenges in understanding its biology and optimal management strategies. Our study aimed to find an association between PD-L1 status and clinicopathological characteristics of penile SqCC, as well as associated prognostic implications. In fact, our results showed a trend towards worse clinical features and worse survival in PD-L1 CPS ≥ 1 patients, as well as a significant association between PD-L1 status and lymph node involvement.

The association between PD-L1 expression and clinicopathological features observed in our study is consistent with previous research in various cancer types, including studies conducted in penile cancer (9, 10).

We found a trend towards larger tumor size, vascular invasion, and perineural invasion in patients with PD-L1 CPS ≥ 1 tumors, although statistical significance was not achieved, probably due to the low number of patients in our cohort. These findings are consistent with the largest cohort, to our knowledge, published by Sabina Davidsson and colleagues, who analysed 222 cases of penile *squamous cell carcinoma* (SqCC) (8). This suggests a potential link between PD-L1 expression and aggressive tumor behaviour, which poses the question regarding the need for a different treatment pathway in these patients.

A novel observation in our study is the significant association between PD-L1 expression and lymph node involvement. We found that a higher proportion of patients with PD-L1 CPS ≥ 1 tumors had lymph node metastases upon lymphadenectomy compared to those with PD-L1 CPS < 1 tumors, with 14 out of 22 PD-L1 CPS ≥ 1 patients (63.6%) having lymph node metastases when lymphadenectomy was performed (p = 0.031) and only 3 out of 12 PD-L1 CPS < 1 patients having lymph node involvement.

These findings are in line with those from Udager and colleagues that were among the first to investigate PD-L1 expression in a cohort of 37 patients with penile SqCC. They discovered that PD-L1 expression was detected in 62.2% of penile SqCC. Despite the limited sample size, they noted a trend towards worse clinical clinicopathological features and worse overall survival but, at least to our knowledge, no statistically significant association has been established yet (9). This finding suggests a potential

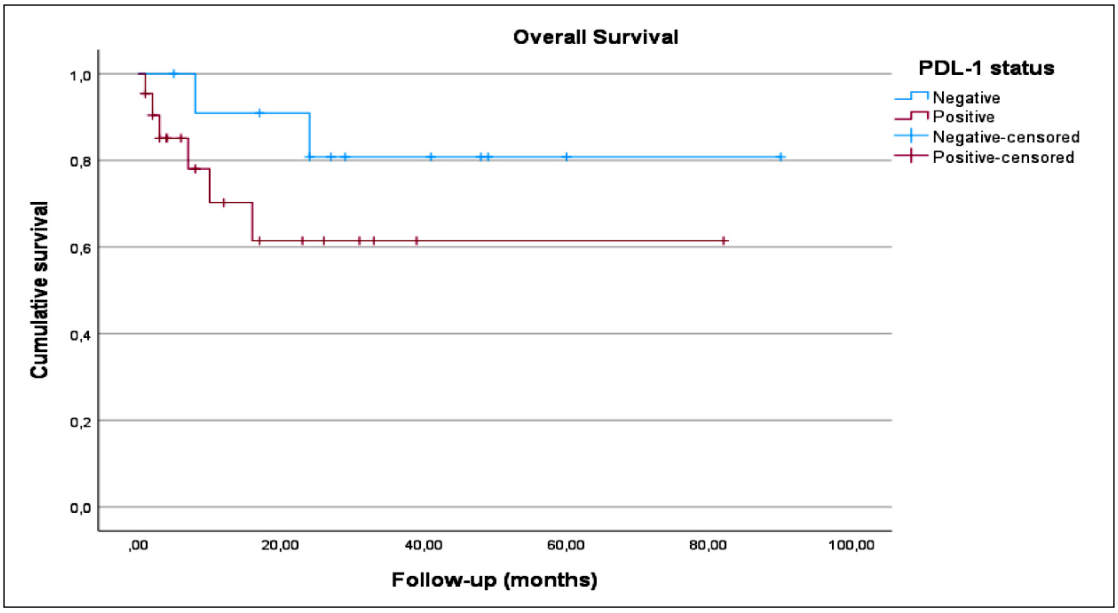

Figure 1.
Overall survival stratified by PD-L1 status.

role for PD-L1 expression in promoting tumor metastasis and aggressiveness. However, the underlying mechanisms driving this association remain unclear and warrant further investigation through mechanistic studies.

The lack of a standard second-line treatment for patients with metastatic penile SCC underscores the urgent need for novel therapeutic strategies. The emergence of immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway has revolutionized cancer treatment across various tumor types. Our study adds to the growing body of evidence supporting the clinical relevance of PD-L1 expression as a potential biomarker for immunotherapy response in penile SqCC.

In our study, we observed a high proportion of patients with PD-L1 CPS ≥ 1 (64,7%), which is in line with the current literature (8, 9). Until now, the main focus has been on HPV related penile carcinoma that in our sample was only 32,4%, which is also in line with the current evidence (3). Given the much higher prevalence of PD-L1 expression there is potential for identifying a novel therapeutic target since the literature indicates that tumors expressing high levels of PD-L1 are more likely to respond to immunotherapy.

Additionally, In the future, it will be of interest to observe the potential impact of male HPV vaccination on the incidence and oncological outcomes of penile cancer. HPV-related penile cancers are believed to have better outcomes (3). If the incidence of HPV-related cancers decreases as a result of vaccination efforts, we may encounter a higher proportion of aggressive cases. Therefore, the importance of novel strategies will become even more significant.

Furthermore, many patients become ineligible for chemotherapy over time, yet they may still be suitable candidates for immunotherapy. Offering such patients an alternative treatment could prevent disease progression.

If clinical benefits are demonstrated in this advanced setting, there is potential to pursue approval for this therapy in earlier stages as an adjuvant treatment for tumors with high-risk features, similar to current discussions regarding kidney cancer treatment protocols.

Despite the promising role of PD-L1 expression as a prognostic biomarker, our study did not find a statistically significant association between PD-L1 expression and overall survival. This may be attributed to the relatively small sample size and short follow-up duration. The survival curves show a trend towards separation, suggesting that with a larger sample size, the survival outcomes would likely achieve statistical significance. Larger studies with longer follow-up periods are warranted to validate our findings and assess the impact of PD-L1 expression on survival outcomes in penile SqCC.

Several limitations should be considered when interpreting our results. First, our study was retrospective and conducted at a single institution, which may introduce selection bias and limit generalizability. Second, the assessment of PD-L1 expression was based on immunohistochemistry, which has inherent variability and subjectivity. Future studies incorporating more robust techniques, such as RNA sequencing or multiplex immunofluorescence, could provide deeper insights into the tumor microenvironment and immune landscape in penile SqCC.

Despite de low number of cases, reflecting the rarity of this tumor, our data comes from a tertiary cancer center in Portugal, where all decisions are based on multidisciplinary tumor boards. Besides this, we exclusively analysed conventional squamous cell carcinoma and omitted other histologies to avoid confounding the results with histologies known to have a different prognosis.

Conclusions

Our study highlights the association between PD-L1 expression and worse clinical outcomes in penile SqCC. It is noteworthy the clinical significance of lymph node involvement in PD-L1 CPS ≥ 1 cases, suggesting a potential role of PD-L1 as a predictive biomarker for metastatic disease.

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