Tumor PD-L1 expression is correlated with increased TILs and poor prognosis in penile squamous cell carcinoma

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\textbf{ABSTRACT}

Despite its rare incidence worldwide, penile squamous cell carcinoma (PeSCC) still presents with significant morbidity and mortality due to the limited treatment options for advanced patients, especially those in developing countries. The program death-1 (PD-1)/PD-1 ligand (PD-L1) axis has been demonstrated to play an important role in tumor immune escape, and immunotherapies targeting this pathway have shown great success in certain cancer types. Here, we analyzed the expression pattern of PD-L1 in tumor cells and tumor-infiltrating lymphocytes (TILs) in PeSCC with a multi-center cohort. We found that the majority of PeSCCs (53.4%) were PD-L1-positive and that high PD-L1 expression in tumor cells was associated with a poor prognosis. Notably, PD-L1 expression in tumor cells was significantly associated with the extent of TILs and CD8\textsuperscript{+} TILs. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) showed that PD-L1 was positively correlated with interferon-gamma (IFN\textgamma) and CD8\textsuperscript{+} gene expression. Moreover, we defined the constitutive and inducible surface expression of PD-L1 in newly established primary PeSCC cell lines. Interestingly, two PeSCC cell lines had high intrinsic PD-L1 expression. Another cell line showed low PD-L1 expression, but the PD-L1 expression could be induced by IFN\textgamma stimulation. Overall, our data showed that high PD-L1 expression in penile tumor cells indicated a poor prognosis. The upregulation of PD-L1 in PeSCC included both extrinsic and intrinsic mechanisms. These findings indicated that the PD-1/PD-L1 axis might be a potential therapeutic target for patients with penile squamous cell carcinoma.

\textbf{Abbreviations:} CSS, cancer specific survival; FFPE, formalin fixed paraffin embedded; IFN\textgamma, interferon-gamma; IHC, immunohistochemistry; LN, lymph node; PD-L1, programmed death ligand 1; PeSCC, penile squamous cell carcinoma; qPCR, quantitative polymerase chain reaction; TIL, tumor-infiltrating lymphocyte

\textbf{Introduction}

Penile squamous cell carcinoma (PeSCC) is a rare cancer with an incidence of 1–10 per 100,000 men worldwide. However, in less developed areas, such as Africa, Southeast Asia and South America, PeSCC is much more common and may account for up to 1–2% of male malignancies. Poor hygiene, phimosis, smoking, lack of circumcision and human papillomavirus (HPV) infection are risk factors for PeSCC. The 5-y overall survival in patients with or without lymph node (LN) involvement is 51.1% and 95.7%, respectively. Due to the lack of experimental and clinical studies on PeSCC, treatment options for advanced patients are limited. As a result, it is important to identify new and efficient therapeutic targets for PeSCC.

The emergence and continuing growth of cancer cells are thought to result from immune tolerance and cancer cell escape. Multiple mechanisms have been demonstrated to be used by tumor cells to limit the effectiveness of the immune response. Among these, the programmed cell death-1 (PD-1)/PD-1 ligand (PD-L1) pathway is believed to play a significant role. PD-L1 on tumor cells or in infiltrating immune cells can interact with its receptor, PD-1, on T-cells, inducing T-cell anergy or exhaustion, thus, inhibiting local immune response. Clinical trials evaluating the efficiency of anti-PD-1/PD-L1 therapy (also called immune checkpoint blockade therapy) in several solid cancers have shown success. Nivolumab and pembrolizumab, two different anti-PD-1 drugs, have recently been approved by the US Food and Drug Administration (FDA) in patients with advanced melanoma, non-small-cell lung cancer and renal cell carcinoma.
Previous studies have indicated that higher expression of PD-L1 in tumor cells or tumor-infiltrating immune cells (TILs) is associated with different pathological features and clinical outcomes across different cancer types.\textsuperscript{10-12} Importantly, it has been reported that the likelihood of response to anti-PD-1 therapy is correlated with tumor PD-L1 expression.\textsuperscript{13,14} Thus, to predict the possible utility of immune checkpoint blockade therapy in PeSCC, it is essential to understand the expression pattern of PD-L1. Recently, Udager et al. published a study that analyzed the PD-L1 expression in a cohort of 37 patients with PeSCC.\textsuperscript{15} According to their findings, primary tumor PD-L1 expression was significantly associated with lymph node metastasis and decreased cancer-specific survival (CSS). However, there were several flaws in that study. First, the cohort was relatively small with only 37 patients, and the majority of the patients (69%) were diagnosed with high clinical stage (II–IV). Moreover, the rate of lethal events was too low, thus preventing the ability to perform survival analysis. Thus, the results from their study may not reflect the expression pattern of PD-L1 in PeSCC patients.

In the present multi-center study, which encompassed a much larger cohort, we characterized the expression pattern of PD-L1 in PeSCC tumor cells and TILs as well as their association with common clinicopathological features and CSS. In addition, several newly established primary PeSCC cell lines were used for the first time to better understand the intrinsic and extrinsic expression of PD-L1 in PeSCC.

Results

Patient characteristics

The baseline patient characteristics are listed in Table S1. A total of 116 patients without previous treatment were included in the evaluation of PD-L1 expression. The median age at surgery was 53.0 y (range 24–86 y). At the end of the last follow up, 32 patients had died of penile cancer. The details of treatment regimens are presented in Table S1. Patients who underwent total penectomy and/or radical lymphadenectomy were mainly diagnosed with higher clinical stage. To adjust for baseline differences, we intended to utilize a propensity score analysis. However, due to the limited cases in each group, the propensity analysis could not be accomplished. The HE staining sections of each patient were reviewed by a pathologist to evaluate the pTNM and G staging. The pTNM staging was assessed according to the American Joint Committee on Cancer (AJCC) 2009 TNM classification for penile cancer.

![Figure 1](image.png)

Figure 1. IHC analysis of PD-L1 expression on the tumor cell membranes or TILs. (A) An isotype control for PD-L1 staining in human placenta tissue (200×). (B) A positive control of PD-L1 staining in human placenta tissue (200×). (C) Positive membrane staining of PD-L1 on penile tumor cell membrane (100×). (D) 200× magnification of the boxed area shown in (C). (E) Positive staining of PD-L1 on TILs (100×). (F) 200× magnification of the boxed area shown in (E).
**Table 1.** Clinicopathological characteristics and univariate log-rank test for CSS.

| Variables               | N (%)   | 3-y CSS (95% CI) | p-value |
|-------------------------|---------|------------------|---------|
| Age at surgery (y), median (range) | 53 (24–86) | 60.9 (44.8–81.2) | 0.992 |
| <53                     | 54 (46.6) | 63.0 (44.8–81.2) |         |
| ≥53                     | 62 (53.4) | 63.2 (46.3–78.1) |         |
| T stage                 |         |                  |         |
| <T2                     | 44 (37.9) | 93.0 (83.4–100)  | 0.001  |
| ≥T2                     | 72 (62.1) | 43.3 (27.6–59.0) |         |
| N stage                 |         |                  |         |
| NO/Nx                   | 74 (63.8) | 86.5 (75.1–97.9) | 0.001  |
| N+                      | 42 (36.2) | 19.4 (2.9–35.9)  |         |
| G grade                 |         |                  |         |
| <G2                     | 71 (61.2) | 82.9 (68.4–97.4) | 0.001  |
| ≥G2                     | 45 (38.8) | 45.1 (28.2–62.0) |         |
| PD-L1 on tumor cells    |         |                  |         |
| Negative                | 54 (46.6) | 77.4 (62.9–91.9) |         |
| Positive                | 62 (53.4) | 50.6 (34.9–66.3) |         |
| PD-L1 on TILs           |         |                  | 0.085  |
| Negative                | 49 (43.0) | 55.4 (38.7–71.7) |         |
| Positive                | 65 (57.0) | 66.5 (50.0–83.0) |         |
| Extent of TILs          |         |                  | 0.177  |
| Negative                | 23 (20.2) | 76.1 (49.8–100.0)|         |
| Positive                | 91 (79.8) | 59.3 (47.0–71.8) |         |
| CD8⁺ TILs               |         |                  | 0.001  |
| Low                     | 60 (52.6) | 74.3 (58.8–89.8) |         |
| High                    | 54 (47.4) | 47.9 (31.6–64.2) |         |

T⁺: <T2, including Ta, Tis and T1; ≥T2, including T2, T3 and T4.
G⁺: >G2, including G2, G3 and G4.
N⁺: N+, including pN1, pN2 and pN3.

**PD-L1 expression in tumor cells or TILs**

Fig. 1A includes an isotype control of PD-L1, whereas Fig. 1B shows a positive control using a term human placenta tissue section. PD-L1 was mainly found on the membrane of tumor cells (Fig. 1C and D), and 62 (53.4%) patients had positive PD-L1 staining in tumor cells. TILs were considered positive in 91 (79.8%) patients (Table 1). The detailed scorings of the extent of TILs and CD8⁺ TILs are presented in Table S1. The representative pictures of each score are depicted in Figs. S1 and S2. Two patients could not be evaluated for TIL extent or PD-L1 staining in TILs due to technical limitations. PD-L1 staining in TILs was positive in 65 patients (57.0%) and negative in 49 patients (43.0%) (Table 1; Fig. 1E and F).

**Correlation of PD-L1 expression with extent of TILs and CD8⁺ TILs**

To evaluate the association of PD-L1 expression and tumor biology, clinicopathological features were compared with PD-L1 expression in tumor cells or TILs. The expression of PD-L1 in TILs was significantly correlated with pN (p = 0.010), G grade (p = 0.012), extent of TILs (p = 0.002) and CD8⁺ TILs (p = 0.001, Table 2). However, no significant correlation was found between the positivity of PD-L1 in tumor cells and common clinicopathological characteristics (Table 2). Interestingly, we found that PD-L1 expression in tumor cells was significantly correlated with the extent of TILs (p = 0.018) and CD8⁺ TILs (p = 0.001, Table 2).

**Correlation between PD-L1 expression and IFNγ in PeSCC**

The regulation of PD-L1 expression in squamous cell carcinoma has been demonstrated to be involved in two distinct aspects, namely intrinsic and extrinsic. Cytokines, especially interferon-gamma (IFNγ), that are expressed by activated T cells can induce PD-L1 expression in the surrounding tumor cells via specific signaling pathways. To better elucidate the correlation between PD-L1 expression and TILs in PeSCC, the mRNA expression levels of PD-L1, IFNγ, and CD8⁺ were evaluated in 24 primary penile cancer specimens. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed a significant positive correlation between PD-L1 and IFNγ or CD8⁺ (Fig. 2A and B). Intrinsic and extrinsic expression of PD-L1 in penile cancer cell lines

Three newly-established primary PeSCC cell lines were used to define the expression pattern of PD-L1 in PeSCC cell lines. Penl2 and I49Rca were two new cell lines established using the same method used to establish Penl1. The STR profiles of these two cell lines confirmed their uniqueness (Table S2). The HE staining of the corresponding inoculated tumors was confirmed to be squamous cell carcinoma (Fig. S3). The protein and mRNA levels of PD-L1 in three PeSCC cell lines (Penl1, Penl2 and I49Rca) and the immortalized human keratinocyte cell line, HaCaT, were analyzed by western blot and qPCR. The PD-L1 protein levels in the PeSCC cell lines were significantly higher than in the normal keratinocyte cell line (Fig. 3A). Notably, Penl1 and I49Rca had much higher expression than Penl2, which was consistent with the PD-L1 mRNA results (Fig. 3B). The different PD-L1 expression levels in the penile cancer cell lines, Penl1 and Penl2, were confirmed using flow cytometry (Fig. 3C). To investigate if PD-L1 expression in penile cancer cells could be stimulated by IFNγ, Penl2 cells, which have low intrinsic PD-L1 expression, were incubated with different IFNγ doses (0, 10, and 50 ng/mL). After 48 h of treatment, the levels of PD-L1 expression were significantly increased in a dose-dependent manner (Fig. 3D).
stimulation, PD-L1 expression was significantly elevated (Fig. 3D).

**Association of PD-L1 expression and CD8^+ TILs with CSS in PeSCC patients**

Consistent with a previous report, inguinal lymph node metastasis was significantly correlated with worse clinical outcome (p = 0.001, Table 1). Moreover, positive PD-L1 expression in tumor cells was associated with a shorter CSS (p = 0.003, Table 1; Fig. 4A). However, neither PD-L1 expression in TILs nor the extent of TILs was associated with CSS (Table 1; Fig. 4B and C). Although 36 (69.2%) patients with PD-L1-negative tumors presented with positive TILs, no significant association was observed between TILs and CSS in this subpopulation of patients (p = 0.906, Fig. S4). Of note, patients with higher CD8^+ TILs showed shorter CSS (p = 0.001, Table 1; Fig. 4D). Multivariate analysis, as shown in Table 3, showed that LN status (HR = 13.30, 95% CI 3.98–44.40, p = 0.001) and CD8^+ TILs (HR = 2.30, 95% CI 1.05–8.57, p = 0.04), but not tumor PD-L1 expression (HR = 1.14, 95% CI 0.37–3.46, p = 0.82) were independent predictors of shorter CSS.

**Discussion**

It has been well demonstrated that the PD-1/PD-L1 axis plays a crucial role in tumor immune evasion. PD-L1 can be found on tumor cells or infiltrating immune cells, which contribute to the inhibition of local immune response. However, the prognostic effect of tumor PD-L1 expression varies across different cancer types and in different studies. In addition, some studies have demonstrated the correlation of PD-L1 expression in TILs with clinical outcome. Recently, a study with 37 penile cancer patients indicated that high level of PD-L1 expression in tumor cells is correlated with LN metastasis and poor CSS. This previous study was the first report to demonstrate the expression of PD-L1 in...
PeSCC, but both the number of patients and lethal rate were relatively low. Moreover, the majority of patients in the previous study were diagnosed with high clinical stage (II–IV). Overall, this previous study provides evidence that may be insufficient to completely demonstrate the role of PD-L1 expression in PeSCC. Here, we conducted a multi-center study, including 116 patients, to analyze the expression pattern of PD-L1 in both tumor cells and TILs. The anti-PD-L1 antibody (clone E1L3N, Cell Signaling Technology) used in our study is a newly validated commercial antibody. The specificity and reproducibility of this antibody have been confirmed in previous studies.28-30 Similar to the previous report, we found that 53.4% of the patient samples showed positive tumor membrane PD-L1 staining, and the higher PD-L1 expression in tumor cells was associated with longer CSS. However, neither PD-L1 positivity in TILs nor the extent of TILs was correlated with CSS. Of note, higher CD8+ TIL expression was correlated with shorter CSS. When adjusting for the pT, pN and G stages, Multivariate Cox Regression analysis showed that CD8+ TILs but not PD-L1 expression was independently correlated with CSS, which was likely due to the association between PD-L1 expression and CD8+ TILs.

Interestingly, the expression of PD-L1 in tumor cells was correlated with the extent of TILs and CD8+ TILs. qRT-PCR analysis showed that PD-L1 was positively correlated with IFNγ and CD8+ gene expression. Several researches also described similar association.23,31,32 This correlation was in accordance with the previous finding that IFNγ, expressed by activated CD8+ T cells, can induce PD-L1 expression in the surrounding tumor cells.33 Moreover, seven patients (Table 2) with absent or focal TILs still presented PD-L1 positivity. Thus, we hypothesized that PD-L1 expression in tumor cells can be constitutive and/or induced by TILs in PeSCC. To confirm this hypothesis, we evaluated the baseline levels of PD-L1 in three primary PeSCC cell lines and in the normal human keratinocyte cell line, HaCaT. To our knowledge, this is the first study to evaluate PD-L1 expression in PeSCC cell lines. The normal keratinocyte cell line, HaCaT, had no detectable PD-L1 expression. Two PeSCC cell lines, namely Penl1 and 149RCa, had a high baseline PD-L1 level, while the Penl2 cell line had relatively low PD-L1 expression. Of note, after IFNγ stimulation, PD-L1 was significantly increased in Penl2 cells. These results collectively indicated that both intrinsic and extrinsic expression of PD-L1 exist in PeSCC cells.

Recently, Ottenhof et al.34 published an article discussing the relationship between HPV status and PD-L1 expression, and they reported that high risk HPV-negative patients tend to have positive PD-L1 expression in tumor cells. However, although it has been reported that HPV DNA can be detected in approximately half of penile cancers, the prevalence of HPV infection in penile cancer patients from Asia is relatively low with only 13.4%.35 Therefore, a larger cohort is required to demonstrate the correlation between HPV status and PD-L1 expression in Chinese penile cancer patients.

Table 3. Multivariable Cox regression analyses for CSS.

| Variables | HR   | 95% CI      | p-value |
|-----------|------|-------------|---------|
| pT stage  | <T2 VS ≥T2 | 2.58   | 0.54–12.19 | 0.23 |
| pN status | NO/Nx vs N+ | 13.30  | 3.98–44.40 | <0.001 |
| G grade   | <G2 vs ≥G2 | 0.93   | 0.38–2.30  | 0.882 |
| PD-L1 on tumor cells | Negative vs positive | 1.14   | 0.37–3.46  | 0.82 |
| CD8+     | Low vs high | 2.30   | 1.05–8.57  | 0.04 |

Abbreviations: CI, confidence interval of the estimated HR; HR, hazard ratio.

Figure 4. PD-L1 expression on tumor cells and CD8+ TILs is associated with shorter CSS. Kaplan–Meier curves for the analysis of tumor PD-L1 expression (A), TILs PD-L1 expression (B), extent of TILs (C) and CD8+ TILs (D). p-values were calculated by log-rank test.
An increasing number of studies are focusing on the regulation mechanisms of PD-L1 expression in tumor cells to identify a more effective approach to block this pathway. Various oncogenic pathways contribute to the intrinsic expression of PD-L1, such as the EML4-ALK oncoprotein in non-small cell lung cancer and loss of PTEN in glioma.36,37 Extrinsic PD-L1 expression is thought to be induced by cytokines, especially IFNγ, in the tumor environment via various downstream pathways.17-19 Because PD-L1 expression in PeSCC cells can be either intrinsic or extrinsic, studies investigating the precise pathways involved are currently underway in our laboratory.

There are also some limitations in our study. First, although we conducted a multi-center study with a large cohort, this was a retrospective study which had inevitable selection bias. Second, due to the rareness of PeSCC, the follow-up period was relatively short for some patients. Third, clinical data were retrospectively collected, resulting in the lack of analysis of some clinical features, such as smoking and circumcision status. Finally, this multi-center cohort was heterogeneous, as the treatment indications, surgical templates or adjuvant therapies might differ among each center.

In conclusion, we analyzed the PD-L1 expression pattern in PeSCC in a multi-center cohort. High PD-L1 expression in tumor cells was correlated with increased CD8+ TILs and worse clinical outcome. In addition, we evaluated PD-L1 expression in PeSCC cell lines for the first time, and we identified that both intrinsic and extrinsic expression existed in PeSCC. Our findings provide a fundamental rationale for further investigation into immune checkpoint blockade therapy in PeSCC.

**Materials and methods**

**Patients and samples**

A total of 116 patients with squamous cell carcinoma of the penis from three institutions (Sun Yat-sen University Cancer Center, Guangdong; The First People’s Hospital of Chenzhou, Hunan; and the Affiliated Oncological Hospital of Xinjiang Medical University, Xinjiang) from August 2002 to August 2015 were included. Patients with previous treatment, including surgery, chemotherapy or radiotherapy, were excluded. All patients underwent partial or radical penectomy with or without bilateral inguinal lymphadenectomy. Formalin-fixed paraffin-embedded (FFPE) tissues of the primary tumors were retrieved from the Department of Pathology. The Institutional Ethical Boards of all three hospitals approved this study. Written informed consents were obtained from all patients. After incubating with secondary antibodies (Envision, Dako), DAB was used to visualize the staining.

**Scoring of PD-L1 expression**

All slides were evaluated by two independent pathologists (Dr. Ping Yang and Dr. Shumei Yan) without prior knowledge of the clinical data of the specimens. The scores of PD-L1 expression in tumor cells and TILs were assessed as previously reported.25,27 Briefly, PD-L1 positivity was identified as ≥ 5% cells with membrane staining, and this 5% threshold was based on a previous phase I trial of anti-PD-1 agents.13 The scoring method of CD8+ TILs was in accordance with a previous report.38 CD8+ TILs were assessed as absent (0), sparse (1+), moderately dense (2+), dense (3+), and very dense (4+) with 0 and 1+ considered negative. The extent of TILs was evaluated in HE slides using the same criteria above.

**Penile cancer cell lines and IFNγ stimulation**

The Penl1 human PeSCC cell line was previously established by our group.20 Two other cell lines, Penl2 and 149RCA, were also established in our institution. The HaCaT immortal keratinocyte cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were routinely cultured in DMEM supplemented with 10% FBS (Gibco, Life Technologies), 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C with humidified air and 5% CO2.

For IFNγ stimulation, cells were seeded at 10,000 cells/well in six-well plates and then cultured with IFNγ (Proteintech) for 48 h at a final concentration of 50, 50 or 100 ng/mL.

**Western blotting**

Approximately 30 μg of protein was separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore). The membranes were incubated with the specific primary antibodies, PD-L1 E1L3N™ (1:1,000, Cell Signaling Technology) and GAPDH (1:2,500, Cell Signaling Technology). After incubation with the corresponding secondary antibody (Santa Cruz Biotechnologies), bands were visualized via Chemidoc Touch (Bio-Rad), and bands were quantified relative to GAPDH by using ImageJ software.

**RNA extraction and qPCR**

The total RNA was extracted from cells and RNaLater (Invitrogen) preserved tissues with TRIzol (Invitrogen). cDNA was synthesized with the GoScript™ Reverse Transcription System (Promega), and qPCR was conducted using GoTaq® qPCR Master Mix (Promega). The primer sequences used are the following:

**PD-L1**

Forward: 5’- CCTACTGGCATTGTGCTGAACGCAT-3’
Reverse: 5’- ACCATAGCTGATCATGCAGCGGTA-3’
**Statistical analysis**

The correlation between the clinicopathological characteristics and PD-L1 expression in tumor cells or TILs was estimated with Fisher’s exact test. Spearman’s rank correlation was used to evaluate the correlations between the mRNA expression of PD-L1 and CD8+ or IFNγ. CSS was defined as the length of time from the date of surgery to death or the last visit of patient. CSS based on PD-L1 expression or other clinicopathological characteristics was estimated by the Kaplan–Meier method and compared by the log-rank test. Cox proportional hazards model was used for multivariate survival analyses.

Statistical analyses were performed using SPSS 19.0. A p-value (two-sided) less than 0.05 was considered statistically significant.

**Disclosure of potential conflicts of interest**

The authors declare that they have no competing interests to disclose.

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