RESEARCH ARTICLE

PD-L1 expression as predictor of immunotherapy eligibility in penile squamous cell carcinoma patients [version 1; peer review: awaiting peer review]

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Abstract

Background: Penile cancer is a rare malignancy and potentially lethal disease with an incidence of 0.6-2.1 per 100,000. Squamous cell carcinoma (SqCC) is the most commonly found penile malignancy. PD-L1 is a tumor marker that co-stimulates the receptor PD-1 to suppress T-cell-mediated antitumor immunity.

Methods: This study is a retrospective cohort study with a total sampling method. The slides taken from the biopsies of seventy-six male patients from Haji Adam Malik Hospital diagnosed with penile squamous cell carcinoma who have already undergone penile biopsy were re-examined for this study, and PD-L1 levels were measured accordingly. Statistical methods were used to assess the association between PD-L1 levels and with SqCC stage.

Results: A total of 76 male patients are the subjects of this study. PD-L1 positivity is identified in 25 patients with +1 intensity in 10 patients (13.2%), +2 in 7 patients (9.2), and +3 intensity in 8 patients (10.5%). There are 36 patients (47.4%) diagnosed with stage T3 SqCC, 35 patients (46.1%) with stage N2 SqCC, and 10 patients (13.2%) with stage M1 SqCC. There is significant correlation between PD-L1 expression and metastasis ($p=0.022$). However, there is no significant correlation between PD-L1 expression and stage N tumor ($p=0.167$).

Conclusions: PD-L1 highly expressed in advanced stage penile SqCC (32.9%), which is associated with high-risk clinicopathologic features and poor clinical outcomes. These findings showed a potential usage of immunotherapy in advanced penile SqCC treatment.
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Introduction
Penile cancer is a rare malignancy due to its low incidence rate. The age-standardized incidence rate is 0.84 cases per 100,000 person-years (by the world standard population). In Western countries, penile cancer is a potentially lethal disease with incidence of 0.6-2.1 per 100,000. Squamous cell carcinoma (SqCC) is a lethal disease which accounts for the most common penile malignancies.

Local excision or laser therapy is the primary treatment for patients with low stage tumor. One of the most important prognostic factors is the involvement of regional lymph node which decreases survival from >90% to ~50% when metastases of lymph nodes are present. Patients with metastases such as lymph node metastases have worse prognosis compared to patients with carcinoma in situ, which are associated with substantial mortality and morbidity.

Tumor cells use immunosuppressive mechanisms to avoid antitumor immune response, thus it is now considered one of the cancer hallmarks. One of the tumor hallmarks is PD-L1 which co-stimulate the receptor PD-1 to down-regulate the T-cell-mediated antitumor immunity. PD-L1 expression can also be aberrant in different malignancies. This characteristic of PD-L1 also provides new immunotherapy treatments for some solid tumors. For example, it is also possible to augment tumor cell killing by inhibiting PD-1/PD-L1 interaction.

Membranous PD-L1 has been investigated in different malignancies including breast cancer, melanoma, head and neck cancer, and non-small-cell lung cancer. However, only a limited number of studies have investigated the expression of PD-L1 in penile squamous cell carcinoma.

A number of studies have reported that positive expression of PD-L1 is associated with poor clinic-pathological features and worse outcome in other organs malignancies, such as bladder cancer. Based on these findings, PD-L1 expression may be a predictive biomarker to predict treatment response and oncological outcome in other malignancies, such as penile squamous cell carcinoma.

In this study, we investigated the correlation between PD-L1 expression and poor prognosis. We examined the correlation between PD-L1 expression and survival of penile SqCC and analyzed the adjusted hazard ratios (HR) of the patients with PD-L1 expression compared to anatomical stage of the tumor.

Methods
Sample and data collection
The design of this study is retrospective cohort with total sampling method. Fifty male patients from Haji Adam Malik Hospital diagnosed with penile squamous cell carcinoma who have already undergone penile biopsy and/or penectomy in year 2014 – 2019 are the subjects of this study.

The slides taken from the biopsies were re-examined for the purposes of this study. We also took data about the overall survival and the status of their survival. We were tracing the subjects for 36 months and taking notes of their conditions at that time to obtain the data needed for survival analysis. The data obtained are listed in a table consisting of age, tumor stage, PD-L1 intensities, overall survival, and the status of the patients (dead or alive).

Sample processing
Immunohistochemistry staining method done in this study was HE stain. Reagent used to stain PD-L1 in this IHC staining was MD21R clone (Medaysis CA), ready to use (rabbit PD-L1 antibody). The positive control in this staining is placenta tissue. PD-L1 antibody is diluted with ratio 1 uL PD-L1: 100 uL IHC diluent and then mixed for 5 minutes.

Pretreatment was done with EDTA pH8.0, 15 minutes using a Pressure Cooker, or 30 – 60 minutes using a water bath at 95° – 99°C. Deparaffination and rehydration were done by using Bond Dewax Solution for 3 times, alcohol for 3 times, and Bond Wash Solution for 3 times. We retrieved the antigen by using Bond Epitope Retrieval Solution 1 for 4 times. We mixed the solution with PD-L1 (Medaysis) primary antibody for 1 hour. The detection was done by using post primary procedure for 8 minutes, Bond Wash Solution for two times, and polymer for eight minutes. We used DAB mixture for staining and hematoxylin for counterstaining.

PD-L1 expression was assessed on cytoplasm and/or tumor cell membranes and TILs. The positive control used was the placenta. PD-L1 expression is considered positive if it has a score of +2 or +3 and is considered negative if it has a score of +1 and 0. Semi-quantitative assessment by using the H-score. PD-L1 expression was graded as low (0-150) or high (151-300).
PD-L1 staining was performed on 500 tumor cells and 100 TILs. Semi-quantitative assessment refers to research by Chovanec et al. who used histoscore (H-score). The percentage of stained cells was assessed on a scale of 0-100%. Intensity measurement then given a score of 0-3 (0 = none; 1 = weak; 2 = moderate; and 3 = strong).

The dominant staining intensity rated in 10 large fields view with 400× magnification (3+ indicate strongly stained, 2+ indicate moderately stained, 1+ indicate weakly stained, and 0 indicate unstained). Percentage of stained tumor cells A = percentage of tumor cells intensity in 3+ \[\{3 \times (\% \text{Cells } 3+)\}\], B = percentage of tumor cells intensity in 2+ \[\{2 \times (\% \text{Cells } 2+)\}\], C = percentage of tumor cells intensity in 1+ \[\{1 \times (\% \text{Cells } 1+)\}\]. Total Histo-score values calculated by A+B+C

\[
H \text{ – Score} = \{1 \times (\% \text{Cells } 1+) + 2 \times (\% \text{Cells } 2+) + 3 \times (\% \text{Cells } 3+)\}
\]

Total value obtained ranges from 0 to 300, thus PD-L1 expression is categorized into “1” for negative (0 – 99) and “2” for positive (100 – 300).

The protocol of the sample processing in this study is available from Protocols.io DOI: http://doi.org/10.17504/protocols.io.bp2k69b4klqe/v1.

**Statistical analyses**

We analyzed the subjects and made a table describing the characteristics of the subjects. Association between expression of PD-L1 and poor prognosis tumor was examined using Fisher’s exact test. Poor prognosis tumor parameters examined in this study are the N tumor staging for lymph nodes infiltration and M tumor staging for metastasis. All statistical analyses were completed using SPSS version 20, and statistical significance was defined as \(P<0.05\)

**Ethics**

This study has been given permission from the Health Research Ethics Committee (KEPK) of the Universitas Sumatera Utara (USU; No. 381/KEPK/USU/2012, dated April 22, 2022). Informed consent from participants was waived by the Ethics Committee as the samples were taken from the previously collected biopsies slides.

**Results**

Total of 76 male patients are the subjects of this study. The median age of diagnosis was 50.4 years. Patient characteristics including age, stage T tumor, stage N tumor, metastasis, and PD-L1 intensity are summarized in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Patient characteristics.</th>
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</thead>
<tbody>
<tr>
<td><strong>Patient characteristics</strong></td>
</tr>
<tr>
<td>Age (median)</td>
</tr>
<tr>
<td>Stage T (n%)</td>
</tr>
<tr>
<td>T1</td>
</tr>
<tr>
<td>T2</td>
</tr>
<tr>
<td>T3</td>
</tr>
<tr>
<td>T4</td>
</tr>
<tr>
<td>Stage N (n%)</td>
</tr>
<tr>
<td>N0</td>
</tr>
<tr>
<td>N1</td>
</tr>
<tr>
<td>N2</td>
</tr>
<tr>
<td>N3</td>
</tr>
<tr>
<td>Stage M (n%)</td>
</tr>
<tr>
<td>M0</td>
</tr>
<tr>
<td>M1</td>
</tr>
</tbody>
</table>
The tumor stage T is classified as T1, T2, T3, and T4. Most patients are diagnosed with SqCC stage T3 (47.4%). Lymph node infiltrating tumor is stated as stage N. There are 35 patients (46.1%) diagnosed with stage N2 SqCC. Metastasis was found in 10 patients (13.2%). Anatomic stage shows the tumor progression described by the combination of stage T, N, and M. Anatomic stage 0 – 2 accounts for stage T0-3 with N0 and M0. Anatomic stage 3 – 4 accounts for stage T1 – 4, stage N1 – 3, and stage M0 – 1.

PD-L1 positivity is identified in 25 patients with +1 intensity in 10 patients (13.2%), +2 in 7 patients (9.2) and +3 intensity in 8 patients (10.5%). Poor prognosis parameters we examined in this study are stage N and stage M tumor. Stage N tumor indicates if the tumor has already infiltrated lymph nodes and its severity is classified as N0, N1, N2, and N3. Stage M is classified as M0 and M1. M0 indicates no metastasis and M1 indicates metastasis.

In this study, we examined the correlation between PD-L1 expression and poor prognosis by using Fischer’s exact tests. We found out that there is significant correlation between PD-L1 expression and metastasis ($P=0.022$). However, there is no significant correlation between PD-L1 expression and stage N tumor ($P=0.167$). The correlation between PD-L1 expression and poor prognosis is summarized in Table 2.

**Discussion**

It has been demonstrated that PD-1/PD-L1 plays an important role in anti-tumor immune response evasion. PD-L1 can be detected on tumor cells, which contribute to inhibit local immune response. Other studies have also demonstrated the correlation between PD-L1 expression and clinical outcome.

Our study revealed low presentation of tumor PD-L1 expression (19.7%) compared to other studies on penile SqCC (range 40 – 62%). Discrepancies in these findings may be due to smaller sample size in our study compared with other studies. This also might be caused by the low prevalence of penile cancer in Asian men in general, compared to other races. Larger study is needed to compare PD-L1 expression between races and its relation to metastasis and survival rate in each group.

**Table 1. Continued**

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Total n=76</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anatomic stage</strong></td>
<td></td>
</tr>
<tr>
<td>Stage 0 – 2 (T0-3,N0,M0)</td>
<td>17 (22.4)</td>
</tr>
<tr>
<td>Stage 3 – 4 (T1-4,N1-3,M0-1)</td>
<td>59 (77.6)</td>
</tr>
<tr>
<td><strong>PD-L1 intensity</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>51 (67.1)</td>
</tr>
<tr>
<td>+1</td>
<td>10 (13.2)</td>
</tr>
<tr>
<td>+2</td>
<td>7 (9.2)</td>
</tr>
<tr>
<td>+3</td>
<td>8 (10.5)</td>
</tr>
</tbody>
</table>

**Table 2. Expression of PD-L1 linked to nodal and distant metastasis.**

<table>
<thead>
<tr>
<th>Poor prognosis</th>
<th>PD-L1 positive n=15 (19.7%)</th>
<th>PD-L1 negative n=61 (80.3%)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>1</td>
<td>16</td>
<td>0.167</td>
</tr>
<tr>
<td>N1</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>7</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td>5</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Stage M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>10</td>
<td>56</td>
<td>0.022</td>
</tr>
<tr>
<td>M1</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
In our study we found out that there is a significant correlation between PD-L1 expression and stage M tumor ($P=0.022$). This result show similarities with the study conducted by Udager et al., which reported that there is strong correlation between PD-L1 expression and metastases.\textsuperscript{21}

In addition, Udager et al. (2016) reported that there is also significant correlation between PD-L1 in tumor cells and positive lymph node status.\textsuperscript{21} However, in our study, we examined that there is no significant correlation between PD-L1 expression and stage N tumors.

PD-L1 expression in tumor cells is used as a predictive biomarker in lung cancer.\textsuperscript{24–26} Unfortunately, no clinical data about the response to checkpoint inhibitors in the penile SqCC setting. However, the squamous histology of penile SqCC resembles that of lung SqCC. Based on these data, we can assume that PD-L1 expression in tumor cell might be a predictive biomarker in penile SqCC.\textsuperscript{22} Evidently, as a predictive biomarker for response to therapy, PD-L1 expression is organ dependent. While PD-L1 expression associated with response to anti-PDL1 agents in bladder cancer patients, no correlation found between PD-L1 expression and response to anti PDL-1 agent in lung cancer.\textsuperscript{28}

There are several compounds that should be considered to give anti-PD-L1 immunotherapy. Each compound is associated with a specific aspect diagnostic PD-L1 IHC assay. These assays use different antibodies and have unique cut-offs for determining PD-L1 positive expression. Different antibodies used in diagnostic method will determine the positive expression of PD-L1 differently.\textsuperscript{21}

Further study should be done to investigate the necessity of giving immunotherapy to patients with PD-L1 positive penile SqCC. Several studies reported that clinical trials using the anti-PD-1 nivolumab have demonstrated clinical response which is independent of PD-L1 expression.\textsuperscript{25,26} On the other hand, Su et al. reported that patient their studies responded well to immunotherapy. They suggested that immunotherapy could be a great option for the treatment of metastatic penile SqCC. Nowadays, there are also ongoing trials by using various monoclonal antibodies, such as atezolizumab, pembrolizumab, and avelumab to investigate immunotherapeutic effects in PD-L1 positive penile SqCC.\textsuperscript{30} Based on these findings, we suggest further study about immunotherapy due to the PD-L1 positive expression in penile SqCC.

The major strength of this study is the integration of PD-L1 expression with pre-existing data. Our study is focused in examining the correlation between PD-L1 expression and poor prognosis linked to survival. The major weakness of this study is low presentation of tumor PD-L1 expression (19.7%) compared to other studies on penile SqCC (range 40–62%) due to small size samples. Several slides used in this study also have decreased in quality due to long period research.

**Conclusion**

PD-L1 positively expressed by several cases of penile SqCC in our cohort Indonesian patient subjects is associated with poor clinical outcome. We found out the significant correlation between PD-L1 expression and metastases. In our study, we also examined that PD-L1 expression correlates strongly with survival, makes it may benefit from immunotherapy.

**Data availability**

**Underlying data**

Figshare: Data of PD-L1 in Penile Squamous Carcinoma.xlsx, https://doi.org/10.6084/m9.figshare.21378411.v1.\textsuperscript{31}

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

**References**


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