Programmed death-ligand 1 expression in tumor cells and tumor-infiltrating lymphocytes are associated with depth of tumor invasion in penile cancer

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Received January 6, 2023; Accepted April 25, 2023

DOI: 10.3892/br.2023.1627

Abstract. The present study aimed to demonstrate the proportion of the programmed death-ligand 1 (PD-L1) expression in penile cancer patients and the association with clinicopathological parameters. Formalin-fixed paraffin-embedded specimens were obtained from 43 patients with primary penile squamous cell carcinoma treated at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, between 2008 and 2018. PD-L1 expression was evaluated by the immunohistochemistry using an SP263 monoclonal antibody. PD-L1 positivity was defined as >25% tumor cell staining or >25% tumor-associated immune cell staining. The correlation between PD-L1 expression and clinicopathological parameters was analyzed. A total of eight of 43 patients (18.6%) were identified as positive for PD-L1 expression in tumor cells and tumor-infiltrating lymphocytes. In the PD-L1 positive group, there was a significant association with pathological T stage (P=0.014) with a higher percentage of PD-L1 positive tumors in T1 stage compared with T2-T4 stage. In this cohort, there was a trend towards longer survival in patients with positive PD-L1 expression (5-year OS: 75% vs. 61.2%, P=0.19). Lymph node involvement and the location of tumor at the shaft of penis were two independent prognostic factors for survival. In conclusion, the PD-L1 expression was detected in 18% of penile cancer patients and high expression of PD-L1 was associated with the early T stage.

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Key words: penile cancer, PD-L1, programmed death-ligand 1, squamous cell carcinoma of penis

Introduction

Primary penile squamous cell carcinoma (SCC) is an uncommon neoplasm in men. In Europe and the US, the reported incidence is ~<1% per 100,000 person-year (1). By contrast, in some countries such as India or Brazil, the prevalence is high ranging from 2.3-8.3 per 100,000 person-year (2-4). In Thailand, according to data from 2008-2012, the age-standardized rate of penile cancer incidence is high (1.4 per 100,000) and ranked top three in the world (5).

Penile cancer is an aggressive tumor with limited systemic treatment options in a locally advanced and advanced stage (1,3). Therefore, identifying prognostic biomarkers is important and could be applied to predict treatment outcomes and planning.

Programmed cell death ligand-1 (PD-L1) is a T-cell regulatory protein expressed on the surface of tumor and tumor-infiltrating lymphocytes. The PD-L1/PD-1 pathway is important in cancer progression (6). The binding between PD-L1 of cancer cells with PD-1 of immune cells helps cancers evade the host immune response and prevents cancers from being killed by cytotoxic T lymphocyte (6,7). In the past few decades, major advances in immunotherapy, especially the use of immune checkpoint inhibitors of anti-PD1 or anti-PDL1 have changed the treatment paradigm in a number of types of cancer. Expression of PD-L1 by tumor cells and tumor-infiltrating lymphocytes has been described in various types of cancer, such as renal cell carcinoma, bladder, and lung cancer (8-11), and has been identified as both a prognostic and predictive marker.

Earlier studies reported high PD-L1 expression positivity in penile cancer, in endemic and non-endemic areas (12-15). However, the results for its prognostic role remain contradictory.

The present study examined the clinicopathological characteristics of PD-L1 expression in penile cancer and the association between PD-L1 expression in tumor cells and immune cells in an endemic area.

Materials and methods

Patients and clinicopathological data. The present study was a retrospective study. All penile SCC patients who were diagnosed and underwent surgical resection in Srinagarind Hospital between 2008 and 2018 were included. The unavailable formalin-fixed paraffin-embedded (FFPE) tissue and those surviving <30 days were excluded from the present study. Finally, the FFPE tissues from 43 patients which were all primary penile SCC, were available for the present study.

Demographic data were collected including age, performance status, and survival time of patients according to Eastern Cooperative Oncology Group (ECOG) (16). The histologic subtype, histologic grading, lymphovascular invasion (LVI) status, and perineural invasion (PNI) status were evaluated using 2016 WHO classification standard templates (17). The pathological staging was performed according to the 8th edition American Joint Committee on Cancer (AJCC) staging system (18). The pre-treatment immune profiles including hemoglobin, total white blood cells, neutrophil count, lymphocyte count, and platelet count were recorded. Neutrophil-lymphocyte ratio and platelet-lymphocyte ratio were calculated.

The present study was approved by the Institutional Review Board of the Khon Kaen University Ethics Committee for Human Research based on the Declaration of Helsinki and the ICH Good Clinical Practice Guidelines (HE611509). For this type of study, formal consent was not required in accordance with institutional guideline.

PD-L1 immunohistochemistry. For all tissue, PD-L1 immunohistochemistry was performed on a representative block of the whole slide section. Tumor sections (4 μm) were deparaffinized and stained with an anti-PD-L1 antibody (VENTANA PD-L1 clone SP263; Roche Diagnostics, cat. no. 790-4905 (prediluted). PD-L1 was stained using Ventana Benchmark XT IHC staining module (incubated at 37˚C for 1 h) and detected by OptiView DAB Detection kit. The sections were counterstained by hematoxylin followed by bluing reagent. The slides were subsequently removed and rinsed, dehydrated, cleared and mounted.

The percentage of tumor cells with membranous staining was assessed separately by two evaluators (one senior resident-in-training and one board-certified pathologist) blinded to the patient's clinicopathological parameters. The tumor and immune cells were considered PD-L1 expression status according to the interpretation guide for the VENTANA PD-L1 (SP263) Assay Scoring (Table I).

Statistical analysis. SPSS software version 27 (IBM Corp.) was used to analyze the association between PD-L1 expression and clinicopathological parameters (including tumor size, histological grading, histologic subtype, staging and survival time) with χ^2 or Fisher's exact test as appropriate. The differences in continuous data between the two dependent groups were analyzed by either an independent t-test (parametric test) or Mann-Whitney test (non-parametric test). Values were presented as the mean ± SD. The survival analysis was conducted and analyzed using Kaplan-Meier estimation with Log-rank and Cox regression tests. The analysis time was restricted to a 10-year period due to the late crossover event. The present study selected a 10-year period as it is the reasonable duration to declare the cure of the disease (19). P<0.05 was considered to indicate a statistically significant difference.

Results

PD-L1 expression and clinicopathological features. The present study included 43 patients with penile SCC. No patient received neoadjuvant chemotherapy or radiotherapy before complete resection. A total of eight out of 43 cases (18.6%) were identified with positive PD-L1 expression, while 35 cases (81.4%) exhibited negative PD-L1 expression (Fig. 1). PD-L1 immunoreactivity in tumor cells (TC) >5% was found in 23 patients (53.5%).

The correlation of PD-L1 status with clinic-pathological characteristics is shown in Table II. Briefly, the median age was 58 years. The PD-L1 expression showed no significant difference between primary tumor locations including tip and shaft (P=0.390).

PD-L1 expression was not significantly correlated with histological grade (P=0.390), lymphovascular or perineural invasion (P=0.340 and 0.530, respectively). Moreover, no association was observed between PD-L1 expression and nodal involvement (P=0.330). Notably, the present study found that pathological T staging, which represented the depth of primary tumor invasion, displayed a statically significant correlation with PD-L1 positivity; 75% of pT1 stage SCC patients were PD-L1 positive, while only 25% of patients with pT2-pT4 were positive for PD-L1 expression (P=0.014; Table II).

Univariate and multivariate analysis of PD-L1 expression and clinicopathological features in the survival of penile SCC patients. At the time of data analysis, 24 patients (55.8%) had succumbed and the median follow-up time was 89.7 months. The median survival time was 7.4 years (95% Confidence Interval 3.7-9.7). The 1, 5, and 10-year OS rates were 78.6, 63.6, and 25% respectively.

The survival rate was analyzed using Kaplan-Meier estimation with a log-rank test. There was a statistically significant difference in clinicopathological features such as tumor location [Hazard ratio (HR)=4.76, P=0.003], histological grade (HR=4.25, P=0.005), LVI (HR=4.89, P=0.002), PNI (HR=4.75, P=0.02), T category (HR=4.31, P=0.002) and lymph node metastasis (HR=3.56, P=0.003) compared with their references. While there was no statistical significance in the patient's age, ECOG score and PD-L1 expression. The significant clinicopathological features of survival analysis were further analyzed to identify independent prognostic factors using the Cox regression test. The result showed that tumor at shaft and positive lymph node metastasis were independent factors for poor survival of SCC patients (HR=4.81 and 2.59, P=0.015 and 0.009, respectively; Table III and Fig. 2).

Expression of PD-L1 in tumor cells and immune cells. The immune profile and inflammatory markers were compared among PD-L1 positive and negative tumors as shown in Table IV. The number of peripheral white blood cell count, total lymphocytes, neutrophil-lymphocyte ratio, and platelet-lymphocyte ratio were comparable. No statistically

Table I. The scoring criteria of PD-L1 status.

PD-L1 interpretation	Staining description
High/positive	PD-L1 status is considered high/positive if any of the following are met: • ≥25% of TC express membrane (any intensity above background) in invasive area; or, • ICP >1% and IC+ ≥25%; or, • ICP=1% and IC+=100%.
Low/negative	PD-L1 status is considered low/negative if: • None of the criteria for PD-L1 high status are met.

PD-L1, programmed cell death ligand-1; TC, tumor cells; ICP, Immune cells present within tumor area; IC+, Immune cells positive PD-L1 expression.

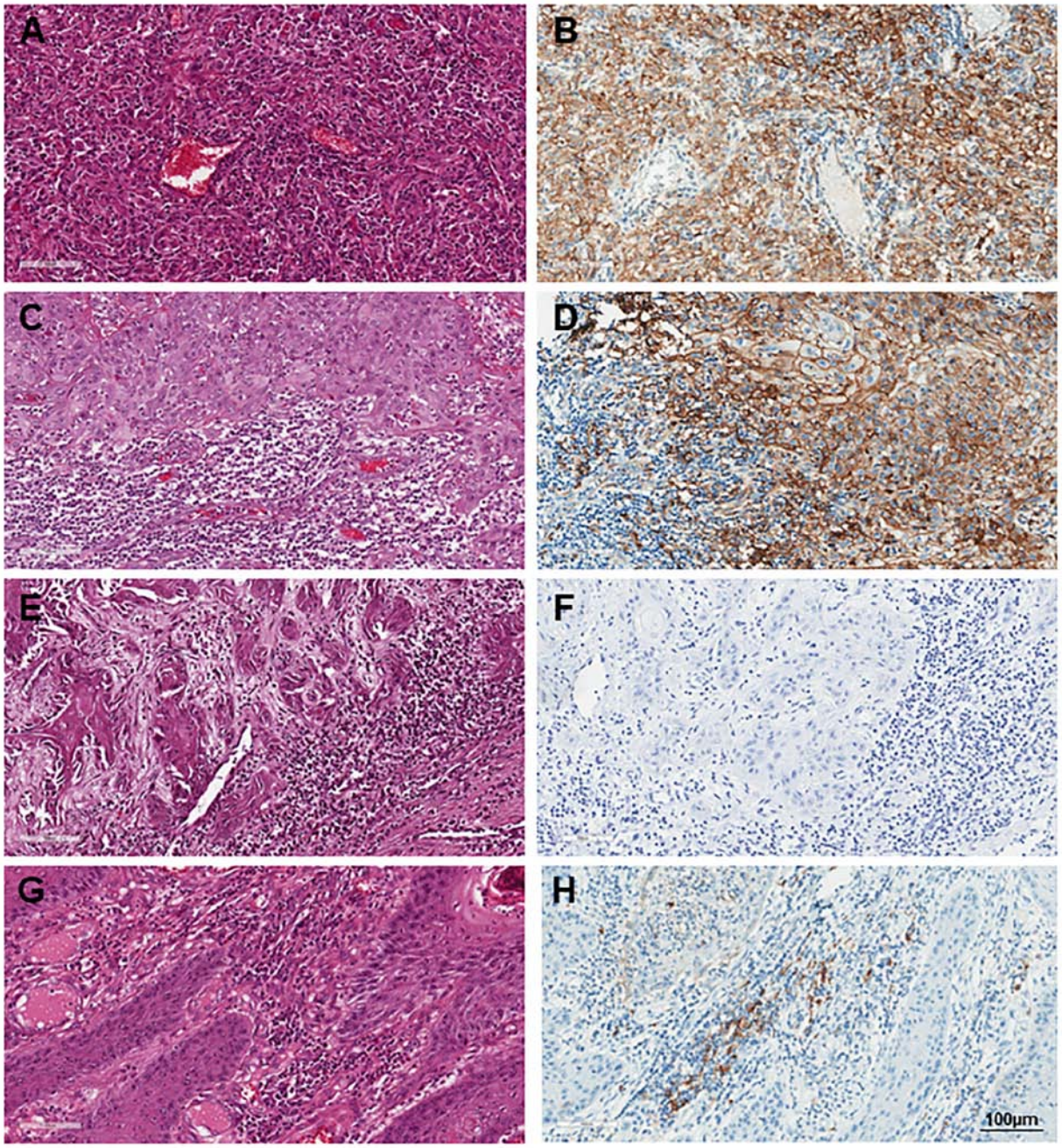

Figure 1. H&E and PD-L1 staining in penile SCC tissues. (A-D) H&E staining and positive PD-L1 expression in same area of tissue. (E-H) H&E staining and negative PD-L1 expression in the same area of tissue. Scale bar, 100 μm (magnification, x40). H&E, hematoxylin and eosin; PD-L1, programmed cell death ligand-1; SCC, squamous cell carcinoma.

Table II. Clinicopathological characteristics of PD-L1 positive and negative in tumors.

Features	All patients	PD-L1 positive (n=8)	PD-L1 negative (n=35)	P-value
Age, median (range)	58 (26-84)	58; 48-84	58; 26-80	0.490
ECOG, n (%)				
0	36 (83.7)	6 (75)	30 (85.7)	0.390
1	7 (16.3)	2 (25)	5 (14.3)	
Location				
Tip	33 (76.7)	7 (87.5)	26 (74.3)	0.390
Shaft	10 (23.3)	1 (12.5)	9 (25.7)	
Histological grade				
1	36 (83.7)	6 (75)	30 (85.7)	0.390
2-3	7 (16.3)	2 (25)	5 (14.3)	
LVI positive	5 (11.6)	0	5 (14.3)	0.340
PNI positive	3 (7)	0	3 (8.6)	0.530
T stage				
T1	14 (32.6)	6 (75)	8 (22.9)	0.014
T2-4	29 (67.4)	2 (25)	27 (77.1)	
Lymph node metastasis (N stage)				
Negative	27 (62.8)	4 (50)	23 (65.7)	0.330
Positive	16 (37.2)	4 (50)	12 (34.3)	
Stage				
I-II	24 (55.8)	4 (50)	20 (57.1)	0.510
III-IV	19 (44.2)	4 (50)	15 (42.9)	

PD-L1, programmed cell death ligand-1; ECOG, Eastern Cooperative Oncology Group; LVI, Lymphovascular invasion; PNI, Perineural invasion; HR, hazard ratio; CI, confidence interval.

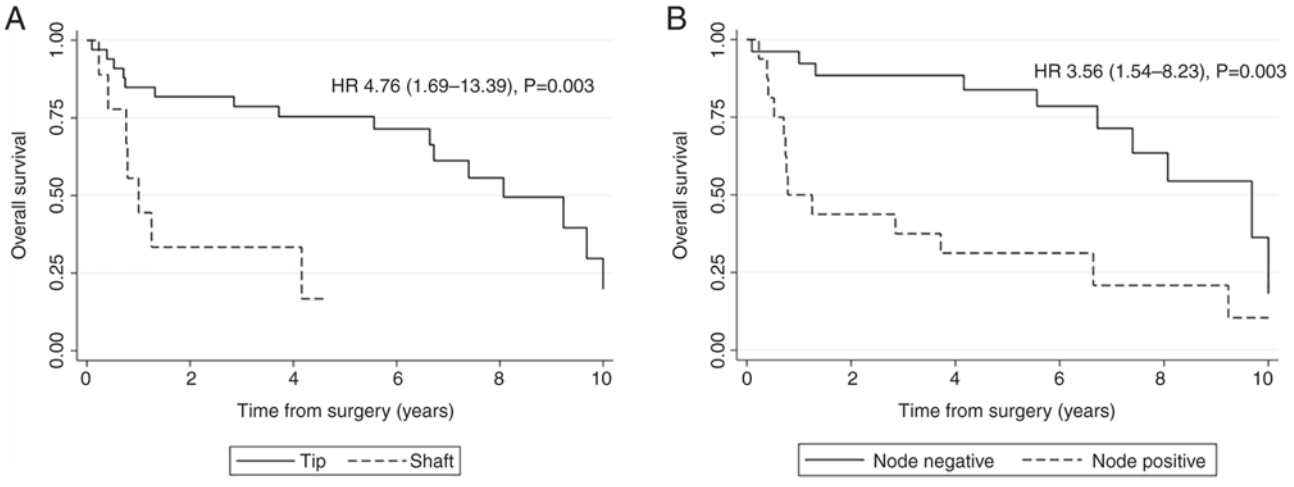

Figure 2. The significant survival analysis of clinicopathological features of SCC patients. (A) tumor locations. (B) lymph node metastasis status. SCC, squamous cell carcinoma; HR, hazard ratio.

significant difference was observed in the immune profile and inflammatory markers between PD-L1 positive and negative.

Comparing PD-L1 positive and negative cases, the percentage of PD-L1 expression was higher in both tumor cells (57.5 vs. 3%; P=0.0001) and immune cells (12.5 vs. 3%; P=0.012) (Table IV and Fig. 3). Moreover, the correlation analysis between PD-L1 expression in tumor and immune cells has shown that there was a high positive correlation between tumor cells and immune cells PD-L1 expression, R^2=0.55, P<0.001 as in Fig. 4.

Table III. Univariate and multivariate analysis for overall survival.

Characteristic	Median survival (years)	Univariate		Multivariate	
		HR (95%CI)	P-value	HR (95%CI)	P-value
Age					
<58	8.07	Reference		-	
>58	6.64	1.33 (0.58-3.03)	0.5	-	-
ECOG					
0	7.40	Reference		-	
1	0.74	4.18 (0.92-19.0)	0.06	-	-
Histological Grade					
1	8.07	Reference		Reference	
2-3	0.79	4.25 (1.54-11.75)	0.005[a]	1.37 (0.27-6.84)	0.70
Location					
Tip	8.07	Reference		Reference	
Shaft	1.00	4.76 (1.69-13.39)	0.003[a]	4.81 (1.35-17.16)	0.015[a]
T stage					
T1-2	9.23	Reference		Reference	
T3-4	1.32	4.31 (1.71-10.85)	0.002[a]	2.39 (0.67-8.46)	0.18
Lymph node metastasis (N)					
Node negative	9.68	Reference		Reference	
Node positive	0.79	3.56 (1.54-8.23)	0.003[a]	2.59 (0.87-7.73)	0.09[a]
LVI					
Negative	8.07	Reference		Reference	
Positive	1.25	4.89 (1.62-14.81)	0.005[a]	1.40 (0.31-6.41)	0.66
PNI					
Negative	8.07	Reference		Reference	
Positive	1.00	4.73 (1.29-17.41)	0.02[a]	2.06 (0.43-9.86)	0.37
PD-L1 status					
Negative	6.72	Reference		-	
Positive	NR	0.41 (0.09-1.76)[b]	0.23	-	-

[a]P<0.05. [b]Estimation by Cox regression test; HR, hazard ratio; CI, confidence interval; NR, not reached.

Discussion

Immune checkpoint inhibitors have been shown to possess significant benefits in various types of cancer (6). The percentage of PD-L1 expression in tumor and immune cells is the prognostic and predictive biomarker for PD-1/PD-L1 blockade agents in several tumors including non-small cell lung cancer and gastric carcinoma (9,20). However, in certain types of cancer, such as renal cell carcinoma, bladder cancer and melanoma, PD-L1 expression does not predict the benefit of an anti-PD1 agent (21-23). Even patients identified as PD-L1 negative may derive benefits from therapy.

In the present study in an endemic area, the PD-L1 status of tumor cells and immune cells in a cohort of primary SCC of the penis was evaluated using the SP263 antibody. It was found that 18.6% of the tumors were identified as PD-L1 positive. The PD-L1 positive rate in penile cancer varies greatly according to the type of antibody and the cut-off value. Studies have validated PD-L1 in penile cancer and report a positive rate ranging from 7.3-87% (12,15). Udager *et al* (15) were among the first to report the PD-L1 expression in 37 patients with penile SCC and the PD-L1 expression was positive in 62.2% of cases. The lower reported positive rates in this study could be the higher cut-off point in tumor cells; TC ≥25% in this study vs. TC ≥1-5% in other studies. When the cut-off value to TC >5% was re-examined in the present study, the PD-L1 positive rate was 53.5% which is comparable to the results from China and Brazil (4,13).

Montella *et al* (24) demonstrated the highest proportion of positive PD-L1 expression in tumor cells and immune cells using either SP142 or SP263 antibody in T1 stage and lower PD-L1 positivity in T2, T3, and T4 accordingly. Similarly the present study also found a statistically significant correlation between PD-L1 expression and pT staging in which

Table IV. Comparison of immune profile between the PD-L1 expression status of SCC patients.

Immune profile	PD-L1 positive (n=8)	PD-L1 negative (n=35)	P-value
PD-L1 expression on tumor cells (%) median, IQR	57.5, 35-67.5	3, 0.5-15	<0.001[a]
PD-L1 expression on immune cells (%) median, IQR	12.5, 4-18.7	3, 0.5-7.5	0.012[a]
Percent of tumor-associated immune cells in the tumor area (%) median, IQR	52.5, 28.7-65	25, 12.5-50	0.091
Hb (g/dl) median, IQR	13.5, 11.7-14	12.5, 11-13.6	0.250
White blood cells ($10^3/\mu$l) median, IQR	10.8, 7.8-17.9	9.2, 7.2-12.4	0.430
Total PMN ($10^3/\mu$l) median, IQR	5.6, 4.4-13.8	5.8, 4.2-9.0	0.640
Total lymphocyte ($10^3/\mu$l) median, IQR	2.4, 1.8-2.8	2.1, 1.4-2.9	0.640
Platelet ($10^3/\mu$l) median, IQR	274, 217-356	297, 249-391	0.280
Neutrophil-lymphocyte ratio median, IQR	2.6, 1.9-8.2	2.7, 2.0-5.2	0.840
Platelet-lymphocyte ratio median, IQR	111.1, 69.4-395.5	133, 86.8-241.5	0.660

[a]P<0.05. PD-L1, programmed cell death ligand-1; SCC, squamous cell carcinoma; IQR, interquartile range.

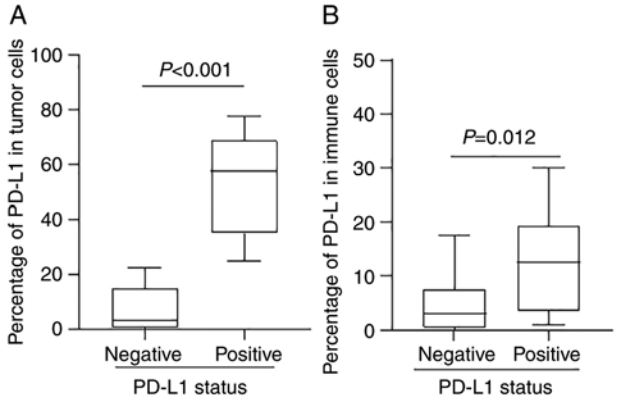

Figure 3. The comparison of PD-L1 levels between negative and positive in tumor and immune cells. (A) The expression of PD-L1 in tumor cells. (B) The expression of PD-L1 in immune cells. PD-L1, programmed cell death ligand-1.

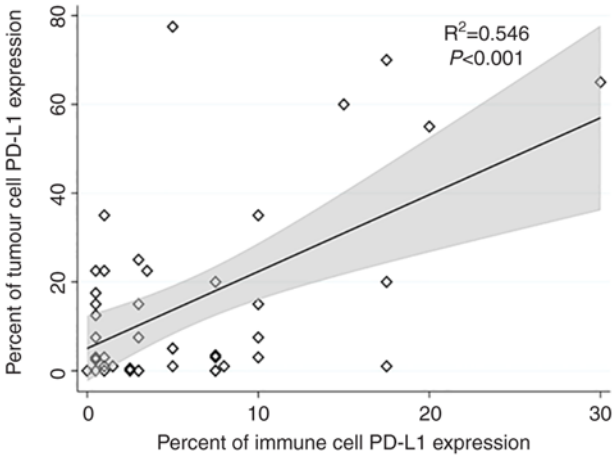

Figure 4. The association of PD-L1 expression between tumor and immune cells. X-axis is PD-L1 expression in immune cells and Y-axis is PD-L1 expression in tumor cells. PD-L1, programmed cell death ligand-1; R^2, Pearson's correlation coefficient.

75% of SCC patients with PD-L1 positive correlated with pT1 stage.

PD-L1 expression has been associated with regional lymph node metastasis and decreased cancer-specific survival in several studies (12,15,25,26). By contrast, in this cohort, PD-L1 positivity did not show worse survival outcomes when compared with negative patients. The present study further examined the PD-L1 expression by tumor cells at the cut-off value of 1 and 5%, but no survival difference was found between those with positive or negative PD-L1.

In a recent meta-analysis, higher PD-L1 expression was associated with shorter cancer-specific survival in Caucasians but not in Asians (Chinese study). Furthermore, it was not associated with overall survival (27). The different races of patients and etiology of penile cancer along with different PD-L1 antibody, detection technique and cut-off level could explain the variations of the results. Further standardization of the technique designated for penile cancer is warranted.

With limited data, immune checkpoint inhibitors, either single agent anti PD-1 or a combination of anti PD-1/anti CTLA4, did not provide an impressive outcome compared with other types of tumor (28,29). PD-L1 expression as a predictive biomarker for ICIs in advanced penile cancer remains controversial. However, more data regarding ICI combined with chemotherapy or radiotherapy in the future is expected (30).

The major strength of the present study included the use of whole tissue sections, which has significant advantages over tissue microarray (TMA), as whole tissue sections allow a more comprehensive assessment of tumor protein expression. This is especially important for PD-L1 immunochemistry, which can reduce interpretative bias from tumor heterogeneity compared with the tissue microarray technique. Moreover, the present study was the first conducted in the high incidence area of southeast Asia. The present study had a long follow up period and a high proportion of node positive disease.

There are a few limitations in the present study. First, the number of PD-L1 positive cases was small and only single PD-L1 antibody was used. Second, *in vitro* experiments for PD-L1 positivity were not performed. Third, the present study did not evaluate the HPV status of p16 expression in the samples. Larger number of tumor samples and *in vitro* validations are needed in future studies.

In summary, PD-L1 expression was found in 18% of primary penile SCC and PD-L1 positivity (high expression) was more common in the early pT stage (pT1).

Acknowledgements

Not applicable.

Funding

The present study was supported by the Faculty of Medicine, Khon Kaen University, Thailand (grant no. IN63132).

Authors' contributions

SS and JC conceived the present study. SS, NK, WS, UR, PK, AS, KW, PT, PW and JC were responsible for data curation. SS, NK, WS, UR, PK, AS, KW, PT, PW and JC were responsible for experiments. SS, NK, WS, UR, PK, AS, KW, PT, PW and JC were responsible for methodology. JC and SS confirm the authenticity of all the raw data. SS, NK, WS and JC were responsible for writing, reviewing and editing the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of the Khon Kaen University Ethics Committee for Human Research based on the Declaration of Helsinki and the ICH Good Clinical Practice Guidelines (HE611509). For this type of study, formal consent was not required in accordance with institutional guideline.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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