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ORIGINAL ARTICLE

Up-regulation of indoleamine 2,3-dioxygenase 1 (IDO1) expression and catalytic activity is associated with immunosuppression and poor prognosis in penile squamous cell carcinoma patients

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Abstract

Background: Indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan (Trp) catabolism have been demonstrated to play an important role in tumor immunosuppression. This study examined the expression and catalytic activity of IDO1 in penile squamous cell carcinoma (PSCC) and explored their clinical significance.

Methods: IDO1 expression level, serum concentrations of Trp and kynurenine (Kyn) were examined in 114 PSCC patients by immunohistonchemistry and solid-phase extraction-liquid chromatography-tandem mass spectrometry. The survival was analyzed using Kaplan-Meier method and the log-rank test. Hazard ratio of death was analyzed via univariate and multivariate Cox regression. Immune cell types were defined by principal component analysis. The correlativity was assessed by Pearson's correlation analysis.

List of abbreviations: A/G, albumin/globulin; AJCC, American Joint Committee on Cancer; CD8, cluster of differentiation 8; CI, confidence interval; CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T lymphocyte-associated protein 4; DSS, disease-specific survival; HR, hazard ratio; IDO1, Indoleamine 2,3-dioxygenase 1; IDO2, Indoleamine 2,3-dioxygenase 2; IFNγ, interferon-gamma; IHC, Immunohistochemistry; KTR, Kyn/Trp ratio; Kyn, Kynurenine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MDSC, myeloid-derived suppressor cell; NLR, neutrophil to lymphocyte ratio; PD-1, programmed cell death protein-1; PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2; PSCC, penile squamous cell carcinoma; pTNM, pathological tumor-node-metastasis; ROC, receiver operating characteristic; SPE, solid-phase extraction; TAM, tumor-associated macrophage; TDO, tryptophan 2,3-dioxygenase; TIIC, tumor-infiltrating immune cell; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T lymphocyte; Trp, Tryptophan; WBC, white blood cell.

Results: The expression level of IDO1 in PSCC cells was positively correlated with serum Kyn concentration and Kyn/Trp radio (KTR; both $P < 0.001$) but negatively correlated with serum Trp concentration ($P = 0.001$). Additionally, IDO1 up-regulation in cancer cells and the increase of serum KTR were significantly associated with advanced N stage (both $P < 0.001$) and high pathologic grade ($P = 0.008$ and 0.032, respectively). High expression level of IDO1 in cancer cells and serum KTR were associated with short disease-specific survival (both $P < 0.001$). However, besides N stage (hazard radio [HR], 6.926; 95% confidence interval [CI], 2.458-19.068; $P < 0.001$) and pathologic grade (HR, 2.194; 95% CI, 1.021-4.529; $P = 0.038$), only serum KTR (HR, 2.780; 95% CI, 1.066-7.215; $P = 0.036$) was an independent predictor for PSCC prognosis. IDO1 expression was positively correlated with the expression of interferon-γ (IFNγ, $P < 0.001$) and immunosuppressive markers (programmed cell death protein 1, cytotoxic T-lymphocyte-associated protein 4 and programmed death-ligand 1 and 2; all $P < 0.05$), and the infiltration of immune cells (including cytotoxic T lymphocytes, regulatory T lymphocytes, tumor-associated macrophages, and myeloid-derived suppressor cells; all $P < 0.001$) in PSCC tissues. Furthermore, the expression of IDO1 was induced by IFNγ in a dose-dependent manner in PSCC cells.

Conclusions: IFNγ-induced IDO1 plays a crucial role in immunoediting and immunosuppression in PSCC. Additionally, serum KTR, an indicator of IDO1 catabolic activity, can be utilized as an independent prognostic factor for PSCC.

KEYWORDS

cytotoxic T-lymphocyte-associated protein 4, immunosuppression, indoleamine 2,3-dioxygenase 1, interferon-gamma, kynurenine/tryptophan ratio, penile cancer, programmed cell death protein 1, programmed death-ligand 1, tumor-infiltrating immune cells

1 | BACKGROUND

Penile squamous cell carcinoma (PSCC) is a rare malignancy with an incidence below 1/100,000 in developed countries, but it accounts for up to 1%-2% of malignancies in South America, Southeast Asia, and parts of Africa [1]. The risk factors accounting for PSCC include human papilloma virus infection, phimosis, cigarette smoking, poor hygiene, and lack of circumcision [2]. Immunosuppression has been demonstrated to facilitate the development and metastasis of penile cancer [3, 4]. Programmed cell death-ligand 1 (PD-L1), a suppressive costimulatory molecule of T cells, was found to be highly expressed in PSCC cells, and PD-L1 expression was significantly associated with metastasis and poor prognosis of PSCC [4, 5].

Indoleamine 2,3-dioxygenase 1 (IDO1), an important enzyme catabolizing tryptophan (Trp) into kynurenine (Kyn), is mainly expressed in macrophages, dendritic cells, and tumor cells [6]. Kyn metabolites interact with aryl hydrocarbon receptor as a ligand and consequently suppress the antitumor immune response by promoting the differentiation of Foxp3$^+$ regulatory T lymphocytes (Tregs) [6-9]. Trp depletion can act as a potent regulatory signal via molecular stress-response pathways involving general control nonrepressed 2 kinase, which leads to cell cycle arrest and functional anergy in cytotoxic T lymphocytes (CTLs) and promotes the differentiation and activation of Tregs [6]. Other enzymes catabolizing Trp into Kyn include indoleamine 2,3-dioxygenase 2 (IDO2) and tryptophan 2,3-dioxygenase (TDO) [10-12]. Nonetheless, IDO1 plays an important role in Trp catabolism due to its substantially increased enzyme activity compared with IDO2 and TDO [11] and initiates the Kyn pathway in a majority of settings [12].

Previous studies have indicated that IDO1 is expressed in many human cancers, and the high expression of IDO1 is associated with poor prognosis in a variety of cancer types [13-16]. Both Trp depletion and Kyn accumulation provide a favorable microenvironment for tumor cells to escape from immune surveillance [6, 8]. IDO1 catalytic activity, characterized by the Kyn/Trp ratio (KTR), is positively associated

with tumor development, metastasis, and poor prognosis [13-15]. However, IDO1 expression and activity in PSCC have not been reported. In this study, we examined the expression level and catalytic activity of IDO1 in PSCC and tried to explore the effect of IDO1 on the immune microenvironment and the correlation of IDO1 with clinicopathological characteristics and prognosis in PSCC patients.

2 | MATERIALS AND METHODS

2.1 | Patient selection

For this retrospective study, PSCC patients treated at Sun Yat-sen University Cancer Center (Guangzhou, China) between January 2009 and January 2016 were selected according to the following inclusion criteria: (1) patients had newly diagnosed PSCC; (2) they had undergone partial or radical penectomy with or without bilateral inguinal lymphadenectomy. This study was approved by the institution's ethics committee and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. The pathological tumor-node-metastasis (pTNM) and pathological grade were re-evaluated by two pathologists according to the eighth edition of TNM staging system from the American Joint Committee on Cancer (AJCC) [17]. Regular outpatient or telephone follow-ups were carried out, all patients underwent follow-up every 3 months for the first 2 years after surgery, every 6 months in the following two years, and every year thereafter. The last follow-up was September 29, 2019. Follow-up examinations included a clinical examination, ultrasound or computed tomography, and tumor marker examination.

2.2 | Measurement of serum Trp and Kyn concentrations

Serum Trp and Kyn concentrations were determined by solid-phase extraction (SPE)-liquid chromatography-tandem mass spectrometry (LC-MS/MS) with deuterated internal standards [13, 14]. Briefly, preoperative serum samples were mixed with acetonitrile (1:3, v/v) and vibrated for 1 h. Then, supernatants were diluted two times, and reversed-phase chromatography was performed in a BEH C18 column (mobile phase: H_2O or acetonitrile, containing 0.01% heptafluorobutyric acid and 0.1% methanoic acid). The concentration of Trp or Kyn was detected in the multiple reaction-monitoring modes using a quadrupole tandem mass spectrometer (5500 Q TRAP LC-MS/MS, Applied Biosystems, Foster City, CA, USA) with positive electrospray ionization. For the analyses of the association of Trp metabolism with clinicopatholog-

ical characteristics and survival, the serum Trp and Kyn concentrations and the KTR were determined to be high or low according to cut-off values (60.4 μmol/L, 2.04 μmol/L, and 0.032, respectively) determined by receiver operating characteristic (ROC) curve analysis.

2.3 | Immunohistochemistry (IHC) staining and scoring

Formalin-fixed, paraffin-embedded tumor tissues were sectioned into 4 μm-thick slides, which were dewaxed and rehydrated according to routine practices, followed by blocking of endogenous peroxidase activity with 3% H_2O_2 and antigen retrieval by boiling as previously described [18]. The slides were incubated with primary antibodies against IDO1 (1:100; #86630; Cell Signaling Technology, Inc. (CST), Danvers, MA, USA) or cluster of differentiation 8 (CD8; 1:200; ZA-0508; ZSGB-BIO Inc., Beijing, China) at 4°C overnight and then incubated with a secondary antibody, followed by visualization with DAB staining using the Dako REAL^{TM} EnVision^{TM} Detection System (K5007, DAKO, Glostrup, Denmark) according to the manufacturer's instructions. Finally, the slides were counterstained with hematoxylin (#14166; CST).

The staining was scored independently by two pathologists. For IDO1 expression in cancer cells, IHC staining intensity was graded as follows: negative (0), weak (1), moderate (2), or strong staining (3) (Supplementary Figure S1A), and the IDO1 expression level in cancer cells was presented as an H-score, which was the sum of each intensity grade multiplied by its corresponding percentage [19, 20]. In contrast, the expression of IDO1 in tumor-infiltrating immune cells (TIICs) and the expression of CD8 in tumor-infiltrating lymphocytes (TILs) were presented as the percentage of positive cells (Supplementary Figure S1B). The expression levels of IDO1 in cancer cells and TIICs (the density of IDO1$^+$ TIICs), and the density of CD8$^+$ TILs were identified as high or low according to cut-off values (1.15, 8.5%, and 9.5%, respectively) determined by ROC analysis.

2.4 | Cell culture

The PSCC cell lines (Penl1, Penl2, and 149Rca), which were previously established by our group [21-23], and the lung squamous carcinoma cell line H596 (ATCC HTB-178, American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C with 5% CO_2.

2.5 | Western blotting

Western blotting was performed as previously described [24, 25]. Briefly, the cells mentioned above were collected and lysed in lysis buffer [24] and then concentrated by a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Roche Diagnostics GmbH, Mannheim, Germany). After blocking with nonfat dry milk (5%, w/v), membranes were incubated sequentially with primary antibodies against IDO1 (1:1000; #86630; CST) or β-actin (1:1000; #4970; CST) and with horseradish peroxidase-linked anti-rabbit IgG (1:2000; #7074, CST); the bands were visualized with enhanced chemiluminescence substrate (Bio-Rad, Hercules, CA, USA), and the signals were captured via a ChemiDoc^{TM} Touch Imaging System (Bio-Rad).

2.6 | RNA extraction, reverse transcription, and semi-quantitative real-time PCR (qPCR)

Total RNA was extracted from PSCC cells or RNA later-preserved cancer tissues using TRIzol reagent (Takara, Kusatsu, Japan) and then reversely transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). qPCR was performed on a CFX Connect^{TM} Real-Time System (Bio-Rad) using SYBR$^{®}$ Premix Ex Taq^{TM} (Takara) with specific primers (Supplementary Table S1) according to the manufacturer's instructions. The relative expression of genes was calculated via the $2^{-\Delta Ct}$ method (normalized to *GAPDH* expression) [26, 27].

2.7 | Statistical analysis

Statistical analysis was performed with SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA). Cut-off values for IDO1 level, CD8$^+$ TILs, serum Kyn and Trp concentrations and KTR, albumin/globulin (A/G) ratio, counts of neutrophils and lymphocytes in blood, and neutrophil to lymphocyte ratio (NLR) were determined by ROC curve analysis, while those for age and white blood cell (WBC) count were defined according to medians. The event for disease-specific survival (DSS) was death from PSCC. The duration was calculated from surgery date to the date of event occurrence, the last follow-up or die of other non-PSCC causes. The DSS results were analyzed using Kaplan-Meier method and the log-rank test. Univariate and multivariate Cox regression analyses were performed for hazard ratio (HR) of death. Principal component analysis was used to define immune cell types by their corresponding signature markers. Pearson's correlation analysis was employed for correlation assessment. Analysis of variance was used for comparisons between multiple groups. Two-sided P values of less than 0.05 were considered to indicate statistical significance.

3 | RESULTS

3.1 | IDO1 expression and its catalytic activity in PSCC patients

A total of 114 PSCC patients were selected, median age is 54 years (range, 24-86 years) and the detailed information on the involved patients is shown in Supplementary Table S2. We first examined IDO1 expression in their PSCC tissues by IHC and found that IDO1 was expressed in both cancer cells and TIICs (Supplementary Figure S1). Thirty-three (28.9%) patients had high levels of IDO1 expression in cancer cells, while 38 (33.3%) patients had high densities of IDO1$^+$ TIICs (Supplementary Table S2). Correspondingly, serum concentrations of Kyn and Trp were also detected using a SPE-LC-MS/MS method. Correlation analysis showed that the expression level of IDO1 in cancer cells, but not that in TIICs, was positively correlated with serum Kyn concentrations and the KTR (both $P < 0.001$), and negatively correlated with Trp concentrations ($P = 0.001$; Supplementary Figure S2), though IDO1 in both cancer cells and TIICs catalyze Trp into Kyn, consequently influencing serum concentrations of Kyn and Trp and the KTR.

3.2 | The relationships between IDO1 expression level or Trp catabolism and clinicopathological and hematologic parameters in PSCC

We analyzed the relationships between IDO1 expression level or Trp catabolism and clinicopathological parameters. We found that the expression of IDO1 in cancer cells, but not that in TIICs, was significantly associated with N stage ($P < 0.001$), pathologic grade ($P = 0.008$) and CD8$^+$ TIL density ($P = 0.001$; Table 1). Meanwhile, the serum Kyn concentration was significantly associated with N stage ($P < 0.001$) and CD8$^+$ TIL density ($P = 0.012$), while the KTR was associated with N stage ($P < 0.001$), pathologic grade ($P = 0.032$), and peripheral blood lymphocyte count ($P = 0.031$) and NLR ($P = 0.002$). However, the serum Trp concentration was not associated with the clinicopathological or hematologic parameters (all $P > 0.05$), except CD8$^+$ TIL density ($P = 0.040$; Table 2).

TABLE 1 The associations between IDO1 expression and clinicopathological characteristics of 114 PSCC patients

Characteristic	IDO1 H-score in cancer cells			IDO1$^+$ TIIC density		
	Low [cases (%)]	High [cases (%)]	P value	Low [cases (%)]	High [cases (%)]	P value
Total	81 (71.1)	33 (28.9)		76 (66.7)	38 (33.3)	
Age			0.302			0.427
< 54 years	43 (75.4)	14 (24.6)		40 (70.2)	17 (29.8)	
≥54 years	38 (66.7)	19 (33.3)		36 (63.2)	21 (36.8)	
Phimosis			0.265			0.080
Absent	21 (63.6)	12 (36.4)		26 (78.8)	7 (21.2)	
Present	60 (74.1)	21 (25.9)		50 (61.7)	31 (38.3)	
T stage			0.189			0.779
T_1	30 (78.9)	8 (21.1)		26 (68.4)	12 (31.6)	
T_{2-4}	51 (67.1)	25 (32.9)		50 (65.8)	26 (34.2)	
N stage			<0.001			1.000
N_0	57 (86.4)	9 (13.6)		44 (66.7)	22 (33.3)	
N_{1-3}	24 (50.0)	24 (50.0)		32 (66.7)	16 (33.3)	
Pathologic grade			0.008			0.341
G_1	56 (80.0)	14 (20.0)		49 (70.0)	21 (30.0)	
G_{2-3}	25 (56.8)	19 (43.2)		27 (61.4)	17 (38.6)	
CD8$^+$ TIL density			0.001			0.596
Low	48 (85.7)	8 (14.3)		36 (64.3)	20 (35.7)	
High	33 (56.9)	25 (43.1)		40 (69.0)	18 (31.0)	
WBC count			0.302			0.112
$< 7.805 \times 10^9$/L	38 (66.7)	19 (33.3)		34 (59.6)	23 (40.4)	
$\geq 7.805 \times 10^9$/L	43 (75.4)	14 (24.6)		42 (73.7)	15 (26.3)	
Neutrophil count			0.626			0.334
$< 4.22 \times 10^9$/L	28 (68.3)	13 (31.7)		25 (61.0)	16 (39.0)	
$\geq 4.22 \times 10^9$/L	53 (72.6)	20 (27.4)		51 (69.9)	22 (30.1)	
Lymphocyte count			0.194			0.421
$< 1.75 \times 10^9$/L	31 (64.6)	17 (35.4)		30 (62.5)	18 (37.5)	
$\geq 1.75 \times 10^9$/L	50 (75.8)	16 (24.2)		46 (69.7)	20 (30.3)	
NLR			0.359			0.270
< 3.25	54 (74.0)	19 (26.0)		46 (63.0)	27 (37.0)	
≥3.25	27 (65.9)	14 (34.1)		30 (73.2)	11 (26.8)	
Serum A/G ratio			0.340			0.114
< 1.7	54 (68.4)	25 (31.6)		49 (62.0)	30 (38.0)	
≥1.7	27 (77.1)	8 (22.9)		27 (77.1)	8 (22.9)	

Abbreviations: IDO1, indoleamine 2,3-dioxygenase 1; CD8, cluster of differentiation 8; TIIC, tumor-infiltrating immune cell; WBC, white blood cell; NLR, neutrophil to lymphocyte ratio; A/G, albumin/globulin.

3.3 | Prognostic significance of IDO1 expression level and Trp catabolism in PSCC patients

Next, we analyzed the prognostic significance of the IDO1 expression level and Trp catabolism in PSCC patients using univariate Cox regression model. In addition to T stage (HR, 2.403; 95% confidence interval [CI], 1.049-5.503; $P = 0.032$), N stage (HR, 14.424; 95% CI, 5.558-37.433; $P < 0.001$),

and pathologic grade (HR, 4.608; 95% CI, 2.251-9.434; $P < 0.001$), the IDO1 expression level in cancer cells (HR, 4.956; 95% CI, 2.524-9.729; $P < 0.001$), serum Kyn concentration (HR, 4.548; 95% CI, 2.220-9.315; $P < 0.001$), Trp concentration (HR, 4.458; 95% CI, 0.228-0.921; $P = 0.025$) and KTR (HR, 6.818; 95% CI, 2.960-15.705; $P < 0.001$), CD8$^+$ TIL density (HR, 3.366; 95% CI, 1.575-7.194; $P = 0.001$), peripheral blood neutrophil count (HR, 2.804; 95% CI, 1.152-6.822; $P = 0.023$), lymphocyte count (HR, 0.424; 95% CI,

TABLE 2 The associations between Trp catabolism and clinicopathological characteristics of 114 PSCC patients

Characteristic	Serum Kyn concentration (µmol/L)			Serum Trp concentration (µmol/L)			KTR		
	<2.04 [n (%)]	≥2.04 [n (%)]	P value	<60.4 [n (%)]	≥60.4 [n (%)]	P value	<0.032 [n (%)]	≥0.032 [n (%)]	P value
Total	68 (59.6)	46 (40.4)		58 (50.9)	56 (49.1)		61 (53.5)	53 (46.5)	
Age			0.127			0.708			0.851
< 54 years	38 (66.7)	19 (33.3)		28 (49.1)	29 (50.9)		30 (52.6)	27 (47.4)	
≥54 years	30 (52.6)	27 (47.4)		30 (52.6)	27 (47.4)		31 (54.4)	26 (45.6)	
Phimosis			0.478			0.931			0.271
Absent	18 (54.5)	15 (45.5)		17 (51.5)	16 (48.5)		15 (45.5)	18 (54.5)	
Present	50 (61.7)	31 (38.3)		41 (50.6)	40 (49.4)		46 (56.8)	35 (43.2)	
T stage			0.589			0.508			0.507
T_1	24 (63.2)	14 (36.8)		21 (55.3)	17 (44.7)		22 (57.9)	16 (42.1)	
T_{2-4}	44 (57.9)	32 (42.1)		37 (48.7)	39 (51.3)		39 (51.3)	37 (48.7)	
N stage			<0.001			0.174			<0.001
N_0	51 (77.3)	15 (22.7)		30 (45.5)	36 (54.5)		46 (69.7)	20 (30.3)	
N_{1-3}	17 (35.4)	31 (64.6)		28 (58.3)	20 (41.7)		15 (31.3)	33 (68.8)	
Pathologic grade			0.096			0.164			0.032
G_1	46 (65.7)	24 (34.3)		32 (45.7)	38 (54.3)		43 (61.4)	27 (38.6)	
G_{2-3}	22 (50.0)	22 (50.0)		26 (59.1)	18 (40.9)		18 (40.9)	26 (59.1)	
CD8+ TIL density			0.012			0.040			0.059
Low	40 (71.4)	16 (28.6)		23 (41.1)	33 (58.9)		35 (62.5)	21 (37.5)	
High	28 (48.3)	30 (51.7)		35 (60.3)	23 (39.7)		26 (44.8)	32 (55.2)	
WBC count			0.445			0.454			0.851
< 7.805×10^9/L	36 (63.2)	21 (36.8)		31 (54.4)	26 (45.6)		31 (54.4)	26 (45.6)	
≥7.805×10^9/L	32 (56.1)	25 (43.9)		27 (47.4)	30 (52.6)		30 (52.6)	27 (47.4)	
Neutrophil count			0.829			0.403			0.420
< 4.22×10^9/L	25 (61.0)	16 (39.0)		23 (56.1)	18 (43.9)		24 (58.5)	17 (41.5)	
≥4.22×10^9/L	43 (58.9)	30 (41.1)		35 (47.9)	38 (52.1)		37 (50.7)	36 (49.3)	
Lymphocyte count			0.073			0.328			0.031
< 1.75×10^9/L	24 (50.0)	24 (50.0)		27 (56.3)	21 (43.8)		20 (41.7)	28 (58.3)	
≥1.75×10^9/L	44 (66.7)	22 (33.3)		31 (47.0)	35 (53.0)		41 (62.1)	25 (37.9)	
NLR			0.076			0.656			0.002
< 3.25	48 (65.8)	25 (34.2)		36 (49.3)	37 (50.7)		47 (64.4)	26 (35.6)	
≥3.25	20 (48.8)	21 (51.2)		22 (53.7)	19 (46.3)		14 (34.1)	27 (65.9)	
Serum A/G ratio			0.642			0.463			0.912
< 1.7	46 (58.2)	33 (41.8)		42 (53.2)	37 (46.8)		42 (53.2)	37 (46.8)	
≥1.7	22 (62.9)	13 (37.1)		16 (45.7)	19 (54.3)		19 (54.3)	16 (45.7)	

Abbreviations: IDO1, Indoleamine 2,3-dioxygenase 1; CD8, cluster of differentiation 8; TIL, tumor-infiltrating lymphocyte; WBC, white blood cell; NLR, neutrophil to lymphocyte ratio; A/G, Albumin/Globulin; Kyn, kynurenine; Trp, tryptophan; KTR, Kyn/Trp ratio.

0.209-0.859; $P = 0.017$), and NLR (HR, 3.070; 95% CI, 1.512-6.234; $P = 0.002$), but not the IDO1+ TIIC density (HR, 1.546; 95% CI, 0.791-3.024; $P = 0.199$), were significantly associated with DSS in PSCC patients (Table 3).

Kaplan-Meier analysis suggested that high IDO1 level in cancer cells, high CD8+ TIL density, high serum Kyn concentration and KTR (all $P < 0.01$), low Kyn concentration ($P = 0.019$; Figure 1), high neutrophil count and NLR and low lymphocyte count (all $P < 0.05$; Supplementary Figure S3) were significantly associated with short DSS. Additionally, the cumulative 5-year DSS rates were markedly lower in the patients with high IDO1 expression in cancer cells (34.1% vs. 80.7%), high CD8+ TIL density (53.5% vs. 82.0%), high serum Kyn concentration (43.1% vs. 82.9%), or high KTR (42.2% vs. 87.8%) than in their counterparts. Whereas the cumulative 5-year DSS rate was markedly higher in the high serum Trp concentration group than in the low serum Trp concentration group (58.3% vs. 76.9%).

TABLE 3   Univariate and multivariate Cox regression analyses for disease-specific survival in PSCC patients

Characteristics	Univariate analysis		Multivariate analysis[#]	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (≥54 *vs.* < 54 years)	1.075 (0.554-2.088)	0.830	N/A	N/A
Phimosis (present *vs.* absent)	0.899 (0.634-1.274)	0.549	N/A	N/A
T stage (T_{2-4} *vs.* T_1)	2.403 (1.049-5.503)	0.032	N/A	N/A
N stage (N_{1-3} *vs.* N_0)	14.424 (5.558-37.433)	<0.001	7.130 (2.570-19.784)	<0.001
Pathologic grade (G_{2-3} *vs.* G_1)	4.608 (2.251-9.434)	<0.001	2.203 (1.042-4.658)	0.039
IDO1 H-score in cancer cells (≥1.15 *vs.* < 1.15)	4.956 (2.524-9.729)	<0.001	N/A	N/A
IDO1$^+$ TIIC density (≥8.5% *vs.* < 8.5%)	1.546 (0.791-3.024)	0.199	N/A	N/A
CD8$^+$ TIL density (≥9.5% *vs.* < 9.5%)	3.366 (1.575-7.194)	0.001	2.033 (0.928-4.454)	0.076
Peripheral blood WBC count (≥7.805 × 10^9/L *vs.* < 7.805 × 10^9/L)	1.424 (0.708-2.864)	0.321	N/A	N/A
Peripheral blood neutrophil count (≥4.22 × 10^9/L *vs.* < 4.22 × 10^9/L)	2.804 (1.152-6.822)	0.023	N/A	N/A
Peripheral blood lymphocyte count (≥1.75 × 10^9/L *vs.* < 1.75 × 10^9/L)	0.424 (0.209-0.859)	0.017	N/A	N/A
Peripheral blood NLR (≥3.25 *vs.* < 3.25)	3.070 (1.512-6.234)	0.002	2.780 (1.066-7.215)	0.036
Serum Kyn concentration (≥2.04 μmol/L *vs.* < 2.04 μmol/L)	4.548 (2.220-9.315)	<0.001	N/A	N/A
Serum Trp concentration (≥60.4 μmol/L *vs.* < 60.4 μmol/L)	0.458 (0.228-0.921)	0.025	N/A	N/A
Serum KTR (≥0.032 *vs.* < 0.032)	6.818 (2.960-15.705)	<0.001	2.773 (1.134-6.778)	0.025
Serum A/G ratio (≥1.7 vs. < 1.7)	0.664 (0.286-1.540)	0.34	N/A	N/A

Abbreviations: IDO1, Indoleamine 2,3-dioxygenase 1; TIIC, tumor-infiltrating immune cell; CD8, cluster of differentiation 8; TIL: tumor-infiltrating lymphocyte; Kyn, kynurenine; Trp, tryptophan; KTR, Kyn/Trp ratio; WBC, white blood cells; NLR, Neutrophil to lymphocyte ratio; A/G, Albumin/Globulin; HR, hazard radio; CI, confidence interval; PSCC, penile squamous cell carcinoma; N/A, not applicable.

[#]Method = Backward stepwise (conditional LR). Variates with *P* value ≤0.05 in univariate analysis, serum Kyn and Trp concentrations (covered by serum KTR), and blood neutrophil and lymphocyte counts (covered by blood NLR) were all excluded from multivariate Cox regression analysis. IDO1 H-score in cancer cells and T stage were included in multivariate Cox regression analysis, but they were, in turn, removed out of analysis in the procedure of backward stepwise.

Multivariate analysis suggested that, besides N stage (HR, 6.926; 95% CI, 2.458-19.068; $P < 0.001$) and pathologic grade (HR, 2.194; 95% CI, 1.021-4.529; $P = 0.038$), only KTR (HR, 2.780; 95% CI, 1.066-7.215; $P = 0.036$), but not CD8$^+$ TIL density (HR, 2.033; 95% CI, 0.928-4.454; $P = 0.076$) or IDO1 H-score in cancer cells, was independent predictors for PSCC prognosis (Table 3).

3.4 | IDO1 expression is associated with immune-related gene expression, immune cell infiltration, and hematological parameters

Considering the possible immunosuppressive role of IDO1, we investigated the correlation between the expression of IDO1 and the genes that affect the immune response in PSCC tissues. Linear regression analyses showed that the expression of *IDO1* at mRNA level was positively correlated with the mRNA levels of programmed cell death protein-1 (*PD-1*), cytotoxic T lymphocyte-associated protein 4 (*CTLA-4*),

PD-L1 and programmed death-ligand 2 (*PD-L2*) in PSCC tissues (all $P < 0.05$; Figure 2A).

We also tested the association between *IDO1* expression and immune cell infiltration in PSCC tissues by comparing the mRNA expression levels of *IDO1* and characteristic immune cell markers. The results shown that *IDO1* mRNA levels were positively correlated with the infiltration of CD3$^+$CD8$^+$ CTLs, CD3$^+$CD4$^+$CD25$^+$FOXP3$^+$ Tregs, CD14$^+$CD163$^+$CD206$^+$ tumor-associated macrophages (TAMs), and CD14$^+$CD11b$^+$CD33$^+$ myeloid-derived suppressor cells (MDSCs; all $P < 0.001$, Figure 2B). These results indicated that the immunosuppressive role of IDO1 may be associated with the expression of immunosuppressive factors and the infiltration of immune cells, especially suppressive immune cells, in PSCC tissues.

Similar to the results of χ^2 test described above (Tables 1 and 2), linear regression analyses revealed that the IDO1 protein levels in cancer cells (but not those in TIICs) and its catalytic activity (as indicated by serum Kyn and Trp concentrations and KTR) were also significantly correlated with
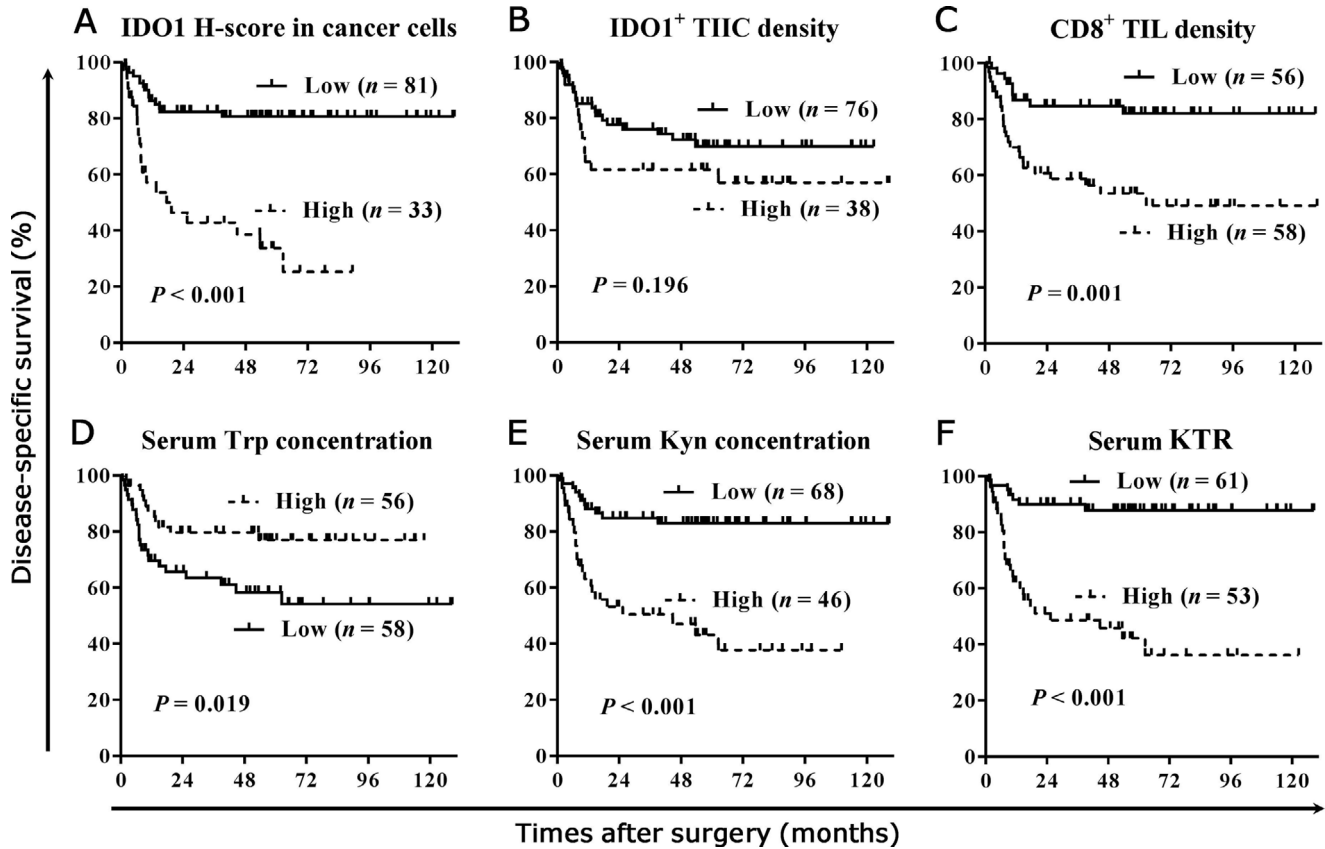
FIGURE 1   Kaplan-Meier DSS curves stratified by different variables in 114 patients with PSCC. DSS curves stratified by IDO1 H-score in cancer cells (A), IDO1$^+$ TIIC density (B), CD8+ TIL density (C), serum Trp concentration (D), Kyn concentration (E) and KTR (F). DSS was different among subgroups stratified by all variables (all $P < 0.05$) except IDO1$^+$ TIIC density ($P = 0.196$). Abbreviations: DSS, disease-special survival; IDO1, Indoleamine 2,3-dioxygenase 1; PSCC, penile squamous cell carcinoma; Kyn, kynurenine; Trp, tryptophan; TIIC, tumor-infiltrating immune cell; KTR, Kyn/Trp ratio

CD8$^+$ TIL density (IDO1 protein level in cancer cells, serum Kyn concentration and KTR, all $P < 0.001$; Trp concentration, $P = 0.040$; IDO1 protein level in TIICs, $P = 0.786$; Supplementary Figure S4).

3.5 | IDO1 expression is regulated by interferon-gamma (IFNγ) in PSCC cells

Most immune cells secrete IFNγ, which has been reported to stimulate *IDO1* expression [11, 28]. Since IDO1 expression was positively correlated with immune cell infiltration in PSCC, we examined IDO1 expression with respect to the mRNA levels of *IFNγ* in PSCC tissues and found that there was a significant relationship between the expression of *IDO1* and *IFNγ* ($P < 0.001$, Figure 3A). Western blotting assay revealed that the endogenous expression of IDO1 was almost undetectable in PSCC cell lines Penl1, Penl2, and 149RCa (Figure 3B), but IFNγ remarkably induced IDO1 expression in a dose-dependent manner in PSCC cells lines Penl1 and 149RCa (Figure 3C). These data indicated that the

up-regulation of IDO1 expression was likely induced by TIIC-secreted IFNγ in PSCC tissues.

4 | DISCUSSION

It has been verified that IDO1 plays a crucial role in immuno-suppression in cancers [6, 8, 9]. Up-regulation of IDO1 expression and activity are associated with poor prognosis in various cancers [13-16]. However, there is no information about IDO1 expression and its clinical significance in PSCC. In the present study, we reported that up-regulation of IDO1 expression in cancer cells and IDO1 catalytic activity, as determined by elevated serum Kyn concentration and KTR, were associated with worse clinical outcomes, and KTR, N stage, and pathologic grade acted as independent predictors for the clinical outcomes of PSCC patients.

Although both cancer cells and TIICs in PSCC tissues express IDO1, which contributes to Trp depletion and Kyn accumulation, only the expression level of IDO1 in cancer cells, but not that in TIICs, was related to serum Trp and Kyn
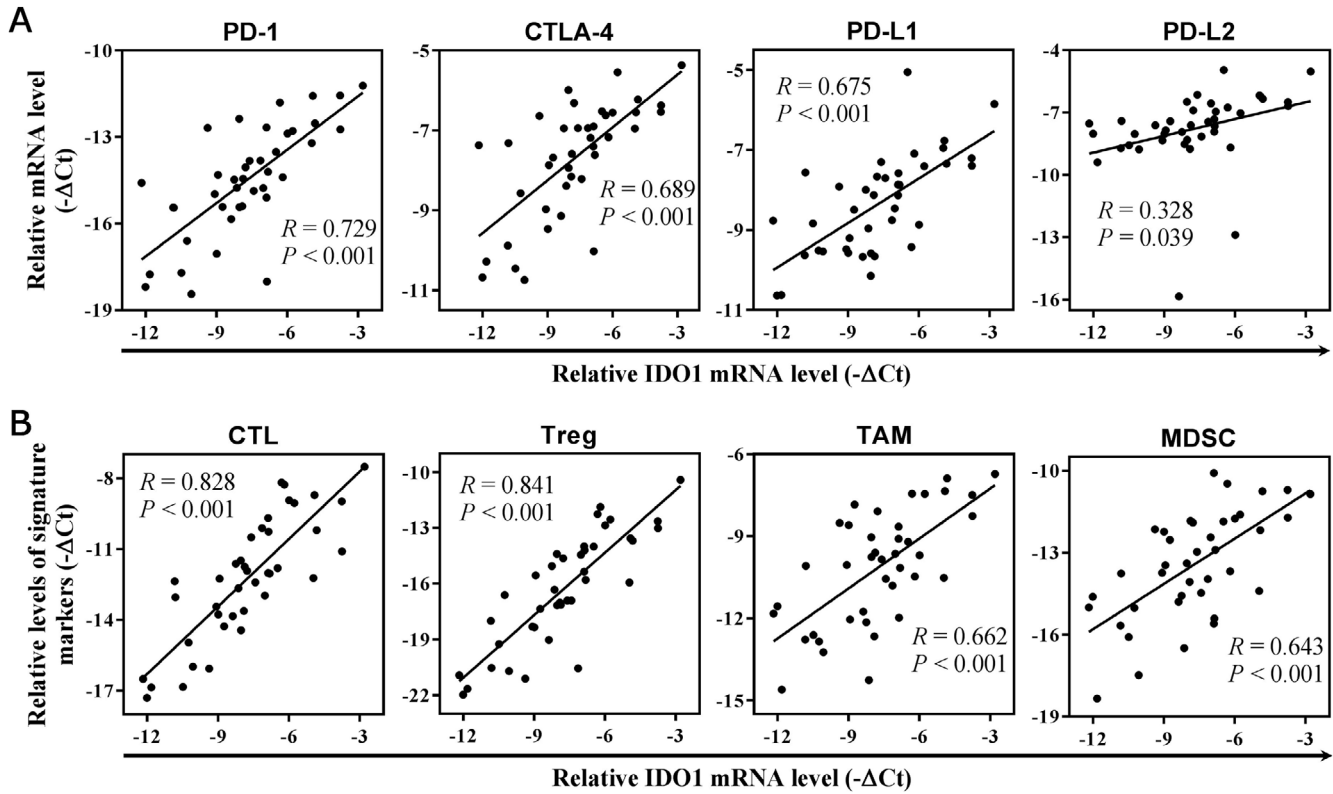
FIGURE 2 IDO1 expression is associated with immune-related gene expression and immune cell infiltrates in PSCC. Pearson's correlation analysis between the mRNA levels of IDO1 and the mRNA levels of immune-related gene PD-1, CTLA-4, PD-L1, and PD-L2 (A) and the infiltration of CTL, Treg, TAM and MDSC (B), defined by a principal component analysis of the corresponding signature marker genes in PSCC. Abbreviations: IDO1, Indoleamine 2,3-dioxygenase 1; PSCC, penile squamous cell carcinoma; PD-1, programmed cell death protein-1; CTLA-4, cytotoxic T lymphocyte-associated protein 4; PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2; CTL, cytotoxic T lymphocyte; Treg, regulatory T lymphocyte; TAM, tumor-associated macrophage; MDSC, myeloid-derived suppressor cell
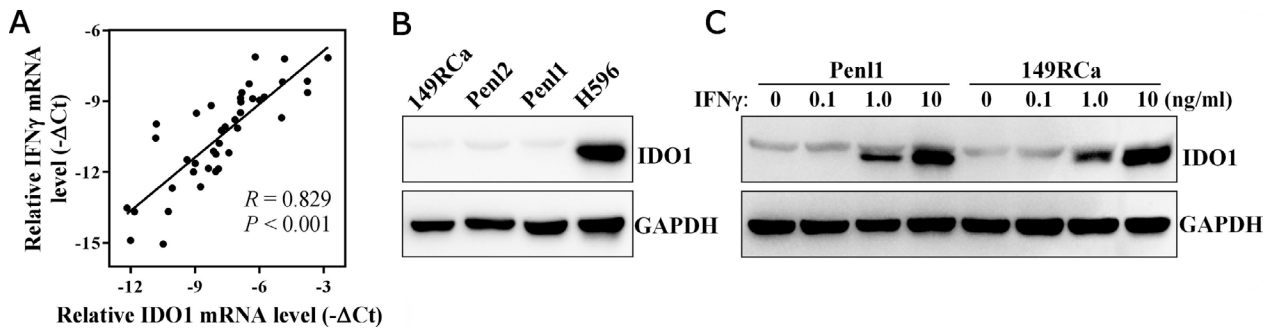
FIGURE 3 IFNγ induce IDO1 expression in PSCC cells lines. (A) Pearson's correlation analysis between the mRNA levels of IDO1 and IFNγ. (B) Western blotting analysis of the intrinsic IDO1 expression in PSCC cell lines (H596 cells were served as positive control). (C) The inducible IDO1 expression in Penl1 and 149RCa cells treated with IFNγ for 48 hours. Abbreviations: IDO1, Indoleamine 2,3-dioxygenase 1; PSCC, penile squamous cell carcinoma; IFNγ, interferon-gamma

concentrations and KTR. We suspected that this phenomenon might result from IDO1 expression in cancer cells being much higher than that in TIICs of PSCC tissues. Surprisingly, KTR, an index of IDO1 catalytic activity, rather than IDO1 expression levels, played an independent prognostic role in PSCC. In our view, the main reasons for the different performance

between IDO1 expression levels in cancer cells and KTR in PSCC prognosis prediction might be explained as follows: (1) Kyn accumulation and Trp depletion rather than IDO1 itself induce immunosuppression in PSCC; (2) not only IDO1 in cancer cells but also IDO1 expressed in other cell types (such as TIICs and endothelial cells) are involved in Trp catabolism,

which affects the tumor immune microenvironment, although IDO1 expressed in other cells played a secondary role in the present study; (3) IDO2 and TDO also catabolize Trp into Kyn [10-12], although IDO1 plays a quantitatively superior role in Trp catabolism [11, 12]. That is, serum KTR reflects the catalytic activities of IDO1, IDO2 and TDO. Finally, serum KTR represents systemic Trp metabolic activity, which certainly influences systemic immunity beyond the tumor immune microenvironment.

In the present study, we found that IDO1 expression was positively correlated with the expression of the inhibitory checkpoint molecules PD-L1, PD-L2, PD-1, and CTLA-4, as well as the infiltration of suppressive immune cells, including Tregs, TAMs, and MDSCs, in agreement with previous reports in various solid tumors [29-34]. Unexpectedly, the up-regulation of IDO1 *expression* was positively correlated with an increased density of CD8$^+$ TILs in PSCC, which may be related to a poor prognosis. CD8$^+$ TILs are generally considered to have an antitumor effect and associated with good clinical outcomes in many types of cancers [29, 35]. However, this view is inconsistent with and contradictive to our findings in PSCC and several recent findings [5, 30, 36, 37]. Thus, we assume that there is a strong suppressive immune microenvironment in PSCC, which eliminates the antitumor effect of CD8$^+$ TILs.

Considering our findings that the mRNA expression levels of IDO1 and IFNγ are positively correlated and IFNγ induces IDO1 expression in PSCC cells, we present a profile of immunoediting in PSCC tissues below. According to the theory of tumor immunoediting proposed by Dunn GP *et al.* [38], the innate immunity recognizes transformed cells (original tumor cells) in the initiation phase of tumor and secrets IFNγ, which starts a cascade of innate immune reactions. Then, innate and tumor-specific immunity are consequently activated, which provides immune surveillance to eliminate tumor cells and control tumor growth. Meanwhile, the Darwinian selection of tumor variants may ultimately lead to immune escape and the development of clinically apparent tumors when tumor variants have acquired insensitivity or resistance to immune surveillance. However, in addition to functioning as an antitumor factor via antiangiogenesis [39], antiproliferation [40, 41], apoptosis induction [42] and antitumor immunity activation [43, 44], IFNγ secreted by immune cells induces the expression of many immunosuppressive factors, such as PD-L1 [5, 45], PD-L2 [46] and IDO1 [30, 47], and in turn inhibits the antitumor function of CD8$^+$ TILs in the tumor microenvironment. This hypothesis was supported by our previous [5] and present study, which shown that IFNγ stimulated PD-L1 and IDO1 expression in PSCC cells *in vitro* and CD8$^+$ TILs density was negatively correlated with the prognosis of PSCC patients, although whether IDO1 actually led to the dysfunction of CD8$^+$ TILs needs further investigation. Thus, IFNγ plays an important role in cancer immunoediting [48], which plays dual roles in facilitating host protection against cancer and promoting cancer escape from immune destruction [38].

Because of the negative feedback of immunoregulation, it is urgently needed to develop clinically feasible treatment regimens to break existing immunosuppression and shift the equilibrium in cancer immunoediting from tumor tolerance to eradication [49]. Promisingly, several IDO1 inhibitors [50], such as indoximod [51], epacadostat [52] and navoximod, have been developed and entered clinical trials. We expect that IDO1 inhibitors will benefit comprehensive therapy for advanced PSCC.

5 | CONCLUSIONS

The present study revealed that serum KTR, an index of IDO1 catalytic activity, is associated with IDO1 expression levels in cancer cells. Additionally, high KTR is associated with poor prognosis in PSCC. Furthermore, the IDO1 expression level is significantly correlated with the expression levels of IFNγ, PD-1, CTLA-4, and PD-L1/2 and the infiltration of immune cells, especially suppressive immune cells, in PSCC tissues. Given that IDO1 expression in PSCC cells is induced by IFNγ, we speculate that IFNγ-induced IDO1 plays a crucial role in the formation of immunosuppressive tumor microenvironment in PSCC, and the catalytic activity of IDO1, as represented by serum KTR, could be utilized as an independent predictive factor for the prognosis of PSCC patients.

AUTHORS' CONTRIBUTIONS
Conceptualization and funding acquisition: HH and RYL; Data analysis: QHZ and JBL; Data interpretation: QHZ, ZSL, JPC, KBH, TYL and KY; Acquisition of data: ZWL, ZKQ, YHL, SJG and YLY; Performed experiments: CZD; Project administration and supervision: FJZ and HH; Writing the manuscript: QHZ, JBL and RYL; Review and editing the manuscript: ZSL and HH.

All authors read and approved the final manuscript.

DECLARATIONS
ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Institutional Ethical Boards of Sun Yat-sen University Cancer Center. Written informed consent was obtained from all patients.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The key raw data is available in the Research Data Deposit public platform (www.researchdata.org.cn) with the approval number RDDB2019000700.

COMPETING INTERESTS

The authors declare that they have no potential conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.