Expression and Prognostic Value of Indoleamine 2,3-dioxygenase in Pancreatic Cancer

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Abstract

Background: Indoleamine 2,3-dioxygenase (IDO), an enzyme for tryptophan metabolism through the kynurenine pathway, exhibits an immunosuppressive effect and induces immune tolerance in tumor cells. The effects of IDO on pancreatic cancer are poorly understood. This study aimed to investigate the expression and prognostic significance of IDO in pancreatic cancer.

Methods: We evaluated the protein expression of IDO in PANC-1, CFPAC-1, and BxPC-3 cell lines with or without 48 h treatment by 500 U/ml interferon-γ (IFN-γ). We performed immunohistochemical staining and Western blot analysis for IDO expression in both pancreatic cancer and normal pancreas tissues obtained from Chinese PLA General Hospital from July 2012 to December 2013. Survival analysis was performed to correlate IDO expression and histopathologic parameters with overall survival. The Kaplan-Meier method and Cox proportional hazards regression model were conducted.

Results: PANC-1, CFPAC-1, and BxPC-3 cell lines expressed IDO at the protein level, and the relative expression amount increased after stimulation with 500 U/ml IFN-γ. Immunohistochemical analysis results revealed that high IDO expression was observed in 59% of pancreatic adenocarcinoma tissues. Compared with normal pancreatic tissues, pancreatic adenocarcinoma showed significantly higher IDO expression levels, especially among patients with high tumor node metastasis (TNM) stages ($\chi^2 = 4.550$, $P = 0.030$), poor histological differentiation ($\chi^2 = 5.690$, $P = 0.017$), and lymph node metastasis ($\chi^2 = 4.340$ $P = 0.037$). Kaplan-Meier survival curves showed that high IDO expression was correlated with low survival rates (hazard ratio [HR] = 0.49 $P = 0.009$). Multivariate analysis using Cox proportional hazards model indicated that lymph node metastasis ($HR = 0.35$ $P = 0.010$) and IDO expression ($HR = 0.42$ $P = 0.020$) were two independent prognostic predictors of pancreatic adenocarcinoma.

Conclusions: The study confirmed that high IDO expression in pancreatic adenocarcinoma was related to poor prognosis of patients. These findings provided evidence that IDO was involved in pancreatic adenocarcinoma progression and might serve as a relevant therapeutic target.

Key words: Immunohistochemistry; Indoleamine 2,3-dioxygenase; Pancreatic Neoplasms; Prognosis

INTRODUCTION

Pancreatic cancer is a highly common malignancy worldwide and is considered the 9th leading cause of cancer death in China.[1] The disease is mainly characterized by rapid progression and metastasis. It is reported that about 80% of patients present with unresectable stages at diagnosis with distant metastases or locally advanced tumors.[2] Surgery remains as the standard treatment for early-stage pancreatic cancer. With the development of medical techniques, novel treatment options can improve the prognosis of patients with unresectable pancreatic tumors.[3,4] However, the 5-year survival rate for all patients with pancreatic cancer is less than 5%. Even patients who had undergone margin-negative (R0)

surgical resection attained a 5-year survival rate of no more than 20%.[5] Several studies elucidated that local immune suppression of the tumor microenvironment was the key for cancer growth, metastasis, and even tumor immune escape.[6,7] Hence, elucidation of the involved molecules and the mechanisms in the generation, development, and metastasis of pancreatic cancer might

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contribute to disease prognosis and provide targets for therapeutic intervention.

Indoleamine 2,3-dioxygenase (IDO) is a rate-limiting enzyme that catabolizes tryptophan into a stable metabolite under the kynurenine pathway. Tryptophan is an essential amino acid for cell survival and cannot be synthesized *in vivo*.[8] IDO activity considerably influences immune regulation. IDO exerts its immunosuppressive effect in several ways, such as by inducing immune tolerance to tumor antigens, suppressing T and natural killer cells by tryptophan deletion and generation, and activating regulatory T-cells (Tregs) which is characterized by immune suppression and dysfunction.[6,9,10] Overexpression of IDO has been identified correlating with poor clinical outcome in some cancer types, including breast, gastric, nasopharyngeal, and liver cancers.[11-14] However, the expression of IDO in pancreatic cancer and its prognostic value on pancreatic cancer has not been intensively studied.

To explore the effect of IDO in pancreatic cancer progression, we identified IDO in tumor tissues and analyzed the correlation of this molecule and clinicopathologic features of patients with overall survival.

METHODS

Cell lines and culture
PANC-1, CFPAC-1, and BxPC-3 cells were obtained from China Infrastructure of Cell Line Resources. The cells were maintained in Dulbecco's modified Eagle's medium (Hyclone Co., Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone Co., Logan, UT, USA) and 100 U/ml penicillin and streptomycin. The cells were grown at 37°C in a humidified 5% CO_2 air incubator and subcultured twice a week. For treatment, the cells were washed twice with phosphate-buffered saline (PBS) and 1×10^6 cells were incubated in cell-culture plates with or without 500 U/ml interferon-γ (IFN-γ; Sigma Co., St. Louis, MO, USA) for 48 h.

Patient selection and follow-up
The study enrolled 80 patients with primary pancreatic adenocarcinoma from the Department of Hepatobiliary and Pancreatic Surgical Oncology, Chinese People's Liberation Army General Hospital from July 2012 to December 2013. All the eighty pancreatic adenocarcinoma tissues were obtained from the patient at the time of surgery. The exclusion criteria were acute and severe postoperative complications and lack of clinical information. Histologic features were reviewed by two pathologists based on the current WHO criteria. Clinical data including age, gender, tumor size, tumor differentiation, tumor location, tumor node metastasis (TNM) stage, lymph node metastasis, and survival data were obtained from electronic medical records. All the patients were followed up until April 2016, with a median follow-up time of 40 months. Overall survival was defined as the time from the date of surgery to the date of death or last visit. This study was approved by the Ethics Committee of the People's Liberation Army General Hospital and carried out in accordance with the approved guidelines. The principle of informed consents on the use of clinical specimens from each enrolled person was satisfied.

Immunohistochemistry
Eighty pancreatic adenocarcinoma tissue samples and five normal pancreas tissue samples were used for Immunohistochemistry staining. Initially, 5 μm tissue sections were mounted on silanized slides, dewaxed, and rehydrated. For antigen retrieval, the sections were placed in 0.01 mmol/L sodium citrate buffer (pH 6.0), heated in a microwave oven at the maximum power for 15 min, and cooled for 30 min. The sections were then washed with 0.05 mmol/L PBS. Nonspecific endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 min at room temperature. Nonspecific binding was blocked by incubation in 10% normal goat serum with 0.05% Triton X-100 (Sigma Co., St. Louis, MO, USA) in PBS. The sections were incubated at 4°C overnight with IDO antiserum (Abcam Co., Cambridge, MA, USA) diluted at 1:200 in PBS with 5% bovine serum albumin (BSA; Amresco Inc., Solon, OH, USA). The sections were then washed three times with PBS and incubated with the goat anti-mouse secondary antibody (GAM, Abcam Co., Cambridge, MA, USA) 1:200 for 1.5 h at room temperature. The sections were washed with PBS, incubated with the streptavidin-peroxidase complex (SP-HRP, Abcam Co., Cambridge, MA, USA) 1:200 for 1.5 h, and washed again in PBS. IDO immunoreactivity was visualized with diaminobenzidine and H_2O_2 for 2 min, followed by hematoxylin counterstaining. Finally, the sections were dehydrated in ethanol and mounted. Tissue sections incubated with PBS buffer instead of the IDO antiserum was used as negative control. Tissue sections of normal lymph node which was considered a positive IDO expression tissue were used as positive controls.

Evaluation of Immunohistochemistry staining
IDO expression was assessed by semi-quantitative evaluation of staining intensity and the percentage of positive tumor cell in each tissue core. The proportion score represented the percentage of tumor cells by IDO staining (0: none; 1: <25%; 2: 25–50%; 3: >50%). The intensity score signified the staining intensity in positively stained cells (0: None; 1: Weak; 2: Moderate; and 3: Strong). The overall IDO expression score in each sample was determined by multiplying the proportion (0–3) and intensity score (0–3). Staining was analyzed in two or more cores to standardize the analysis. Six photomicrographs of independent areas were chosen for each core. In the present study, the IDO expression score >4 was defined as high IDO expression and ≤4 as low expression.

Western blotting
Pancreatic cancer cell lines, five tissue samples of early stage (Stages I/II) pancreatic adenocarcinoma, five tissue samples of advanced pancreatic adenocarcinoma

(Stages III/IV), and five normal pancreas tissue samples were used for Western blot analysis. Proteins were extracted from cancer tissues using nondenaturing lysis buffer. Protein concentrations were determined using Bradford protein assay reagent (Sigma Co., St. Louis, MO, USA) with BSA as a standard. Protein lysate (150 μg) was then subjected to electrophoresis on 12.5% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% (w/v) nonfat milk and 0.01% (v/v) Tween-20 (Sigma Co., St. Louis, MO, USA) in tris-buffered saline (TBS; pH 7.6) for 3 h at room temperature and incubated with IDO and glyceraldehyde-3-phosphate dehydrogenase antiserum (Abcam Co., Cambridge, MA, USA) diluted at 1:1000 in TBS overnight at 4°C. The membrane was washed for 30 min with three changes in TBS. Subsequently, a 1:5000 dilution of the goat anti-rabbit/mouse IgG alkaline phosphatase-linked secondary antibody (Sigma Co., St. Louis, MO, USA) was incubated at room temperature for 3 h. The membrane was again washed for 30 min with three changes in TBS and processed for development using an alkaline phosphatase substrate, namely, bromochloroindolyl phosphate/nitro blue tetrazolium (Sigma Co., St. Louis, MO, USA), at room temperature. The relative levels of intensity of each blot were densitometrically scanned and analyzed.

Statistical analysis

SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used. Data were expressed as mean ± standard error (SD). Student's t-test was used to compare data between two groups, and one-way analysis of variance (ANOVA) was employed for data comparison among multiple groups. Pearson Chi-square test or Fisher's exact test was used to comparing categorical variables. Survival analysis was performed in accordance with Kaplan-Meier method. Univariate and multivariate analyses were conducted to relate IDO expression and histopathologic parameters with survival of patients with pancreatic adenocarcinoma using the Cox proportional hazards regression model. Log-rank test was employed for comparison of survival among groups. A value of $P < 0.05$ was considered statistically significant.

Results

Indoleamine 2,3-dioxygenase expression in PANC-1, CFPAC-1, and BxPC-3 cell lines

Western blotting results displayed that all the three pancreatic cancer cell lines expressed IDO at the protein level [Figure 1a]. IDO expression relatively increased after stimulation with 500 U/ml IFN-γ [Figure 1b].

Immunohistochemical expression of indoleamine 2,3-dioxygenase in pancreatic adenocarcinoma and normal pancreatic tissues

IDO expression was examined in eighty pancreatic adenocarcinoma tissue samples and five normal pancreas tissue samples by immunohistochemical staining. IDO protein expression was observed in pancreatic adenocarcinoma tissue samples at various levels in the cytoplasm of tumor cells [Figure 2]. IDO protein expression was not detected in normal pancreatic tissues. High IDO expression was found in 47 cases, with a prevalence rate of 59%. The 33 (41%) remaining cases displayed low or undetectable IDO expression.

Correlation of indoleamine 2,3-dioxygenase expression with clinicopathological factors in pancreatic adenocarcinoma tissues

The correlations of IDO expression with clinicopathological factors in pancreatic adenocarcinoma are shown in Table 1. The high expression of IDO was significantly correlated with histological differentiation ($\chi^2 = 5.690$, $P = 0.017$), TNM stage ($\chi^2 = 4.550$, $P = 0.030$), and lymph node metastasis ($\chi^2 = 4.340$, $P = 0.037$), but not with the age ($\chi^2 = 0.025$, $P = 0.870$), patient gender ($\chi^2 = 0.018$, $P = 0.890$), tumor size ($\chi^2 = 0.003$, $P = 0.950$), and location ($\chi^2 = 3.030$, $P = 0.080$).

Indoleamine 2,3-dioxygenase expression in pancreatic adenocarcinoma tissues with different tumor node metastasis stages

Western blot analysis was performed to determine IDO protein expression in five tissue samples of early-stage (Stages I/II) pancreatic adenocarcinoma, five tissue samples of advanced (Stages III/IV) pancreatic

Table 1: Relationship between IDO expression and clinicopathological factors in pancreatic adenocarcinoma tissues ($N = 80$)

Variables	n	IDO expression, n		χ^2	P
		High	Low		
Gender					
Male	49	29	20	0.018	0.890
Female	31	18	13		
Age (years)					
≥60	36	21	15	0.025	0.870
<60	44	26	18		
Tumor size (cm)					
≥3.0	27	11	6	0.003	0.950
<3.0	53	36	17		
Tumor location					
Head	58	38	20	3.030	0.080
Body and tail	22	9	13		
Histological differentiation					
Well and moderately	37	16	21	5.690	0.017
Poorly	43	31	12		
TNM stage					
I, II	62	32	30	4.550	0.030
III, IV	18	15	3		
Lymph node metastasis					
Positive	39	28	11	4.340	0.037
Negative	41	19	22		

IDO: Indoleamine 2,3-dioxygenase; TNM: Tumor node metastasis.
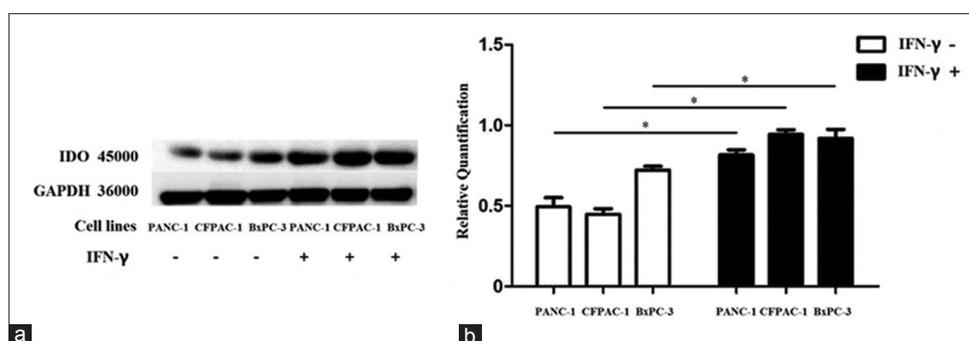
Figure 1: IDO protein expression in pancreatic cancer cell lines with (+) or without (−) interferon-γ stimulating assessed by Western blotting. (a) IDO expression in different pancreatic cancer cell lines. (b) The relative protein level of IDO in different pancreatic cancer cell lines. *$P < 0.05$. IDO: Indoleamine 2,3-dioxygenase.
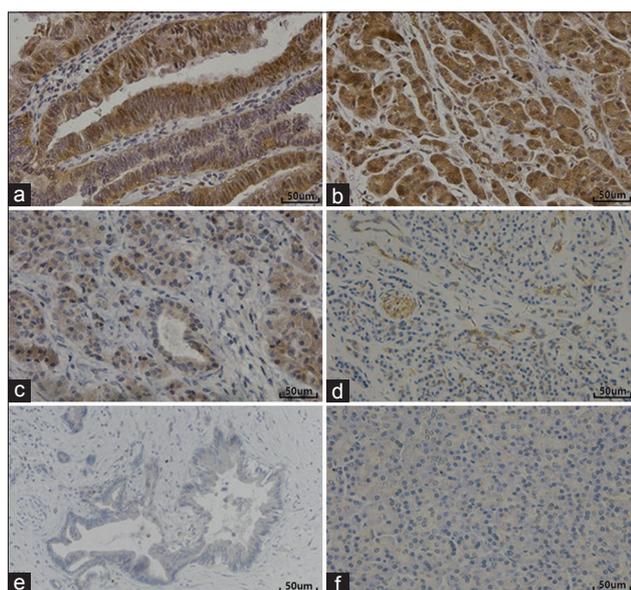
Figure 2: IDO protein expression in pancreatic adenocarcinoma tissues and normal pancreas tissues shown by immunohistochemistry. (a and b) High expression of IDO in pancreatic adenocarcinoma tissues. (c-e) Low expression of IDO in pancreatic adenocarcinoma tissues. (f) IDO expression in normal pancreas. IDO: Indoleamine 2,3-dioxygenase.

adenocarcinoma, and five tissue samples of normal pancreas. IDO protein expression level was higher in pancreatic adenocarcinoma tissues than that in normal pancreatic tissues ($n = 5$; $P < 0.010$) [Figure 3]. Comparison of different TNM stages of pancreatic adenocarcinoma showed that IDO expression significantly differed between Stages I/II and III/IV of pancreatic adenocarcinoma ($n = 5$; $P < 0.050$). IDO protein expression was positively correlated with pancreatic adenocarcinoma progression.

Correlation of overall survival rate with indoleamine 2,3-dioxygenase expression and clinicopathological factors

Survival curves were constructed using Kaplan-Meier method to elucidate the effect of IDO expression and clinicopathological factors on patient prognosis. The results are shown in Figure 4. Patients with low IDO expression showed significantly increased overall survival rate ($P < 0.01$) compared to patients with high IDO expression. Moreover, univariate and multivariate analyses were conducted using the Cox proportional hazard model to analyze the correlation of IDO expression and other clinicopathological factors with patient prognosis. The results of univariate analysis indicated that TNM staging (hazard ratio [HR] = 4.82, $P = 0.001$), lymph node metastasis ($HR = 0.38$, $P = 0.003$), and IDO expression ($HR = 0.49$, $P = 0.009$) were related to the overall survival of patients with pancreatic adenocarcinoma [Table 2]. However, overall survival rate was not correlated with gender, age, tumor size, tumor location, and histological differentiation. Multivariate analysis revealed that lymph node metastasis ($HR = 0.35$, $P = 0.010$) and IDO expression ($HR = 0.42$, $P = 0.020$) were two independent prognostic predictors of pancreatic adenocarcinoma.

DISCUSSION

Recently, a series of studies focused on the role of IDO in cancer prognosis, and their results indicated that this immunosuppressive enzyme was closely related to clinical outcomes.[15-17] On the basis of the clinical evidence for poor prognosis in tumors with high IDO expression, there has been increasing scientific interest in IDO as a molecular target for the development of immunotherapy drugs. IDO-inhibiting drugs might be used as an adjunctive treatment approach for cancers with IDO expression. Up to now, 1-methy-DL-tryptophan which is one of the IDO competitive inhibitors has been widely studied *in vitro* and *in vivo*, and underwent the phase I study for bioavailability and safety.[18] However, the prognosis value and therapeutic potential of IDO in pancreatic adenocarcinoma need further investigations.

In the present study, the *in vitro* results showed that PANC-1, CFPAC-1, and BxPC-3 cells expressed the IDO protein, and the expression level increased after stimulation with 500 U/ml IFN-γ for 48 h. IFN-γ is a cytokine which is critical for innate and adaptive immunity. Inflammatory reaction in the tumor microenvironment could enable T-cells to release IFN-γ. Moreover, this cytokine could
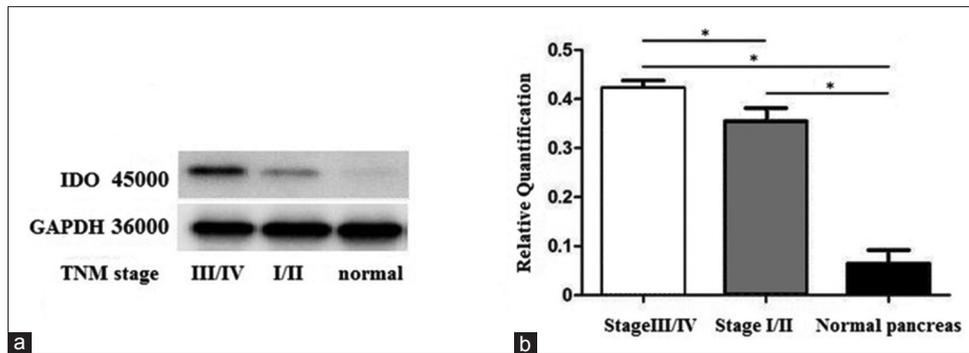
Figure 3: IDO protein expression in early stage (Stages I/II, *n* = 5) pancreatic adenocarcinoma tissue, advanced pancreatic adenocarcinoma tissue (Stages III/IV, *n* = 5), and normal pancreas tissue (*n* = 5) shown by Western blotting. (a) Expression of IDO examined by Western blotting. (b) The relative protein level of IDO in pancreatic adenocarcinoma tissues with different TNM stages and normal pancreas tissues. **P* < 0.05. IDO: Indoleamine 2,3-dioxygenase; TNM: Tumor node metastasis.
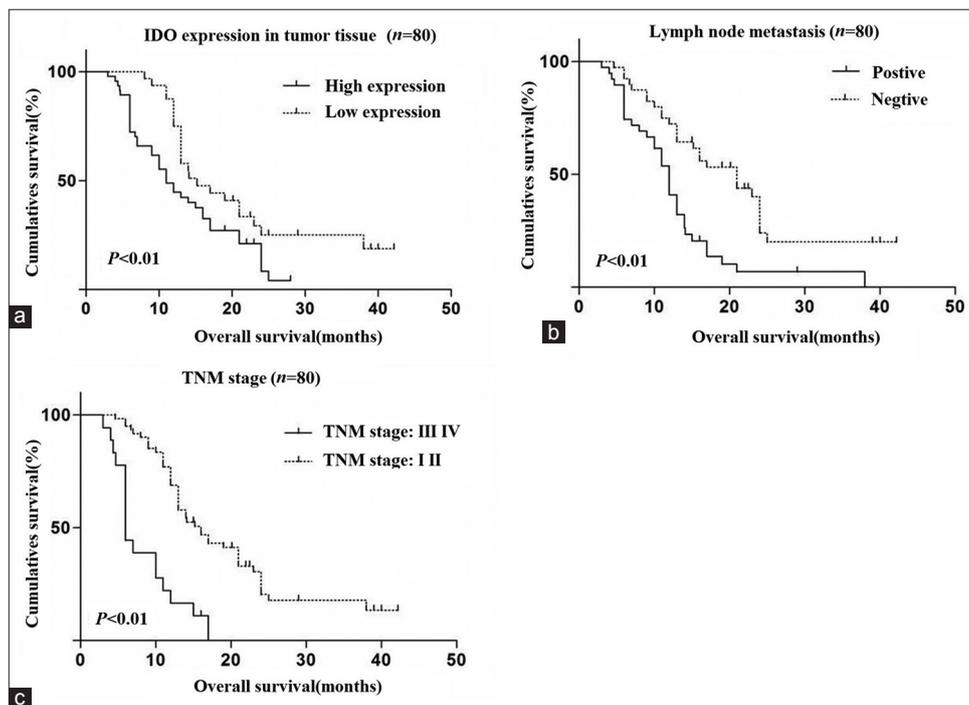
Figure 4: Kaplan-Meier survival curves according to IDO expression levels (a), lymph node metastasis (b), and TNM stage (c). IDO: Indoleamine 2,3-dioxygenase; TNM: Tumor node metastasis.

Table 2: Univariate and multivariate analysis of overall survival in pancreatic adenocarcinoma (*n* = 80)

Variables	Univariate analysis			Multivariate analysis		
	HR	95% *CI*	*P*	*HR*	95% *CI*	*P*
Gender	0.74	0.44–1.23	0.250	–	–	–
Age	1.39	0.84–2.29	0.200	–	–	–
Tumor size	0.64	0.38–1.07	0.090	–	–	–
Tumor location	0.61	0.34–1.12	0.110	–	–	–
Histological differentiation	0.77	0.47–1.29	0.320	–	–	–
TNM stage	4.82	2.63–8.85	0.001	1.61	0.65–4.01	0.300
Lymph node metastasis	0.38	0.23–0.64	0.003	0.35	0.17–0.74	0.010
IDO	0.49	0.29–0.84	0.009	0.42	0.20–0.88	0.020

HR: Hazard ratio; *CI*: Confidence interval; IDO: Indoleamine 2,3-dioxygenase; –: Not available; TNM: Tumor node metastasis.

exhibit immunomodulatory effects through the regulation of IDO expression in tumor microenvironment. In this study, we analyzed the correlation of IDO expression with the clinicopathological factors of pancreatic adenocarcinoma.

High IDO expression level was significantly correlated with histological differentiation, lymph node metastasis, and TNM staging. These three clinicopathological factors represent degrees of malignancy; thus, we speculated that IDO was linked with cancer progression in pancreatic adenocarcinoma. Other studies demonstrated that high levels of IDO were present in the advanced stages of ovarian carcinoma and colorectal cancer.[19,20] Moreover, IDO expression was found in all invasive uterine cervical cancers, whereas noninvasive tumors presented a lower expression level.[21,22] These findings suggest that IDO might enhance the proliferation, invasion, and metastatic abilities of cancer cells to represent a high degree of malignancy through various mechanisms. These tumor immune escape mechanisms include depletion of tryptophan from tumor microenvironments, toxic effects of kynurenine pathway metabolites, maintenance of inflammation in the tumor microenvironment, development of immune tolerance to tumor antigens, inhibition of T cells, generation and activation of Tregs, and promotion of tumor angiogenesis.[23-26]

We further evaluated the effect of IDO expression and clinicopathological factors on patient prognosis. Patients with low IDO expression exhibited a significantly higher overall survival rate. Univariate analysis indicated that TNM stage, lymph node metastasis, and IDO were related to the overall survival rate of patient with pancreatic adenocarcinoma. Multivariate analysis revealed that lymph node metastasis and IDO expression were two independent prognostic predictors of pancreatic adenocarcinoma. This finding is consistent with recent reports on other tumors, such as colorectal cancer, ovarian cancer, hepatocellular carcinoma, and esophageal squamous cell cancer.[14,19,20,27] Although TNM staging is also a prognostic predictor for cancer patients, this factor did not exhibit statistically significant correlation with overall patient survival as detected through multivariate analysis in the present study. This observation might be explained mainly by the notion that the statistical results were limited by the low number of cases and short observation period.

There are several limitations in this study. The number of enrolled patients was limited in this study. A large-scale multicenter study is needed to confirm these results. Another limitation was the noninclusion of the IDO action mode in pancreatic cancer progression. IDO could play an immunosuppressive role in tumor through multiple signaling pathways including mammalian target of rapamycin complex 1, general control nonderepressible 2 (GCN2), and aryl hydrocarbon receptor.[28-31] The exact mechanism of IDO in the progression of pancreatic cancer will be analyzed in the future work.

In conclusion, the study confirmed that IDO expression in pancreatic adenocarcinoma is related to patient prognosis and might be used as an independent prognostic indicator. We hope that IDO could also be a therapeutic target for the treatment of pancreatic adenocarcinoma in the near future.

Conflicts of interest
There are no conflicts of interest.

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