

Original Article

Expression and function analysis of indoleamine 2 and 3-dioxygenase in bladder urothelial carcinoma

Chenggang Yang^{1*}, Yongchun Zhou^{2*}, Lijuan Zhang¹, Congguo Jin², Mei Li¹, Lijuan Ye¹

¹Department of Pathology, The Tumor Hospital of Yunnan Province, The Third Affiliated Hospital of Kunming Medical University, Kunming City 650118, China; ²Cancer Institute, The Tumor Hospital of Yunnan Province, The Third Affiliated Hospital of Kunming Medical University, Kunming City 650118, China. *Equal contributors.

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Abstract: Indoleamine 2, 3-dioxygenase (IDO) is a rate-limiting enzyme for tryptophan metabolism inducing immune tolerance of tumors. The purpose of this study is to investigate IDO expression and its prognostic significance in bladder urothelial carcinoma (BUC). In this study, immunohistochemical staining for IDO expression in BUC tissues (n = 84) and normal bladder tissues (n = 22) was performed. The mRNA expression levels of IDO in BUC and normal bladder were analyzed by quantitative RT-PCR. Survival analysis was performed for the correlation of IDO expression and clinicopathological factors with disease-free survival. Positive expression of IDO was found in 48 of 84 cases in BUC tissues and was significantly correlated with histological classification, histological grade and TNM stage. While IDO expression in normal bladder tissues was expressed in only 4 of 22 (18.2%) cases. Moreover, IDO mRNA levels of BUC were significantly higher than that of normal bladder. We also found that IDO, histological grade and TNM stage were closely associated with DFS. These results indicated that IDO was related to the progression of BUC and might be one of the crucial prognostic factors for BUC.

Keywords: Indoleamine 2, 3-dioxygenase, bladder urothelial carcinoma, immunohistochemistry, disease-free survival, prognosis

Introduction

Bladder urothelial carcinoma (BUC) is one of the most common malignancies in the urinary system and accounts for approximately 15,000 deaths annually in the United States [1]. Most cases of BUC are superficial bladder tumors, but carcinomas in situ carry a high probability of turning into an invasive carcinoma [2, 3]. The recurrence rate of BUC is high after surgical removal, so BUC is still a difficult conundrum for clinical treatment [3, 4]. As BUC has a high recurrence rate, the effective prognostic biomarkers are crucial for the treatment of this disease. Angiogenesis and p53 have been considered as the potential biomarkers of progression to invasive tumors in BUC [4, 5]. However, a confirmatory study showed that the activation of angiogenesis and loss of p53 were insufficient to facilitate the progression of BUC [3]. Therefore, new biomarkers are needed for BUC.

Invasion and metastasis are the special biological characteristics of advanced tumors, which

are also the principle factors affecting the prognosis of tumors [6]. The immune resistance mechanisms are supposed to be involved in the progression and metastasis of tumors [7, 8]. It has been reported that tumors are able to escape the host immune surveillance by several methods [9, 10], but the detailed mechanisms of immune resistance are still unclear. Indoleamine 2, 3-dioxygenase (IDO) is a kind of intracellular enzymes that catalyzes the initial and rate limiting steps in the metabolism of the tryptophan along the kynurenine pathway [11]. Evidence for the immunosuppressive role of IDO was first revealed by Munn et al. [12] who proved that IDO prevents rejection of the allogeneic fetus by depleting tryptophan locally and producing tryptophan metabolites. In 2003, Uyttenhove et al. [13] demonstrated that most human tumors constitutively expressed IDO which protected tumors from host immune attack by catalyzing tryptophan degradation. Moreover, it was shown that IDO inhibitors potentiated the antitumor activity by improving

responses to cancer chemotherapy [14]. Therefore, IDO may have an essential role in the progression and metastasis of tumors, and it may be a new prognostic marker for malignancy. Recently, IDO has been reported to be important in the prognosis of various human tumors, such as ovarian cancer [7], endometrial cancer [15] and colorectal cancer [16]. However, the correlation between IDO expression and BUC has not yet been well studied.

In order to explore the role of IDO in the progression of BUC, we performed immunohistochemical analysis for IDO expression in 84 BUC tissues and 22 normal bladder tissues first. Then the mRNA levels of IDO were detected to further verify the relationship between IDO expression and BUC. Finally, the correlation analysis of disease-free survival (DFS) with IDO expression and clinicopathological factors was conducted to investigate the prognostic implication of IDO in BUC.

Materials and methods

Patients and case selection

This study included 84 BUC samples from patients who underwent surgical tumor resection at the Tumor Hospital of Yunnan Province in China between January 2007 and July 2011. All the patients (70 males and 14 females) have received exactly pathological diagnosis. The mean age of the patients was 52.5 years and ranged from 20 to 85. All patients were staged according to the 1997 Union for International Cancer Control (UICC) TNM classification criteria: 30 were stage Ta, 20 were stage T1, 20 were stage T2, and 14 were stage T3T4. Histological grade was assigned according to the criteria of the 1999 World Health Organization (WHO) classification: 18 were G1, 32 were G2, and 34 were G3. Based on the histological classification of WHO, the patients were divided into two types: 54 were infiltrating types and 30 were non-infiltrating types. In addition, 22 normal bladder tissues of healthy donors were collected as controls. This study was approved by the institutional review boards at the Tumor Hospital of Yunnan Province.

Immunohistochemistry

Informed consent was obtained from individual patients for the use of their tissue samples. The formalin-fixed and paraffin-embedded sp-

ecimens were cut at a thickness of 4 μm . Immunohistochemical staining was performed using the streptavidin-peroxidase (SP) method. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 10 min after deparaffinization and rehydration. For heat-induced antigen retrieval, tissue sections were soaked in citric acid buffer (PH 6.0) and incubated at 95°C for 15 min in a microwave oven. Then nonspecific immunoglobulin binding was blocked by incubation with normal goat serum for 20 min. The sections were incubated at room temperature for 1 h with primary antibody against IDO. After rinsing thrice in phosphate buffer saline (PBS), sections were incubated at 37°C for 30 min with biotinylated second antibody followed by staining with 3, 3'-diaminobenzidinetetrahydrochloride in 0.01% H_2O_2 for 10 min. Finally, slides were counterstained with hematoxylin. As a negative control, the primary antibody was replaced with PBS. The known cervical cancer sections were used as positive control.

Quantification method

IDO expression levels were classified semi-quantitatively according to the percentage of tumor cells with IDO staining and the staining intensity. The proportion score reflects the percentage of tumor cells with IDO staining (score 0, none; score 1, < 25%; score 2, 25%-50%; score 3, > 50%). The intensity score represents the estimated staining intensity (score 0, no staining; score 1, weakly stained; score 2, moderately stained; score 3, strongly stained). The final IDO expression score was defined as follows: IDO- if the sum of the proportion score and the intensity score was 0, IDO 1+ if the sum was 1 to 2, IDO 2+ if the sum was 3 to 4, and IDO 3+ if the sum was 5 to 6. In present study, the IDO expression score > 2 (IDO 2+ and IDO 3+) was defined as the positive expression of IDO.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from 100 mg frozen tissues using RNA extraction kit (Qiagen) according to the manufacturer's recommendations. After DNase I treatment (Invitrogen), one microgram of total RNA was used for cDNA syntheses by reverse transcription using the Reverse Transcription Enzyme (Invitrogen). Quantification of expression levels of IDO were determined

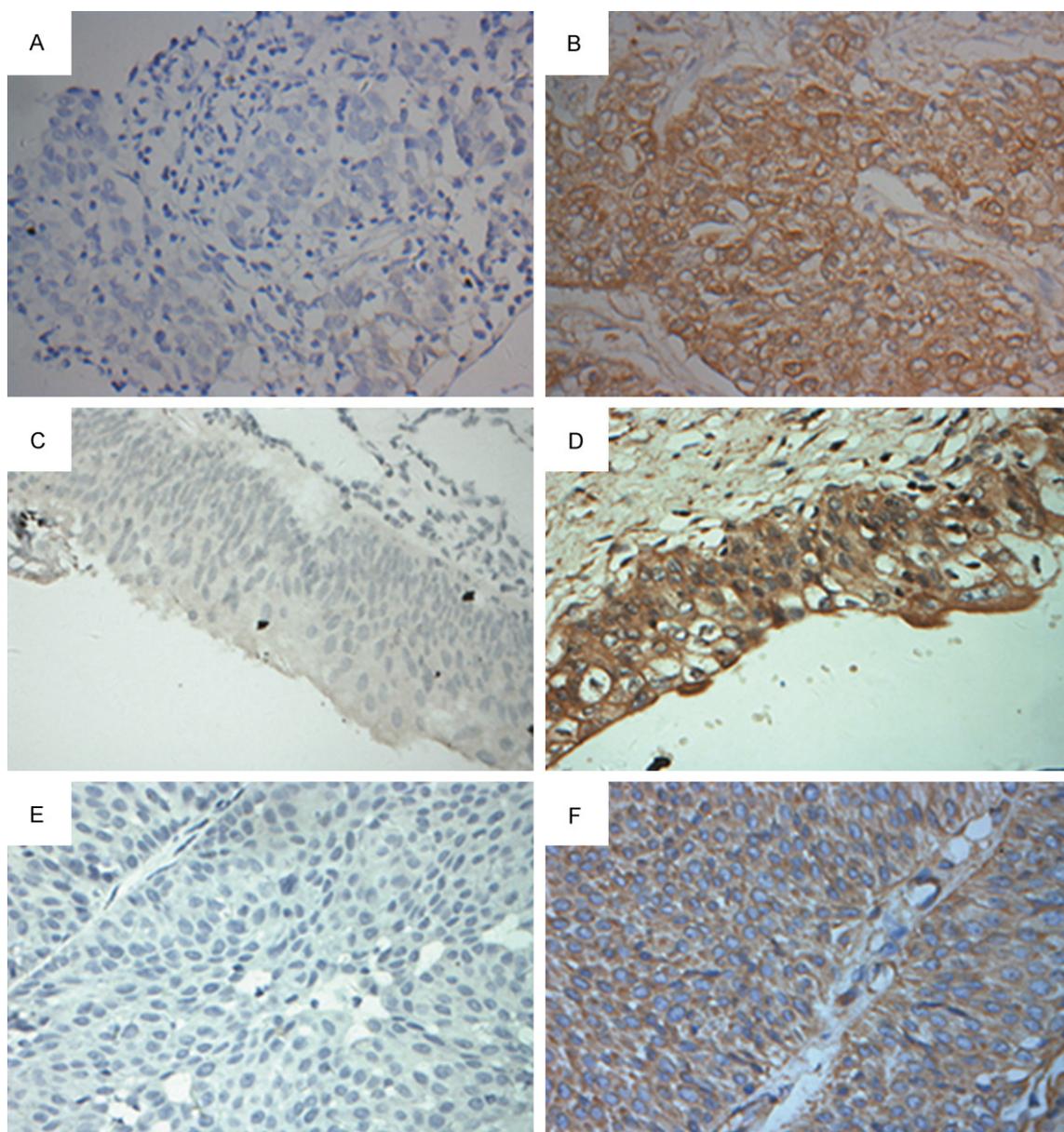


Figure 1. Representative immunohistochemical staining for IDO expression. A. Negative control; B. Positive control; C. Normal bladder tissues with negative expression of IDO; D. Normal bladder tissues with positive expression of IDO; E. Negative expression of IDO in bladder urothelial carcinoma; F. Positive expression of IDO in bladder urothelial carcinoma. Original magnification, 400 × in A-F.

by quantitative RT-PCR using SsofastEvaGreenSupermix Kit (BIO-RAD) and ABI 7500 system. The IDO primers for quantitative RT-PCR were as follows: Sense primer: 5'-GATGAAG-AAGTGGGCTTTGC-3'. Antisense primer: 5'-TCC-AGTTTGCCAAGACACAG-3'.

In addition, the reaction conditions were: 95°C for 5 min; 95°C for 20 s; 55°C for 30 s; 40 cycles. Housekeeping gene GAPDH was used

for normalization and all reactions were run in triplicate. The relative mRNA expression of IDO was analyzed with the $2^{-\Delta\Delta Ct}$ method [17].

Statistical analysis

Pearson χ^2 test and Fisher's exact test were used to analyze the correlation of IDO expression with various clinicopathologic factors. Comparison of IDO mRNA expression among different tissues, histological grades and TNM

IDO used in prognosis of BUC

Table 1. Correlation of IDO expression with clinicopathologic factors and different tissues

Variables	cases	IDO expression (n)		χ^2	P-value
		negative	positive		
Tissues					
Normal bladder	22	18	4		
BUC	84	31	53	5.992	0.022
Clinicopathologic factors					
Age (years)					
> 60	44	18	26		
≤ 60	40	18	22	0.143	0.705
Gender					
Male	70	28	42		
Female	14	8	6	1.400	0.237
Histological classification					
Infiltrating	54	18	36		
Non-infiltrating	30	18	12	5.600	0.018
Histological grade					
G1	18	14	4		
G2	32	16	16		
G3	34	6	28	20.268	< 0.001
TNM stage					
Ta	30	18	12		
T1	20	11	9		
T2	20	5	15		
T3T4	14	2	12	12.075	0.007

IDO represents indoleamine 2, 3-dioxygenase; BUC represents bladder urothelial carcinoma.

stages was done with the t test, respectively. DFS was calculated from the date of surgery to the date of recurrence or metastasis. Survival analysis was performed according to the Kaplan-Meier method. Comparison of the survival between groups was performed with the log-rank test. SPSS version 16.0 (Chicago, IL, USA) was used for all the statistical analysis, and $P < 0.05$ was regarded as significant.

Results

Immunohistochemical expression of IDO in BUC tissues and normal bladder tissues

We examined the IDO expression in 84 BUC tissues and 22 normal bladder tissues by immunohistochemical staining. The IDO protein expression was observed in both BUC and normal bladder with predominantly cytoplasmic staining based on **Figure 1C-F**. As shown in **Table 1**, of the 84 BUC tissues examined, the positive expression of IDO was found in 48 cases and

the positive rate was 57.1%. While only 4 cases of 22 normal tissues (18.2%) were detected with positive expression of IDO. The IDO expression of BUC was significantly higher than that of normal bladder ($P = 0.022$).

Correlation of IDO expression with clinicopathological factors in BUC

The correlations of IDO expression with clinicopathologic variables in BUC were summarized in **Table 1**. The result showed that the positive expression of IDO was significantly correlated with histological classification ($P = 0.018$), histological grade ($P = 0.001$) and TNM stage ($P = 0.007$), but not with the age ($P = 0.705$) and gender of patients ($P = 0.237$).

mRNA expression of IDO in BUC and normal bladder

To investigate whether IDO expression was accompanied by the progression of BUC, quantitative RT-PCR was used in this study. From **Figure 2A**, we found that the IDO expression levels of BUC were significantly higher than those of normal bladder tissues ($P < 0.05$). By comparing different histological grades of BUC, there have significant difference ($P < 0.05$) in the IDO mRNA levels between high grade BUC (G3) and low/moderate grade BUC (G1 and G2). Furthermore, IDO mRNA levels of Ta-T1 stage were significantly lower than those of T2-T4 stage according to the TNM stage (**Figure 2B**).

Correlation of DFS with IDO expression and clinicopathological factors

The DFS of BUC was 39 months on the basis of the follow-up data. In one year, the DFS rates of patients were 90.5% and the rates dropped to 25% within 5 years.

To evaluate the impact of IDO expression and clinicopathological factors on patient prognosis, DFS curves were constructed using Kaplan-Meier method and the results were showed in **Figure 3**. Based on **Figure 3A**, we found that

IDO used in prognosis of BUC

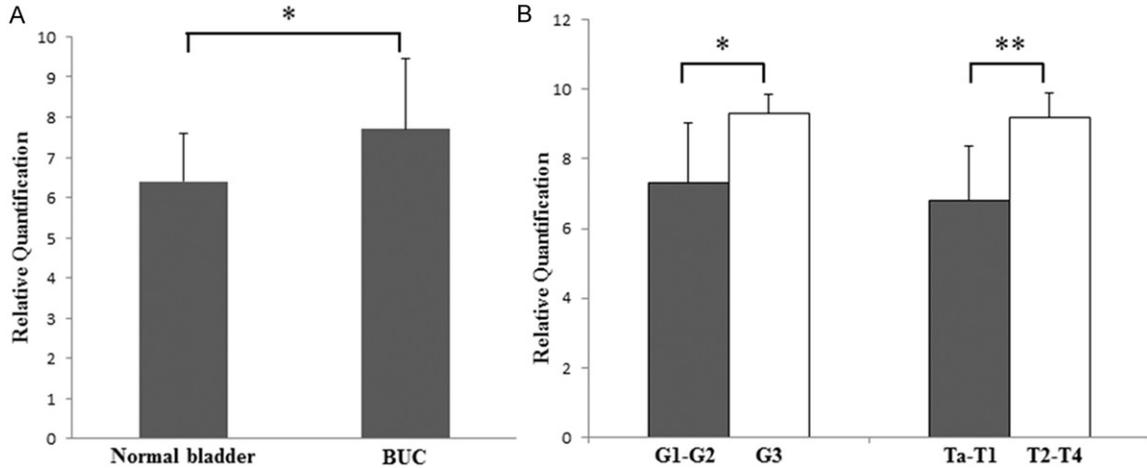


Figure 2. IDO mRNA levels in normal bladder and bladder urothelial carcinoma (BUC). A. The IDO mRNA levels in BUC were significantly higher than that in normal bladder ($P < 0.05$). B. IDO expression of G3 showed the significant difference in comparison with G1-G2 ($P < 0.05$) according to the histological grade. The IDO expression of T2-T4 was significantly higher than that of Ta-T1 ($P < 0.01$) based on TNM stage.

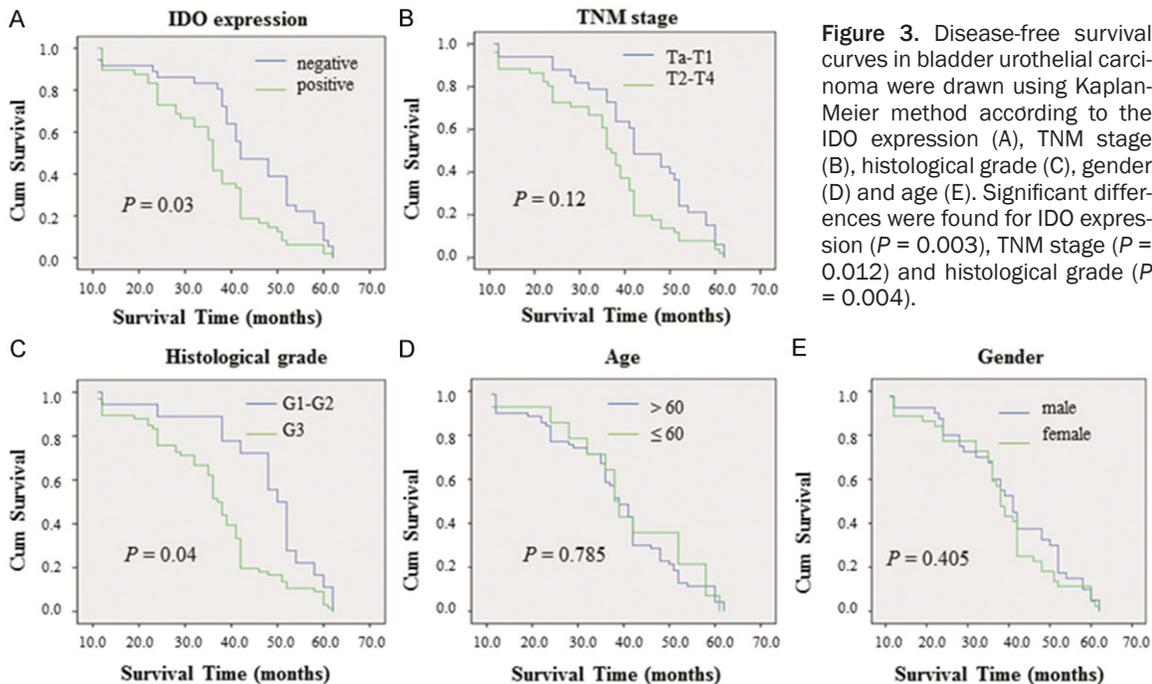


Figure 3. Disease-free survival curves in bladder urothelial carcinoma were drawn using Kaplan-Meier method according to the IDO expression (A), TNM stage (B), histological grade (C), gender (D) and age (E). Significant differences were found for IDO expression ($P = 0.003$), TNM stage ($P = 0.012$) and histological grade ($P = 0.004$).

patients with positive IDO expression had significantly impaired DFS ($P = 0.003$) as compared with patients with negative expression of IDO. Moreover, we analyzed the correlation of histological grade and TNM stage with DFS and found that both TNM stage ($P = 0.012$) and histological grade ($P = 0.004$) were closely related to the DFS of BUC, as shown in **Figure 3B, 3C**. However, no significant differences were detected between DFS and gender ($P = 0.785$), age ($P = 0.405$) (**Figure 3D, 3E**).

Discussion

Recent studies have reported that the immunosuppressive enzyme IDO is closely related to poor clinical outcome and immune escape of various human tumors [18-20]. But there were few reports about the role of IDO in BUC. In the present study, to explore the expression and significance of IDO in BUC, we analyzed the expression of IDO in BUC using surgical specimens from 84 patients, and found that the

positive expression of IDO was significantly correlated with disease progression and DFS of BUC.

Previous study showed that the IDO mRNA levels were relatively low in normal bladder tissues and IDO-positive cells were mostly found in the muscularis layer of bladders [21]. Thus, positive expression of IDO was detected in normal bladder tissues by immunohistochemical staining in our study. But IDO was expressed in only 4 of 22 (18.2%) cases. The study of Uyttenhove et al. [13] showed that IDO was expressed in a variety of human tumor types, including bladder carcinomas. Moreover, IDO-expressing tumors escaped the host immune surveillance by blocking T-lymphocyte proliferation. Subsequently, the immunosuppression functions of IDO in tumors have been proved in many cancer types [22, 23]. In present study, the frequency of IDO expression in BUC was high. Indeed, IDO was positively expressed in 48 cases (57.14%) of all patients. Furthermore, the expression of IDO in BUC was significant higher than that in normal bladder, and the result was further validated by quantitative RT-PCR (**Figure 2A**). These evidences indicated that IDO might play an important role in BUC.

Then we analyzed the correlation of IDO expression with the clinicopathological factors of BUC. The results showed that positive IDO expression was significantly correlated with histological classification, histological grade and TNM stage. Thus, we speculated the IDO was linked with cancer progression of BUC due to these factors represented degrees of malignancy. Other studies have demonstrated that high levels of IDO have been detected in advanced stages of ovarian carcinoma and nasopharyngeal carcinoma [24, 25]. Moreover, IDO expression was found in all invasive uterine cervical cancers, whereas non-invasive tumors presented a much lower expression of IDO [26]. In this study, quantitative RT-PCR was used to detect the IDO levels of different histological grades and TNM stages in BUC. The results also showed that advanced BUC was accompanied with high expression of IDO. However, the mechanism about the correlation between IDO expression and tumor progression is still unknown, further studies are still needed.

Many studies on the IDO expression in human cancers revealed that patients with high expression of IDO had decreased progression-

free survival [27-29]. For example, IDO expression evaluated by immunohistochemistry (IHC) and enzymatic activity was inversely correlated with progression-free survival in endometrial cancer and malignant melanoma [15, 30]. The present data demonstrated that the patients with positive IDO expression had a poor clinical outcome by analyzing the rates of DFS. Moreover, the histological grade and TNM stage were also closely associated with DFS by log-rank test, as expected. These findings suggested that IDO might be used as a prognostic parameter of BUC.

In summary, we demonstrated here that IDO was closely involved in the progression of BUC and might be used as a novel prognostic indicator of BUC. Furthermore, IDO could be a therapeutic target for the treatment of BUC. However, there are some limitations in this study. First, multivariate analysis was needed to explore if IDO was an independent prognostic factor for DFS. Second, the protein level of IDO in BUC was not detected by western blot. In the future, further studies will be performed to validate the key role of IDO in BUC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lijuan Zhang, Department of Pathology, Tumor Hospital of Yunnan Province, Third Affiliated Hospital of Kunming Medical University, 519 Kunzhou Road, Xishan District, Kunming City 650118, China. Tel: +86-871-68185656-2112; Fax: +86-871-68185656-2112; E-mail: zhlijuanzh@163.com

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IDO used in prognosis of BUC

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