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Indoleamine-pyrrole 2,3-dioxygenase might be a prognostic biomarker for patients with renal cell carcinoma

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ABSTRACT

Objective: To explore the role of indoleamine-pyrrole 2,3-dioxygenase (IDO), an immunomodulatory enzyme, in renal cell carcinoma (RCC).

Methods: A total of 40 patients diagnosed as RCC in the Second Xiangya Hospital were included in this study. All patients received nephrectomy. The histopathological features of samples were assessed semi-quantitatively. IDO mRNA level in RCC and non-RCC renal tissues was determined by real-time quantitative PCR (RT-qPCR). And the expression of IDO protein in endothelial cells was examined by immunohistochemistry; a Kaplan-Meier survival curves was calculated on the basis of IDO mRNA level.

Results: Level of IDO mRNA in RCC samples was significantly higher than that in tumor-free samples with $P<0.001$. Patients with high IDO expression had an significantly longer survival time than those with low IDO expression ($P=0.01$). There was a statistically significant inverse correlation between IDO and proliferation marker Ki67. Patients with high IDO level were of low Ki67 level, and vice versa ($P<0.01$).

Conclusion: IDO might be a prognostic biomarker for patients with RCC.

KEY WORDS indoleamine-pyrrole 2,3-dioxygenase; renal cell carcinoma; endothelial cell; prognostic biomarker

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吲哚胺吡咯 2，3- 双加氧酶与肾细胞癌预后的预测

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[摘要] 目的：探讨吲哚胺吡咯 2，3- 双加氧酶 (IDO，一种具有免疫调节作用的酶) 在肾细胞癌 (RCC) 患者中的作用。**方法：**在中南大学湘雅二医院诊断为 RCC 的患者 40 例，全部接受肾切除术。肾组织的免疫病理检查采用半定量方法评估。实时定量 PCR(RT-qPCR) 检测 RCC 与非肾癌肾组织 IDO 的 mRNA 水平。免疫组织化学的方法检测血管内皮细胞的 IDO 蛋白表达。同时依据 IDO 的 mRNA 水平计算出患者的 Kaplan-Meier 生存曲线。**结果：**RCC 肿瘤组织中 IDO mRNA 的表达明显高于正常肾组织 ($P<0.001$)。在 RCC 患者中，IDO 高表达的患者比那些低 IDO 表达的患者具有明显较长的生存时间 ($P=0.01$)。统计学分析显示：RCC 肿瘤组织中 IDO 的水平和肿瘤增殖标志物 Ki67 之间呈负相关，IDO 水平高的患者 Ki67 水平低，反之亦然 ($P<0.01$)。**结论：**IDO 可能作为预测 RCC 患者预后的生物标志物。

[关键词] 吲哚胺吡咯 2，3- 双加氧酶；肾细胞癌；内皮细胞；预后生物标志物

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Although tumors present a variety of antigens, malignant tumor tissue is usually not recognized by the immune system as the attack target[1-3]. Therefore, the tumor seems able to suppress the immunological response thus allowing tumors to grow undisturbed and destroy adjacent healthy tissues. This pathological state of tolerance on the part of the immune system is known as the immune escape of malignant tumors.

But what mechanism allows the renal cell carcinoma (RCC) to escape the attack of the immune system? Researchers have demonstrated that the tumor is infiltrated by T-cells, B cells, natural killer cells and monocytes[4-5]. Despite the presence of tumor antigens, infiltrating immune cells and the evidence of induced immune response in situ the RCC tissue remains immunologically tolerant. It seems as if the malignant cell itself induces the inactivity of tumor-infiltrating lymphocytes, resulting in the tolerance towards the tumor. Researchers have demonstrated decreased cytotoxic activity of $CD8^+$ T-cells in vivo in RCC[6]. Some studies have shown that an extract of RCC cells leads to inhibition of T-cell proliferation[7-8]. In fact, more anti-inflammatory cytokines might also inhibit T-cells in RCCs [9]. In addition to the above-mentioned immune escape mechanisms, some tumors have reduced surface expression of Fas receptor enabling them to escape T cell-induced apoptosis. The expression of Fas ligand can trigger T cell-induced apoptosis, a so-called counter attack [10].

Besides the above-mentioned immune escape mechanisms, indoleamine-pyrrole 2,3-dioxygenase (IDO), an immunomodulatory enzyme, also plays an immune regulatory role. IDO can develop an immunosuppressive effect and thus promotes tumor progression. This study aimed to explore the role of IDO, an immunomodulatory enzyme, in RCC.

1 Materials and methods

1.1 Patients

From June 2010 to January 2011, a total of 40 patients, who were diagnosed as RCC and underwent a nephrectomy in the Second Xiangya Hospital, were included in this study (Table 1). Tissue biopsies were stored at −80℃. As part of the staging, all patients underwent a diagnostic assessment. This included medical history, physical examination, urine and blood tests, ultrasound of the abdomen, X-ray of the chest or magnetic resonance imaging. If necessary for differential diagnosis, a further treatment plan and additional investigations were carried out.

Table 1 Clinical characteristics of the patients (n=40)

Indexes	No.(%)
Age/year	
⩾60	16 (40)
<60	24 (60)
Gender	
Male	24(60)
Female	16 (40)
Tumor stage (T)	
T1	8 (20)
T2	8 (20)
T3	22 (55)
T4	2(5)
Lymph node metastases (N)	
N0	28(70)
N1	2(5)
N2	6(15)
Nx	4 (10)
Metastases (M)	
N0	16 (40)
N1	24 (60)
Nx	0
Histological differentiation (G)	
G1	4(10)
G2	14(35)
G3	22(55)

1.2 RNA isolation from tissue

In order to extract the total RNA from tissue samples, the RNA Midi Kit® (Qiagen, Hilden, Germany) was used. The sample was crushed by a disintegrator (MicroDismembrator, Braun Instrumente, Melsungen, Germany), and lysed in 3.8 mL RLT buffer . The lysed sample was centrifuged for 5 min at 4000 r/min. 3.8 mL 70% ethanol was added into the supernatant, then the supernatant was panned and mixed for one minute. To fix the RNA in the membranes of the kit columns, several wash steps were followed according to the manufacturer's protocol. In order to elute the RNA from the membrane, the filter was transferred to a new 15 mL collection tube and washed twice with 250 mL of RNase-free water. In order to determine the concentration, an aliquot was taken with 1/50 dilution in RNase-free water from the kit, and the absorbance measured at 260 and 280 nm photometrically (HACH LANGE DR 2800 VIS photometer, Germany).

1.3 Reverse transcription

For the transcription of messenger RNA (mRNA) into complementary DNA (cDNA), a reverse transcription reagent system was used. In this case, total cellular RNA was transcribed by the addition of retroviral reverse transcriptases (RTs) in the first-strand reaction in single-stranded cDNA. This process was triggered by the complementary binding of random hexamer primers to mRNA. The extension of the primers was performed by the addition of further nucleotides to the RNA-dependent DNA polymerase in 5'→3' direction. The non-specific random primers bind to multiple sites within the mRNA, thus resulting varying length and partial cDNA copies. The reverse transcription was performed by the manufacturer's protocol and the conditions were the same: 1 mg of RNA sample with RNase-free water to a total volume of 9.9 mL in the denaturation solution, followed by incubation at 70 °C for 10 minutes.

1.4 Real-time quantitative PCR

The purification of total RNA from the cells was performed using the NucleoSpin RNA Kit I (Macherey-Nagel, Germany) according to the manufacturer's recommendations. cDNA corresponding to 50 ng of RNA was added to the SYBR-Green JumpStart Taq Ready Mix (Sigma-Aldrich, St. Louis, MO). Primers and a DNA intercalating dye were added. This fluorescent dye was deposited in the DNA (intercalated) and binds to the double-stranded DNA. Thus the measured fluorescence was directly proportional to the amount of PCR product, which increased exponentially during the PCR from cycle to cycle. The sequences of the primers used are listed in Table 2.

Table 2 PCR primers

Gene	Orientation	Sequence(5'→3')
IDO	Forward	GGT CAT GGA GAT GTC CGT AA
	Reverse	ACC AAT AGA GAG ACC AGG AAG AA
β-actin	Forward	AAG GTG AAG GTC GGA GTC AAC
	Reverse	AGT GGG TGT CGC TGT TGA AGT C

1.5 Immunohistochemical methods

To demonstrate IDO at the protein level, immunohistochemical staining was carried out. Peroxidase staining was performed using an indirect two-step method: First, an unconjugated primary antibody is bound to the antigen to be stained, then a second enzyme-labeled, secondary antibody directed against the Fc part of the primary antibody is added. Finally, the substrate-chromogen was performed.

2 Results

2.1 IDO expression in RCC and tumor-free kidney tissue

Quantitative measurements were performed in a total of 58 samples. Eighteen of them were from tumor-free kidney tissue, 40 from RCC tissue. β-actin cDNA was measured in each sample by real-time PCR separately, as a control for normalization. Figure 1B shows the crossing point (CP) values for the quantification of β-actin cDNA. According to the number of cycles, the CP value was achieved during the amplification process. The average was 25.63, and the standard deviation was 0.82 cycles. Figure 1A shows the CP value for IDO. The average was 38.34 in the tumor-free kidney tissue and 33.23 in the tumor tissue. The RCC samples had a lower average CP value, implying more IDO mRNA expressed in the tumor-free kidney tissue. The statistical analysis of data gained through the RT-qPCR results was carried out using the Wilcoxon test. A highly significant difference was found in the IDO mRNA level between the RCC tissue and the tumor-free kidney tissue (P<0.001), shown in Figure 1C.

To characterize the IDO expression more accurately in RCC endothelial cells, staining was performed with a CEACAM1-detecting monoclonal antibody. CEACAM1 is mainly synthesized in the tumor endothelial cells[11–12]. The antibody, therefore, serves as a marker of neo-angiogenesis within the newly formed and not-yet stabilized blood vessels[13–14]. From a total of 40 RCC samples, 12 samples were assigned to four high and eight low expression groups. In the rest of the RCC samples, IDO could not be detected by immunohistochemistry. A comparison of immunohistochemical staining with the PCR results showed that PCR-negative samples also had no IDO-positive structures visible immunohistochemically. Figure 2A to 2C show three examples of immunohistochemistry in the tissue of three RCC patients. Figure 2D shows that staining with the anti-IDO antibodies correlates well with IDO mRNA levels from the same tumor samples.

2.2 Follow-up

Immunohistochemical staining with a peroxidase-NEM

IDO-specific monoclonal antibody confirmed that IDO was formed in the RCC tissue but not in tumor-free kidney tissue. Since the level of IDO in the tested samples varied, it seemed plausible that the IDO expression in renal cell carcinoma might impact the survival probability of patients. The results show in fact that those patients with high IDO expression had a significantly higher survival probability. Figure 3 depicts Kaplan-Meier survival curves of the patients studied.

Because IDO was expressed primarily in the endothelial cells and a significant correlation of IDO mRNA level with survival rate was detected, we hypothesized that the expression of IDO in the endothelial cells caused a decreased flux of tryptophan into the surrounding tissue. The local lack of tryptophan, an essential amino acid for protein synthesis, in the tumor cells would inhibit their proliferation. To confirm this hypothesis, immunohistochemical staining with the Ki67 antibody was performed in RCC tissues. Ki67 is formed in nuclear cells and regarded as a prognostic marker for RCC patients. A high Ki67 level was associated with a low survival rate[15]. A highly significant inverse correlation was observed between the number of IDO-positive tumor-endothelial cells and the number of tumor cells with Ki67-positive nuclear cells (P<0.01, Figure 4). Patients with high IDO expression consequently showed low Ki67 levels, and vice versa. That is, high IDO expression was correlated with a high survival rate.

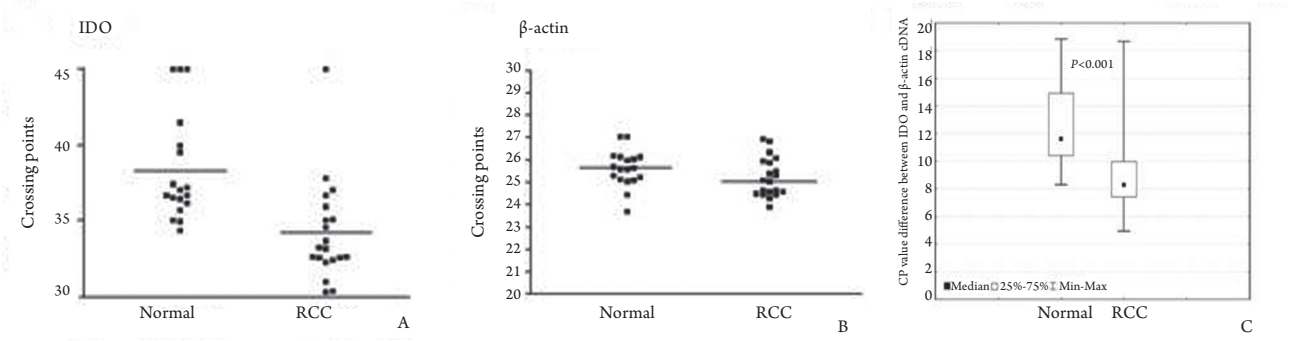

Figure 1 IDO mRNA level in tumor-free and RCC samples by real-time PCR. A: Scatter plot of IDO mRNA level determined by real-time PCR, shown as crossing point (CP) values; B: Scatter plot of β-actin mRNA; C: IDO mRNA level in tumor-free and RCC samples after correction with β-actin mRNA.

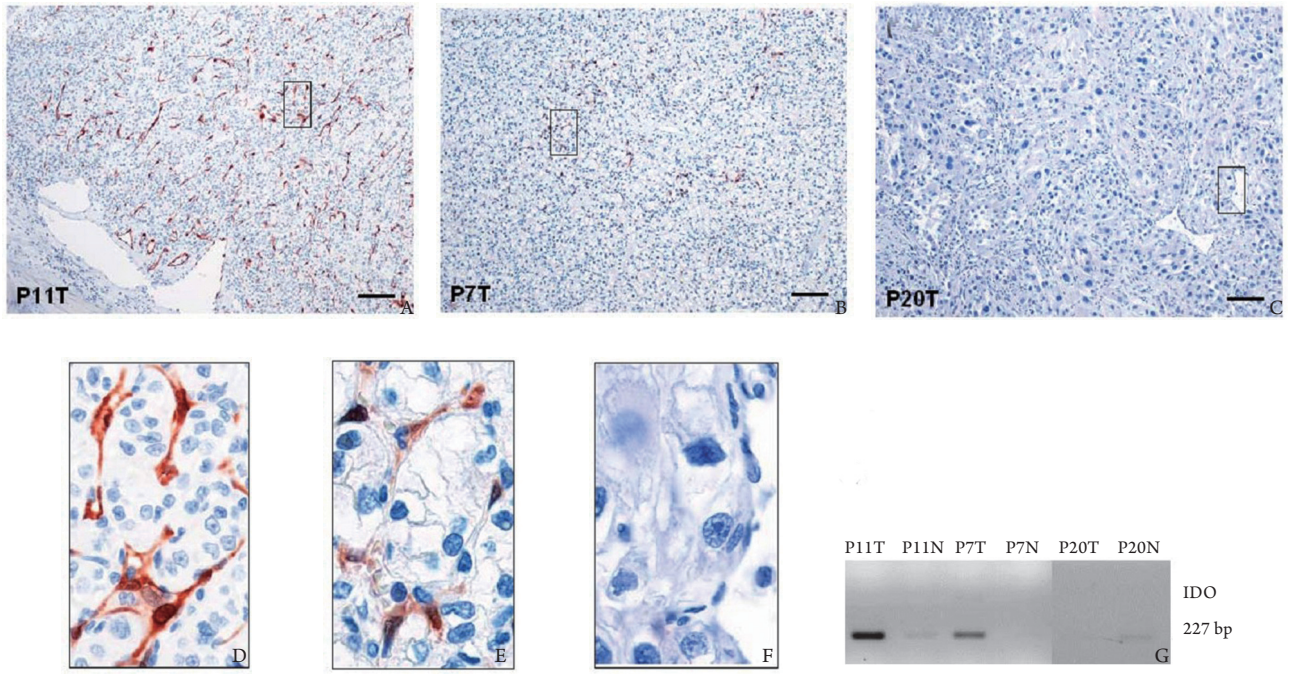

Figure 2 Correlation of IDO mRNA and protein levels in RCC samples. A: High IDO protein expression; B: Low IDO protein expression; C: No detectable IDO protein expression; D–F: Magnification (×400) of A–C, respectively; G: The staining with the anti-IDO antibodies correlates well with IDO mRNA levels from the same tumor samples.

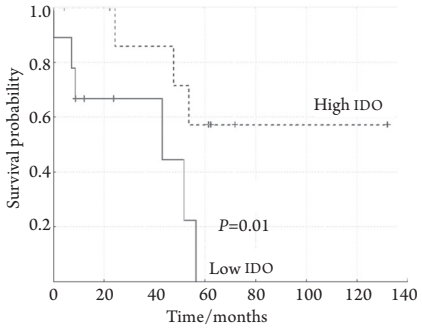

Figure 3 Kaplan-Meier survival analysis according to IDO mRNA level.

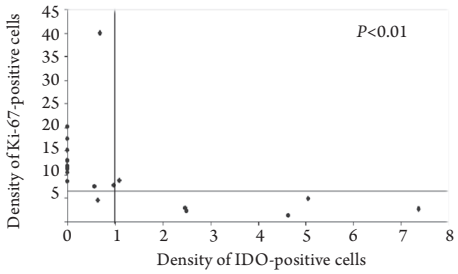

Figure 4 Inverse correlation between IDO expression in tumor endothelial cells and the proliferation marker Ki67 in tumor cells.

3 Discussion

The enzyme, indoleamine 2,3-dioxygenase (IDO), as a catabolic enzyme of the essential amino acid tryptophan, is endowed with a very powerful immunosuppressive activity. In pregnant mice, IDO expression in the placenta is fundamental to preventing the mother's immune system from attacking the semi-allogenic fetus: inhibition of IDO activity results in the loss of all allogenic fetuses [16]. It has also been demonstrated in vitro that IDO expression can be induced in both macrophages and dendritic cells (DCs), which thereafter acquire the capacity to inhibit cell proliferation. As result of IDO activity, in both T and NK cells, reverses their commitment to cell cycle progression and the cells enter an arrested state, in which both adaptive and innate immunity are consequently affected. It has been suggested that IDO may be a microenvironmental factor that plays a role in tumor evasion of T cell–mediated rejection[17]. Growing evidence suggests that various types of human tumor cells express IDO, and inflammatory mediators, especially interferon-γ (IFN-γ), have the specific ability to induce IDO expression [18]

But the role of IDO in malignant tissue is controversial[19–21]. In several experiments, IDO has been ascribed a T-cell-inhibiting property. Through this action, the IDO-immune response works against the malignant tissue and inhibits the progression of the tumor. This property has been confirmed by the following observations: patients with high IDO level in ovarian adenocarcinomas, colorectal adenocarcinomas, endometrial and esophageal cancer have a poorer prognosis [20–23]. However, it has also been reported that patients with high IDO expression in non-small cell lung cancer and hepatocellular carcinoma have the longer survival [24–25].

This study has dealt with the role of IDO in RCC tissue. We found that more than one third of patients (16/40) hasa relatively high IDO mRNA expression and, simultaneously, had a longer survival. This trend was confirmed by the highly significant inverse correlation between the density of IDO-positive endothelial cells and the number of proliferating Ki67-positive tumor cells. Ki67 is a prognostic marker for RCC patients. A lower Ki67 expression in the nuclei cells of RCC is associated with longer survival. An increase in survival probability is presumably because IDO is expressed primarily in the neoangiogenesis of newly formed endothelial cells. We present the following hypothesis: the expression of IDO in endothelial cells is inhibited by the local deprivation of the essential amino acid tryptophan (or by the tumor-toxic breakdown products of tryptophan) which limits the proliferation of the tumor. This hypothesis is supported by the fact that RCC patients with high IDO expression has the longer survival. Thus, IDO has a certain prognostic value in RCC. However, to strengthen the hypothesis and IDO's prognostic value further research with much larger RCC samples is required.

IDO can induce a kind of immunogenic tolerance in tumor cells, as well as in dendritic cells and in lymph nodes. This immunosuppressive effect of IDO, which

inhibits an immune response against the malignant tissue, suggests a potential use of IDO inhibitors to circumvent the immune escape of tumors. For example, Friberg et al[26] found that LLC cells (Lewis lung carcinoma cells) stimulated a more robust allogeneic T cell response in the presence of a competitive inhibitor of IDO. Similar results were achieved by Uyttenhove et al [19]. In addition, in other mouse models, an increasing use of IDO inhibitors has vouched for the efficiency of such chemotherapeutic agents [27–28].

Since radiotherapy and systemic therapy for RCC have only limited effectiveness, radical tumor surgery remains paramount. In addition to the surgical treatment, immunotherapy has been established. Immunotherapy is based on the immunogenicity of the RCCs and is induced by stimulation of the immune system to eliminate tumor cells. Many cytokines can serve as therapeutic agents, including the cytokines interferon-α (IFN-α-2a and -2b) and interleukin-2 (IL-2). These cytokines induce the expression of IDO. In this study, it was shown that high IDO expression is associated with the long survival. The findings recommend the potential of IDO inhibitors in the treatment of RCCs. This opportunity represents a new therapeutic approach to RCC and requires a further and deeper research.

In summary, our findings suggest that high IDO expression is correlated with the high survival rate, and that IDO might be a prognostic biomarker for patients with RCC.

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