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Original Article

Clinical significance of IL-35 expression in the progression of clear cell renal cell carcinoma

Jun Zhang[1*], Linkun Hu[1*], Lei Lei[4*], Yuwei Gu[1], Xuefeng Zhang[1], Kerong Wu[3], Xuedong Wei[5], Haiyan Liu[2], Jianquan Hou[1]

[1]Department of Urology, The First Affiliated Hospital of Soochow University, 188 Shizi Rd, Suzhou 215006, People's Republic of China; [2]Immunology Programme, Life Sciences Institute and Department of Microbiology and Immunology, National University of Singapore, 117456, Singapore; [3]Center of Uro-nephrological Disease, Ningbo First Hospital, NO. 59 Liuting Avenue, Haishu District, Ningbo 315000, People's Republic of China; [4]Institute of Blood and Marrow Transplantation, Soochow University, 199 Renai Rd, Suzhou 215006, People's Republic of China; [5]Department of Urology, The Changshu Hospital Affiliated to Soochow University, 1 Shuyuan Rd, Changshu 215006, People's Republic of China. [*]Equal contributors.

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Abstract: Interleukin (IL)-35 is a novel inhibitory cytokine produced by regulatory T cells. There is accumulating evidence that IL-35 plays an important role in tumor immunity. This study aims to investigate the expression of IL-35 in clear cell renal cell carcinoma (ccRCC) patients and the association between IL-35 and disease progression. Enzyme-linked immunosorbent assay (ELISA) was used to analyze plasma IL-35 levels and quantitative real-time PCR was used to detect the relative IL-35 mRNA expression in peripheral blood mononuclear cells (PBMCs) and tissues. Immunofluorescence was used to detect IL-35 expression in tumor tissues. Plasma IL-35 levels were found to be significantly higher in ccRCC patients than healthy controls (106.39 ± 57.53 pg/mL vs. 65.18 ± 18.23 pg/mL, $P < 0.0001$). Similarly, mRNA expression levels of both subunits of IL-35 in PBMCs were higher in ccRCC patients (EBI3, $P < 0.0001$; p35, $P < 0.0001$); the expression of IL-35 was also higher in tissues from ccRCC patients, as determined by RT-PCR and immunofluorescence. Furthermore, plasma IL-35 levels correlated positively with lymph node metastasis ($P < 0.0001$) and later tumor stage ($P < 0.0001$). In conclusion, plasma IL-35 levels are significantly higher in ccRCC patients, suggesting that IL-35 could be a potential biomarker for ccRCC disease progression.

Keywords: Interleukin-35, clear cell renal cell carcinoma, tumor marker, diagnosis

Introduction

Approximately 209,000 people worldwide are newly diagnosed with renal cell carcinoma (RCC) each year, accounting for 2-3% of adult solid tumors. The incidence of RCC has increased in recent decades [1]. Clear cell renal cell carcinoma (ccRCC) accounts for approximately 70% of all adult epithelial kidney tumors in surgically resected series [2]. However, despite valid surgical resection of localized RCC, 30% to 40% of patients subsequently experience recurrence, resulting in an estimated 102,000 deaths annually [3]. A series of clinical trials has been initiated to investigate adjuvant therapy for high-risk patients, to prevent recurrence. There is increasing evidence that ccRCC is associated with an imbalance of anti-inflammatory and pro-inflammatory cytokines that significantly contribute to tumor formation, progression, and metastasis [4-7].

IL-35, which belongs to the IL-12 family of cytokines, is a heterodimeric anti-inflammatory cytokine [8] comprised of the subunit Epstein-Barr virus-induced gene 3 (EBI3) and the p35 subunit of IL-12 [9]. It is predominantly expressed by Foxp3+ regulatory T-cells (Tregs) and is required for Treg-mediated immunosuppression [10]. Recent studies have shown that IL-35 is widely distributed and exerts biological effects both *in vitro* and *in vivo* via several immune cell subsets, including suppressors of T cell proliferation [8]. IL-35 induces the conversion of naïve T cells into regulatory T cells (iTr35 cells), downregulates Th17 cell develop-

ment and differentiation [11], and suppresses autoimmune inflammation. Furthermore, Wang discovered that IL-35 may serve to induce the production of autologous Breg and IL-35+ Breg cells, which could play a role in the treatment of autoimmune and inflammatory diseases [12].

Recent gene-expression analyses suggested a broader tissue distribution of IL-35 beyond Tregs [13]. Immunohistochemical analysis further revealed that EBI3 and p35 are highly expressed in a range of tumor tissues including lung cancer, colon cancer, esophageal carcinoma, hepatocellular carcinoma and cervical carcinoma [14, 15]. In the tumor microenvironment, Foxp3+ Tregs and other Tregs are considered the primary IL-35 producers [16]. IL-35 produced by Tregs potently suppress the function of Th1, Th17 and Th2 cells [11], and IL-35 expression is associated with immunosuppression, tumor progression and poor prognosis. However, the association between plasma IL-35 levels and clinical characteristics of patients in ccRCC has not been reported. In this study, we investigated the clinical significance of plasma IL-35 levels in patients with ccRCC and assessed the potential for clinical application as a prognostic biomarker for ccRCC.

Materials and methods

Study subjects

Sixty-four newly diagnosed ccRCC patients enrolled at the First Affiliated Hospital of Soochow University from January 2015 to December 2015 were included in the study as the patient group. Diagnosis of ccRCC was histologically confirmed. Patients were excluded if they had comorbidities such as chronic or acute inflammatory disease, autoimmune disease, or secondary or multiple malignancies. The control group comprised 53 age- and sex-matched healthy volunteers. The clinicopathological features of the patients and controls are shown in **Table 1**. Written informed consent was obtained from all subjects. The study was approved by the Ethics Committees of the First Affiliated Hospital of Soochow University.

Sample collection and processing

All peripheral blood samples collected from ccRCC patients and healthy volunteers were obtained prior to treatment. All blood samples (5 mL) were obtained in the morning after overnight fasting and were collected in tubes containing EDTA. The samples were processed by centrifugation for 15 min at 3,000 rpm within 2 h of collection, and the plasma samples were stored at -80°C until processed for the ELISA assay. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (Solarbio, Beijing, China), according to the manufacturer's instructions. Both cancer and normal tissues (> 5 cm away from cancer tissue) were obtained from operative specimens and stored at -80°C until use. All procedures were identical for both the experimental group and control group.

ELISA assay for IL-35

Plasma IL-35 concentrations were measured by a specific human IL-35 ELISA (Cloud-Clone Corp, Wuhan, China) according to the manufacturer's instructions. Each sample was assayed in duplicate, and the average calculated.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from PBMCs, cancer tissues, adjacent cancer tissue and normal tissue using TRIzol Reagent (TaKaRa, Dalian, China). Total RNA was used for first-strand cDNA synthesis, with the First-Strand Synthesis System for reverse-transcription PCR. Real-time PCR was performed to quantify 1 μg of cDNA using appropriate primer pairs with SYBR Green PCR Master mix (TaKaRa, Dalian, China). Each sample was processed in triplicate. **Table 2** shows forward and reverse primer sequences. The thermal cycle profile was as follows: 30 s at 94°C, 5 s at 94°C, and 30 s at 60°C for 42 cycles. The amplification specificity of each primer set was confirmed by melting curve analysis. The Ct value of each sample was determined, and relative gene expression levels were calculated using the ΔCt method normalized to the endogenous gene *β*-Actin. The data are expressed as n-fold relative to the control.

Immunofluorescence

Tissue sections (4 μM) were prepared from frozen tissue blocks, including 20 cancer sections and 16 normal sections. The tissues were as-

Table 1. Clinical characteristics of ccRCC patients and controls

Variables	ccRCC	Control	*p*-value
Subject, N	64	53	
Age, years	58 (23-85)	58 (34-80)	0.9084
Sex, M/F	40 (62.5%)/24 (37.5%)	33 (62.3%)/20 (37.7%)	0.979
Mean tumor size, cm, mean ± SD	4.4 ± 2.19		
TNM classification T staging			
T1/T2/T3/T4	38/14/10/2		
Tumor stage			
I/II/III/IV	38/12/12/2		

Table 2. Forward and reverse primers for real-time PCR

Primer		Sequence
Ebi3	Forward	GCT CCC TAC GTG CTC AAT GT
Ebi3	Reverse	CCC TGA CGC TTG TAA CGG AT
p35	Forward	TCC TCC CTT GAA GAA CCG GA
p35	Reverse	TGA CAA CGG TTT GGA GGG AC
β-Actin	Forward	CCT GGG CAT GGA GTC CTG TG
β-Actin	Reverse	AGG GGC CGG ACT CGT CAT AC

sessed by two pathologists, in order to confirm tumorigenicity and to determine the proportion of tumor cells in each section. For immunostaining, tissue sections were subjected to antigen retrieval with sodium citrate (pH 6.0), followed by staining with antibodies against IL-12 p35 (ab131039, Abcam, UK) and EBI3 (ab-118500, Abcam, UK) overnight at 4°C. After washing, the sections were stained with goat anti-rabbit IgG labeled with FITC (BS, Bioworld, China) and goat anti-rat IgG labeled with TRITC (BS, Bioworld, China), then assessed for p35 and EBI3 expression under a confocal laser scanning microscope (Nikon Japan).

Statistical analysis

All statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, USA) and GraphPad5 (GraphPad Software, USA). Data are presented as the mean ± standard deviation (SD). Clinical characteristics were compared by χ^2 test for categorical variables. For continuous variables, the Kolmogorov-Smirnov test was employed to examine whether the acquired data fitted a normal distribution curve. Comparisons between groups using the Mann-Whitney test or Student's t-test were performed and ANOVA was performed to evaluate the difference among groups more than three. Two-sided *p* values were calculated; the differences were considered statistically significant at $P < 0.05$.

Results

Basic clinicopathological characteristics of patients

A total of 64 pathologically confirmed, newly diagnosed ccRCC patients and 53 age- and sex-matched healthy controls participated in the study. The clinical characteristics of the patients and healthy volunteers are summarized in **Table 1**.

IL-35 plasma levels and mRNA levels in ccRCC patients and controls

As shown in **Figure 1**, plasma IL-35 levels were significantly higher in ccRCC patients than in healthy controls (106.39 ± 57.53 pg/mL vs. 65.18 ± 18.23 pg/mL, $P < 0.0001$). We analyzed the mRNA levels of EBI3 and p35 (two subunits of IL-35) in blood samples by real-time PCR. Consistent with the ELISA results, the mRNA levels of EBI3 and p35 were significantly higher in ccRCC patients than in healthy controls. Furthermore, the mRNA levels of both subunits of IL-35 were higher in cancer tissue than in tissue adjacent to cancer and in the normal tissue samples.

IL-35 is highly expressed in ccRCC tissues

IL-35 expression was detected in ccRCC tissues by immunostaining of EBI3 and p35 (**Figure 2**). The sections were stained with FITC-conjugated anti-p35 and TRITC-conjugated anti-EBI3 antibodies, then sections were assessed under a confocal microscope with 200× amplification. Co-localization of EBI3 and p35

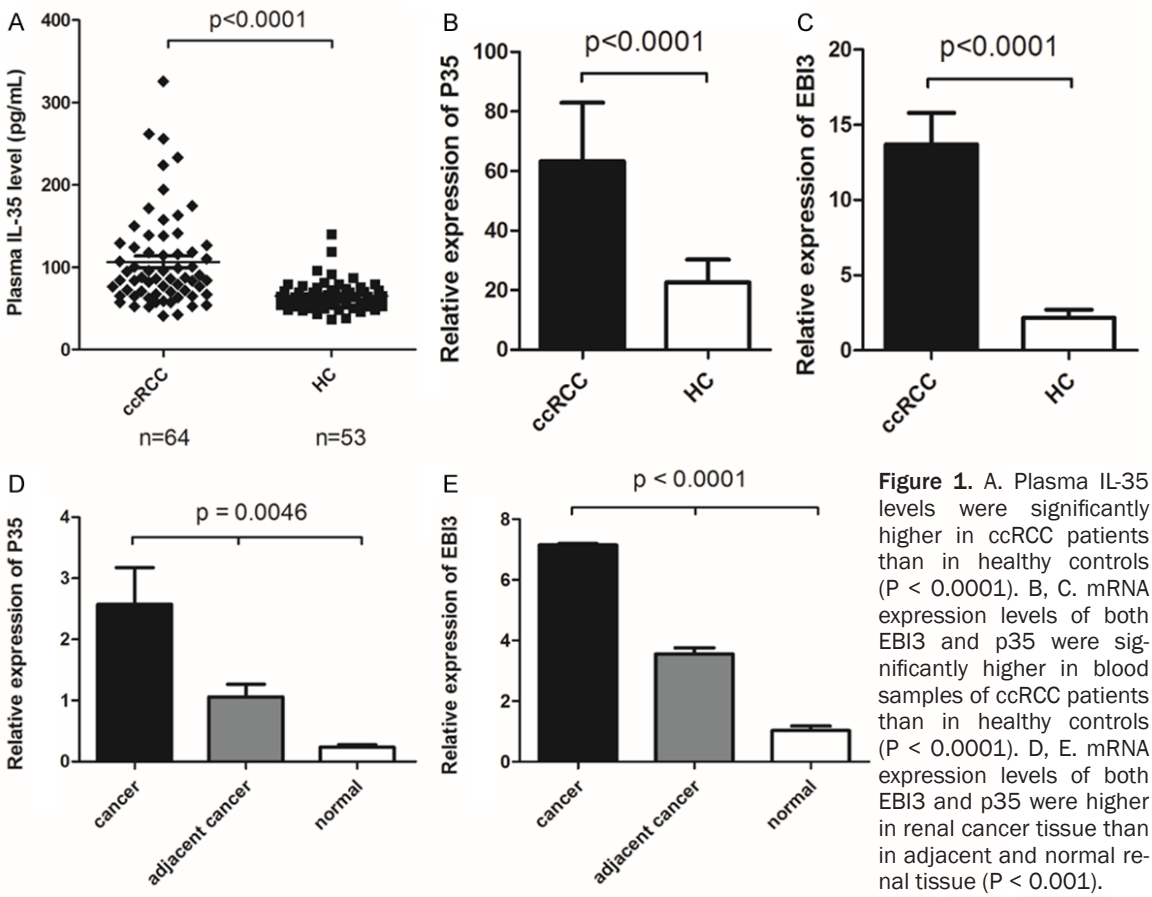

Figure 1. A. Plasma IL-35 levels were significantly higher in ccRCC patients than in healthy controls ($P < 0.0001$). B, C. mRNA expression levels of both EBI3 and p35 were significantly higher in blood samples of ccRCC patients than in healthy controls ($P < 0.0001$). D, E. mRNA expression levels of both EBI3 and p35 were higher in renal cancer tissue than in adjacent and normal renal tissue ($P < 0.001$).

immunofluorescence was observed in all ccRCC sections, but normal tissue exhibited less co-localization, indicating that expression of IL-35 is higher in ccRCC tissue.

Relationship between plasma IL-35 levels and clinical characteristics

Plasma IL-35 expression levels were associated with lymph node metastasis (**Table 3** and **Figure 3**). Patients with lymph node metastasis had higher IL-35 expression levels than patients without lymph node metastasis (178.5 ± 74.84 pg/mL vs. 89.9 ± 30.80 pg/mL, $P < 0.0001$). Furthermore, IL-35 expression positively correlated with tumor stage (I-IV, $P < 0.0001$). These results suggest that IL-35 plasma levels correlate with disease progression in ccRCC patients.

Receiver operating characteristic (ROC) curves for the prediction of ccRCC

The area under the receiver operating characteristic (ROC) curve was 0.78 (**Figure 4**). At the best cutoff value of 80.13 pg/mL, the diagnostic sensitivity, specificity and accuracy of plasma IL-35 in ccRCC was 60.9%, 90.6% and 72.6%, respectively. These findings suggest the possibility of using IL-35 as a tumor marker in the diagnosis of ccRCC.

Discussion

IL-35 is a member of the IL-12 family and is a suppressive cytokine consisting of an α-chain p35 and a β-chain EBI3 [8]. These subunits are also components of other cytokines - p35 binds with p40 to form IL-12, whereas EBI3 associates with p28 to form IL-27. IL-35 is an important anti-inflammatory cytokine and plays a substantial role in the regulation of allograft rejection and autoimmune disease [17, 18]. More recently, IL-35 has been identified as playing a significant role in the pathogenesis of tumor development. In particular it has been associated with immunosuppression and a poor prognosis, although, the exact role of IL-35 in tumorigenesis, particularly in ccRCC progression and prognosis, has not been fully elucidated.

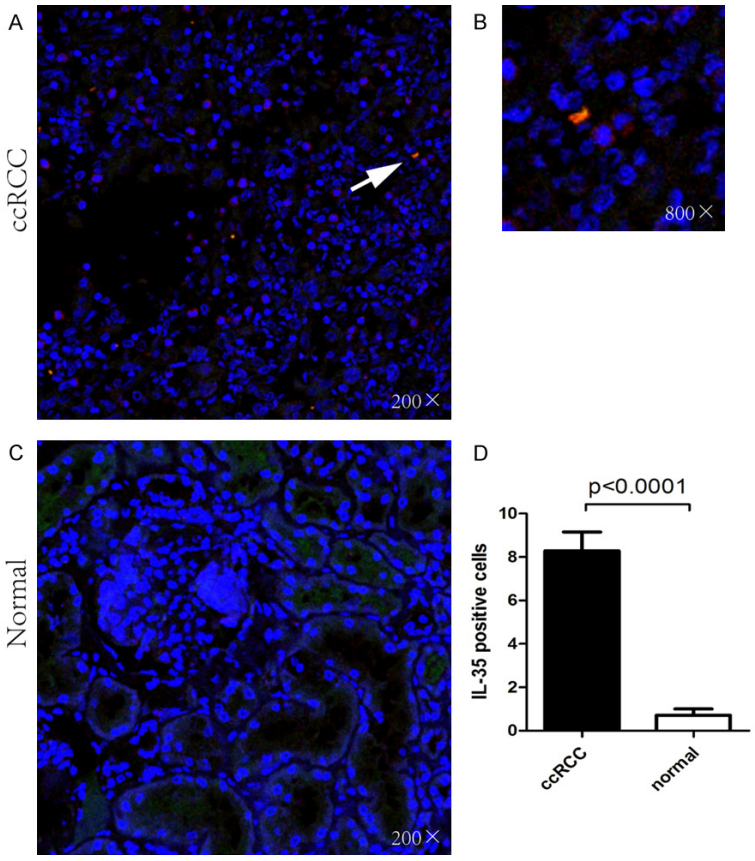

Figure 2. Detection of IL-35 expression in ccRCC tissue by immunostaining of EBI3 and p35. Co-localization of the immunofluorescence of EBI3 and p35 was observed in all ccRCC sections, but normal tissue exhibited less co-localization, suggesting that expression of IL-35 is higher in ccRCC tissue than in normal tissue.

Table 3. Association of plasma IL-35 levels with clinical factors in patients with clear cell renal cell carcinoma

Factors	N (%)	IL-35 (pg/mL)	*p*-value
Age (years)			
< 60	31 (48.4)	105.3 ± 48.60	0.589
≥ 60	33 (51.6)	113.1 ± 64.52	
Sex			
Male	40 (62.5)	111.2 ± 59.44	0.747
Female	24 (37.5)	106.4 ± 53.94	
TNM stage			
I	38 (59.3)	82.6 ± 30.28	< 0.0001
II	12 (18.8)	113.2 ± 19.12	
III-IV	14 (21.9)	178.5 ± 74.84	
Lymph node metastases			
Yes	14 (21.8)	178.5 ± 74.84	< 0.0001
No	50 (78.1)	89.9 ± 30.80	

In this study, we investigated plasma IL-35 levels in ccRCC patients by ELISA and determined that levels were significantly higher in ccRCC patients than in healthy volunteers, suggesting that IL-35 could be a predictor for ccRCC. Furthermore, we found that plasma IL-35 expression levels were positively associated with lymph node metastasis, and later tumor stage. These results are consistent with previous findings in other carcinomas. Circulating IL-35 was found to be significantly increased in pancreatic ductal adenocarcinoma patients, as well as non-small cell lung cancer and in this case also correlated with a poor prognosis [19, 20]. Incolorectal cancer tissue, high expression of IL-35 recruits Treg cells into the tumor microenvironment, promoting tumor growth [21].

Previous evidence suggested that IL-35 is secreted from Tregs and is required for their function both *in vitro* and *in vivo*. In vivo, IL-35 is secreted by Tregs and drives the conversion of naïve T cells to regulatory T cells (iTr35 cells) [11]. More recently, gene expression analysis revealed that IL-35 may have a broader tissue distribution beyond Tregs. EBI3 is expressed in Hodgkin lymphoma cells [22] and acute myeloid leukemia cells [23]. Immunohistochemical analysis further revealed that EBI3 and p35 are highly expressed in tumor tissues from lung cancer, colon cancer, esophageal carcinoma, hepatocellular carcinoma and cervical carcinoma [15]. We observed that IL-35 is highly expressed in ccRCC tissue. Thus, IL-35 not only mediates the biological function of Treg cells but also contributes substantially to the tumor environment and has been specifically shown to limit tumor immunity [24]. However, contradictory results have also been reported. Therefore, further studies of the immunological mechanisms of IL-35 in tumors are needed.

In this study, plasma IL-35 levels correlated with tumor stage, suggesting this could be a prognostic factor for poor outcome in ccRCC patients. Although higher levels of IL-35 in the

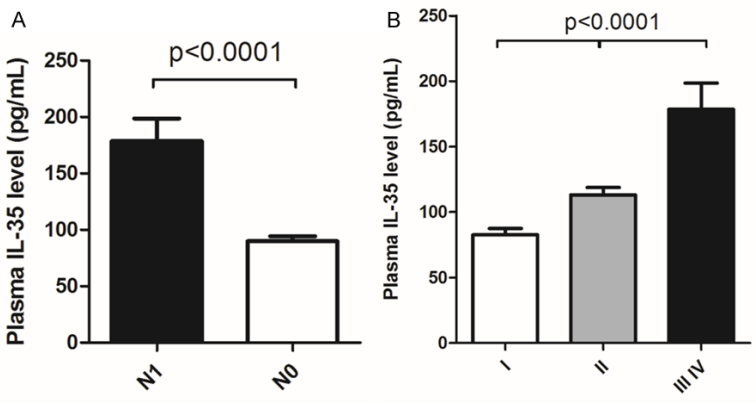

Figure 3. A. Plasma IL-35 levels were significantly increased in patients with positive lymph node metastasis (n = 14) compared to patients with negative lymph node metastasis (n = 50) (P < 0.0001). B. Plasma levels of IL-35 in patients of all stages. The difference between tumor stage was significant (P < 0.0001).

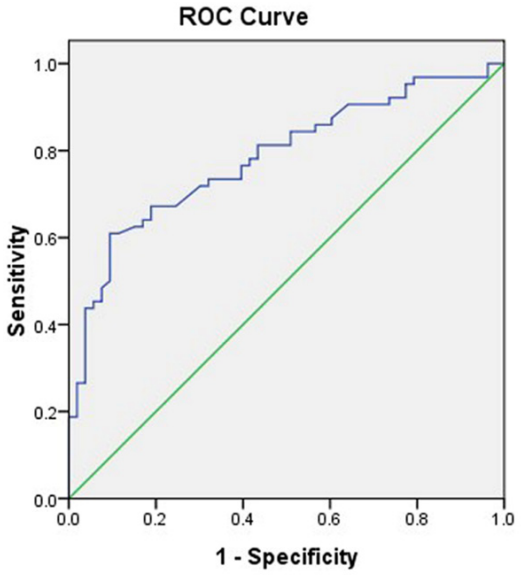

Figure 4. ROC curve analysis using IL-35 to discriminate ccRCC patients from healthy volunteers.

tumor environment are likely to play a more significant role in ccRCC progression and metastasis, in clinical practice, obtaining tumor tissue requires invasive techniques and may not always be feasible. Therefore, monitoring plasma IL-35 levels could allow the prediction of disease progression and the identification of risk factors in patients prior to the determination of TNM stage.

In summary, IL-35 levels are increased in ccRCC patients and tissues. Plasma IL-35 levels were positively associated with the clinical stage of ccRCC. Therefore, IL-35 could be a potential biomarker for prognosis in ccRCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Jianquan Hou, Department of Urology, The First Affiliated Hospital of Soochow University, 188 Shizi Rd, Suzhou 215006, People's Republic of China. E-mail: 15850115650@163.com

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