

## ORIGINAL ARTICLE

# Expression and significance of FOXP1, HIF-1a and VEGF in renal clear cell carcinoma

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## Summary

**Purpose:** To investigate the expressions of FOXP1, hypoxia inducible factor (HIF)-1a and vascular endothelial growth factor (VEGF) in renal cell carcinoma of the clear type (CCRCC) and their relationship with the patient clinicopathological features.

**Methods:** The expressions of forkhead box-P1 (FOXP1), HIF-1a and VEGF in 55 cases of CCRCC tissues were determined using immunohistochemistry. Then, their correlations with clinical stage, histological grade and lymph node metastasis were analyzed using chi-square test.

**Results:** Thirty-seven of the 55 cases (67.3%) of CCRCC expressed FOXP1 with an abnormal expression rate of 38.2% (21/55), in which there were 10 cases with positive FOXP1 both in the nucleus and the cytoplasm and 11 cases

with positive FOXP1 in cell membrane. The abnormal expression rate of FOXP1 in high grade CCRCC (G3/G4) was significantly higher than that in low grade CCRCC (G1/G2,  $p < 0.05$ ). FOXP1 expression was significantly correlated with the expression of HIF1 and VEGF ( $r = 0.54$ ,  $p < 0.01$  and  $r = 0.37$ ,  $p < 0.05$ , respectively), but was not obviously correlated with clinical stage, lymph node metastasis and 5-year overall patient survival ( $p > 0.05$ ).

**Conclusion:** Abnormal expression of FOXP1 and its deficiency are common events in CCRCC. Abnormal expression of FOXP1 may create progression of tumor from low grade to high grade by regulating the HIF-1-VEGF pathway.

**Key words:** clear cell renal cell carcinoma, forkhead transcription factor-1, hypoxia-inducible factor-1, vascular endothelial growth factor

## Introduction

Renal cell carcinoma (RCC) is the most common malignant kidney tumor in adults, and about 1/3 of the patients have metastasis on first presentation. Although surgical resection can cure 60-70% of localized RCC, there are still about 50% of patients with postoperative recurrence [1,2]. On histology, RCC is classified as clear cell, papillary, chromophobe, collecting duct and other unclassified types, among which CCRCC is the most common. Cytogenetic analysis shows that there are different gene expressions in different subtypes of RCC, even in the same types of RCC. For example, the VHL gene at 3p25-p26 is not only expressed

in sporadic CCRCC (the rate of loss-of-heterozygosity/LOH is up to 80-90%), but also in chromophobe RCC or other types of RCC [3,4]. The mutation diversity of the putative RCC gene may determine the diversity of its biological behavior. Therefore, understanding the genetic variation of RCC and improving the prognostic evaluation level have great significance for the treatment of this disease.

LOH of chromosome 3 (3pLOH) is the most common chromosomal variation for RCC. It was found that in this region there are many tumor suppressor genes, such as VHL (3p25), DUTTI/ROBO1 (3p12-13), FHIT (3p14.1) and FOXP1 (3p14.1). VHL variation is a characteristic genetic

change of CCRCC, and the function of most genes depends on the inactivation of this gene. Therefore, VHL gene is considered as the housekeeping gene. It can degrade HIF and regulate VEGF via its protein product (pVHL), playing a role of tumor suppressor [3,4]. FOXP1 gene at 3p14.1 is a newly discovered tumor suppressor gene. It is widely expressed in human tonsils, lymph nodes, gastrointestinal tract, brain and kidney and other organs and tissues, regulating the cell proliferation and differentiation, tumor formation, immune response and signal transduction [5]. At present, the biological role of FOXP1 in tumors is still being investigated. It is reported that its overexpression in the nucleus of diffuse large B-cell lymphoma indicates poor prognosis [6], and its high expression in the nucleus of MALT-type lymphoma indicates malignant transformation [7]. In breast cancer, the nuclear expression of FOXP1 is closely related with the estrogen receptor, and indicates longer tumor-free survival of patients [8]. The various biological functions of FOXP1 protein mean that there are different regulation mechanisms in different tumors.

Banham et al. [5] reported that each subtype of RCC can express FOXP1, and the nuclear expression is common in hereditary RCC with VHL gene mutation. This suggests that FOXP1 may be involved in the genesis of RCC and has potential impact on tumor progression, and may be related to VHL mutation. The expression of FOXP1 in both prostate cancer and breast cancer are negatively correlated with HIF [8,9], suggesting that FOXP1 may be involved in the PI3K/AKT/HIF pathway and exert its antitumor function. Whether FOXP1 plays a role in RCC via HIF-VEGF pathway has not been reported so far.

The purpose of this study was to investigate the correlation of FOXP1 expression with clinicopathological features including histological type, tumor grade and size, lymph node metastasis and histology, as well as with VEGF and HIF, and discuss the possible mechanism of its effect on the prognosis, to provide more information for gene therapy of RCC.

## Methods

### General data

Fifty-five cases of CCRCC definitely diagnosed in this hospital from 2005 to 2006, including 35 males and 20 females, aged from 13 to 88 years (median 58), were enrolled in this study which was conducted in accordance with the declaration of Helsinki and after

approval from the Ethics Committee of Wenzhou Medical University. Written informed consent was obtained from all participants.

All patients were followed-up by telephone from 25 to 84 months postoperatively. Another 48 cases of fresh CCRCC tissues and their corresponding adjacent tissues were collected from 2009 to 2011 to detect the mRNA expression of FOXP1.

### Histology

All specimens were fixed in 4% neutral formalin and embedded in paraffin blocks. Then, tissues were cut into 4 $\mu$ m-thickness sections and stained with hematoxylin and eosin. Then the slides were observed under light microscope by an experienced pathologist. Tumors were graded according to the Fuhrman histological grading method [10].

### Immunohistochemistry

Immunohistochemistry was performed to determine the expression of FOXP1, HIF-1a and VEGF in the CCRCC tissues using the Envision method [5,10]. The primary monoclonal antibodies for FOXP1 (JC12, a gift from Banham AH, the University of Oxford, diluted in 1:80), HIF-1a and VEGF (both diluted in 1:50, Beijing ZSGB Bio-tech, Beijing, China) were used according to the manufacturer's instruction. Tonsil tissue with reactive hyperplasia was used as a positive control for FOXP1 and PBS served as negative control.

### Evaluation of immunohistochemical staining

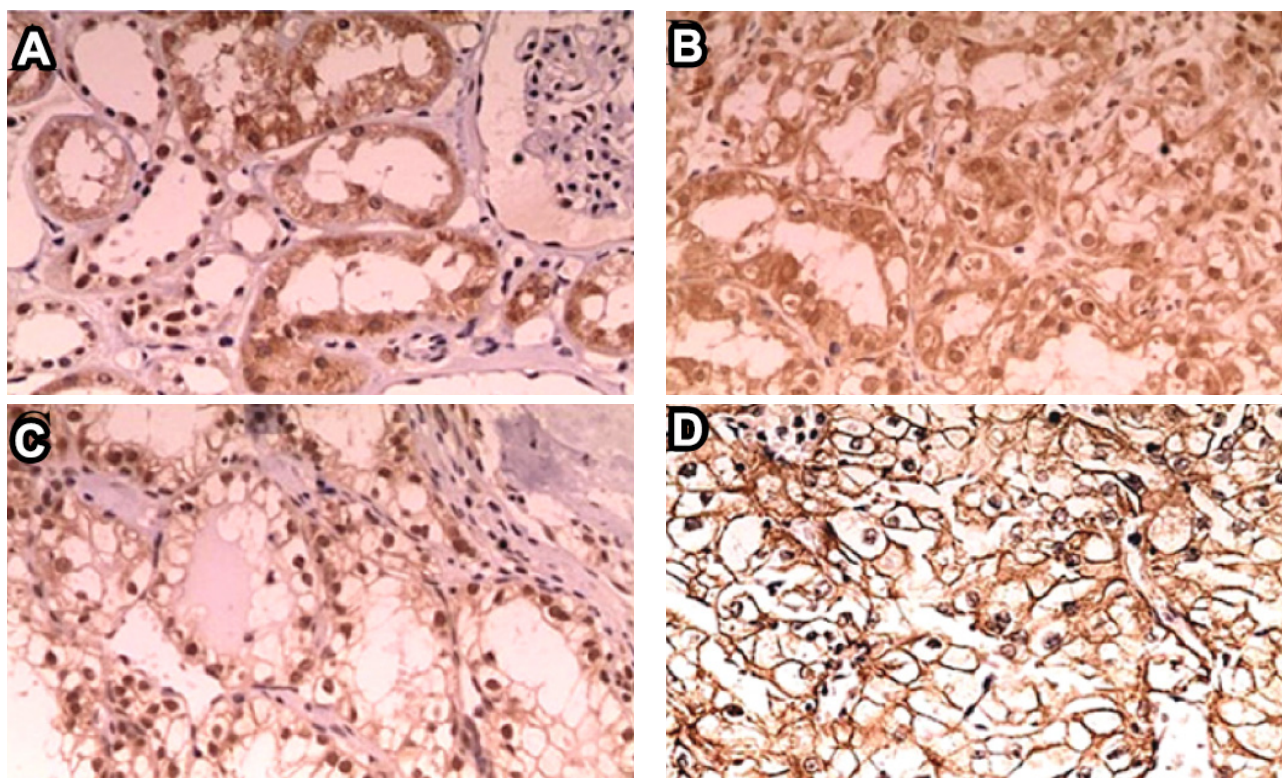
Punctate brown cellular granules observed in the cytoplasm, nucleus or cell membrane could be defined as positive for FOXP1, HIF-1a and VEGF. The number of positive cells in the strongest expression regions of each case was calculated (1-10%: 0, 11-30%: 1, 31-70%: 2, >70%: 3) [11,12]. FOXP1 expressed in both the cytoplasm and nucleus or in cell membrane was defined as abnormal expression [11].

### RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA in the fresh CCRCC tissues was extracted using Trizol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction and was then determined using a spectrophotometer (NanoDrop 1000, USA). Two  $\mu$ g of the total RNA was used as template to synthesize cDNA using the Quantscript RT kit (TaKaRa, Shiga, Japan) following the manufacturer's instruction. qPCR was performed using SYBR Premix Ex Taq polymerase (TaKaRa). Specific primers for FOXP1 and GAPDH genes (Table 1) were designed using the Primer Express 5.0 software and synthesized by Shanghai GeneCore Bio Technologies Co., Ltd. Fluorescence signals were measured after 40 PCR cycles. All experiments were performed in triplicate and normalized to GAPDH. The amplification efficiency of GAPDH

**Table 1.** Gene-specific primer pairs for *FOXP1* and *GAPDH*

Gene	Forward	Reverse	Product size (bp)
OXP1 (Gen Bank AF146696.1)	5'- TGGAAGAATGCAGTGCCTCA-3'	5'- GAAGGGTTACCACTGATCTTTTGT -3'	137
GAPDH (Gen Bank NM_002046.3)	5'- GTCAACGGATTGGTCTGATTG -3'	5'-CTGGAAGATGGTGATGGGATT-3'	213



**Figure 1.** FOXP1 expression in normal kidney tissues and CCRCC tissues (200×). **A:** FOXP1-positive in normal kidney tissue; **B-D:** FOXP1-positive in CCRCC tissues (**B:** FOXP1 expressed in the nucleus, cytoplasm and membrane simultaneously; **C:** simultaneous expression of FOXP1 in the nucleus and membrane; and **D:** simultaneous expression of FOXP1 in the membrane and cytoplasm).

was close to 100%.

#### Statistics

All statistical calculations were carried out using SPSS v12.0 statistical software (SPSS Inc., Chicago, ILL, USA). Correlation comparison between the expression of FOXP-1, pVHL, HIF-1a and VEGF and the clinicopathological parameters was performed using chi-square test. Pairwise correlation was carried out using Spearman's rank correlation analysis. The mRNA expression of FOXP1 was analyzed using ANOVA. For all tests, two-sided  $p < 0.05$  was considered statistically significant.

## Results

#### General data

The clinical manifestations of the patients in-

cluded anorexia and weight loss (N=2), painless hematuria (N=20), low back pain or discomfort (N=3) and the remaining were found on physical examination. There were 32 cases with left and 23 cases with right RCC. The tumor size ranged from a diameter of 1.5cm to 14×12×9 cm. All the tissues included 32 cases of pT1, 12 cases of pT2 and 11 cases of pT3/pT4 (4 cases of pT3 and 7 cases of pT4). As graded pathologically, the tissues included 18 cases of G1, 25 cases of G2 and 12 cases of G3/G4 (9 cases of G3 and 3 cases of G4). Ten cases were found with lymph node metastasis and underwent radical nephrectomy.

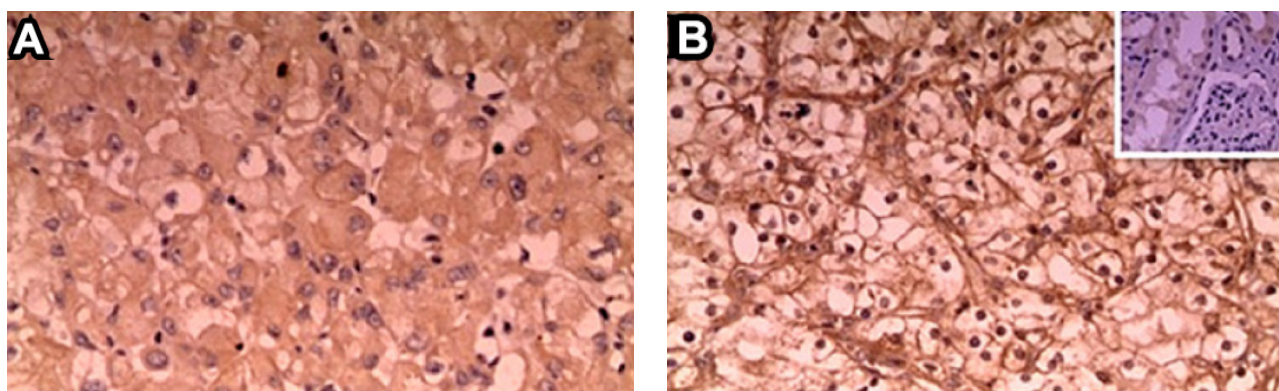
#### Follow-up

Fifty-two patients had follow-up data and 3 were lost to follow-up. Twelve patients died in 5 years and 40 were still alive until the end of fol-

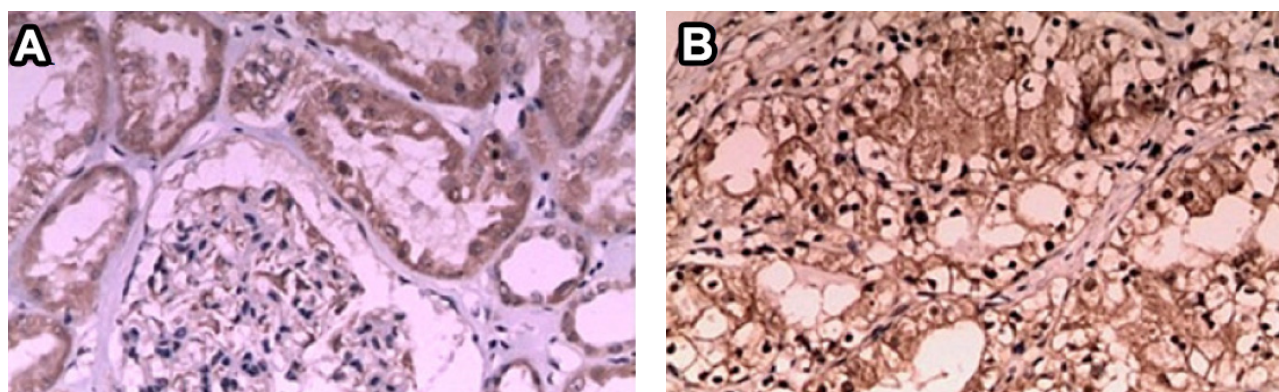
**Table 2.** Correlation between the clinicopathological features and the expression of FOXP1, HIF-1a and VEGF in CCRCC (N=55)

Clinicopathological features	FOXP1 expression			A*	HIF-1a expression			VEGF expression		
	0	1	2 and 3		0	1	2 and 3	0	1	3
T stage										
pT1 (N=32)	12	5	15	13	20	4	8	21	5	6
pT2 (N=12)	3	4	5	4	3	4	5	5	4	3
pT3/pT4 (N=11)	3	3	5	4	1	3	7 <sup>▲</sup>	2	3	6 <sup>▲</sup>
Fuhrman grade										
G1/G2 (N=43)	15	12	16	13	21	10	12	25	8	10
G3/G4 (N=12)	3	0	9 <sup>▲</sup>	8	2	1	9 <sup>▲</sup>	3	4	5 <sup>▲</sup>
Lymph node metastasis										
Yes (N=10)	2	1	7	5	1	4	5	2	2	6
No (N=45)	16	11	18	16	22	7	6 <sup>▲</sup>	26	10	9 <sup>▲</sup>
Survival (5 years)										
Dead (N=12)	2	5	5	7	2	4	6	2	3	7
Alive (N=40)	16	7	17	14	20	8	12 <sup>▲</sup>	23	8	9 <sup>▲</sup>

▲ p<0.05 vs the other group. A\*: abnormal expression



**Figure 2.** HIF-1a expression in renal tissues (200×). The upper right inlet shows HIF-1a expression in normal renal tissue. **A:** Cytoplasmic expression of HIF-1a in CCRCC tissues; **B:** Simultaneous expression of HIF-1a in cytoplasm and membrane.



**Figure 3.** VEGF expression in renal tissues (200×). **A:** VEGF expression in normal renal tissue; **B:** VEGF expression in CCRCC tissue.

low-up.

Expression of FOXP1

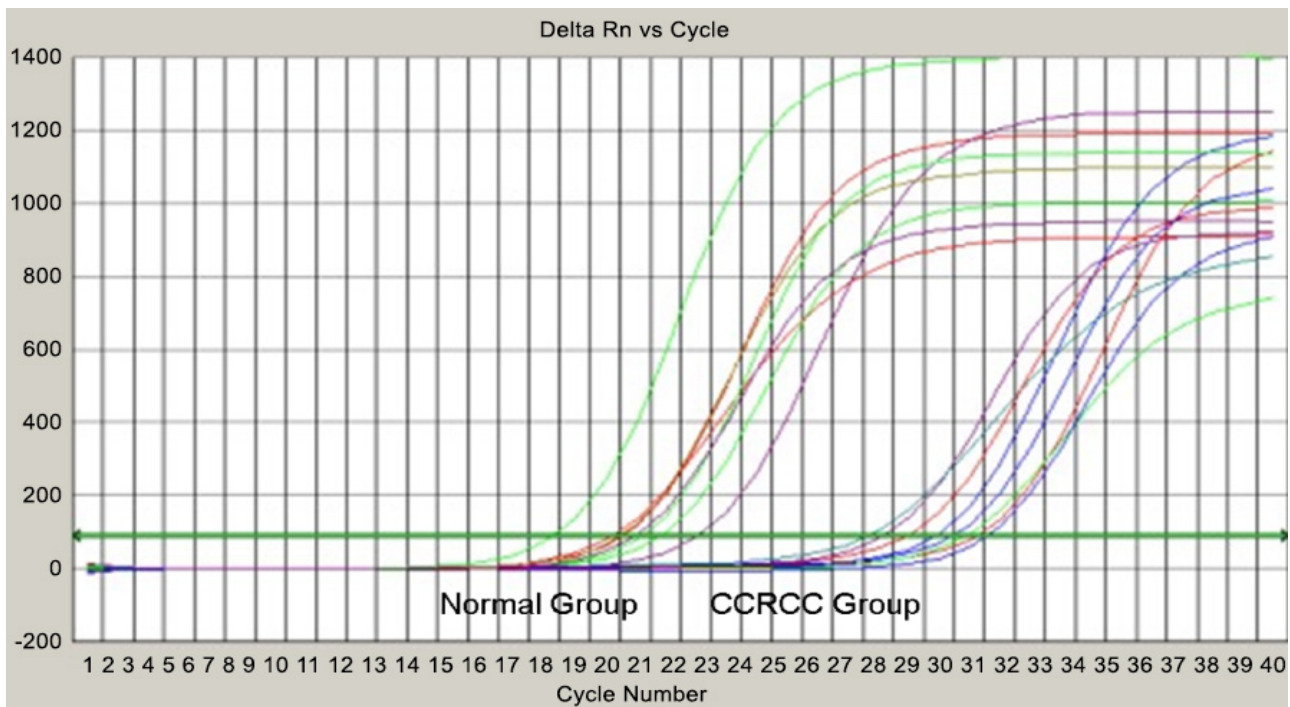
In the normal renal tissues, FOXP1 was expressed in the cytoplasm of the proximal tubules and the nucleus of the distal convoluted tubule

and collecting duct. In the glomerular and thin sections of the loop, FOXP1 was negative or weakly expressed. In the 55 cases of CCRCC, 18 (32.7%) cases were FOXP1-negative, 12 (21.8%) cases were weakly positive, 5 (16.4%) cases were moderately positive and 20 (36.4%) cases were strongly positive. The positive rate of FOXP1 expression was not correlated with the clinical stage, histological grade, lymph node metastasis and 5-year overall survival of patients with CCRCC. Twenty-one (38.2%) cases of CCRCC tissues expressed FOXP1 abnormally, including 10 cases in both nucleus and cytoplasm and 11 cases in the cell membrane (Figure 1). In the 12 cases of G3/G4 CCRCC, there were 8 (66.7%) cases with abnormal expression of FOXP1, while in the 43 cases of CCRCC with G1/G2, there were only 13 (30.23%) cases with abnormal expression of FOXP1, showing significant difference between the two groups ( $\chi^2=5.28$ ,  $p=0.02$ ). The 5-year mortality rate of patients with abnormal expression of FOXP1 was 33.3% (7/21), show-

ing no significant difference from that of the patients with normal expression of FOXP1 (16.13%, 5/31,  $\chi^2=2.64$ ,  $p=0.1$ ). The abnormal expression of FOXP1 was not correlated with the clinical stage, lymph node metastasis and 5-year overall survival rate (Table 2).

#### HIF-1a expression

HIF-1a was weakly expressed in the cytoplasm of normal renal tubular cells, but not in the glomeruli. In the CCRCC tissues, HIF-1a was mostly expressed in the cytoplasm, but to a little degree in both cytoplasm and nucleus or cell membrane (Figure 2). The positive rate of HIF-1a in CCRCC tissues with pT3/pT4 was significantly higher than that in CCRCC tissues with pT1/pT2 ( $\chi^2=6.67$ ,  $p=0.01$ ). It was also significantly higher in the G3/G4 group than in the G1/G2 group ( $\chi^2=3.99$ ,  $p=0.046$ ). In cases with positive HIF-1a, the lymph node metastasis rate and the 5-year mortality rate increased signif-



**Figure 4.** The mRNA expression of FOXP-1 in CCRCC tissues was significantly lower than that in the normal renal tissues.

**Table 3.** Renal cell carcinoma FOXP1 mRNA expression

Groups	Mean Ct of FOXP1	Mean Ct of GAPDH	$\Delta Ct$ FOXP1-GAPDH	$\Delta \Delta Ct$ $\Delta Ct - \Delta Ct_{nor}$	Relative expression of FOXP1
Normal	25.02 $\pm$ 3.67	18.87 $\pm$ 2.74	6.15 $\pm$ 2.39	0	1
CCRCC	26.23 $\pm$ 3.05	17.60 $\pm$ 2.50	8.63 $\pm$ 2.98*	2.48	0.18

\* $p < 0.01$  vs normal group

Ct: circulation time,  $\Delta Ct$ : the difference between the circulation times of two genes (e.g. the difference in the Table was the difference between the circulation times of FOXP1 and internal reference GAPDH),  $\Delta \Delta Ct$ : referred to the difference between the differences of circulation times of FOXP1 and internal reference GAPDH in the experimental and the control group respectively, nor: normal

icantly ( $\chi^2=5.09$ ,  $p=0.024$  and  $\chi^2=7.47$ ,  $p=0.006$ , respectively) (Table 2).

#### *VEGF expression*

Normally, VEGF was moderately expressed in the cytoplasm of renal tubular and glomerular cells. In contrast, in CCRCC VEGF was expressed not only in the cytoplasm, but in both cytoplasm and nucleus or cytoplasm and cell membrane sometimes (Figure 3). The expression intensity of VEGF was closely correlated with the clinical stage, histological grade, lymph node metastasis and 5-year overall survival rate ( $\chi^2=5.89$ ,  $p=0.015$ ;  $\chi^2=4.12$ ,  $p=0.042$ ;  $\chi^2=4.67$ ,  $p=0.031$ ; and  $\chi^2=6.17$ ,  $p=0.013$ , respectively) (Table 2).

#### *Relationship between FOXP1 and HIF-1a and VEGF*

The expression of FOXP1 was positively correlated with the expressions of HIF-1a and VEGF ( $r=0.54$ ,  $p<0.01$  and  $r=0.37$ ,  $p<0.05$ , respectively). The expression of HIF-1a was also positively correlated with VEGF expression ( $r=0.47$ ,  $p<0.01$ ). The positive rate of HIF-1a of the group with abnormal expression of FOXP1 was 90.5% (19/21), significantly higher than that of the group with non-abnormal expression of FOXP1 (58.8%; 20/34,  $\chi^2=6.31$ ,  $p=0.012$ ).

#### *Expression of FOXP1 mRNA*

The mRNA expression of FOXP1 in the CCRCC group was significantly lower than that in the normal renal tissues ( $p=0.00$ ) (Figure 4, Table 3).

## **Discussion**

The results of this study showed that the positive expression rate of FOXP1 in high grade (G3/G4) CCRCC was significantly lower compared to low grade (G1/G2) CCRCC, and the expression level of FOXP1 mRNA in cancer tissue was significantly lower than in normal renal tissue. The abnormal expression rate of FOXP1 in G3/G4 CCRCC was significantly higher than in G1/G2 CCRCC. The abnormal expression of FOXP1 did not show any relation with the clinical stage, lymph node metastasis or 5-year overall survival. In CCRCC, the expression intensity of HIF-1a and VEGF showed obvious correlation with pathological grade, clinical stage, lymph node metastasis and 5-year overall survival rate. Positive correlations among FOXP1, HIF-1a and VEGF, and the positive expression rate of HIF-1a in patients with abnormal FOXP1 expression were significantly higher

than those in patients with normal FOXP1 expression.

In normal tissues, FOXP1 is mainly expressed in the nucleus or cytoplasm, while in tumor cells there is abnormal FOXP1 expression, in cell membrane, or karyolemma [5,7-9]. The results of this study showed that the positive expression rate of FOXP1 protein in CCRCC was 67.3%, and the abnormal expression rate and loss of expression rate were 38.2% and 32.7%, respectively. Toma et al. [12] have analyzed the single nucleotide polymorphism (SNP) in 22 cases of CCRCC and found that the loss of expression rate of FOXP1 gene in 3pLOH 3 was 85%, and the abnormal expression rate of FOXP1 protein was 90%. Loss of FOXP1 expression is common, but it is not correlated with clinical stage, pathological grade or other pathologic parameters. The reported experimental results in this study indicate that the abnormal expression and loss of FOXP1 expression are common events in CCRCC. The abnormal expression rate of FOXP1 in this study was slightly lower than in the reported results, but the loss of expression rate contrasted them. This may be related to the different experimental methods used. The results of this study also showed that, with the increase of pathological grade, the abnormal expression rate of FOXP1 also increased. This indicates that the abnormal expression of FOXP1 is related with the grade of malignancy, and may be involved in the developmental process of the tumor. It has no association with clinical stage, lymph node metastasis or 5-year overall survival rate. This may be related with stage I and II in most cases. Therefore, long-term follow-up is also required, and the significance of FOXP1 in CCRCC needs to be further studied.

Banham et al. [5,9] found that FOXP1 gene is expressed in a variety of normal human and tumor tissues in varying degrees, and its mRNA and protein expression are significantly lower in colon cancer and many other tumor tissues than in their corresponding normal tissues. Therefore, FOXP1 is supposed to be a tumor suppressor gene. In this study, the mRNA expression of FOXP1 in CCRCC tissue was significantly decreased as compared with that in normal kidney tissues, suggesting the reduced expression of FOXP1 mRNA may be associated with the occurrence of CCRCC, which is consistent with the Banham et al. research results. Both the immunohistochemistry result and mRNA determination support that FOXP1 acts as a tumor suppressor gene to inhibit the occurrence of tumors.

HIF-1 $\alpha$  is affected by the oxygen concentration, VHL gene mutation status and activated state of related molecular pathways [3,11,13]. The  $\alpha$  chain of pVHL combines with ElonginB to form the ubiquitin ligase complex, and the  $\beta$  chain binds the HIF-1 $\alpha$  and degrades it with normal oxygen concentration. Hypoxia and VHL inactivation or mutation can lead to reduction of HIF degradation, stability increase and concentration of HIF increase in cells, combining the HIF sites in downstream target genes including VEGF, PDGF and CXCR. It functions as transcription factor, forming the transcription initiation complex, thus initiating the transcription of the target gene VEGF and increasing the corresponding protein. VEGF can induce neovascularization, promote proliferation of tumor cells and contribute to tumor progression [3,4,13]. The results of this study showed the HIF-1 $\alpha$  expression from low grade to high grade CCRCC significantly increased. The expression of HIF-1 $\alpha$  in advanced-stage tumors and cases with lymph node metastasis was significantly higher compared with low stage tumors and cases without lymph node metastasis. The expression rate of VEGF increased with the increase of tumor stage and grade, and was related to lymph node metastasis and mortality. The results also showed that HIF-1 $\alpha$  is positively related to VEGF, which further confirms the function of HIF-1 $\alpha$  in regulating the downstream target gene VEGF.

There are inconsistent reports about the effect of HIF-1 $\alpha$  on the prognosis of CCRCC. Lidgren et al. [11] used Western blot assay to detect 66 cases of CCRCC and found that the expression of HIF-1 $\alpha$  in tumor was significantly higher than in normal kidney tissue. Although HIF-1 $\alpha$  expression is not correlated with tumor size, pathological grade or stage or venous invasion, it is an independent prognostic factor concerning 5-year overall survival. CCRCC with high expression of HIF-1 $\alpha$  can bear a better prognosis. However, the immunohistochemistry results showed that survival in patients with high HIF-1 $\alpha$  expression was not significantly different from that of patients with low HIF-1 $\alpha$  expression.

Most reports show that the high expression of HIF-1 $\alpha$  mainly occurs in high grade and high stage CCRCC, with poor prognosis and short survival time [14]. It is believed that these different results are due to the different experimental methods. The location of HIF-1 $\alpha$  in tumor cell (cytoplasm or nucleus) affects the exertion of its function. HIF-1 $\alpha$  is a nuclear transcription factor, and it is activated in the nucleus. Its high expres-

sion in the cytoplasm means that it has been transcribed into the cytoplasm, with relatively good prognosis [3,11,14]. In the present study immunohistochemistry was used, and the results showed that among 55 cases of CCRCC, there were 10 cases of nuclear expression and 21 cases of cytoplasmic expression. The nuclear expression was relatively higher in high grade CCRCC. Whether the HIF-1 $\alpha$  nuclear expression indicates poor prognosis still needs to be further explored.

In fact, it is proved that PI3K/AKT signaling can regulate the activity of HIF-1 $\alpha$ , thereby promoting tumor angiogenesis, erythropoiesis and glucose metabolism [15]. PI3K/AKT signal pathway negatively regulates the FOXO transcription factor subfamily. FOXO regulates the HIF-1 $\alpha$  protein level via the pVHL-independent protein degradation mechanism, and inhibits the response of VEGF to HIF-1 $\alpha$  [16]. The nuclear expression of FOXP1 protein (JC12) in endometrial carcinoma is decreased significantly with abnormal cytoplasmic expression. The cytoplasmic expression of FOXP1 is positively correlated with HIF-1 $\alpha$  expression, and is also associated with the muscle layer infiltration depth of endometrial carcinoma [17]. The FOXP1 protein sequence contains PI3K activation site. Combined with the results of this study, it is found that, FOXP1 has obvious correlation with HIF-1 $\alpha$  and VEGF. The positive expression rate of HIF-1 is significantly higher than that in patients with normal FOXP1 expression (59.8%). It is speculated that FOXP-1 may have an action mechanism similar with FOXO, negatively regulating the HIF-1 $\alpha$  activity. The abnormal expression of FOXP-1 causes loss of normal regulation function, leading to increased HIF-1 $\alpha$  protein content in tumor tissue, which up-regulates the expression of downstream target genes and promotes tumor angiogenesis, thus accelerating the proliferation, invasion and metastasis of tumor cells.

In conclusion, FOXP1 exists in normal kidney tissue as a tumor suppressor gene. The reduction of its mRNA expression may be closely related to the occurrence and development of CCRCC. Abnormal expression of FOXP1 protein is a common event in CCRCC, which may promote the process where tumor progresses from low grade to high grade by regulating the HIF-1-VEGF pathway. The combined detection of FOXP1, HIF-1 and VEGF can be used as an effective biological molecular indicator for evaluating the prognosis of patients with CCRCC.

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