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Original Article

Expression of FOXC2 in renal cell carcinoma and its relationship to clinical pathological features

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Abstract: Objective: This study aimed to investigate expression level of FOXC2 and its relationship to clinical pathological features of renal cell carcinoma (RCC). Methods: The expression levels of FOXC2 in RCC tissues and normal renal tissues (62 samples, respectively) were detected by immunohistochemistry and PCR Array. Statistics analyses were done with SPSS to compare the differences between RCC tissues and normal renal tissue, and to explore the relationship between the expression level of FOXC2 and the clinical pathological features of RCC. Results: Expression level of FOXC2 in RCC tissues was significantly higher than in normal renal tissues, and other related cancer genes also highly expressed in RCC tissues. FOXC2 expression was positively associated with clinical stage and pathological grade ($P < 0.05$), but not significantly related to the gender and age ($P > 0.05$). Conclusion: The expression of FOXC2 in renal cell carcinoma was significantly higher than that in normal renal tissues. It is suggested that FOXC2 might play a crucial role in the occurrence and development of RCC and could be an important prognostic indicator for clinical therapy.

Keywords: Renal cell carcinoma, FOXC2, immunohistochemisty, PCR array

Introduction

Renal cell carcinoma (RCC), a common malignant tumor of urinary system, originates from renal tubular epithelial cells and is so-called as "kidney cancer" [1]. RCC accounts for about 3% of adult malignancies, and the cancer mortality accounts for about 2% of cancer deaths [2]. In recent years, studies about RCC have made some achievements from clinical pathology to molecular mechanism. However, the morbidity and mortality of RCC have an increasing tendency nowadays. When diagnosed with RCC, most patients were at end-stages with local invasion and distant metastasis [3]. Radiotherapy and chemotherapy were not effective for end-stage RCC, which make early diagnosis and effective judgment important. Studies have shown that Forkhead Box C2 (FOXC2) expresses highly in tumor endothelial cells, and is strongly associated with formation, invasion and metastasis of tumors [4, 5]. In human esophageal squamous cell carcinoma, FOXC2 can be used as a prognostic indicator, which could indicate the malignant grades of the tumors and provide effective help for clinical treatments [6]. At the same time, in the basal-like subtype of invasive breast carcinoma, highly expressed FOXC2 promotes invasion and metastasis of the tumor, acting as an effective molecular marker of this cancer [7]. However, there were few researchers studying about FOXC2 in RCC. In this study, the expression levels of FOXC2 and other relevant genes were detected in both RCC tissues and normal renal tissues by immunohistochemistry and PCR. In order to provide a possible novel indicator for clinical treatment, the relationship between FOXC2 and the clinical pathological features was analyzed to explore the role of FOXC2 in formation and development of RCC.

Methods and materials

Samples

62 RCC samples were collected from patients with RCC after radical nephrectomy from January 2014 to December 2014 in Henan province people's hospital. There were 35 male patients and 27 female patients, with a mean age of 54 ± 7 years old. All cases were diag-

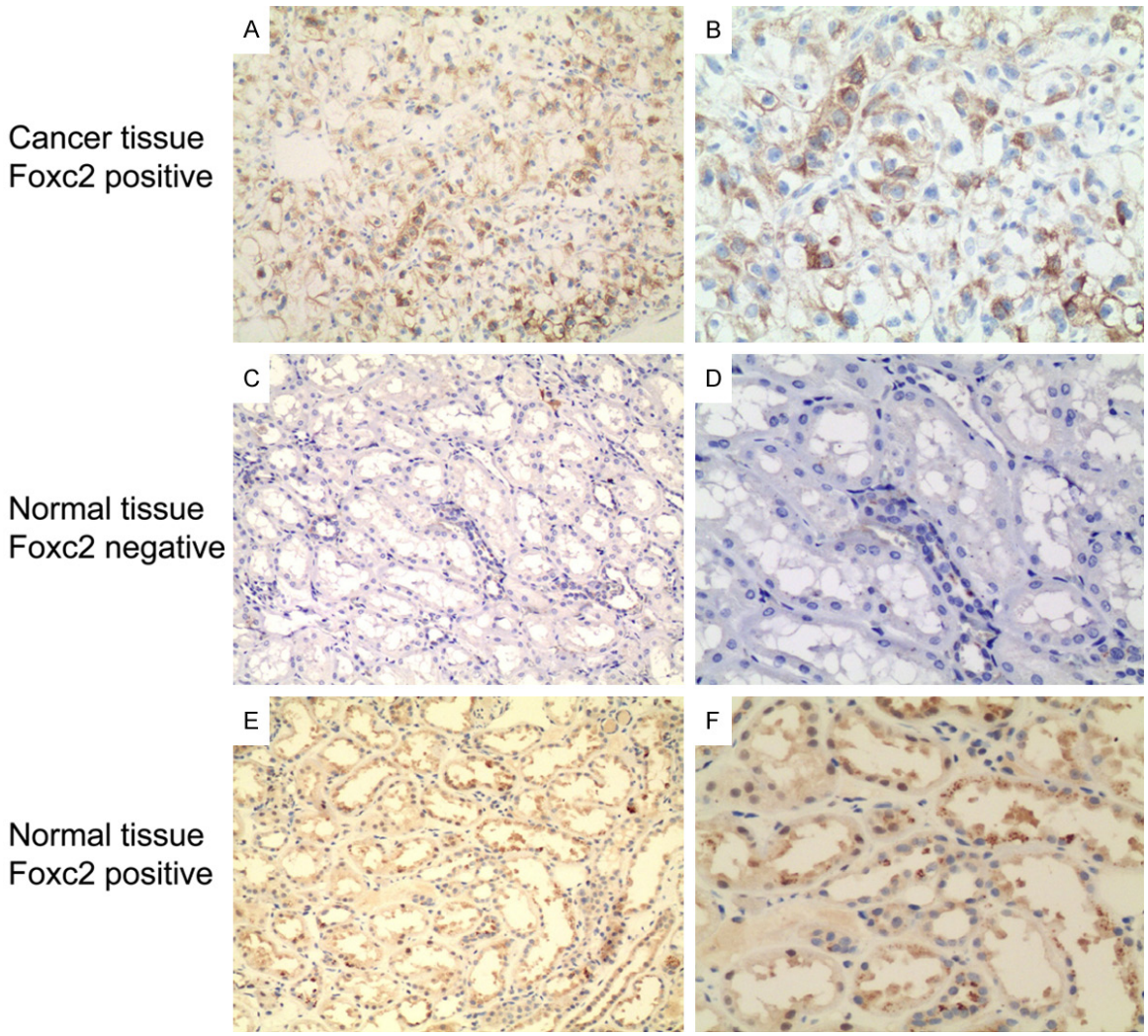

Figure 1. Immunohistochemical staining of FOXC2 expression in RCC and normal renal tissues. The top two panel is representative immunohistochemical images of RCC tissue (A, × 100 and B, × 200), demonstrating diffuse cytoplasmic staining for FOXC2. Negative (C, × 100 and D, × 200) or positive (E, × 100 and F, × 200) FOXC2 expression with cytoplasmic dot-like staining pattern in about 17.74% normal renal tissues.

nosed through pathology, and treated wiht surgery. Before surgery, no case got chemotherapy and radiotherapy. After surgery, all cases finished complete follow-up records. According to the standard of American Joint Committee on Cancer tumor-node-metastasis (AJCC-TNM, 2009), these cases were classified as follows: 10 cases in stage I, 13 cases in stage II, 23 cases in stage III and 16 cases in stage IV. According to the standard of Fuhrman grading system, these cases were classified as follows: 7 cases in high differentiation grade, 15 cases in mediate differentiation grade and 10 cases in low differentiation grade. All samples were fixed in 10% formaldehyde, embedded by paraffin, cut into sections and stained by immunohistochemistry. At the same time, some of the samples were put into tubes containing RNAlater, quickly frozen by liquid nitrogen, and kept at -80°C for the following extraction of RNA.

Agents

The antibodies used in this study were monoclonal antibodies against human FOXC2 (Abcam, USA). And the immunohistochemistry kit was also purchased from Abcam Company. Human Cancer Pathway Finder PCR Array was PAHS-033, purchased from SABiosciences Company.

Immunohistochemistry

Immunohistochemical kit was used to detect the expression of FOXC2 in both RCC tissues

Table 1. The relationship between FOXC2 expression and clinical pathological features of RCC

Clinical features	Cases	FOXC2 expression		Percentage of positive cells	χ^2	*P value*
		Positive	Negative			
Gender		50	12			
Male	35	27	8	77.14%	0.6316	0.5242
Female	27	23	4	85.19%		
Age						
≤ 50	21	16	5	76.19%	0.4037	0.7431
> 50	41	34	7	82.93%		
Differentiation grade*						
High	7	4	3	57.14%	10.1746	0.0065
Mediate	15	9	6	60.00%		
Low	40	37	3	92.50%		
TNM Stage*						
Stage I + Stage II	23	13	10	56.52%	13.6322	0.0005
Stage III + Stage IV	39	37	2	94.87%		

Footnotes: *P < 0.05. Chi-square test (χ^2) was used to analyze the statistical significance of the comparison between two groups.

and normal renal tissues. The manipulation was based on the kit introduction, and 3'3 diaminobenzidine tetrahydrochloride was used for color development. Phosphate buffered saline was used instead of the antibody as a negative control, and RCC tissues served as positive controls compared with normal renal tissues. Two senior pathologists were invited to observe the stained sections. Five random vision fields of each section were observed under high power lens (× 400). The ratio of positively stained cells and tumor cells was recorded. 0%~25% was taken as negative expression, and > 25% was taken as a positive expression.

Gene expression by PCR array

The total RNA was extracted by TRIzol (Invitrogen, USA), and reversely transcribed into cDNA by SMARTscribe reverse transcriptase (Clontech). The expressions of relevant genes were detected by SYBR green-based real-time quantitative PCR assay with 96-well plates. Both RCC tissues and normal renal tissues were divided into three groups and repeatedly detected three times. Five housekeeping genes, ACTB, B2M, GAPDH, HPRT1 and RPLP0, were taken as internal standards. The quantitative expression of target gene was calculated by the method of $2^{\Delta\Delta Ct}$, and was analyzed by RT2 Profiler PCR Array Data Analysis website. DAVID was used for annotation, visualization and functionally analysis for those significantly changed genes.

Statistical analysis

SPSS17.0 was used to analyze acquired data. Chi-square test and Fisher's exact test were used to compare the difference between two groups. $P < 0.05$ was considered as statistically significant.

Results

Expression of FOXC2

Immunohistochemical staining showed that FOXC2 positive staining areas were concentrated in the cytoplasm in RRC tissue. The expression of FOXC2 in RCC tissues (74.42%) was significantly higher than that in normal renal tissues (17.74%), ($P < 0.05$) (**Figure 1**).

The relationship between FOXC2 and clinical pathological features of RCC

The expression of FOXC2 was irrelevant with gender and age ($P > 0.05$), while significantly relevant with the differentiation grade and AJCC-TNM stage of RCC ($P < 0.05$), shown in **Table 1**.

PCR array

There were a total number of 96 cancer relevant genes detected, including 34 (among them FOXC2 was highly expressed) significantly upregulated genes and 14 significantly downregulated genes (more than 2 times, $P < 0.05$).

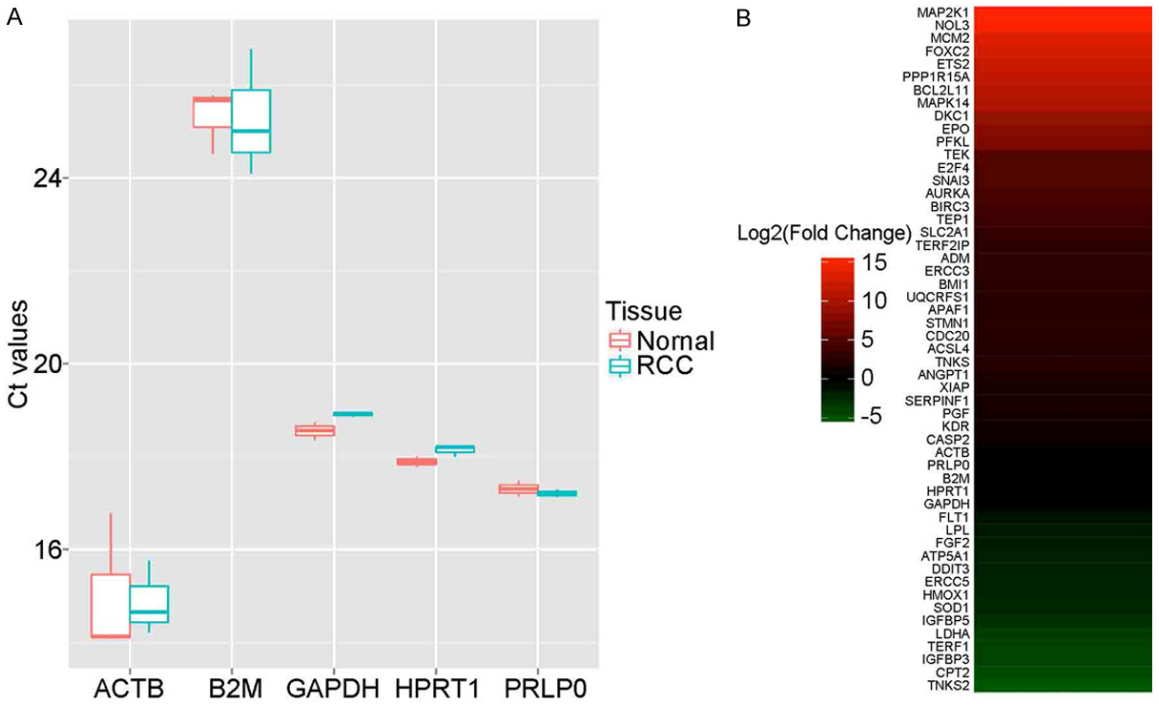

Figure 2. A. Expressions of housekeeping genes in both RCC tissues and normal renal tissues. B. Expression patterns of cancer relevant genes (0 means the expression level of target gene in RCC tissues was as the same as that in normal renal tissues).

Among them, five housekeeping genes as internal standards in both groups were not significantly different. Analyzed by Gene Ontology (GO), most upregulated genes acted to inhibit cell apoptosis, maintain telomere, negatively regulate cell death and so on, while most downregulated genes worked on the regulation of cell cycle, as well as cell differentiation (**Figure 2**).

Discussion

FOXC2 is a transcription factor of human forkhead family, which expresses in normal adipose tissue and bone tissue, melanoma, esophageal cancer, colorectal cancer, breast cancer and other malignant tumors, and relates to many diseases [5]. Studies confirmed that FOXC2 directly induced the transcription of two cell surface molecules: CXC-chemokine receptor 4 (CXCR4) and integrin β3. FOXC2 combines with and promoters CXCR4 and integrinβ3 via fox-binding element, activates the transcription and expression of them, increases the expression of VEGF, and then promotes angiogenesis [8].

The expression of FOXC2 was disturbed in FOXC2 mutant mice. Compared with wild type mice, the growth speed of tumors and the number of new blood vessels decreased in FOXC2 mutant mice [9]. FOXC2 promoted epithelial-mesenchymal transition (EMT) of tumor cells. EMT was a key process of tumor development and metastasis by increasing the growth of blood vessel in tumors and promoting the growth, invasion and metastasis of tumor cells [10-12]. Recent studies showed that FOXC2 highly expressed in human esophageal squamous cell carcinoma and breast cancer, and significantly related to the survival rate of patients, indicating that FOXC2 might be taken as a potential new prognostic factor of tumors.

In this study, the expression level of FOXC2 in RCC tissues was detected with qualitative and semi-quantitative analyses through methods of immunohistochemical and PCR Array. At the same time, the expressions of other relevant genes were also detected and compared between normal renal tissues and RCC tissues. Results indicated that FOXC2 abnormally highly expressed in RCC tissues, and was highly relevant to clinical pathological features and the differentiation grade of RCC. High expression of FOXC2 was significantly relevant to high malignant degree of RCC, including end-stage clinical pathological features and low differentia-

tion of RCC, while not significantly relevant to gender and age. Most of the upregulated genes in RCC worked on the inhibition of cell apoptosis, telomere maintenance, negative regulation of cell death and so on, promoting the growth and development of the tumors.

Conclusion

This study indicates that FOXC2 might be involved in the occurrence and development of RCC, and related to the process of further invasion and metastasis. FOXC2 might be an effective indicator for early diagnosis and an important molecular marker for prognosis, laying a theoretical foundation for targeted therapy for RCC in clinics.

Disclosure of conflict of interest

None.

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