Forkhead box J1 expression is upregulated and correlated with prognosis in patients with clear cell renal cell carcinoma

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Abstract. The forkhead box (FOX) family of transcription factors are considered to have a role in tumorigenesis. FOXJ1 is a member of the FOX family; however, its function in human renal cell carcinoma (RCC) has remained to be elucidated. Therefore, the present study evaluated the expression of FOXJ1 in human clear cell RCC and the effect of FOXJ1 on the proliferative ability of RCC cells. The RCC specimens analyzed in the present study were obtained from 286 patients with RCC who underwent nephrectomy. FOXJ1 mRNA expression levels were determined using reverse transcription-quantitative polymerase chain reaction, and FOXJ1 protein expression levels were determined using immunohistochemistry and western blot analysis. To determine the effect of FOXJ1 on the proliferative ability of RCC cells, the expression of FOXJ1 was decreased using small interfering (si)RNA, and a FOXJ1 vector was stably transfected into RCC cell lines. The proliferative ability of RCC cells was then examined using a WST-1 assay and xenograft experiments with BALB/c nude mice, where the association between FOXJ1 expression and patient survival was determined using Kaplan-Meier analysis. FOXJ1 expression was significantly higher in RCC tissues compared with that of healthy renal tissues. Furthermore, FOXJ1 expression was associated with tumor stage, histologic grade and size. In addition, FOXJ1 significantly enhanced the proliferation of RCC cells *in vitro* and *in vivo*. The present study identified that FOXJ1 expression was upregulated in RCC and enhanced the proliferative ability of RCC cells. Therefore, FOXJ1 may serve as an independent prognostic marker and a therapeutic target for the treatment of patients with RCC.

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Introduction

Human renal cell carcinoma (RCC) is the most common type of malignant kidney tumor in adults worldwide, and ~85% of RCCs are clear cell RCC (CCRCC) (1). RCC is regarded as a localized disease in the early stages, however, 30% of patients with RCC that present with localized disease at the time of diagnosis develop metastatic disease within three years (2). Furthermore, the prognosis for metastatic RCC is poor (3) as RCC is resistant to traditional chemotherapy (4,5) and alternative therapeutic strategies for advanced RCC are limited. At present, novel strategies for the treatment of advanced RCC include molecular targeted therapy (6), monoclonal antibodies (7), immunotherapy (8) and the suppression of signaling pathways (9). Although specific markers predicting the prognosis of advanced RCC and its potential therapeutic response to treatment have been investigated, the molecular mechanisms underlying the progression and development of RCC have remained elusive.

The forkhead box (FOX) family comprises numerous proteins with a wide spectrum of biological processes, including differentiation, metabolism, apoptosis, proliferation, migration and invasion. FOX proteins contain conserved transcriptional factors defined by a common DNA-binding domain (10). Furthermore, the FOX family is divided into 19 subclasses and consists of 50 genes in the human genome (11). Previous studies have determined that FOX proteins are associated with carcinogenesis and the progression of malignancies. For example, the expression of FOXM1 was increased in a variety of types of tumor, including basal cell and hepatocellular carcinoma, as well as lung, breast, prostate and colorectal cancer (12-17). FOXM1 may be associated with carcinogenesis due to its role as a key regulator in the G1/S and G2/M phases of the cell cycle (18-21). In addition, FOXO has been reported to be dysregulated in various types of tumor, including prostate and breast cancer, leukemia, glioblastoma and endometrial carcinoma (22-28); FOXA1 was overexpressed in thyroid, lung and esophageal cancer (29,30); and FOXC2 appears to be a key gene involved in tumor progression and angiogenesis (31).

FOXJ1 is a transcription factor that is significant in the central nervous and reproductive systems (32-34). Previous studies have demonstrated that abnormal expression of

FOXJ1 is associated with autoimmune diseases and certain inflammatory diseases (35,36). This association appears to be due to the ability of FOXJ1 to suppress T cell activity, resulting in spontaneous autoimmunity (37). In addition, FOXJ1 inhibits the humoral immune response in B cells, with FOXJ1 deficiency in B cells being associated with germinal center formation and the development of autoantibodies (38). A previous study proposed that FOXJ1 expression was decreased in breast cancer, thus, functioning as a tumor suppressor gene (39). However, FOXJ1 expression was increased in hepatocellular carcinoma and was associated with poor prognosis. Furthermore, overexpression of FOXJ1 appears to be involved in proliferation and cell-cycle progression. In brief, little is known regarding the potential roles of FOXJ1 in carcinogenesis (40).

The expression of FOXJ1 and its function in human RCC is unclear. Therefore, the current study aimed to determine the expression of FOXJ1 in human RCC and its effect on the proliferative ability of human RCC cells.

Materials and methods

Patients and samples. The current study included 286 patients with RCC that had undergone radical nephrectomy in the Department of Urology of the Affiliated Hospital of Yanbian University (Yanji, China) between April 2002 and March 2003. The histological cell type of all specimen slices was determined by experienced pathologists and all samples were diagnosed as conventional CCRCC. The clinical tumor stages and characteristics were classified according to the tumor node metastasis (TNM) classification system (41), and the nuclear grade was evaluated according to the Fuhrman grading system of malignant tumors (42). RCC tissue samples and corresponding healthy kidney tissues located at a maximal distance from the tumor were collected immediately following surgical resection. The samples were formalin-fixed (Sigma-Aldrich, St. Louis, MO, USA), dehydrated and paraffin-embedded (Sigma-Aldrich). All tissue samples were maintained in liquid nitrogen (Sigma-Aldrich) prior to protein and RNA extraction. The patients were followed up every three months for a period of 120 months. The present study was approved by the Ethics Committee of the Affiliated Hospital of Yanbian University and written consent was obtained from all patients.

Immunohistochemistry. All paraffin-embedded tissue sections (4 μm) were deparaffinized in xylene (Sigma-Aldrich) and rehydrated. Subsequently, endogenous peroxidase activity was blocked by treatment with 0.4% hydrogen peroxide (Sigma-Aldrich) for 20 min followed by blocking with rabbit serum (Sigma-Aldrich) for 30 min. The sections were then incubated with primary FOXJ1 monoclonal mouse anti-rat antibody (cat. no. sc-53139; 1;1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37˚C for 1 h. The sections were washed with Tris buffer prior to incubation with biotinylated polyclonal goat anti-mouse antibody (cat. no. E0433; 1:2,000; Dako, Glostrup, Denmark) at 37˚C for 2 h. Detection of the antibody reactions was performed using the standard streptavidin-biotin complex technique (43). The tissue sections were immunohistochemically examined under a light microscope (ZX-117M; Shenzhen Zhongxun Optics Instrument Co., Ltd., Shenzhen, China), with FOXJ1 expression semi-quantitatively determined according to staining intensity (-, negative; +, weak; ++, moderate; and +++, strong).

Western blot analysis. Western blot analysis was performed according to the manufacturer's instructions. Briefly, total protein was isolated from the CCRCC and healthy tissue samples using lysis buffer (Sigma-Aldrich) as previously described (44), and the total protein concentration was determined using a Bradford dye-binding protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, 10% SDS-PAGE (Bio-Rad Laboratories, Inc.) was performed. FOXJ1 monoclonal mouse anti-rat antibody (cat. no. sc-53139; 1;1,000; Santa Cruz Biotechnology, Inc.) was applied as the experimental antibody and anti-β-actin monoclonal mouse anti-human antibody (cat. no. ab6276; 1:5,000; Abcam, Cambridge, UK) was applied as a loading control at 37˚C for 2 h. The immune complexes were evaluated using an enhanced chemiluminescence system (GE Healthcare Life Sciences, Chalfont, UK).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the CCRCC and healthy kidney tissues using an illustra™ QuickPrep mRNA purification kit (GE Healthcare, Life Sciences), according to the manufacturer's instructions, and RT was performed using a First-Strand complementary (c)DNA synthesis kit (GE Healthcare Life Sciences). The PCR conditions were determined according to the manufacturer's instructions as follows: Denaturation at 95˚C for 5 min, annealing for 30 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 1 min and extension at 72˚C for 10 min. RT-qPCR was performed using LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Indianapolis, USA) and the PCR products were detected by agarose gel electrophoresis, followed by quantification of the products using LightCycler (Roche Diagnostics GmbH). The primer sequences were as follows: FOXJ1 forward, 5'-TCGAGATGGCGGAGAGCTGG-3' and reverse, 5'-GATCCCAAGAAGGCCCCCAC-3'; GAPDH forward, 5'-ATCAAGAAGGTGGTGAAGCAG-3' and reverse, 5'-TGGAGGAGTGGGTGTCGC-3'. All RT-qPCR experimental procedures were conducted in accordance with Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (45).

Cell culture. Four RCC cell lines (Caki-1, NC65, ACHN, and A498) were purchased from the American Type Culture Collection (Manassas, VA, USA). The RCC cell lines were cultured in complete medium consisting of RPMI-1640 medium (Gibco Bio-Cult Diagnostics Ltd., Glasgow, Scotland, UK) supplemented with 25 mM HEPES, 2 mM glutamine, 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 5% non-essential amino acids (all obtained from Sigma-Aldrich). All RCC cell lines were maintained as monolayers in 10-cm petri dishes (Corning Inc., Corning, NY, USA) and cultured in an incubator with a humidified atmosphere of 5% CO_2 at 37˚C.

RNA interference (RNAi) and transfection

RNAi. RCC cells were incubated in culture dishes with complete medium at 37˚C until cell confluence reached 30-50%. Subsequently, the RCC cells were transfected

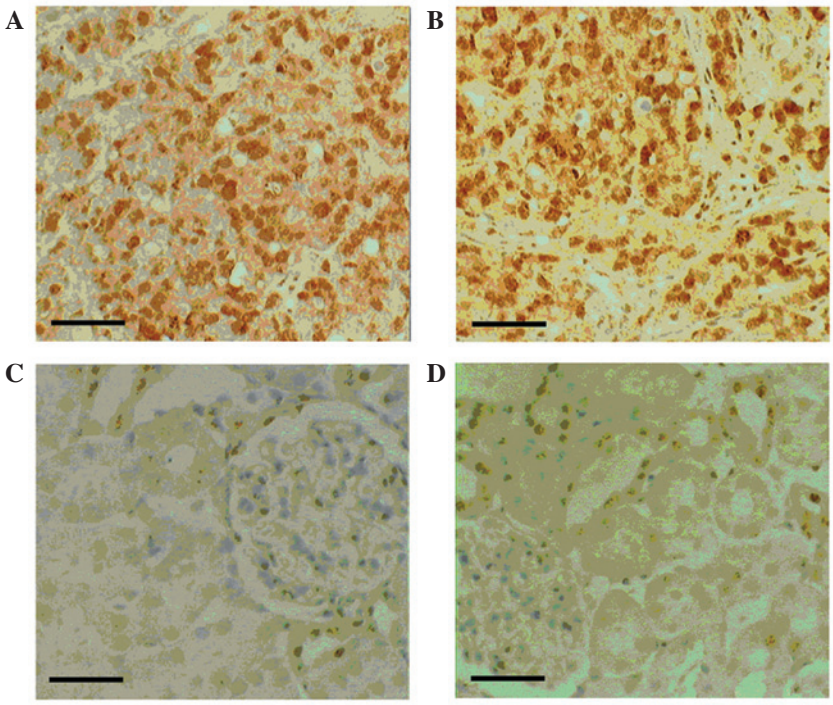

Figure 1. FOXJ1 is highly expressed in CCRCC tissues. Immunohistochemical analysis of two paired renal tissue samples, indicating high FOXJ1 expression in human CCRCC tissues obtained from a (A) stage $T_1N_0M_0$ and (B) stage $T_2N_0M_0$ patient and no or low FOXJ1 expression in the corresponding healthy kidney tissues of the (C) stage $T_1N_0M_0$ and (D) stage $T_2N_0M_0$ patients (magnification, x400). Tumor staging was performed according to the to the American Joint Committee on Cancer tumor node metastasis (TNM) classification system for renal cell carcinoma (41). Scale bars, 20 μM. FOXJ1, Forkhead box J1; CCRCC, clear cell renal cell carcinoma.

with 50 ng/ml small interfering (si)RNA oligonucleotides against FOXJ1 using Lipofectamine® 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). The siRNA oligonucleotide sequences were designed using siDirect software (http://sidirect2.rnai.jp). Following incubation for 48 h, FOXJ1 expression was evaluated by RT-PCR.

Transfection. The coding sequence of normal human FOXJ1 was synthesized by RT-PCR using HK-2 (healthy kidney cell line) cDNA (American Type Culture Collection) as the substrate. The PCR products of FOXJ1 were then subcloned into the pcDEF3 vector (Sigma-Aldrich) as described previously (46). The expression vector containing full-length FOXJ1 cDNA was stably transfected into the four RCC cell lines using Lipofectamine 2000. G418 (Sigma-Aldrich) was used to select RCC cells successfully transfected with FOXJ1, and FOXJ1 expression was evaluated by RT-PCR.

Proliferative ability analysis. The effect of FOXJ1 on the proliferative ability of RCC cells was analyzed using a WST-1 assay. In brief, exponentially-growing RCC cells were obtained and seeded into a 96-well microtiter plate. Following incubation for 24, 48 and 72 h, 10 μl WST-1 (Roche Diagnostics GmbH, Penzberg, Germany) was added to each well, and incubated for an additional 2 h. The absorbance, which represents the cell count in each well, was examined using a microculture plate reader (Immunoreader NJ-2000; Japan Intermed Co., Ltd., Tokyo, Japan) at a wavelength of 450 nm.

RCC xenograft mouse models. Thirty BALB/c nude mice (age, 3-4 weeks; Affiliated Hospital of North Sichuan Medical College, Nanchong, China) were randomly divided into two groups (control and FOXJ1 vector groups). The mice were kept in pathogen-free conditions, at temperatures of 26-28˚C and 30-40% humidity and were exposed to 12 h light/dark cycles with free access to food and water. A total of $4x10^8$ RCC cells were administered via subcutaenous injection into the lumbar region of each mouse. All mice were observed continuously for five weeks and the volume of each tumor was measured once a week. Following five weeks, all mice were sacrificed under deep anesthesia and the final volume of each tumor was recorded. Tumor volumes (v) were calculated using the following formula: $v = ab^2\pi / 6$, where a is the longest diameter and b is the longest perpendicular diameter.

Statistical analysis. Statistical calculations were performed using SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA). All experiments were performed in triplicate and the results are presented as the mean ± standard deviation. Statistical significance was determined using a Student's t-test, and the χ^2 test was performed to analyze the association between FOXJ1 expression and clinicopathological characteristics. In addition, survival curves were plotted using Kaplan-Meier analysis. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The present cohort included 192 male and 94 female patients (age range, 51-84 years; median age, 67 years), with a tumor diameter of 1-17 cm (median size, 4.6 cm). The TNM staging distribution was as follows: Stage I, 147 patients; stage II, 73 patients; stage III, 41 patients;

Table I. Association between characteristics of patients with CCRCC and FOXJ1 expression, detected using quantitative polymerase chain reaction and immunohistochemistry.

				FOXJ1 protein expression				
Characteristic	n	FOXJ1 mRNA expression, mean ± SD	P-value	-	+	++	+++	P-value
Kidney disease state			<0.05					<0.05
CCRCC	286	2.64±0.35		24	61	112	89	
Healthy	286	0.71±0.18		156	97	33	0	
Gender			>0.05					>0.05
Male	192	2.67±0.25		16	42	74	60	
Female	94	2.58±0.28		8	19	38	29	
Age, years			>0.05					>0.05
<60	159	2.67±0.23		13	34	62	50	
≥60	127	2.61±0.24		11	27	50	39	
Tumor size, cm			<0.05					<0.05
≤7	147	2.07±0.22		18	53	50	26	
>7	139	3.25±0.33		6	8	62	63	
Histological grade[a]			<0.05					<0.05
I	124	1.85±0.18		21	42	59	2	
II	97	2.76±0.27		3	17	35	42	
III	65	3.98±0.36		0	2	18	45	
Tumor stage			<0.05					<0.05
I	147	2.07±0.22		18	53	50	26	
II	73	2.74±0.26		6	7	38	22	
III	41	3.47±0.32		0	1	17	23	
IV	25	4.35±0.41		0	0	7	18	

[a]Determined using the Fuhrman grading system. CCRCC, clear cell renal cell carcinoma; FOXJ1, forkhead box J1; SD, standard deviation.

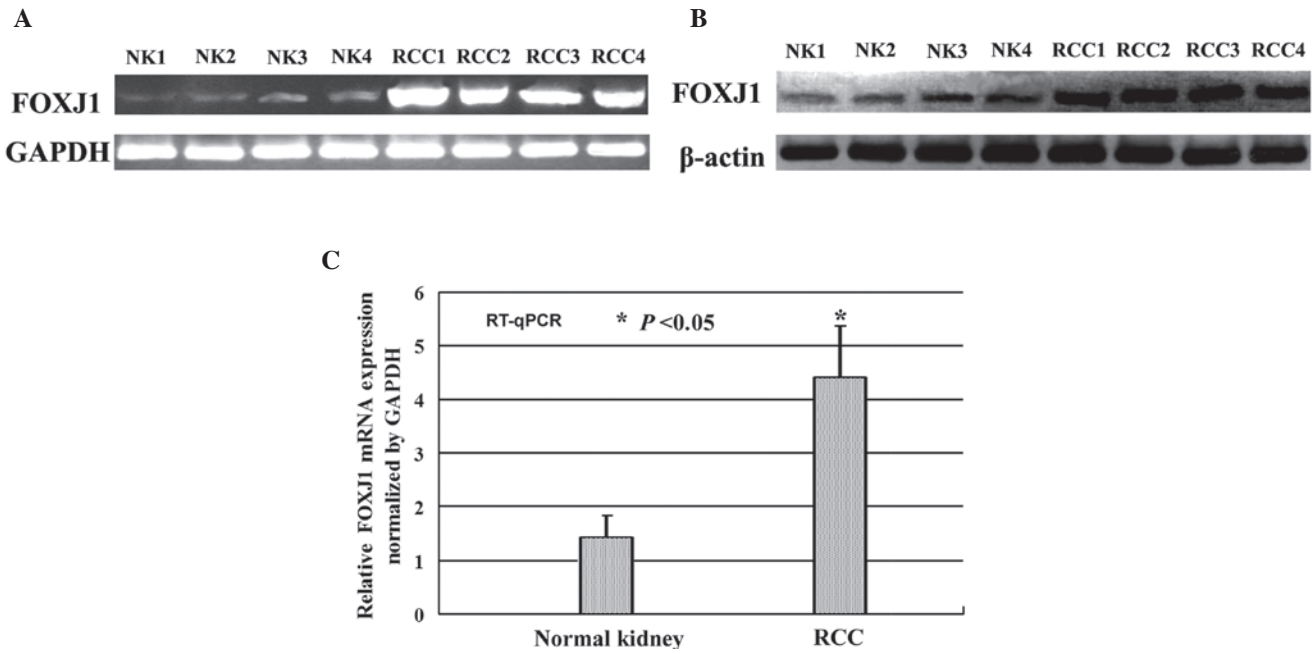

Figure 2. FOXJ1 is highly expressed in CCRCC tissues, compared with that of NK tissue. FOXJ1 expression detected by (A) RT-PCR, (B) western blot analysis and (C) RT-qPCR in four human CCRCC and corresponding NK tissues. All experiments were performed in triplicate and values are presented as the mean ± standard deviation. NK, normal kidney; CCRCC, clear cell renal cell carcinoma; FOXJ1, forkhead box J1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

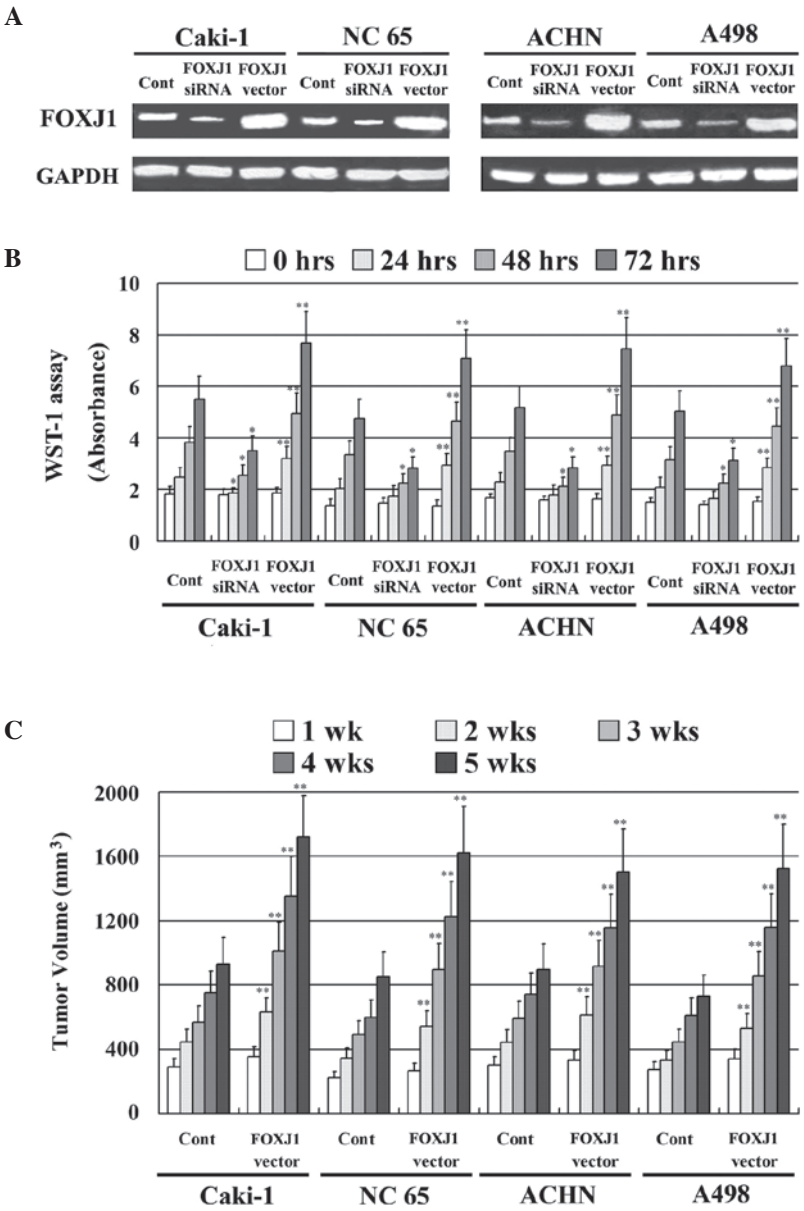

Figure 3. FOXJ1 expression enhances the proliferation of RCC cells. (A) FOXJ1 expression was decreased using siRNA and increased by stable transfection of a FOXJ1 expression vector in RCC cell lines. Successful transfections were confirmed using reverse transcription-polymerase chain reaction. The proliferative ability of RCC cells was detected using (B) an *in vitro* WST-1 assay and (C) an *in vivo* xenograft investigation using BALB/c nude mice. *P<0.05 and **P<0.01 vs. Cont. FOXJ1, forkhead box J1; Cont, control; siRNA, small interfering RNA; RCC, renal cell carcinoma.

and stage IV, 25 patients. In addition, the Fuhrman staging distribution was as follows: Grade I, 124 patients; grade II, 97 patients; and grade III, 65 patients (Table I). The presenting symptoms included hematuria (28 patients), flank pain (36 patients) and palpable masses (19 patients). RCC was an incidental finding during the routine examination of 108 patients. Furthermore, laboratory analysis indicated an elevated erythrocyte sedimentation rate in 64 patients at the time of diagnosis, while thrombocytopenia, erythrocytosis and anemia existed in four patients each. Forty-nine patients exhibited one or more concomitant diseases, including angina, urolithiasis, diabetes mellitus and valvular heart disease; 12 patients with CCRCC had previously been treated with radical nephrectomy on the contralateral side; and 29 patients exhibited metastatic CCRCC at the time of diagnosis.

FOXJ1 protein expression in RCC. FOXJ1 protein expression in human CCRCC and healthy kidney tissues was investigated by immunohistochemical analysis. FOXJ1 expression appeared to be increased in CCRCC tissues (Fig. 1A and B) compared with that of corresponding healthy kidney tissues (Fig. 1C and D). FOXJ1 staining was detected in the cytoplasm and nuclei of 262/286 CCRCC samples (91.6%), but in only 130/286 (45.4%) healthy kidney tissue samples. A significant association was detected between increased FOXJ1 protein expression levels and various clinicopathological characteristics using χ^2 analysis, including advanced tumor stage, high histological grade and tumor size ($P \leq 0.05$). However, the other investigated characteristics, including gender and age, did not exhibit a significant association with FOXJ1 protein expression ($P > 0.05$; Table I). These results indicate that FOXJ1 may be involved in the carcinogenesis and progression of human CCRCC.

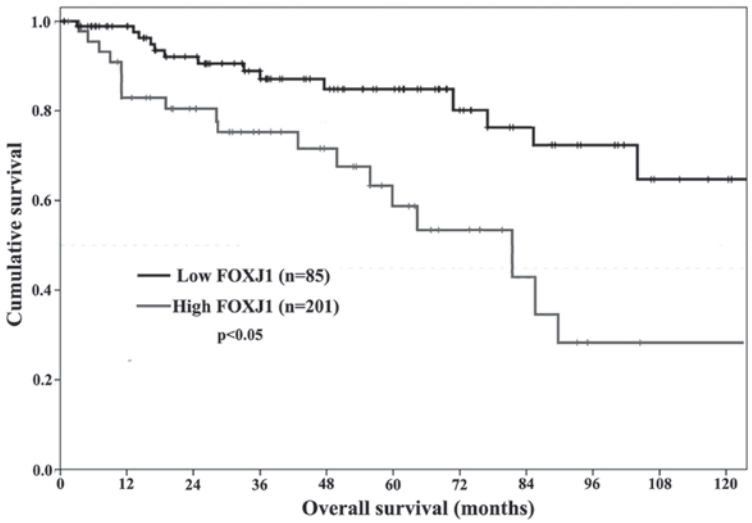

Figure 4. Kaplan-Meier analysis reveals an association between FOXJ1 expression and the survival of patients with clear cell renal cell carcinoma. High expression of FOXJ1 was significantly associated with the poor prognosis of patients with RCC and FOXJ1 expression acted as an independent marker for determining the prognosis of patients with RCC. FOXJ1, forkhead box J1; RCC, renal cell carcinoma.

Evaluation of FOXJ1 expression using RT-PCR, western blotting and RT-qPCR. To clarify the elevated FOXJ1 protein expression observed in CCRCC by immunohistochemistry, RT-PCR (Fig. 2A) and western blot analysis (Fig. 2B) were performed to determine FOXJ1 expression levels in human CCRCC and healthy kidney tissues. The relative level of FOXJ1 expression was analyzed by RT-qPCR with reference to an internal control (Fig. 2C). The results indicated that FOXJ1 expression was significantly increased in CCRCC tissue compared with that of corresponding healthy kidney tissues, and FOXJ1 was expressed at levels similar to those detected by immunohistochemistry. The results of four pairs of CCRCC and corresponding healthy kidney tissue samples are indicated in Fig. 2.

Effect of FOXJ1 on the proliferation of RCC cells. A pcDEF3 vector containing full-length FOXJ1 cDNA was stably transfected into Caki-1, NC65, ACHN and A498 cell lines. Additionally, FOXJ1 expression was suppressed using siRNA. Successful transfections were confirmed using RT-PCR, where FOXJ1 expression was markedly increased by the FOXJ1 vector insert and markedly decreased by siRNA (Fig. 3A). The effect of FOXJ1 on the proliferation of RCC cells was determined by performing a WST-1 assay. RCC cells expressing high levels of FOXJ1 exhibited a significantly increased proliferative ability compared with that of the control cells. By contrast, RCC cells expressing low levels of FOXJ1 exhibited lower proliferative ability compared with that of the control cells (Fig. 3B). The observed increase in proliferation associated with increased FOXJ1 expression was supported by identical results obtained from the *in vivo* xenograft investigations of BALB/c nude mice (Fig. 3C).

Prognostic significance of FOXJ1 expression. Due to the significant association identified between FOXJ1 expression, and clinical stage and pathologic grade in CCRCC, the present study aimed to determine whether FOXJ1 was able to be regarded as a prognostic marker in human CCRCC. In the current cohort, 15 patients succumbed to myocardial infarction and 11 patients succumbed to advanced malignant disease. Kaplan-Meier analysis was performed to calculate the association between FOXJ1 expression and survival in CCRCC. It was demonstrated that the survival time of patients with CCRCC significantly differed between the low and high FOXJ1 expression groups ($P<0.05$; Fig. 4). Furthermore, following 10 years of follow-up, it was determined that patients expressing immunohistochemically low levels of FOXJ1 (- and +) lived significantly longer compared with patients in whom immunohistochemical staining demonstrated high FOXJ1 expression (++ and +++). These results indicated that FOXJ1 expression may serve as an independent marker for predicting the prognosis of patients with CCRCC.

Discussion

Various members of the FOX family, including FOXM1, FOXO, FOXA1 and FOXC2, have been studied, with the results indicating that FOX sub-families may be important in the tumorigenesis and progression of certain carcinomas (10). However, the function of FOXJ1 in carcinogenesis has remained unclear. To date, the role of FOXJ1 has generated considerable attention in certain types of tumor, with a number of studies analyzing its expression in human tumors. For example, a recent study indicated that FOXJ1 expression was increased and associated with aggressive characteristics in hepatocellular carcinoma. Thus, FOXJ1 was proposed as a prognostic marker in patients with hepatocellular carcinoma (40). By contrast, a previous study proposed that FOXJ1 was decreased and may function as a tumor suppressor gene in breast cancer (39). The expression of FOXJ1 and its role in RCC has remained to be determined.

To the best of our knowledge, the current study was the first to investigate FOXJ1 expression in human RCC. FOXJ1 expression levels were determined in human CCRCC samples using RT-PCR, western blot analysis and RT-qPCR. These methods identified that FOXJ1 expression levels were similar to those

detected by immunohistochemistry. Additionally, the current study revealed that FOXJ1 expression was significantly increased in CCRCC compared with that of healthy kidney tissues. Furthermore, the expression of FOXJ1 was significantly associated with tumor stage, histological grade and tumor size. These findings indicated that FOXJ1 may function as a significant gene that is key in the tumorigenesis and progression of CCRCC. *In vitro* and *in vivo* analysis of the effect FOXJ1 expression on RCC cell proliferation indicated that FOXJ1 significantly enhanced the proliferation of RCC cells. Similar results were detected in xenograft investigations using BALB/c nude mice. The present study also used Kaplain-Meier analysis to investigate the association between FOXJ1 expression and the survival of patients with CCRCC. The results indicated that high expression of FOXJ1 was associated with poor prognosis in patients with CCRCC. Thus, it was proposed that FOXJ1 may be considered as an oncogene and an independent marker for predicting prognosis in patients with RCC. In addition, the FOXJ1 gene may be important in the tumorigenesis of renal cancer in adults and high expression levels of FOXJ1 may accelerate the progression of human RCC. The effects of FOXJ1 observed in both CCRCC tissues and RCC cells indicate that the conclusions drawn from these results are likely to apply to RCC in general. Thus, future studies should analyze the detailed molecular mechanisms regulated by FOXJ1 in human RCC.

In conclusion, the current results indicate that FOXJ1 expression was increased in human RCC and that FOXJ1 enhanced the proliferation of RCC cells. These findings indicate that FOXJ1 is a significant gene that may be crucial in the tumorigenesis and progression of human RCC. Thus, silencing of FOXJ1 expression may present a novel treatment strategy for patients with RCC.

Acknowledgements

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