

Expression and clinical significance of RCDG1 in renal cell carcinoma: A novel renal cancer-associated gene

ZUHU YU¹⁻³, LIANGCHAO NI¹, DUQUN CHEN^{1,3}, ZHENGMING SU^{1,4}, WENSHUI YU^{1,4},
QIANG ZHANG¹, YADONG WANG¹, CAILING LI², YAOTING GUI^{1,2} and YONGQING LAI^{1,2}

¹Department of Urology, Peking University Shenzhen Hospital, Shenzhen, Guangdong 518036; ²The Key Laboratory of Stem Cell Biology, Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Institute of Urology, Peking University Shenzhen Hospital, Shenzhen PKU-HKUST Medical Center, Shenzhen, Guangdong 518036; ³Anhui Medical University, Anhui, Hefei 230032; ⁴Shantou University Medical College, Shantou, Guangdong 515041, P.R. China

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Abstract. Recently identified molecular tumor markers have numerous potential applications in the diagnosis, therapy and prognostic prediction of renal cell carcinoma (RCC). Through bioinformatics-based screening approaches together with validation of western blot and immunohistochemical data, the present study identified a novel renal cancer-associated gene, preliminarily named Renal Cancer Differentiation Gene 1 (RCDG1), originally known as chromosome 4 open reading frame 46 (C4orf46). RCDG1 expression was evaluated by western blot analysis of RCC and adjacent normal tissues, renal cancer cell lines and normal kidney HEK293T cells. Additionally, RCDG1 expression was assessed in 124 RCC paraffin sections, including 92 paired adjacent normal tissues, by immunohistochemistry. The results showed that RCDG1 was significantly downregulated in RCC tissues as compared with normal adjacent tissues ($P < 0.001$), and the expression of RCDG1 in clear cell (cc) RCC tissues was significantly lower as compared with that of non-ccRCC tissues ($P = 0.005$). Furthermore, statistical analysis revealed RCDG1 expression was negatively correlated with the Fuhrman grade in ccRCC ($P = 0.008$). A reduction in RCDG1 expression may be associated with the oncogenesis of RCC and the differentiation of

ccRCC. Further studies may provide more information about the function of RCDG1 gene in RCC.

Introduction

Renal cell carcinoma (RCC) is the most common malignant tumor of the adult kidney. It accounts for ~3% of adult malignancies and ~90% of all renal neoplasms (1). RCC is a highly vascularized tumor that originates in the renal cortex, with a steadily increasing annual incidence rate of 2.6% (2). Approximately 30% of patients have metastasis when initially diagnosed with RCC and up to 30% of patients with clinically localized disease develop cancer recurrence following surgery (3,4). Although novel therapeutic strategies have improved the treatment of RCC, the prognoses of patients with RCC remain unfavorable, particularly for those with advanced tumors (5,6).

According to the World Health Organization International Histological Classification of Kidney Tumors, RCC was subdivided into clear cell renal cell carcinoma (ccRCC), papillary RCC, chromophobe RCC, collecting duct carcinoma and unclassified renal carcinoma, based on histological and genetic differences (2,7). Approximately 80% of RCCs are classified as clear cell RCC (ccRCC), which is the most aggressive form of RCC with the highest rate of metastasis and poorest survival among common renal malignancies (8,9). Although great progress has been made, the underlying molecular mechanisms of tumorigenesis and progression of ccRCC are still not entirely clear (10-13).

Molecular markers are able to provide information on the occurrence and aggressiveness of RCC, allowing for the development of more targeted and effective strategies for early detection and treatment of RCC (14,15). Current markers are inadequate to substantially alter existing diagnostic, therapeutic and prognostic strategies of RCC. Therefore, it is essential to identify novel renal cancer-associated genes. By means of bioinformatic approaches, a novel renal cancer-associated gene newly named Renal Cancer Differentiation Gene 1 (RCDG1) in the present study [previously known as chromosome 4 open reading frame 46 (C4orf46); gene ID: 201725], was identified. The expression of RCDG1 in RCC, ccRCC and

Correspondence to: Professor Yongqing Lai, Department of Urology, Peking University Shenzhen Hospital, Institute of Urology of Shenzhen PKU-HKUST Medical Center, 1120 Lianhua Road, Shenzhen, Guangdong 518036, P.R. China
E-mail: yqlord@163.com

Professor Yaoting Gui, The Key Laboratory of Stem Cell Biology, Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Institute of Urology, Peking University Shenzhen Hospital, Shenzhen PKU-HKUST Medical Center, 1120 Lianhua Road, Shenzhen, Guangdong 518036, P.R. China
E-mail: guiyaoting2007@yahoo.com.cn

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normal kidney tissues was examined using western blot and immunohistochemical analyses. Statistical analysis was then performed to identify any correlation between RCDG1 levels and clinicopathological characteristics of the patients.

Materials and methods

Patients and tissue specimens. The tissue specimens used in the present study, including the paraffin sections of 124 RCC and 92 paired adjacent normal tissues (located 2.0 cm outside of visible RCC lesions) and fresh RCC and adjacent normal tissues, were collected from patients who underwent radical nephrectomy at the Department of Urology, Peking University Shenzhen Hospital (Shenzhen, China). Clinical and pathological characteristics of these 124 RCC patients are listed in Table I. The fresh RCC tissues and adjacent normal tissues, including eight ccRCCs and four papillary carcinomas, were stored at -80°C following dissection. All of these tissue specimens were clinically and pathologically confirmed to be RCC-positive or normal tissues, by experienced pathologists of the Pathology Department, Peking University Shenzhen Hospital (Shenzhen, China). All tissue samples were classified according to the American Joint Committee for Cancer (AJCC) and Fuhrman nuclear grading (16). The study was approved by the Ethics Committee of Peking University Shenzhen Hospital (Shenzhen, China). Written informed consent was obtained from all patients and the study was reviewed and approved by the Hospital Ethics Committees.

Perl programming to screen candidate genes in silico. To screen for novel renal cancer-associated genes *in silico*, the following steps were performed as previously described (17). A secondary classification database for expressed sequence tag (EST) libraries was generated based on the Cancer Genome Anatomy Project (CGAP) information of EST libraries (18). The CGAP EST libraries were classified into two classes: Libraries from nonfetal, nongermlinal and nonplacental normal tissues (NT), and libraries from renal cancer. Furthermore, Unigene clusters with <20 ESTs from NT libraries and >2 ESTs from renal cancer libraries were screened. The frequency of the best serial analysis of gene expression (SAGE) tag in NT for each candidate gene was counted based on CGAP SAGE data and Unigene clusters with <20 SAGE tags from NT were retained for further analysis. Finally, the candidate genes were analyzed manually using an Affymetrix HG-U133AB microarray data of normal tissues downloaded from the University of California at Los Angeles public core.

Cell lines and culture condition. Human renal cancer cell lines, 786-O, ACHN, 769-P, Caki-2 and human kidney HEK293T cells were obtained from the Key Laboratory of Male Reproductive Medicine and Genetics (Guangdong, China) and were used for western blot analysis of RCDG1 expression in this study. These cells were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, at 37°C in a humidified incubator containing 5% CO_2 .

Quantitative polymerase chain reaction (qPCR) evaluation for the mRNA of RCDG1. Total RNA from 12 paired

renal cancer tissues and adjacent normal tissues was extracted using TRIzol solution (Invitrogen, Carlsbad, CA, USA), treated with Revert Aid First Strand cDNA Synthesis kit (MBI Fermentas Inc., Burlington, ON, Canada) to obtain the cDNA templates according to the manufacturer's instructions. The cDNA was then subjected to qPCR for evaluation of the relative mRNA levels of RCDG1 and GAPDH (as an internal control) with the corresponding primer pairs: Sense: 5'-GGAGACGCAGCCTTTTCATTA-3' and antisense: 5'-GTCCCGCCACGTTTTAAGGA-3' for RCDG1; and sense: 5'-CACCAGGGCTGCTTTTAAGTC-3' and antisense: 5'-GAAGATGGTGATGGGATTTTC-3' for GAPDH. The reaction mixture was set up in a total volume of $20\ \mu\text{l}$, consisting of $1\ \mu\text{l}$ cDNA template synthesized previously, $10\ \mu\text{l}$ SYBR Green master mix (Invitrogen), $1\ \mu\text{l}$ of each primer (sense and antisense primer) and RNase-free water. Cycling parameters were set as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 40 sec. The expression levels were calculated using the $\Delta\Delta\text{Ct}$ method.

Western blot analysis of RCDG1 expression. Collected cells of cell lines used in this study and the frozen fresh samples were homogenised on ice in three volumes of lysis buffer [$150\ \text{mM}$ NaCl, $20\ \text{mM}$ Tris-HCl (pH 7.4), 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 5 mg/ml aprotinin, and 1 mg/ml leupeptin; Thermo Fisher Scientific, Waltham, MA, USA]. Protein concentration was quantified using the Pierce Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific) and protein samples ($100\ \mu\text{g}$) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking the membranes with 10% fat-free milk at room temperature for 2 h, the membranes were incubated in primary antibodies overnight at 4°C . RCDG1 (previously known as C4orf46) and β -actin proteins were identified with the primary antibodies rabbit polyclonal anti-C4orf46 (1:1,000, Sigma, St. Louis, MO, USA) to detect RCDG1 and sc-47778 (1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. The membranes were washed three times with Tris-buffered saline containing Tween-20 and incubated for 2 h with secondary antibody goat anti-rabbit immunoglobulin (Ig)G-horse radish peroxidase (HRP) (sc-2004) or goat anti-mouse IgG-HRP (sc-2005). The protein bands were detected with the Immun-Star™ HRP Chemiluminescence kit peroxide buffer and luminol enhancer (Bio-Rad Laboratories, Hercules, CA, USA). Each assay was repeated at least three times.

Immunohistochemistry (IHC). IHC analysis of RCDG1 was performed according to standard procedures. Briefly, paraffin-embedded samples were cut into $5\ \mu\text{m}$ sections, dewaxed in xylene and rehydrated in a descending ethanol series, followed by incubation in 3% hydrogen peroxide solution for 20 min. Antigen retrieval was performed by boiling the sections in a microwave oven for 2x15 min in 0.01 M citrate buffer (pH 6.0). The sections were washed with phosphate-buffered saline (PBS) three times for 5 min, and the sections were then treated with 10% bovine serum albumin for 30 min at 37°C to block non-specific protein binding. For the immunostaining of RCDG1, the specimens were treated with rabbit polyclonal antibody anti-C4orf46 (1:800, Sigma, USA)

Table I. Clinicopathologic characteristics of 124 patients with RCC.

Characteristics	Cases, n (%)
Age (years)	
<60	62 (50.0)
≥60	62 (50.0)
Gender	
Male	88 (71.0)
Female	36 (29.0)
Histological type	
Clear cell RCC	60 (48.4)
Papillary RCC	34 (27.4)
Chromophobe RCC	28 (22.6)
Collecting duct carcinoma	2 (1.6)
Fuhrman grade	
G1-2	54 (43.5)
G3	43 (34.7)
G4	27 (21.8)
AJCC clinical stage	
T1	78 (62.9)
T2	33 (26.6)
T3-4	13 (10.5)

RCC, renal cell carcinoma; AJCC, American Joint Committee for Cancer.

to detect RCDG1 overnight at 4°C. The samples were then rinsed with PBS three times and treated with the anti-rabbit IHC kit (Maixin Bio; Fujian, China) at 37°C for 30 min. Subsequently, the slides were stained with 3'3-diaminobenzidine tetrahydrochloride (Maixin Bio, Fujian, China) for 3 min, counterstained with hematoxylin (Maixin Bio), dehydrated, and mounted. Negative controls were prepared with omission of the primary antibodies.

Evaluation of the staining was carried out by two independent pathologists who were blinded to the clinicopathologic variables with a two-score system of immunointensity (II) and immunopositivity (IP) (19,20). II was graded as follows: 0, no staining; 1, weakly stained; 2, moderately stained; 3, highly stained. The percentage of cells with IP was graded as follows: 0, ≤1; 1, 2-25; 2, 26-50; 3, 51-75 and 4, ≥75%. All of the paraffin-embedded sections were given final scores based on the multiplications of the II and IP score. A final score of 0-12 was graded as negative (I, 0-1), weak (II, 2-4), moderate (III, 5-8), and strong (IV, 9-12). In case of any discrepancy, the specimens were evaluated by the two observers together until a final score was agreed.

Statistical analysis. Statistical analyses were performed using SPSS 17.0 (IBM, Armonc, NY, USA). The χ^2 test was used to make comparisons between RCC tissues and adjacent normal tissues, as well as the comparison between ccRCC and non-ccRCC tissues. Relationships between the expression of RCDG1 and clinicopathologic variables were

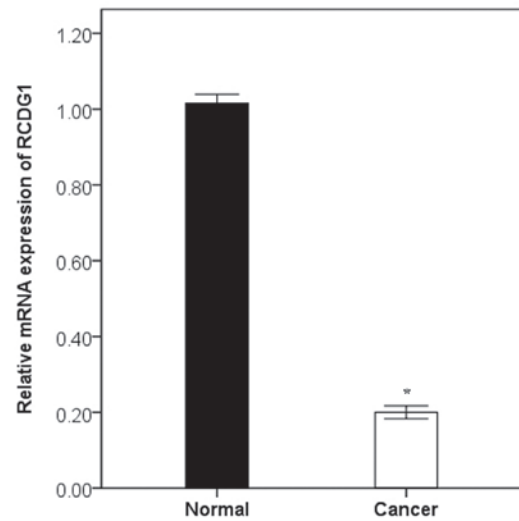


Figure 1. Quantitative polymerase chain reaction evaluation for mRNA expression in 12 paired renal cell carcinoma tissues and adjacent normal tissues (*P<0.001).

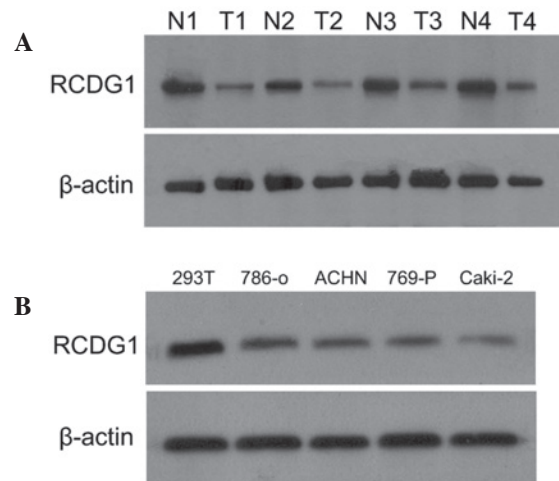


Figure 2. Protein expression of RCDG1 in renal tissues and cell lines, analyzed by western blot. (A) Four representative pairs of renal cell carcinoma and adjacent normal tissues are presented. (B) RCDG1 expression in HEK293T and renal cancer cell lines 786-O, ACHN, 769-P, Caki-2. All results are representatives of three independent experiments. N, adjacent normal tissue; T, tumor tissue.

calculated using the Kruskal-Wallis and Mann-Whitney rank sum tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Results of in silico screening and qPCR evaluation. A total of 32 candidate clusters were first screened out by Perl programming based on EST data. Following secondary analysis using SAGE and microarray data, the data was narrowed to nine clusters, with reconfirmed high expression in normal tissues. The nine clusters were ranked according to the number of EST from renal cancer and qPCR was performed to evaluate the renal cancer specificity. In the first five genes evaluated, C4orf46 (chromosome 4 open reading frame 46) was highly

Table II. Expression of RCDG1 in renal cell carcinoma and normal tissues (χ^2 test).

Histology	Positive cases, n (%)	RCDG1 expression, n (%)				P-value
		I	II	III	IV	
Normal (n=92)	90 (97.8)	2 (2.2)	1 (1.1)	4 (4.3)	85 (92.4)	<0.001
RCC (n=124)	66 (53.2)	58 (46.8)	45 (36.3)	21 (16.9)	0	0.005
ccRCC (n=60)	23 (38.3)	37 (61.7)	15 (25.0)	8 (13.3)	0	
Non-ccRCC (n=64)	43 (67.2)	21 (32.8)	30 (46.9)	13 (20.3)	0	

RCC, renal cell carcinoma; cc, clear cell.

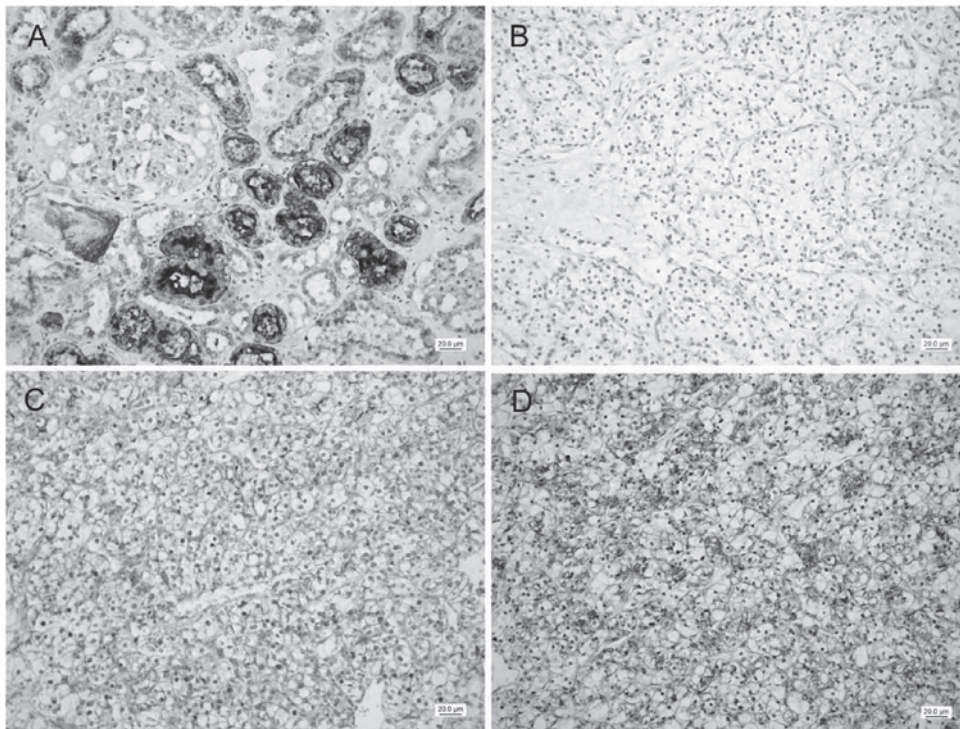


Figure 3. Immunohistochemical staining of RCDG1 in renal cell carcinoma tissues and normal tissues (magnification, x200). (A) Normal kidney tissues showed strong staining. (B) Negative staining of renal cell carcinoma tissues. (C) Weak staining of renal cell carcinoma tissues. (D) Moderate staining of renal cell carcinoma tissues.

specific for renal cancer and it was therefore temporarily termed RCDG1 (Fig. 1).

Western blot analysis of RCDG1 protein levels in RCC tissues and cell lines. Western blotting was performed to determine the expression levels of RCDG1 in RCC and adjacent normal tissues, as well as in RCC cell lines and HEK-293T cells. As shown in Fig. 2A, RCDG1 protein was expressed in both RCC tissues and adjacent normal tissues. The expression levels of RCDG1 in RCC tumor tissues (T) were significantly lower as compared with those of adjacent normal tissues (N). This difference in expression was consistently observed in RCC lines (786-O, ACHN, 769-P and Caki-2) as compared with normal HEK293T cells (Fig. 2B).

IHC analysis of the expression of RCDG1 in RCC tissues and adjacent normal tissues. In total, 124 RCC tissues and 92 cases

of adjacent normal tissues were used for detection of RCDG1 protein expression by IHC. In normal renal tissues, 90 (97.8%) cases showed positive immunostaining (score ≥ 2) with a total average score of 9.6 ± 0.3 and 85 (92.4%) cases showed strong staining (score ≥ 9) of RCDG1. As shown in Fig. 3, epithelial cells in renal tubules, including the proximal tubules and distal convoluted tubules, showed strong cytoplasmic staining of RCDG1. By contrast, 66 (53.2%) cases showed positive staining and none of the tissue samples were found to be strongly stained. Statistic analysis demonstrated RCDG1 protein expression levels in RCC tissues were significantly lower than those in normal tissues ($P < 0.001$ by χ^2 test; Table II).

Twenty-three (38.3%) cases of ccRCC tissues showed positive staining of RCDG1 (Table II). Respectively, 21 cases of papillary RCC (61.8%) showed positive staining and 21 cases of chromophobe RCC (75.0%) showed positive immunostaining (Table III). Of the two cases of collecting duct

Table III. Correlation between RDCG1 expression and clinicopathologic characteristics of patients with non-clear cell renal cell carcinoma.

Characteristics	RDCG1 expression, n (%)			P-value
	I	II	III	
Age (years)				
<60 (n=30)	12 (40.0)	14 (46.7)	4 (13.3)	0.145
≥60 (n=34)	9 (26.5)	16 (47.0)	9 (26.5)	
Gender				
Male (n=49)	18 (36.7)	22 (44.9)	9 (18.4)	0.233
Female (n=15)	3 (20.0)	8 (53.3)	4 (26.7)	
Histological type				
Papillary RCC (n=34)	13 (38.2)	13 (38.2)	8 (23.6)	0.938
Chromophobe RCC (n=28)	7 (25.0)	17 (60.7)	4 (14.3)	
Collecting duct carcinoma (n=2)	1 (50.0)	0	1 (50.0)	
Fuhrman grade				
G1-2 (n=29)	10 (34.5)	15 (51.7)	4 (13.8)	0.310
G3 (n=24)	9 (37.5)	10 (41.7)	5 (20.8)	
G4 (n=11)	2 (18.2)	5 (45.4)	4 (36.4)	
Tumor stage				
T1 (n=35)	15 (42.9)	14 (40.0)	6 (17.1)	0.239
T2 (n=21)	4 (19.0)	13 (62.0)	4 (19.0)	
T3-T4 (n=8)	2 (25.0)	3 (37.5)	3 (37.5)	

RCC, renal cell carcinoma.

Table IV. Correlation between RDCG1 expression and clinicopathologic characteristics of patients with clear cell renal cell carcinoma.

Characteristics	RDCG1 expression, n (%)			P-value
	I	II	III	
Age (years)				
<60 (n=32)	18 (56.3)	9 (28.1)	5 (15.6)	0.359
≥60 (n=28)	19 (67.9)	6 (21.4)	3 (10.7)	
Gender				
Male (n=39)	22 (56.4)	11 (28.2)	6 (15.4)	0.263
Female (n=21)	15 (71.4)	4 (19.1)	2 (9.5)	
Fuhrman grade				
G1-2 (n=25)	10 (40.0)	11 (44.0)	4 (16.0)	0.008
G3 (n=19)	12 (63.2)	4 (21.0)	3 (15.8)	
G4 (n=16)	15 (93.7)	0	1 (6.3)	
Tumor stage				
T1 (n=43)	24 (55.8)	13 (30.2)	6 (14.0)	0.376
T2 (n=12)	10 (83.3)	0	2 (16.7)	
T3-T4 (n=5)	3 (60.0)	2 (40.0)	0	

carcinoma, one of the tissue samples was moderately stained, whereas the other was negative. The results revealed RDCG1 expression levels in ccRCC tissues were significantly lower than those in non-ccRCC tissues ($P=0.005$, χ^2 test, Table II).

Correlation between RDCG1 expression and clinicopathological characteristics in ccRCC and in non-ccRCC samples. It was next investigated whether the expression of RDCG1 correlated to the patients' clinicopathological characteristics

in ccRCC and non-ccRCC tissues. In ccRCC tissues, as shown in Table IV, RCDG1 expression was negatively correlated with the Fuhrman grade and cases with lower RCDG1 expression showed a significantly higher Fuhrman grade ($P=0.008$, Kruskal-Wallis test), while no correlation was found with age, gender and tumor state. No significant correlation was observed between RCDG1 expression and any of the characteristics measured in non-ccRCC tissues (Table III).

Discussion

Although numerous environmental and genetic factors have been associated with RCC, the definitive mechanisms involved in the initiation and progression of RCC have remained elusive (2,21). Recent identification and potential application of molecular tumor markers is expected to reform the clinical staging of RCC and to have an important role in the early diagnosis, individualized treatment and prognostic prediction of RCC patients (22). Until recently, there has been limited use of these molecular markers for RCC (13). Numerous tumor markers have been found to be associated with tumor progression and prognoses of RCC patients. A study by Chuang *et al* (23) showed that tumor necrosis factor- α (TNF- α) was able to promote invasion and epithelial-mesenchymal transition of kidney cancer (23). Mutations of the Von Hippel-Lindau (VHL) gene were considered critical for the initiation of ccRCC and loss-of-function mutations have been shown to be correlated with a poor prognosis for patients with ccRCC (24,25). High expression of carbonic anhydrase IX, which is regulated by the Von Hippel-Lindau (VHL) protein (pVHL), was suggested to be correlated with a favorable prognosis and a greater likelihood of response to systemic treatment for metastatic disease (26,27). However, there is still a need to discover renal cancer-associated genes, which promote the mechanisms of pathogenesis, invasion and metastasis of RCC.

A renal cancer-associated gene was newly identified in the present study through bioinformatic, western blot and immunohistochemical analyses. This gene was preliminarily named RCDG1 in this study, however, it is registered as C4orf46 (chromosome 4 open reading frame 46). RCDG1 is located on 4q32.1 with an mRNA of 3,545 bp which encodes a small, conserved and uncharacterized protein C4orf46 (PRO_0000335689). Immunohistochemical staining showed that the protein was predominantly located in the cytoplasm of epithelial cells in the proximal tubules as well as the distal convoluted tubules.

In the present study, western blotting was performed to evaluate the expression of the RCDG1 protein in RCC and normal kidney tissues, RCC cell lines and a normal kidney cell line. The results demonstrated that RCDG1 was significantly downregulated in RCC tissues and renal cancer cell lines, as compared with normal tissues and cell lines. An IHC assay of RCDG1 in paraffin sections of paired RCC and adjacent normal tissues showed comparable results to the western blot analysis. Furthermore, statistical analysis revealed that RCDG1 had a diverse expression pattern across different types of RCC and the downregulation was more marked in ccRCC tissues as compared with other types of RCC (non-ccRCC) tissues. Further analysis showed RCDG1 expression was statistically correlated with the Fuhrman grade in ccRCC cases but not

in other types of RCC tissues. This suggested that reduced expression of RCDG1 may be involved in the occurrence and differentiation of ccRCC.

The number of samples used in the present study was moderate but sufficient to reveal the statistically significant differences. The functions of RCDG1 in epithelial cells of renal tubules, involvement in cellular pathways, transcriptional control and mechanisms of downregulation in RCC, however, remain to be elucidated. Recent advances in experimental techniques using knockdown and transgenic overexpression of target genes have facilitated further understanding of the pathogenesis, behavior and molecular biology of cancers (28,29). Functional experiments on renal cancer cell lines through RNA interfere and overexpression of RCDG1 may provide further information for understanding the roles of RCDG1 in RCC. Furthermore, comprehensive analysis of the transcriptional regulation of RCDG1 may help identify the mechanisms of the tumorigenesis and progression of RCC, offering a new target for the emerging targeted therapies.

In conclusion, the present study newly identified a renal cancer-associated gene, preliminarily named RCDG1. RCDG1 was shown to be significantly downregulated in RCC tissues, most markedly in ccRCC tissues. RCDG1 expression was shown to be negatively correlated with the Fuhrman grade in ccRCC, suggesting that the downregulation of RCDG1 may be involved in the tumorigenesis of RCC and the differentiation of ccRCC. Further functional analysis of RCDG1 will offer additional information regarding the role of this gene in RCC.

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