

Down-regulated Expression of RhoA in Human Conventional Renal Cell Carcinoma

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Abstract. *Background: Renal cell carcinoma (RCC) is the most common cancer in adult kidney. Delayed diagnosis may result in progression and metastasis. Markers for early detection of RCC are lacking. The small GTPase RhoA has been implicated in the regulation of cell morphology, motility and transformation, but the role of RhoA in tumorigenesis of RCC remains unclear. In this study, the significance of RhoA expression in human RCC was characterized. Materials and Methods: RhoA mRNA and protein expressions were examined by real-time polymerase chain reaction (PCR) and immunohistochemistry respectively in 46 pairs of tissues. Results: Real-time PCR revealed that RhoA mRNA expression was significantly lower in cancerous as compared to normal tissues ($p=0.036$). RhoA protein expression was significantly higher in normal than in cancerous tissues ($p=0.032$). However, RhoA expression did not correlate with age, differentiation grade or TNM stage (all $p>0.05$). Conclusion: RhoA down-regulation may be involved in human renal tumorigenesis.*

Renal cell carcinoma (RCC) is the most common malignancy of the adult kidney and accounts for approximately 3% of all adult cancer cases in Western countries (1). RCC has a highly resistant phenotype to conventional therapeutic methods, including chemotherapy

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and radiotherapy. Only 10% to 20% of patients respond to immunotherapy. However, surgical resection of localized disease has been regarded as the only curative treatment (2). Thus, the treatment of surgically unresectable metastatic RCC continues to represent a therapeutic challenge. Many studies have demonstrated the genetic and environmental factors leading to RCC which arises with a long period of tumorigenesis (3). Therefore, research for the identification and characterization of potential molecular marker alterations occurring during RCC carcinogenesis may provide rapid and effective methods for early detection of RCC (4). Therapy based on these findings may constitute the backbone of new treatments for RCC in the coming century.

It is generally agreed that tumor stage, grade and patient performance status are prognostic indicators in RCC (5). However, stage and grade are not sufficient to predict clinical outcomes. Markers, such as growth factors, Ki-67, p53 mutations and others (6-8), have been recently investigated. Unfortunately, none of these markers appears superior to the traditional staging and grading systems. Efforts to identify more specific markers for RCC are still ongoing.

Rho family proteins are prominent members of the well-known Ras superfamily of small guanosine triphosphatases (GTPases) that can cycle between the inactive GDP-bound state and the active GTP-bound state and that exhibit intrinsic GTPase activities (9). Several Rho GTPases have been shown to regulate diverse signal transduction pathways and are involved in a variety of biological processes, including cell morphology, motility, proliferation and apoptosis (10-12). Recently, a number of reports have shown that RhoA expression was up-regulated in a group of malignancies, including gastric, testicular, pancreatic, ovarian and breast cancer (13-17), and that the expression level of RhoA seemed to be positively correlated with the progress

of these carcinomas, suggesting that RhoA may play an important role in tumorigenesis and tumor progression (18). However, the exact molecular mechanism of this progress remains largely unknown, and, in particular, there are few available results on the expression of RhoA in human renal cell carcinoma. In this study, we examined the significance of RhoA expression and the relationship between RhoA expression and the clinical and histological parameters in patients with RCC.

Materials and Methods

Tissue specimens. This study had a total of 46 patients with RCC, 31 males and 15 females, with a mean age of 64 years. Each pair of tissues included a RCC tumor portion and normal-looking renal cortical tissue from the same patient. These specimens were obtained from nephrectomies carried out at the National Taiwan University Hospital (NTUH). Fuhrman's nuclear grading system from I to IV was used (19). The grade I, II, III and IV classifications were present in 8, 20, 7 and 10 cases, respectively. Tumors were staged according to the TNM system and histologically classified according to the WHO guidelines (20). Tumors were further staged into 31 cases (67%) as being organ-confined (T1-2N0M0), 12 cases (26%) were locally advanced (T3-4N0M0) and 3 cases (7%) were metastatic (any T with N1-2 or M1). Clinicopathological characteristics of the tumors are summarized in Table I. Approval from the Institutional Review Board of NTUH and Kaohsiung Medical University was obtained and informed consent was received from all participating patients.

Total RNA extraction. Total RNA was extracted by REzol™ reagent (PROtech Technologies, NE, USA). Fresh frozen tissue specimens were placed directly in REzol™ reagent (100 mg/ml). One microgram of total RNA was treated with 1U/10 µl of DNase I.

Reverse transcription (RT). RT was performed using an RNA PCR Kit (Promega, WI, USA), with 1 µg of RNA sample being added to 20 µl of a reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM dNTP mixture, 1 U/µl of RNase inhibitor, 0.25 U/µl of ImProm-II™ Reverse Transcriptase, 0.5 µM random primer and 0.5 µM of oligo d(T)-adaptor primer. Using a DNA thermal cycler (ABI PCR System, 9700; Perkin Elmer, Norwalk, CT, USA), the reaction mixture was incubated at 42°C for 60 min, heated at 70°C for 15 min to inactivate the reverse transcriptase, and then cooled to 4°C for 5 min. The final product, cDNA was stored at -20°C for further study.

Real-time PCR. Primers were synthesized to encompass a specific segment of the cDNA sequence of the RhoA (forward primer, 5'-GGCTGGACTCGGATTC-GTT-3'; reverse primer, 5'-CACAGGC TCCATCACCAACA-3'), and GADPH (forward primer, 5'-TCTC CTCTGACTTCAACAGCGAC-3'; reverse primer, 5'-CCCTGTT GCTGTAGCCAAATTC-3'). A master-mix of the following reaction components was prepared to the indicated end concentration: 6.4 µl of water, 1.2 µl of MgCl₂ (4 mM), 1 µl of forward primer (0.4 µM), 1 µl of reverse primer (0.4 µM) and 12.5 µl of LightCycler Fast Start DNA Master SYBR Green I (ABI). Nine microliters of master-mix was added to the 96-well plate; 1 µl volume, containing 50 ng cDNA, was added as a PCR template. The 96-well plates

were closed, centrifuged and placed into the rotor. To improve SYBR Green I quantification, the temperature fluorescence measurement point was set at 72°C. At the completion of cycling, melting curve analysis was performed to establish the specificity of the amplicons produced. A ratio of specific mRNA/GADPH amplification was then calculated to correct for any differences in efficiency. The relative abundance of the mRNAs, expressed as ΔC_T value = C_T RhoA - C_T GADPH. High mRNA expression was indicated by low ΔC_T values and, conversely, low mRNA expression was indicated by high ΔC_T values.

Immunohistochemistry. Immunostaining (IHC) was performed on paraffin sections by the avidin-biotin-peroxidase complex method (BioGenex, CA, USA). In brief, the sections were deparaffinized in xylene and rehydrated through graded alcohols, then boiled in 0.01 M citrate buffer (pH 6.0) for 10 min. Hydrogen peroxide, 0.3%, was added to block any endogenous peroxidase activity. To block nonspecific binding, the sections were incubated with a goat serum blocking solution composed of 10% normal goat serum in phosphate-buffered saline (pH 7.4) and 0.05% sodium azide. The sections were then incubated with anti-RhoA antibody (Santa Cruz, LA, USA) used at a 1:100 dilution or control nonimmunized mouse serum at 4°C overnight. Biotinylated goat anti-mouse IgG was used as a linker. After washing, streptavidin-biotin complex was applied and stained with diaminobenzidine (Golden Bridge Internal Inc., WA, USA). Counterstaining was performed lightly with hematoxylin. Specific staining for RhoA was identified as a brown color in the cytoplasm. Expression of RhoA was evaluated according to the ratio of positive cells per specimen and staining intensity as described elsewhere (16). The ratio of positive cells per specimen was evaluated quantitatively and scored 0 for staining of $\leq 1\%$, 1 for staining of 2 to 25%, 2 for staining of 26 to 50%, 3 for staining of 51 to 75%, and 4 for staining $>75\%$ of the cells examined. Intensity was graded as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong staining. A total score of 0 to 12 was finally calculated as the percentage of positive cells \times staining intensity. The evaluation of immunostaining was performed by one pathologist (W. Y. K), who was unaware of the tissue site and of the fate of the patient.

Statistical analysis. The levels of RhoA mRNA between the normal and RCC tissues were compared using the paired sample *t*-test. Three separate experiments with triplicate data were performed. Data are presented as mean \pm standard error of the means (SEM). Independent-sample *t*-test and one-way ANOVA were used to compare protein expressions determined by IHC analysis. All tests were two-sided with $p < 0.05$ being statistically significant.

Results

Down-regulation of RhoA in RCC. The clinical and pathological characteristics of the 46 patients are given in Table I. Expression of RhoA mRNA in cancerous tissue was detected using real-time PCR. High mRNA expression was indicated by low C_T values and, in contrast, low mRNA expression was indicated by high C_T values. In this study, RhoA mRNA levels were significantly higher in normal (mean $\Delta C_T = 4.99 \pm 0.28$) than in RCC tissues ($\Delta C_T = 5.99 \pm 0.37$; $p = 0.036$) (Figure 1A). According to the AJCC stage classification of RCC, the ΔC_T values of Stage

Table I. Immunostaining expression of RhoA in normal parenchymal and RCC tissues.

Characteristic	Patients No. (%)	RhoA protein expression (mean score±SE)		p-value*
		Normal renal tubular cells	RCC	
Total	46 (100)	8.5±0.4	7.7±0.4	p=0.032
Gender				
Male	31 (67)	8.0±0.5	7.7±0.5	
Female	15 (33)	9.6±0.7	7.5±0.6	
p-value†		0.065	0.835	
Grade				
I	8 (17)	9.8±1.2	8.0±0.8	
II	20 (43)	8.2±0.6	7.6±0.6	
III	7 (15)	7.7±0.8	8.0±0.9	
IV	10 (22)	9.0±0.9	6.9±1.0	
ND	1 (3)	8	12	
p-value		0.423	0.793	
Stage				
Organ-confined (T1-2N0M0)	31 (67)	8.7±0.5	7.5±0.4	
Locally advanced (T3-4N0M0)	12 (26)	8.7±0.9	7.3±0.8	
Metastatic (N1-2 or M1)	3 (7)	6.7±1.3	10.7±1.3	
p-value‡		0.474	0.118	
Histological type				
Conventional	40 (87)	8.8±0.4	7.9±0.4	
Non-conventional	6 (13)	7.0±1.1	6.3±1.8	
p-value†		0.137	0.193	

RCC, renal cell carcinoma; SE, standard error; TNM, tumor-node-metastasis; ND, not determined. Non-conventional types include papillary, chromophobe, sarcomatoid and collecting duct. *Paired sample t-test; †independent-sample t-test for two groups; ‡one-way ANOVA for three or more groups.

I+II and III+IV were 5.91±0.15 and 6.08±0.31, respectively. However, no significant correlation was observed between RhoA transcripts and tumor stage (p=0.11) (Figure 1B).

Immunohistochemical analysis of RhoA expression in paired tissue samples. Representative profiles of immunostaining for RhoA are shown in Figures 2 and 3. Protein expression was examined by immunohistochemistry on a scale from 0 to 12 (percentage of positive cell × staining intensity). The immunostaining score was estimated as the average of all tissue cores analyzed and the results are summarized according to clinicopathological data in Table I. The normal tissues had markedly higher scores (mean 8.5±0.4) when compared to those of cancerous tissues (mean 7.7±0.4, p=0.032) (Table I). The immunostaining of RhoA in normal tissues was weak in 0% (0 cases), moderate in 65% (30 cases) and strong in 35% (16 cases) of the 46 paired specimens. In contrast, RhoA immunostaining in cancerous

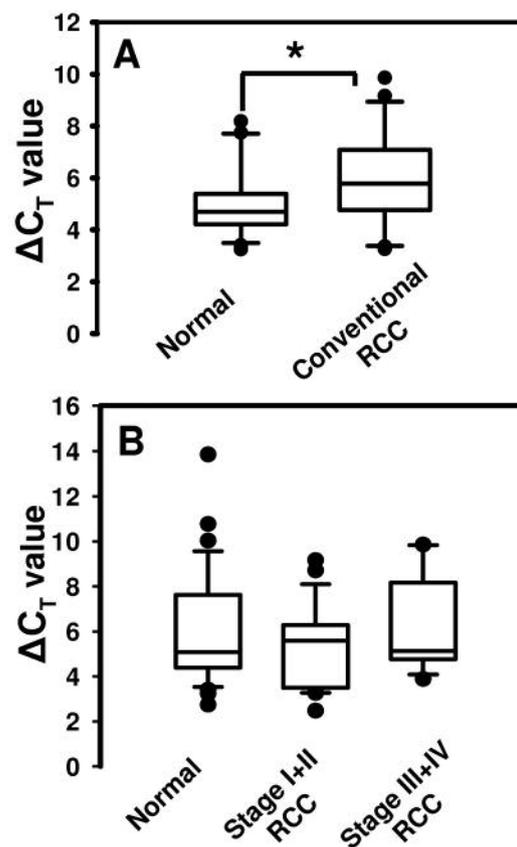


Figure 1. Down-regulation of RhoA gene in conventional renal carcinoma cell. A, Expression of RhoA in normal and renal cell carcinoma tissues by real-time PCR analysis. Paired sample t-test was used to compare RhoA expression in the normal and RCC tissues (*p=0.036). B, Expression of RhoA in different stages of RCC tissues by real-time PCR analysis. ΔC_T value = C_T RhoA - C_T GAPDH.

tissues was weak in 9% (4 cases), moderate in 76% (35 cases) and strong in 15% (7 cases) of these cases (data not shown). There were no cases without immunostaining in normal or RCC tissue specimens. However, RhoA expression did not correlate with sex, differentiation grade or TNM stage, with a statistic p>0.05 in each parameter.

Discussion

Although a number of reports have shown that RhoA expression was up-regulated in a group of malignancies, including gastric, testicular, pancreatic and ovarian cancer (13, 15-17), the role of RhoA in the tumorigenesis of RCC remains unclear. This study was the first examining and characterizing the expression of RhoA in normal kidney and RCC tissues. From our results, we found RhoA expression was significantly down-regulated in RCC compared to that in normal kidney tissues. However, RhoA expression did not correlate with

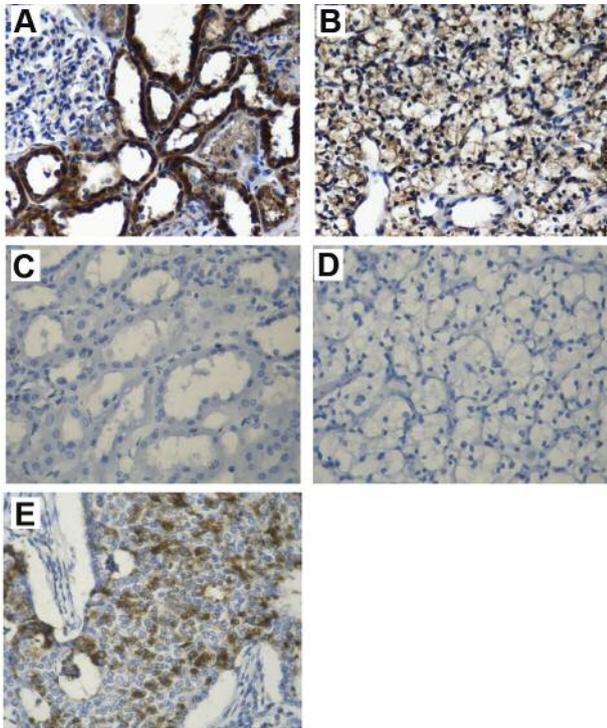


Figure 2. Immunohistochemical staining of RhoA in human conventional RCC. Tissue sections of normal parenchymal tissues (A, C) and RCC (B, D) were from the same patient. The monoclonal mouse anti-RhoA antibody was used to stain paraffin sections (A, B). Negative control was the omission of the primary antibody (C, D). Breast cancer tissue was used as a positive control for indication of RhoA expression staining (E). Original magnification, $\times 200$.

differentiation grade or TNM stage. These results indicated that RhoA was down-regulated in conventional RCC, but was not associated with the progression of the cancer.

Conventional cell carcinoma is a common form of renal tumor among the various subtypes of RCC (21). The Rho family proteins, including Cdc42, Rac1 and RhoA, were shown to be involved in invasion and migration of cancer cells by regulation of cytoskeleton organization (22). Recent studies have demonstrated that down-regulated expression of RhoA may play an important role in enhancing migration and invasion of pancreatic carcinoma cells and cytoskeleton disorganization in RCC (13). Herein, we also found similar results: that RhoA was significantly down-regulated in RCC compared to normal tissues. Taken together, these findings seemed to indicate the down-regulation of RhoA may be an important factor in tumorigenesis or migration of conventional RCC. However, the effects of altered RhoA expression involved in cellular transformation and migration were often cell type-specific, which was reflected by the conflicting results reported in the literature (23). For example, up-regulated expressions of RhoA was associated with tumor progression in ovarian, gastric and testicular cancer cells (15-17).

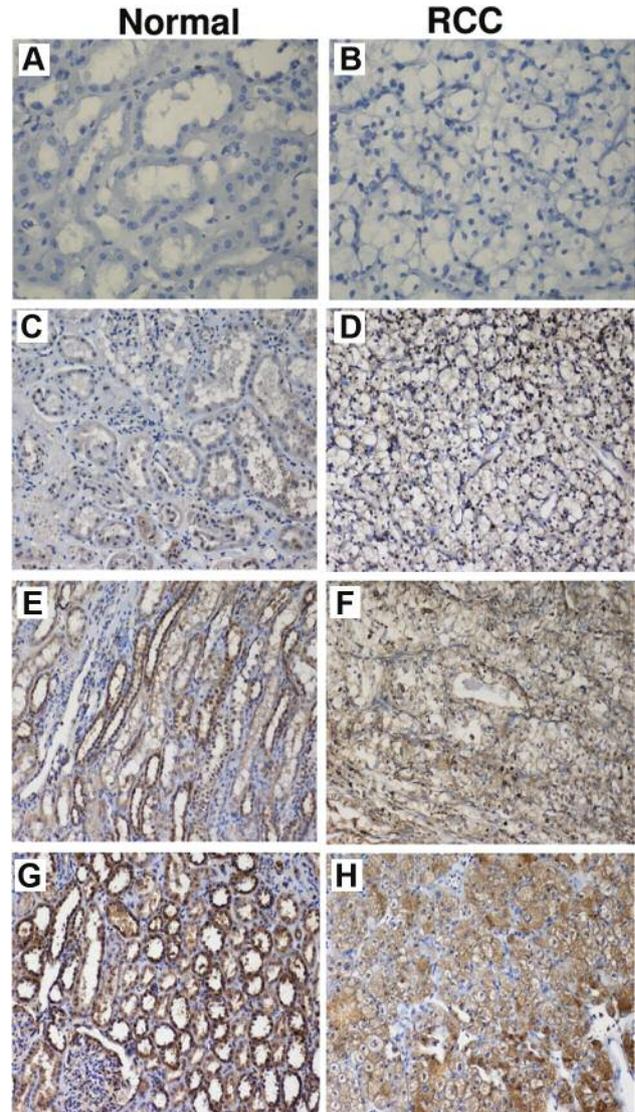


Figure 3. Representative immunohistochemical staining of RhoA expression in normal parenchymal and RCC tissues demonstrating semi-quantitative staining scores. A and B, no signal; C and D, weak staining (1+); E and F, moderate staining (2+); G and H, strong staining (3+); $\times 200$ magnification.

Recent studies have demonstrated that Rho family proteins could be important players in cellular apoptosis (24). The first evidence for a role of Rho family proteins in apoptosis were revealed from *in vivo* tumorigenic experiments in nude mice injected with fibroblasts transformed with rho gene (25). Tumor generated by stable, Rho-transformed cell line showed a high apoptotic feature, suggesting that Rho GTPase proteins could be triggering signals that ultimately led to apoptosis. Accordingly, it was later reported that overexpression of the human genes *rho A*, *rho C* and *rac 1* induced apoptosis upon serum deprivation in NIH3T3 fibroblasts and human erythroleukemia K562 cells (26). The

RhoA-induced cellular apoptosis occurred through a complex mechanism involving concurrent generation of ceramides and the *de novo* synthesis of FasL (10). Therefore, our understanding of the roles the Rho proteins may play in transformation and apoptosis, although incipient, is being strongly established. We suggest the down-regulation of RhoA might play important roles in allowing RCC cells to escape from apoptosis and in enhancing their cell migration.

In summary, this was the first study to show the down-regulated expression of RhoA protein in human conventional RCC tissues *in vivo*. However, further studies are needed to determine the molecular role of RhoA in RCC tumorigenesis.

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