Possible role of the RhoC/ROCK pathway in progression of clear cell renal cell carcinoma

Hideyuki Abe*, Takao Kamai*, Toshihiko Tsujii, Fumihiko Nakamura, Tomoko Mashidori, Tomoya Mizuno, Miho Tanaka, Katsuhisa Tatsumiya, Nobutaka Furuya, Akinori Masuda, Tomonori Yamanishi and Ken-Ichiro Yoshida

Department of Urology, Dokkyo Medical University, Tochigi, Japan.

ABSTRACT

To clarify the role of the Rho small GTP-binding protein (Rho) and its major downstream target, ROCK (Rho-associated serine-threonine protein kinase), in progression of renal cell carcinoma (RCC), we examined mRNA expression for Rho and ROCK genes in surgical specimen of RCC tissues from 78 Japanese patients and in the corresponding non-tumor tissues originating from the same patient using a real-time reverse transcription polymerase chain reaction (RT-PCR). Expression of mRNA for RhoA did not differ between tumor and non-tumor tissues. RhoB mRNA expression was higher in the tumor ($P < 0.05$), but expression was not associated with tumor grade, stage, or prognosis. However, degree of RhoC and ROCK mRNA expression was related to tumor grade ($P < 0.05$) and stage ($P < 0.0001$). A positive relationship was seen between expression of mRNA for RhoC and that for ROCK in tumor tissues ($P < 0.0001$). Kaplan-Meier plots showed high RhoC and ROCK mRNA expression to be negatively associated with overall survival ($P < 0.0001$). Multivariate analysis showed mRNA expression of RhoC and ROCK to be independent poor prognostic factors concerning overall survival. Our findings implicate the RhoC/ROCK pathway in carcinogenesis and progression of RCC, indicating that RhoC/ROCK may be a useful prognostic marker and a possible molecular target for treatment of the disease.

Annual estimates of new diagnoses of renal cell carcinoma (RCC) are increasing steadily (17). RCC is resistant to chemotherapy and radiotherapy (17, 28, 29), while immunotherapy with interferon and/or interleukin (IL)-2 achieves responses in 10% to 20% of advanced RCC (17, 18). Although surgical resection of the primary tumor for patients remains the mainstay of therapy, RCC is characterized by a high proportion of cases with metastases present at diagnosis or appearing as a relapse following nephrectomy. Patients with distant metastases have a poor prognosis, with a 5-year survival rate of less than

10% in stage IV disease (17). Although inhibition of metastases logically would be a promising treatment strategy in advanced RCC, much of the molecular mechanism of progression and metastasis in RCC is yet to be eluidated.

Cell migration is central to metastasis of malignant tumors (20). The Rho small GTP-binding protein (Rho) regulates formation of stress fibers, focal adhesions, and cell migration through reorganization of the actin cytoskeleton (7, 25). Overexpression of Rho in human cancer tissues in comparison to non-tumor tissues has been reported, with higher expression of Rho correlating with higher stage (6, 13, 24). Several lines of evidence directly link Rho to acquisition of a migratory, invasive, and metastatic phenotype (2, 3). Furthermore, Rho-kinase (ROCK), one of the major downstream effectors of Rho, induces stress-fiber formation and assembly for focal contact by regulating contractility of the actin-myo-

Address correspondence to: Takao Kamai, M. D. Ph. D.
Department of Urology, Dokkyo Medical University, 880 Kitakobayashi, Mibu, Tochigi, 321-0293, Japan
Tel: +81-282-87-2162, Fax: +81-282-86-7533
E-mail: kamait@dokkyomed.ac.jp
*H. A. and T. K. were equally contributed to this work.

sin system (9, 16). ROCK has been reported to be involved in progression of the human tumors (8, 10, 12), while a ROCK inhibitor suppressed tumor growth and metastasis (8, 10). These observations suggest that the Rho/ROCK pathway may be associated with progression and metastasis in human tumors. However, we have known of no available data regarding this pathway in RCC. To address the question of whether the Rho/ROCK pathway is involved in renal carcinogenesis and progression of RCC, we compared degree of mRNA expression for Rho and ROCK genes in RCC tissue with expression in corresponding non-tumor parts of the same patient's nephretomy specimen using a real-time reverse transcription polymerase chain reaction (RT-PCR). We also examined the relationship between intensity of expression in tumors and grade, stage, and prognosis. Such information may be useful for predicting the biological behavior of RCC and formulating treatment strategy in individual cases. We then considered the clinical implications of our molecular findings.

MATERIALS AND METHODS

Patients and tissue preparation. Specimens from surgery for newly diagnosed clear cell RCC between 1995 and 2000 from 78 consecutive Japanese patients were studied (46 men and 32 women; mean age, 65.8 years; range, 33–82). All patients underwent imaging studies (CT and/or MRI) prior to surgery to obtain information necessary for staging. Postoperative follow-up ranged from 3 to 78 months (median follow-up duration, 31 months). In all cases three sites from the tumor and three non-tumor samples from the resected kidney were studied (14). Resected tissues were embedded in optimal cutting temperature (OCT) tissue compound (Miles, Elkhart, IN) and stored at −80°C as described previously (13, 24). Clinical stage was determined according to the criteria of the TNM system (23).

Real-time RT-PCR assay. Methods of purification of total RNA and cDNA synthesis were described previously (14). Expression profiles of the Rho and ROCK genes were analyzed with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) using the SYBR Green method. Oligonucleotide primer pairs for Rho and ROCK genes and β2-microglobulin amplification, as well as conditions of PCR, were chosen as described previously (12, 13, 19, 24). A real-time RT-PCR assay was performed on 25 μL of reaction mixture con-

taining 20 ng of sample cDNA, 100 nM sense primer, 100 nM antisense-primer, and 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems). PCR was carried out for 50 cycles of 95°C for 15 sec and 60°C for 1 min. To normalize the amplified products in each sample, we used β2-microglobulin as a quantitative internal control (13, 14). A standard curve for expression of each mRNA was generated using fivefold dilutions of a control RNA sample (25x, 5x, 1x, 0.2x, and 0.04x). Expression of mRNA for Rho and ROCK genes was expressed as a ratio to that of β2-microglobulin, and this relative expression was calculated (13, 14). Mean values from real-time RT-PCR data for the three samples of resected tissues were used for analysis according to a method described previously (13, 14).

Statistical analysis. Results of RT-PCR were analyzed statistically using the Mann-Whitney U test as described previously (13, 14, 24). Prognostic variables were analyzed for overall survival by the Cox proportional hazards model. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. P values less than 0.05 were considered to indicate statistical significance. Data were analyzed with commercial software.

RESULTS

mRNAs of RhoA, RhoB, and RhoC were detected in both tumor and non-tumor tissues. ROCK mRNAs could be detected clearly in tumor tissues, but were only weakly expressed in non-tumor tissues. Relative mRNA expression for RhoA did not show a significant difference between tumor and non-tumor tissues (mean ± S.D. = 1.35 ± 0.55 vs. 1.25 ± 0.35, $P = 0.1716$) (Fig. 1A). Amounts of mRNA expressing RhoB, RhoC, and ROCK were significantly higher in tumor tissues than in non-tumor parts of specimens (RhoB, 1.48 ± 0.87 vs. 1.18 ± 0.57, $P = 0.0483$, Fig. 1B; RhoC, 3.31 ± 2.15 vs 1.19 ± 0.66, $P < 0.0001$, Fig. 1C; ROCK, 1.83 ± 0.95 vs 1.13 ± 0.28, $P < 0.0001$, Fig. 2D).

High mRNA expression for RhoC and ROCK was associated with a poor histologic differentiation grade ($P < 0.0001$; Fig. 2B, C) and higher clinical stage ($P < 0.0001$; Fig. 2E, F). In contrast, degree of mRNA expression for RhoB did not correlate with grade or stage (Fig. 2A, D). Expression of mRNA encoding ROCK in tumors correlated with expression of RhoC (correlation coefficient r = 0.581, $P < 0.0001$, Fig. 3), but this was not true for RhoB
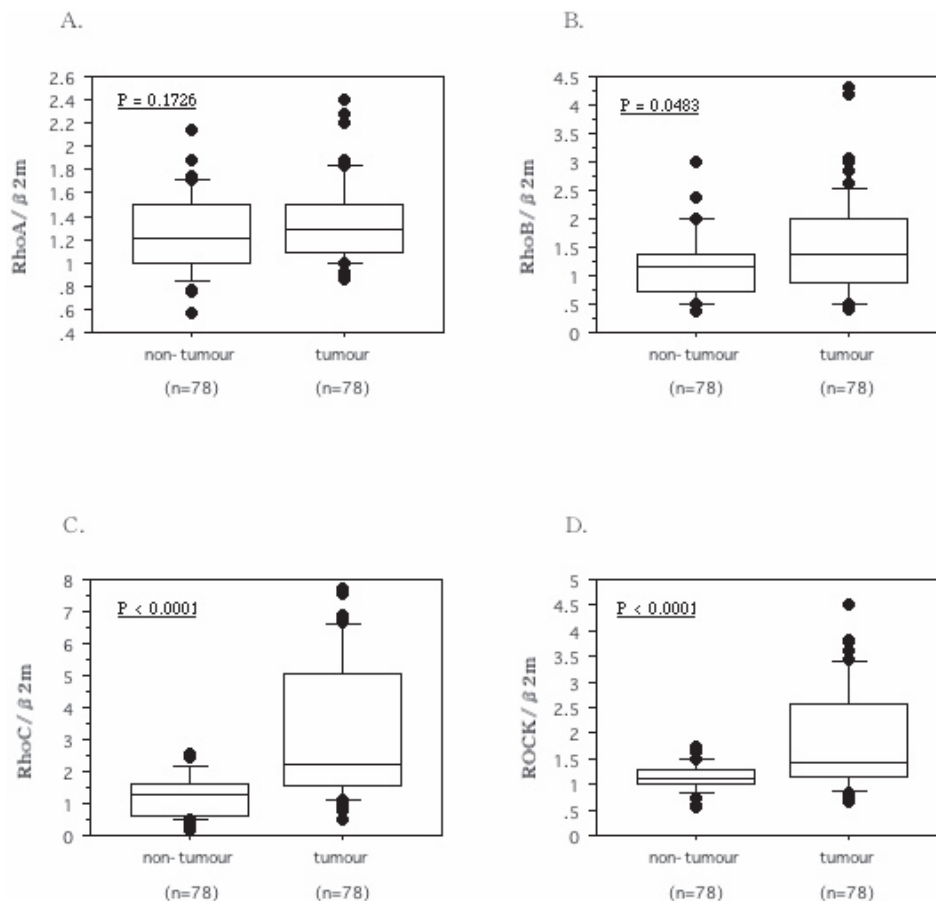
Fig. 1 The relative expression levels for mRNA of RhoA, RhoB, RhoC, and ROCK to those of β2-microglobulin (RhoA/β2m, RhoB/β2m, RhoC/β2m, and ROCK/β2m) in non-tumor and tumor. The median value was seen in the box-plots.

(data not shown).

We analyzed the cancer specific overall survival curve for RCC patients as follows. Mean values of relative mRNA expression for RhoB, RhoC, and ROCK in tumor tissues were 1.48 ($\pm$ 0.87), 3.31 ($\pm$ 2.15), and 1.83 ($\pm$ 0.95), respectively. Cases were divided into two groups with expression above or below these means, high expression or low expression, according to methods described previously (24). Kaplan-Meier plots of survival rate in patients with low vs. high RhoC and ROCK mRNA expression showed that higher expression of RhoC and ROCK was associated with poorer overall survival ($P <$ 0.0001; Fig. 4B, C), while RhoB expression was not (Fig. 4A). Although a univariate analysis using the Cox proportional hazards model identified grade, stage, RhoC, and ROCK independent prognostic factors for overall survival, stage, RhoC, and ROCK remained significant in a multivariate analysis (Table 1).

DISCUSSION

As RCC metastasizes early by a hematogenous route, prognosis is generally unfavorable (17). Rho and its downstream effector, ROCK, are involved in regulation of a variety of cellular processes such as cytoskeletal organization, cell cycle progression, malignant transformation, and metastatic properties (3). To take into account the possibility of inter-individual variation in expression of mRNA for Rho and ROCK genes, tumor tissues in RCC and corresponding non-tumor tissues obtained from the same patient were compared. We found that mRNA expression for RhoC and ROCK were significantly elevated in tumors compared with non-tumor tissues. A positive correlation was present between RhoC and ROCK of mRNA expression in tumors. High RhoC and ROCK mRNA expression in tumor tissues were associated with higher tumor grade and stage. Furthermore, higher mRNA expression for RhoC and ROCK was associated with shortened
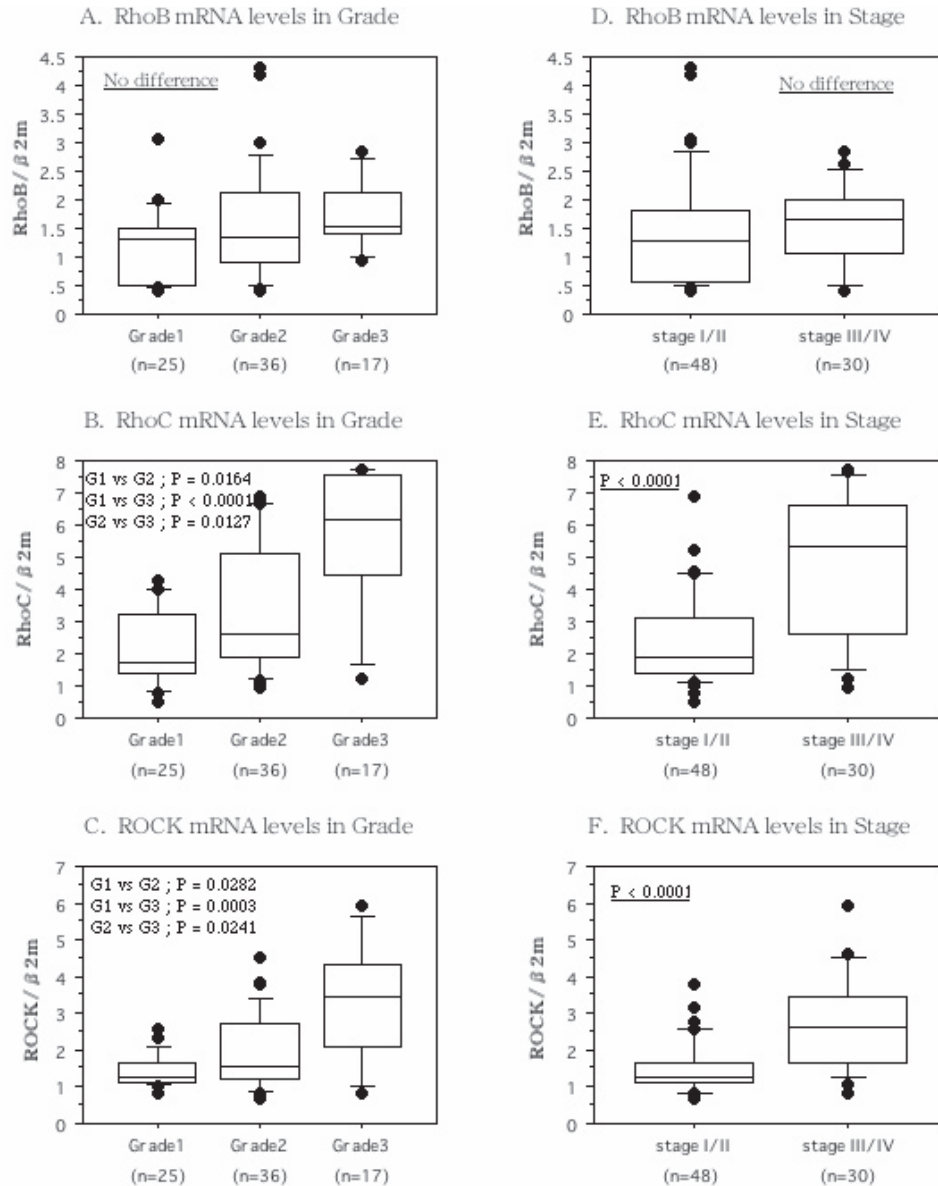
Fig. 2 Expression of RhoB, RhoC, and ROCK mRNAs in grade (A–C) and stage (D–F). The median value was seen in the box-plots.

survival. To our knowledge, this report is the first concerning the relationship between Rho and clinical important characteristics of RCC. The data suggested that the RhoC/ROCK pathway may be involved in progression of RCC.

Although our data showed a strong correlation of intensity of mRNA expression for RhoC with that for ROCK in tumors, this study was not designed to prove direct linkage between RhoC and ROCK expression. On the other hand, our findings demonstrated that mRNA expression for RhoC and ROCK was significantly higher in tumor tissues than in non-tumor tissues, while higher mRNA expression for either was linked with poor histological differentiation, higher stage, and poorer prognosis. Similarly, overexpression of RhoC has been associated with progression of pancreatic ductal adenocarcinoma (24) and inflammatory breast cancer (26). RhoC has been shown to be most associated with metastasis of cancer cells among Rho subfamily members according to studies using high-density DNA microarray (2). Furthermore, neovascularization is a characteristic feature in RCC, while the lung is the most common metastatic site for RCC. Overexpression of RhoC

was reported to lead to increased expression of angiogenic factors (27), while RhoC overexpression also was found to promote the ability of melanoma cells to escape the circulation and colonize the lung (2). However, how RhoC induces metastasis remains unclear. While RhoA, RhoB, and RhoC share common functions in regulating the actin cytoskeleton

Fig. 3 Spearman rank correlation coefficient relationship between expression levels of mRNAs for RhoC and ROCK.

via the major downstream effector ROCK (22), they differ in subcellular location (1) and regulation of expression (5, 11). Therefore, tumor-specific and/or organ-specific roles for RhoA, RhoB, and RhoC may exist in human cancers.

The present observations associating higher expression of RhoC and ROCK mRNAs with advanced stage and poor survival are indicative of a possible role for the RhoC/ROCK pathway in renal carcinogenesis and progression of RCC. A specific ROCK inhibitor (Y-27632) was found to reduce invasive activity and dissemination of tumor cells (8, 10). Taking the various observations together, ROCK inhibitors may represent a potential therapy for prevention of cancer invasion and metastasis. Similarly to RhoC, mRNA expression for RhoB was higher in tumors than in non-tumor tissues; however, RhoB mRNA expression did not correlate with grade, stage, or prognosis. Recent studies indicate that RhoB actually might negatively regulate cell proliferation (4). A negative role in growth control and/or transformation would contrast with the positive effects of RhoA and RhoC in these processes
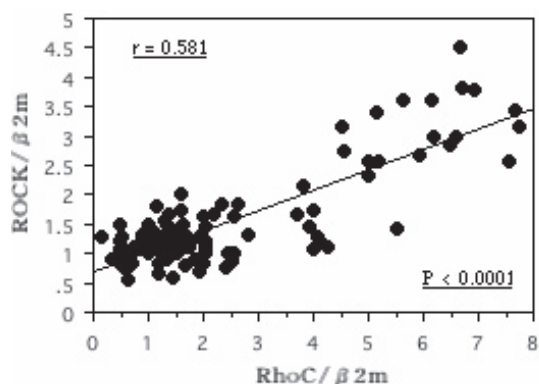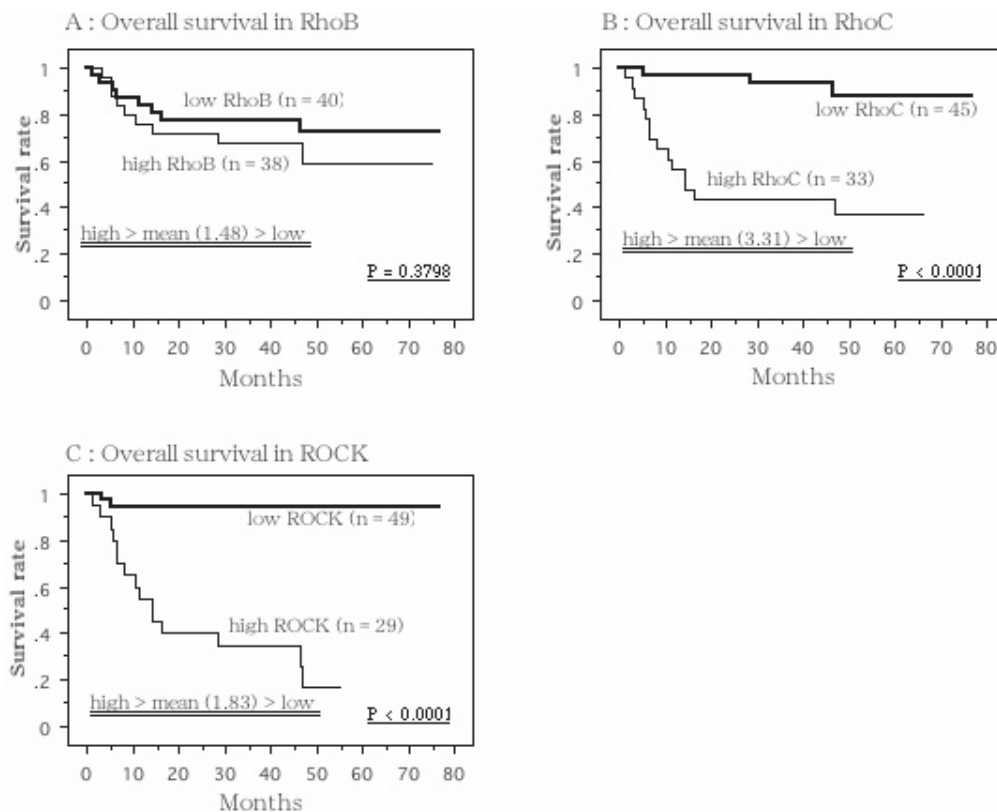

Fig. 4 Overall survival curve based on the mean values of mRNA expression for RhoB, RhoC, and ROCK in tumor tissues; the cases were divided into two groups at the mean values—high and low expression. *P* value was analyzed by log-rank test.

Table 1   *Cox regression analysis for potential prognostic factors in overall survival*

	Variable	Unfavorable/ favorable characteristics	No. of Patients	Relative risk	95 % confidence interval	*P* value
Univariate analysis	Grade	4,3 / 2 / 1	17 / 36 / 25	3.005	1.237 - 7.302	0.0151
	pT*	4,3 / 2,1	30 / 48	7.185	2.052 - 25.157	0.0021
	RhoC	high / low	33 / 45	4.272	1.116 - 9.596	0.0048
	ROCK	high / low	29 / 49	4.327	1.780 - 11.940	0.0023
Multivariate analysis	Grade	4,3 / 2 / 1	17 / 36 / 25	1.499	0.543 - 4.138	0.435
	pT*	4,3 / 2,1	30 / 48	8.182	1.025 - 72.397	0.0204
	RhoC	high / low	33 / 45	6.763	1.497 - 17.248	0.0379
	ROCK	high / low	29 / 49	7.102	1.312 - 23.893	0.0295

pT*: pathological Tumor classification of UICC TNM classification

(15). Recent advances indicate that RhoB is a specialized activator of apoptosis in transformed cells, and participates importantly in mediating cellular responses to farnesyltransferase inhibitors (FTIs) (21). Farnesyltransferase occupies a key position in the mevalonic acid pathway, resulting in conversion of mevalonic acid to Ras and Rho. Therefore, FTIs might prove useful in cancer therapy (21).

Although we presently could not analyze protein expression of Rho and ROCK, such determinations will be needed to more clearly elucidate the role of the RhoC/ROCK pathway and RhoB in a larger number of RCC. Such information may guide study of the effect of ROCK inhibitors and/or FTIs on RCC cells *in vitro* and *in vivo*.

Acknowledgement

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