

Rho-ROCK Expression Predicts the Prognosis in Patients with T3/T4 Gastric Cancer

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A small GTPase Rho protein and an effector ROCK have significant roles in cancer adhesion, metastasis, invasion, angiogenesis and cell mortality. We investigated the expressions of RhoA protein and ROCK-1 protein in 100 patients with macroscopically T3/T4 gastric cancer immunohistochemically. The expression of RhoA was detected in gastric cancer specimens from 39 patients and that of ROCK-1 in specimens from 30 patients. The clinicopathological characteristics of 21 tumors with co-expression of RhoA and ROCK-1 proteins (Rho/ROCK ON) were compared with those of the 79 remaining tumors (Rho/ROCK OFF). The percentage of lymph node metastasis positive cases in the Rho/ROCK ON group (81%) was higher than that in the Rho/ROCK OFF group (66%), but the difference was not significant ($P = 0.183$). However, the prognosis of the 21 patients with Rho/ROCK ON was significantly poorer than that of the 79 with Rho/ROCK OFF ($P = 0.006$). Our results indicate that the evaluation of the protein expression of RhoA and ROCK-1 is useful for predicting the prognosis in patients with T3/T4 gastric cancer.

Key words: gastric carcinoma; prognosis; RhoA; ROCK-1

Small GTP-binding proteins are thought to be involved in signal transduction pathways that control sets of essential cellular functions such as reorganization of the actin cytoskeleton, gene expression, cell cycle progression, and cell-cell adhesion (Malliri and Collard, 2003). The small GTP-binding protein Rho is part of a subfamily of the Ras superfamily that includes Cdc42, Rac and Rho proteins. The Rho family has an important role in cancer development and progression, including cell-cell adhesion, cell motility, transformation, proliferation, invasion, and metastasis (Suwa et al., 1998; Pan et al., 2004; Kamai et al., 2003). Cancer cell migration is central to the process of metastasis. Rearranging the actin

cytoskeletal proteins in response to Rho is important for the ability of tumor cells to metastasize (Chrzanowska-Wodnicka, 1996; Benitah et al., 2004). Activation of Rho results in binding to various effector molecules that elicit downstream responses (Aznar, 2004). The ROCK family, Rho-associated serine-threonine protein kinase, is one of the effector molecules of Rho. ROCK is thought to participate in the induction of focal adhesions and stress fibers in cultured cells (Itoh et al., 1999). In addition, it has been suggested that ROCK-1, one of the isomers of ROCK may play an essential role in the invasion of cancer cells (Kaneko et al., 2002; Yoshinaga, 2003). Rho protein overexpression has been reported in the

Abbreviations: MLC, myosin light chain; PBS, phosphate buffered saline; ROCK, Rho-associated coiled-coil domain kinase

progression of human cancers, suggesting that the Rho/ROCK pathway may be a molecular target for prevention of cancer invasion and metastasis (Sahai, 2003; Somlyo, 2003).

In order to elucidate the malignant potential of gastric cancer, multiple genetic alterations including activation of oncogenes and inactivation of tumor suppressor genes have been investigated vigorously. In studies on the expression levels of RhoA, RhoB, RhoC, Rac1, Rac2, Rac3 and Cdc42, increased expressions of RhoA and Rac1 were related to a higher TNM stage and undifferentiated type in gastric cancer (Pan et al., 2004). Accumulating clinical and experimental data suggest that RhoA working through ROCK protein kinases contributes to the invasive and metastatic abilities of gastric cancer. ROCK inhibitors, such as Y-27632 or WF-536, have been used in some studies (Takamura et al., 2001; Nakajima et al., 2003a). Although these inhibitors are potent and selective, it remains a possibility that these compounds inhibit additional kinases, such as PRK/PKN (Davies et al., 2000). In addition, administration of ROCK inhibitors to whole animals may block tumor cell invasion by enhancing the barrier function of host cell layers (Wojciak-Stothard et al., 2001; Nakajima et al., 2003b). Furthermore, ROCK inhibitors would be useful antiangiogenic chemotherapeutic agents in tumors associated with elevated Rho/ROCK expression (Croft et al., 2004). However, Rho/ROCK expression in gastric cancer and its relationship to a patient's prognosis has yet to be clarified.

Primary T3/T4 gastric cancer frequently metastasizes to the liver and to the peritoneum, and the prognosis of such cases remains unfavorable. In the present study, we immunohistochemically detected the protein expression of RhoA and ROCK-1 in patients with T3/T4 gastric cancer who underwent curative gastrectomy. We also investigated the clinical significance of the Rho/ROCK pathway in advanced gastric cancer with special reference to the mode of recurrence and survival of the patients.

Materials and Methods

Patients

Primary T3/T4 gastric adenocarcinoma specimens were obtained from 100 patients who underwent curative gastrectomy at our institution from 1976 to 1995. The patients' ages ranged from 31 to 91 years (average 64.8 years); 64 were male and 36 were female. The clinicopathological findings were determined according to the Japanese Classification of Gastric Carcinoma by our hospital pathologists (Japanese Research Society for Gastric Cancer, 1995). All patients had undergone either distal, proximal or total gastrectomy with dissection of the level 1 and 2 regional lymph nodes (D2). The patients' follow-up periods for survivors ranged from 2 to 216 months (median, 42 months) after surgery. Informed consent was obtained from all subjects and/or their guardians.

Immunohistochemistry

The methods used for immunostaining for RhoA protein were as described previously (Horiuchi et al., 2003). A streptavidin-biotin kit (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) was used for formalin-fixed and paraffin-embedded specimens. Sections (4 μ m in thickness) were dewaxed in xylene, rehydrated in ethanol and then heated in an autoclave oven (120°C) for 20 min for retrieval of antigens. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 20 min. Tissue sections were then washed twice with phosphate buffered saline (PBS) and pre-blocked for 60 min with 10% rabbit serum. After washing with PBS, the samples were incubated overnight at 4°C with anti-RhoA monoclonal antibody (sc-418, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100. Sections were then washed three times in PBS and incubated with the secondary anti-mouse immunoglobulin (IgG + IgA + IgM) conjugated with biotin for 60 min, followed by incubation with a streptavidin-

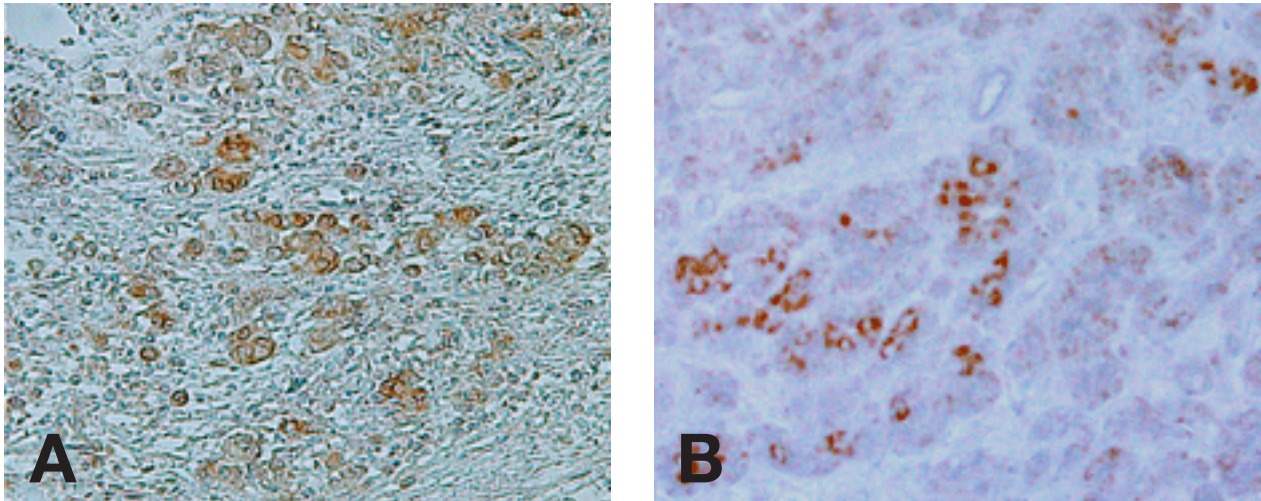


Fig. 1. Immunohistochemical staining pattern of RhoA and ROCK-1 in gastric cancer. Protein expressions of RhoA and ROCK-1 were detected in gastric cancer cell nuclei. **A:** RhoA positive case. **B:** ROCK-1 positive case. Bar = 100 μ m (both in A and B).

peroxidase complex for another 60 min. After three additional washes in PBS, diaminobenzidine tetrahydrochloride working solution was applied. Finally, the slides were counterstained with hematoxylin. Staining for ROCK-1 was performed with an anti-ROCK-1 polyclonal antibody (c-19, Santa Cruz Biotechnology) at a dilution of 1:100, using a similar method to that for the RhoA staining. RhoA and ROCK-1 expressions were not detected in the non-neoplastic region of the resected specimens. Expressions of RhoA and ROCK-1 were evaluated according to the ratio of positive cells per specimen as described previously (Liu et al., 2004). The staining pattern of RhoA or ROCK-1 was classified as follows: negative (negative or equivocal staining or when less than 20% carcinoma cells were detected) and positive (when 20% and more carcinoma cells were detected). Two observers who were unaware of the clinical data or the disease outcome examined all of the histological slides. When the interpretation differed between the two observers, a conference microscope was used for re-evaluation and final decision.

Statistical analysis

The association of factors was evaluated by the χ^2 -test. The significance of difference among means was determined by the Mann-Whitney *U* test. The survival rates were estimated by the Kaplan-Meier method, and statistical analysis was carried out by the log-rank test for equality of the survival curves. The Cox proportional hazard model and a stepwise procedure assessed the influence of each variable on survival. The accepted level of significance was $P < 0.05$.

Results

RhoA/ROCK-1 expression and clinicopathological findings

RhoA and ROCK-1 expressions could not be detected in the non-neoplastic gastric mucosas or in the underlying stromal cells. They were detected only in cancer cells (Fig. 1). We decided that 39 cases (39%) were RhoA positive and 30 cases (30%) were ROCK-1 positive (Table 1). RhoA and ROCK-1 were co-expressed in 21%. No sig-

nificant correlation was detected between these expressions and clinicopathological parameters including age, sex, tumor size, histology, mode of invasion, lymph node metastasis, lymphatic-vascular space invasion and histological stage (data not shown).

The clinicopathological characteristics of 21 patients with tumors co-expressing RhoA and ROCK-1 were compared with those of the other 79 patients. The lymph node metastases were observed in 81% of the former group in comparison to 66% of the latter ($P = 0.183$, Table 2).

Mode of recurrence

Fifty-two patients died of gastric cancer during the follow-up period. Fifteen of 21 patients (71%) with the tumor co-expressing Rho and ROCK-1 died, while 37 of 79 patients (47%) with the tu-

Table 1. Correlation of RhoA and ROCK-1 expression in 100 gastric cancers

	ROCK-1 expression		
	Positive	Negative	Total
RhoA expression			
Positive	21	18	39
Negative	9	52	61
Total	30	70	100

The association of RhoA and ROCK-1 expression: $P < 0.001$.

mor not expressing both Rho and ROCK-1 died as well ($P = 0.045$). Peritoneal metastatic recurrences were frequent in both groups. However, the hematogenic metastases (to liver, lung or bone) tend to be more frequent in patients with Rho and ROCK-1 co-expressing tumors (9/79 [11%], $P = 0.145$, Table 3).

Table 2. Coexpression of RhoA and ROCK-1 proteins and clinicopathological parameters

	RhoA/ROCK-1 expression		<i>P</i>
	Both positive (<i>n</i> = 21)	Else (<i>n</i> = 79)	
Size (cm)	9.0 ± 5.1	8.7 ± 4.6	0.981
Histology			
Differentiated/Undifferentiated	8/13	35/44	0.61
Mode of invasion			
Expansive/Infiltrative	11/10	43/36	0.867
Depth of tumor invasion			
T3/T4	20/1	74/5	0.788
Lymph node metastasis			
Negative/Positive	4/17	27/52	0.183
Lymphatic invasion			
Negative/Positive	3/18	19/60	0.337
Vascular invasion			
Negative/Positive	5/16	26/53	0.423
Histological stage			
II/III/IV	4/16/1	27/44/8	0.233

Table 3. Correlation between RhoA/ROCK-1 protein expression and mode of recurrence

Recurrence pattern	RhoA/ROCK-1 expression		<i>P</i>
	Both positive (<i>n</i> = 21)	Else (<i>n</i> = 79)	
Peritoneal	7 (33%)	18 (23%)	0.321
Hematogenic	5 (24%)	9 (11%)	0.145
Nodal	1 (5%)	4 (5%)	0.955
Remnant stomach or others	2 (9%)	6 (8%)	0.772
Total	15 (71%)	37 (47%)	0.045

Prognosis of patients

The overall 5-year survival rate of 100 patients with T3/T4 gastric cancer was 40%, and the disease-specific 5-year survival rate of these patients was 42%. The disease-specific 5-year survival rate of 21 patients with both RhoA- and ROCK-1-positive tumors (19%) was significantly lower than that of the remaining 79 patients (48%, $P = 0.006$, Fig. 2).

Multivariate analysis was performed with the Cox proportional hazards model using the covariates for histological type, lymph node metastasis, lymphatic invasion, venous invasion and co-expression of RhoA and ROCK-1 (Table 4). The analysis revealed that lymph node metastasis and RhoA/ROCK-1 expression independently contributed to shortened survival.

Discussion

Rho family GTPases are key regulators of many different biological processes including cell motility (Etienne-Manneville and Hall, 2002; Coleman et al., 2002). When bound to GTP, Rho family proteins recruit effector proteins that influence the architecture of the actin cytoskeleton. RhoA and RhoC activate the serine/threonine kinases ROCK-1 and ROCK-2. ROCK activation promotes the stabilization of filamentous actin (F-actin), phosphorylation of regulatory myosin light chains (MLCs), the increase in myosin ATPase activity and the coupling of actin-myosin force

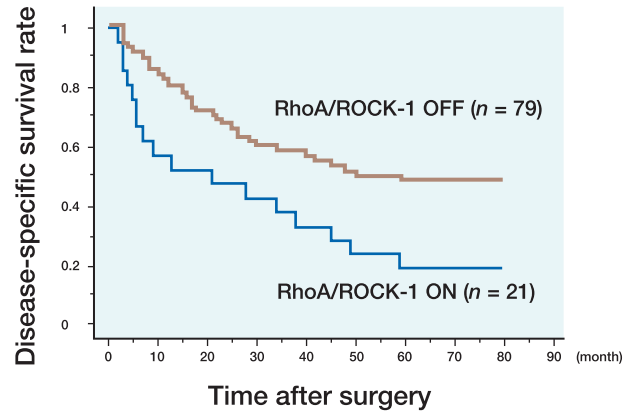


Fig. 2. The disease-specific survival curves of patients with T3/T4 gastric cancer according to RhoA/ROCK-1 protein expression. The disease-specific survival curve of 21 patients with both RhoA- and ROCK-1-positive tumors (Rho/ROCK ON group) was significantly worse than that of remaining 79 patients (Rho/ROCK OFF group, $P = 0.006$).

generation and contractility. In fibroblasts grown in tissue culture, RhoA signals through ROCK-1 and ROCK-2 to promote the formation of stress fibers and focal adhesions that lead to contractile actin-myosin bundles (Leung et al., 1996; Uehata et al., 1997; Riento, 2003). Moreover, genetic experiments in *Drosophila* revealed that elevated Rho signaling in epithelial cells resulted in a loss of epithelial characteristics and increased invasive migratory behavior (Speck et al., 2003).

Co-overexpression of Rho and ROCK proteins in cancer cells has been reported in ovarian cancer, pancreatic cancer, testicular cancer and bladder cancer (Suwa et al., 1998; Kamai et al.,

Table 4. Multivariate survival analysis by the Cox proportional hazards model

Variables	Number of patients	Hazard ratio	95% Confidential interval	P
Tumor histology				
Differentiated/undifferentiated	43/57	0.669	0.387 – 1.156	0.150
Lymph node metastasis				
Positive/negative	69/31	3.000	1.401 – 6.425	0.005
Lymphatic invasion				
Positive/negative	78/22	0.955	0.452 – 2.019	0.904
Vessel invasion				
Positive/negative	69/31	1.180	0.655 – 2.126	0.582
RhoA/ROCK-1 protein expression				
Both positive/else	21/79	1.836	1.033 – 3.263	0.039

2003, 2004). Among Rho family and ROCK proteins, RhoA and ROCK-1 proteins were reported to play important roles in carcinogenesis, cancer cell adhesion, metastasis, invasion and angiogenesis (Itoh et al., 1999; Matsumoto et al., 2001; Igishi et al., 2003; Horiuchi et al., 2003; Shikada et al., 2003). On the other hand, RhoB plays a tumor-suppressive role in human lung cancer (Mazieres et al., 2004). Thus, to investigate the possible prognostic value, we analyzed RhoA and ROCK-1 protein expressions in advanced gastric cancer immunohistochemically.

In studies on gastric cancer concerning the expression levels of RhoA, RhoB, RhoC, Rac1, Rac2, Rac3 and Cdc42, increased expressions of RhoA and Rac1 were related to a higher TNM stage and undifferentiated type in gastric carcinoma (Pan et al., 2004). In the current study, 21% of tumors were positive for both Rho and ROCK-1: they seemed to have a higher tendency for lymph node metastasis, lymphatic invasion, and vascular invasion. They did not show a significantly higher propensity of tumor invasion. However, we found that the prognosis of double-positive patients was significantly poorer than that of the other patients. Also, multivariate survival analysis revealed that the double positive tumor was an independent prognostic factor for advanced gastric cancer as well as lymph node metastasis. To our knowledge, this is the first report to suggest that patients with RhoA/ROCK-1 double positive gastric cancer have a significantly poor prognosis.

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