Significant Association of Rho/ROCK Pathway with Invasion and Metastasis of Bladder Cancer

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ABSTRACT

Purpose: The small GTP-binding protein Rho and its best-characterized downstream effector Rho-associated serine-threonine protein kinase, ROCK, participate in actin cytoskeleton organization, and are linked to pathogenesis and progression of several human tumors. We investigated the roles of Rho and ROCK in bladder cancer.

Experimental Design: Using Western blotting, we quantitated Rho and ROCK protein expression in paired tumor and nontumor surgical samples from 107 consecutive Japanese patients with bladder cancer.

Results: RhoA, RhoC, and ROCK were more abundant in tumors and metastatic lymph nodes than in nontumor bladder and uninvolved lymph nodes (P < 0.0001). Amounts of RhoA and RhoC protein, and ROCK protein expression correlated positively with one another (P < 0.0001). High RhoA, RhoC, and ROCK expression were related to poor tumor differentiation (P < 0.05, P < 0.01, and P < 0.01, respectively), muscle invasion (P < 0.001), and lymph node metastasis (P < 0.05). Kaplan-Meier plots linked high RhoA, RhoC, and ROCK protein expression to shortened disease-free and overall survival (P < 0.0001). By univariate analysis, high RhoA, RhoC, and ROCK protein expression predicted shortened disease-free and overall survival (P < 0.0001). By multivariate analysis, only RhoC was independently influenced in disease-free survival (P < 0.05), and RhoA and RhoC in overall survival (P < 0.001). In contrast, RhoB expression was inversely related to the grade and stage (P < 0.05), and its higher expression is associated with better overall survival (P < 0.05). In superficial tumors (Ta or T1; 63 patients), RhoA, RhoC, and ROCK were unrelated with recurrence-free survival. Overall survival in tumors invading muscle (T2 to T4; 44 patients) was significantly influenced by RhoA, RhoC, and ROCK in a Kaplan-Meier analysis (P < 0.0001, P < 0.0001, and P < 0.01, respectively). Whereas RhoA, RhoC, and ROCK independently predicted shortened overall survival in patients with invasive tumor by univariate analysis (P < 0.0001, P < 0.0001, and P < 0.01, respectively), only RhoC did so by multivariate analysis (P < 0.05).

Conclusion: Rho/ROCK pathway apparently involved in occurrence and progression of bladder cancer may be valuable prognostic markers.

INTRODUCTION

Members of the Rho family of small GTPases are involved in regulation of a variety of cellular processes, including organization of the microfilament network, intercellular contact, and malignant transformation (1). Indeed, these events are interrelated. Specifically, subfamily of Rho family proteins have specialized action on the actin cytoskeleton. The Rho subfamily regulates formation of stress fibers and focal adhesions within cells. The Rac subfamily regulates formation of lamellipodia and membrane ruffling, whereas the Cdc42 subfamily regulates formation of filopodia (1, 2). Lamellipodia and filopodia are seen at the advancing aspect of motile cells, whereas retraction is seen on the opposite side (3). These alterations are accompanied by reorganization of the actin cytoskeleton within the cell. Rho-associated serine-threonine protein kinase, ROCK (4, 5), one of the best characterized downstream effectors of Rho, is activated when it selectively binds to the active GTP-bound form of Rho. Activated ROCK interacts with the actin cytoskeleton to promote stress-fiber formation and assembly of focal contacts (6).

Cancer cell migration is central to the process of metastasis. Here too, rearrangements of the actin cytoskeleton are involved. Rearranging the actin cytoskeletal proteins in response to Rho is important for the ability of tumor cells to metastasize (7). Overexpression of Rho has been linked to progression of human cancers (8–10). Thus, action of the Rho/ROCK pathway takes part in cancer progression by regulating actin cytoskeleton reorganization; indeed, a specific ROCK inhibitor was found to suppress the tumor growth and metastasis (11, 12). These observations suggest that the Rho/ROCK pathway may be a molecular target for prevention of cancer invasion and metastasis. As no data have been reported concerning the Rho/ROCK pathway in progression of bladder cancer, we used Western blotting to quantify protein expression for Rho and ROCK in bladder cancer tissue and non-neoplastic portions of the same resected specimen. Relationships between Rho and ROCK expression, and pathologic features of the tumors were examined.
as were influences of Rho and ROCK expression on patient survival. Such information might lead to improved protocols and case selection for adjuvant therapies.

MATERIALS AND METHODS

Patients and Tissue Preparation. Surgical specimens of bladder cancer obtained between 1994 and 2000 from 107 consecutive Japanese patients with newly diagnosed primary transitional cell carcinoma of the bladder were examined: 68 men and 39 women; 41 to 89 years old; mean age, 67.7 years. All of the patients routinely underwent a imaging studies (computed tomography and/or magnetic resonance imaging) before surgery of disease for preoperative staging. Postoperative follow-up ranged from 3 to 84 months (median, 45 months). Patients underwent surgery before receiving any other therapy. Transurethral resection was performed for superficial bladder tumors and a couple of nontumor epitheliums. During partial or total cystectomy, lymph nodes were resected. In all of the cystectomy cases, three sites of tumor, various portions of adjacent non-neoplastic bladder, and lymph nodes were resected for the study. Grading and staging were carried out according to the criteria of the Tumor-Node-Metastasis classification (13). The study was conducted in accord with the Helsinki Declaration. Institutional Review Board approval was obtained for this investigation. Each patient signed a consent form approved by the Committee on Human Rights in Research of our institution. Intravesical instillation of chemotherapeutic agents (mitomycin C, doxorubicin) and/or immunotherapy with Bacillus Calmette-Guerin was performed in cases where transurethrally resected superficial bladder tumors showed a high histopathologic grade. Systemic postoperative combination chemotherapy using methotrexate, vinblastine, Adriamycin, and cisplatin was administered for invasive disease that showed a high histopathologic grade and/or lymph node involvement.

Western Blotting. We dissected tumors and epitheliums by omitting stromal tissue. Western blotting was carried out as described previously (8). Briefly, 50 μg of cytosolic proteins were separated by SDS-PAGE (12.5% gel) for immunological detection of proteins. After transfer blotting from the gel to a nitrocellulose membrane, bound proteins were stained by Ponceau S to confirm that identical amounts of protein had been transferred. Rho and ROCK protein expression each was analyzed using specific antibodies (each diluted 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with peroxidase-conjugated antirabbit IgG, these proteins were visualized by chemiluminescence. The blotted membrane was scanned densitometrically with a PDI imaging scanner (Agfa Japan, Tokyo, Japan) and analyzed with NIH Image software. For quantitation of proteins, relative amounts of Rho and ROCK in tumors were expressed as a ratio of absorbance of bands from the tumor specimen to those from the corresponding normal tissue, which was set to 1.0, by densitometrical analysis as described previously (8). Mean values from three experiments were obtained for tumor, nontumor, and lymph node tissues, respectively (9).

Immunohistochemistry. Immunohistochemistry, using the same specific antibodies as antibodies for Western blotting, was performed to support the data obtained by Western blotting as described previously (8).

Statistical Analysis. Results of Western blotting were analyzed statistically using the Mann-Whitney U test as described previously (9, 10). The Spearman rank correlation coefficient was used to determine the relationship between protein expression for Rho and ROCK (14). Expression of Rho and ROCK protein, as well as tumor grade and stage of disease, were assessed in terms of survival by the Cox proportional hazards model using univariate and multivariate analysis. The
Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were assessed by the log-rank test. \( P < 0.05 \) were considered significant. Data were analyzed using commercially available software.

**RESULTS**

**Rho/ROCK Expression and Pathologic Characteristics.** RhoA protein was detected in tumor, nontumor bladder, lymph nodes with metastasis, and normal lymph nodes (Fig. 1). The amounts of protein for RhoA were significantly greater in cancerous (mean \( \pm \text{SD} = 3.07 \pm 0.94 \)) than in nontumor bladder tissue, defined as 1.0 (\( P < 0.0001 \); Fig. 2). Expression of protein for RhoA was greater in lymph nodes with metastases (3.66 \( \pm \) 0.98) than in uninvolved lymph nodes, defined as 1.0 (\( P < 0.0001 \); Fig. 2). Increased RhoA protein was associated with high-grade poor differentiation: grade 1, 2.53 \( \pm \) 0.37; grade 2, 2.91 \( \pm \) 0.79; and grade 3, 3.64 \( \pm \) 1.07 (\( P < 0.05 \); Fig. 3A). RhoA protein also was related to higher stage: pTa, 1, 2.61 \( \pm \) 0.41; pT2, 3.01 \( \pm \) 0.64; and pT3,4, 4.24 \( \pm \) 1.05 (\( P < 0.001 \); Fig. 3F).

RhoC, ROCK-I, and ROCK-II expression was significantly greater in primary tumor tissue (3.22 \( \pm \) 1.22, 1.79 \( \pm \) 0.49, and 4.13 \( \pm \) 1.34, respectively) than in nontumor tissue, defined as 1.0 (\( P < 0.0001 \); Fig. 2). RhoC, ROCK-I, and ROCK-II proteins were expressed more strongly in lymph nodes with metastases (4.08 \( \pm \) 0.95, 2.20 \( \pm \) 0.38, and 5.64 \( \pm \) 1.16, respectively) than in normal lymph node tissue, defined as 1.0 (\( P < 0.0001 \); Fig. 2). Higher RhoC and ROCK expression in tumors was associated with poor differentiation (RhoC, in grade 1 lesion, 2.47 \( \pm \) 0.26; grade 2, 3.14 \( \pm \) 1.19; and grade 3, 3.85 \( \pm \) 2.04; \( P < 0.01 \); Fig. 3C; ROCK-I, in grade 1 lesions, 1.42 \( \pm \) 0.28; grade 2, 1.77 \( \pm \) 0.43; and grade 3, 2.08 \( \pm \) 0.49; \( P < 0.01 \); Fig. 3D; and ROCK-II, in grade 1 lesions, 3.06 \( \pm \) 0.48; grade 2, 4.01 \( \pm \) 1.25; and grade 3, 5.04 \( \pm \) 1.35; \( P < 0.001 \); Fig. 3E). High expression also was related to muscle invasion (for RhoC, pTa and 1, 2.53 \( \pm \) 0.38; pT2, 3.31 \( \pm \) 0.84; pT3 and 4, 4.88 \( \pm \) 1.23; \( P <
involved lymph nodes (4.15 \text{ /H11006} abundant in primary tumors that invaded bladder muscle and 
and 4, 5.93 \text{ /H11006} ROCK-II, pTa and 1, 3.20 
 confidential interval; bars, \pm SD.

Fig. 3 Rho and ROCK expression in tumor. A–E, in grade 1 to 3 
tumors. F–J, in < pT1, pT2 and pT3 < tumors. The data show the 95% 
confidential interval; bars, \pm SD.

duties. Expression of ROCK-I and ROCK-II proteins in tumor tissue 
(respective correlation coefficients, r = 0.572, P < 0.0001, and 
and r = 0.643, P < 0.0001; Fig. 5), as well as for RhoC (r = 0.598, 
P < 0.0001, and r = 0.681, P < 0.0001, respectively; Fig. 5), 
but RhoB did not (data not shown).

Whereas RhoA, RhoC, and ROCK proteins were abun-
dantly expressed in tumor cells, these were very weakly ex-
pressed in nontumor cells in immunohistochemistry (Fig. 6). 
RhoB was more abundantly expressed in nontumor cells 
than in tumor cells (Fig. 6), and inversely related to the grade and stage 
(Fig. 3).

Rho/ROCK and Survival. The mean value for protein 
expression of RhoA in tumor samples was 3.07 ( \pm 0.94). As 
described in a previous study (10), cases were divided into two 
groups with expression above and below this mean, high 
expression (34 patients), and low expression (73 patients). Simi-
larly, we divided the cases into groups by the mean values for 
RhoC and ROCK protein expression, for RhoC (3.22 \pm 1.22), 
including 36 patients with high expression and 71 patients with 
low expression; for ROCK-I (1.79 \pm 0.49), including 45 
patients with high expression and 62 patients with low expression; 
for ROCK-II (4.13 \pm 1.34), including 41 patients with high 
expression and 66 patients with low expression.

Univariate disease-free survival according to the Cox pro-
portional hazards model was influenced significantly by RhoA, 
RhoC, ROCK-I, ROCK-II, stage, and grade (Table 1). By mul-
tivariate analysis, RhoC was an independent prognostic factor 
for disease-free survival. With regard to overall survival, 
whereas all of the factors analyzed were statistically significant 
by univariate analysis, only RhoC, RhoA, stage, and grade were 
significant by multivariate analysis (Table 1).

Comparison of Kaplan-Meier survival rate plots in patients 
with low versus high expression of RhoA protein linked high 
RhoA expression with shortened disease-free and overall sur-
(\text{P} < 0.0001; Fig. 7). Similarly, high expression of RhoC, 
ROCK-I, and ROCK-II was associated with poorer disease-free 
survival than low expression (P < 0.0001; Fig. 7). This also was 
true for overall survival (P < 0.0001; Fig. 7).

High expression of RhoB in 54 patients with a mean of 
0.81 ( \pm 0.15) was related to the better overall survival (Table 1; 
Fig. 7).

Cases of superficial disease (Ta and T1; 63 patients) were 
divided into those with RhoA expression above (27 patients) or 
below (36 patients) based on a mean of 2.61 ( \pm 0.41). In these 
superficial cases, a mean of RhoC was 2.53 ( \pm 0.38) with high 
expression in 27 patients and low expression in 36 patients. 
ROCK-I showed a mean of 1.48 ( \pm 0.29) with high expression 
in 35 patients and low expression in 28 patients, whereas for 
ROCK-II the mean was 3.20 ( \pm 0.49) with high expression 
in 29 patients and low expression in 34 patients. Expression of these 
proteins failed to affect recurrence-free survival in superficial 
tumors according to Cox and Kaplan-Meier analyses (data not 
shown).

RhoB, with a mean of 0.85 ( \pm 0.16), low expression in 28 
patients, and high expression in 35 patients, was not related to 
disease-free survival in superficial tumors (data not shown).

In tumor-invading muscle (T2 and higher; 44 patients), 
mean RhoA expression was 3.73 ( \pm 1.08), with high expression 
in 21 patients and low expression in 23 patients. For RhoC, the 
invasive cases showed a mean of 4.20 ( \pm 1.33), with high
expression in 24 patients and low expression in 20 patients; for ROCK-I, the mean was 2.22 (±0.39), with high expression in 26 patients and low expression in 18 patients; for ROCK-II, the mean was 5.45 (±1.29), with high expression in 30 patients and low expression in 14 patients. Kaplan-Meier plots showed that high expression of RhoA, RhoC, ROCK-I, or ROCK-II protein each had a significant influence on disease-free survival in the invasive tumor group (P = 0.0112, P = 0.0109, P = 0.0129,

Fig. 4 RhoA, RhoC, ROCK-I, and ROCK-II expression in metastatic lymph node tissues (mLNs) and its primary muscle invasion tumor (P+), and muscle invasion tumor without lymph node involvement (P−). The data show the 95% confidential interval.

Fig. 5 Spearman rank correlation coefficient relationship between expression levels of proteins for RhoA/RhoC and ROCK.
RhoB expression, with a mean 0.77 (±0.14), low expression in 25 patients, and high expression in 17 patients, was not linked to survival in the invasive tumor (data not shown).

**DISCUSSION**

Rho proteins act as intracellular molecular switches that transduce signals from extracellular stimuli to the actin cytoskeleton and the nucleus, regulating cell migration and malignant transformation. To take into account the possibility of interindividual variation in expression of proteins, we compared protein expression of Rho and ROCK between paired tumor and nontumor tissue samples from bladder and from lymph node. Protein expression for RhoA, RhoC, and ROCK were significantly greater in primary tumors and lymph node with metastases than in non-neoplastic bladder and normal lymph node samples. High RhoA, RhoC, and ROCK protein expression in tumors was associated with poor differentiation, muscle invasion, lymph node metastasis, and shortened survival. In contrast, RhoB expression was inversely related to grade and stage. To our knowledge, this is the first report concerning the relationship between Rho/ROCK and bladder cancer. The data suggest that the Rho/ROCK pathway may be associated with progression of bladder cancer.

Overexpression of Rho has been reported in a number of

<table>
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<th>Variable</th>
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<th>No. of patients</th>
<th>Analysis</th>
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<td></td>
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<td>Relative risk</td>
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<td>Grade</td>
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<td>0.925–2.811</td>
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<td>1.272</td>
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<td>RhoB</td>
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<td>M</td>
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<td>ROCK-I</td>
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<td>ROCK-II</td>
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<td>41/66</td>
<td>M</td>
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<td>2.508–8.017</td>
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* U, univariate.

* M, multivariate.
human cancers (8–10, 15). Furthermore, several lines of evidence directly link Rho to acquisition of a migratory, invasive, and metastatic phenotype (16). Rho is important for the ability of tumor cells to metastasize (7). We showed that RhoA, RhoC, and ROCK, but not RhoB protein expressions were greater in bladder tumors than in parts of the same bladder resection specimen containing no tumor.

RhoA, the prototype of Rho, and ROCK are best characterized in human cancers (11, 12, 14). High expression of RhoA facilitates translocation of this protein from the cytosol to the cell membrane, where it becomes activated and promotes invasion (17). Increased expression of Rho has been linked to higher stage in human cancers (8–10). ROCK mediates responses in the pathway initiated by Rho, and regulates dynamic reorganization of cytoskeletal proteins such as in formation of stress fibers and focal adhesions (18). Although at least two ROCK isoforms, ROCK-I and ROCK-II, may be expressed in the same species (19), differences in their roles in Rho-mediated signaling remain unclear. In the present study, a positive correlation was seen in tumor tissues between expression of RhoA and ROCK. Moreover, higher expressions of ROCK-I/ROCK-II, as well as RhoA in the primary lesion was associated not only with higher stages of disease, but also with poorer survival, in particular in tumors invading muscle. Whereas only weakly detectable in

Fig. 7 Survival curve in patients with bladder cancer based on the mean values of protein expression of Rho and ROCK in tumor tissues, the cases were divided into two groups at this level: high and low expression. Disease-free and overall survival curve in total 107 tumors. P was analyzed by log-rank test.
normal lymph nodes tissue, RhoA and ROCK were abundant in lymph node with metastasis. Furthermore, both were significantly more abundant in tumors invading muscle that showed lymph node involvement than in those without nodal involvement. Ability to enter either blood or lymphatic vasculature is necessary for tumor cells to metastasize to distant sites (20). RhoA and ROCK reportedly are required in both endothelial and the migrating cells for them to cross the vascular endothelium (21, 22).

RhoC, high homology to RhoA, has been shown to be the

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**Fig. 8** Survival curve in muscle invasive bladder cancer based on the mean values of protein expression of Rho and ROCK in tumor tissues, the cases were divided into two groups at this level: high and low expression. Disease-free and overall survival curve in 44 tumors. P was analyzed by log-rank test.
most associated with metastasis of cancer cells among Rho subfamily using high-density DNA microassays (23). Moreover, overexpression of RhoC has been associated with the progression of pancreatic ductal adenocarcinoma (10) and inflammatory breast cancer (24). It has been reported that overexpression of RhoC leads to increased expression of angiogenic factors (25), and that RhoC overexpression also promotes the ability of melanoma cells to exit the blood and colonize the lung (23). Therefore, taken together, the RhoA and RhoC/ROCK pathway may be a useful prognostic marker in bladder cancer.

RhoB also regulates actin organization. It is required for signaling apoptosis in transformed cells that are exposed to farnesyltransferase inhibitors, DNA-damaging agents, or Taxol. Genetic analysis in mice indicates that RhoB is dispensable for normal cell physiology, but that it has a suppressor or negative modifier function in stress-associated process, including cancer (26). As well as in bladder cancer shown in the present study, it has been reported that RhoB expression is readily detected in normal epithelium, carcinomas in situ, and well-differentiated tumors, but it becomes weak to undetectable as tumors become deeply invasive and poorly differentiated in human head and neck squamous cell carcinomas, and RhoA protein levels increase with tumor progression (27). Furthermore, we showed here that higher expression of RhoB was associated with favorable prognosis in bladder cancer. A negative role in growth control and/or transformation would contrast with the positive effects of RhoA and RhoC in these processes (26). These findings give additional support to the notion that RhoB may play a tumor-suppressive role in human cancers.

Although it is likely that RhoA, RhoB, and RhoC share common functions in regulating stress fiber formation (28), these proteins differ in subcellular location (29) and regulation of expression (30, 31). Therefore, abnormal regulation of expression patterns between members of the Rho subfamily in neoplastic cells may be involved in carcinogenesis and cancer progression.

We did not analyze the effect of treatment modalities on outcome of patients in the current study. Despite reports that tumor burden can be reduced for superficial tumors by intravesical chemotherapy and Bacillus Calmette-Guerin immunotherapy or systemic combined methotrexate, vinblastine, Adriamycin, and cisplatin chemotherapy in the case of invasive tumors, these outcomes were poorer than expected (32, 33). In the present study, higher expression of RhoA, RhoC, and ROCK was associated with shorter survival in bladder cancer. New treatment modalities such as cell migration inhibitors may be important possibilities to consider.

A specific ROCK inhibitor, Y-27632 (34, 35), has been reported to block both Rho-mediated activation of actomyosin and invasive activity of cultured rat MM1 hepatoma cells (12). Continuous treatment with this inhibitor reduced dissemination of MM1 cells implanted into the peritoneal cavity of syngeneic rats (11). These results suggested that ROCK inhibition may represent a way to prevent cancer invasion and metastasis by inhibiting cell migration and morphological alterations. We will need to study the effect of ROCK inhibitors on bladder cancer cells in vitro and in vivo. These results may suggest improved treatment strategies.

Mutations of Rho have not been identified in human tumors (10, 36, 37). Although mutational change in Rho genes was not within the scope of the current study, this should be investigated in the future.

Many downstream effectors are likely to be involved in the Rho signaling pathway (4). p140mDia, a mammalian homologue of the Drosophila diaphanous, controls actin polymerization (38). ROCK and p140mDia act cooperatively in the stress fiber formation to mediate Rho effects (39). Other possible downstream effectors and their roles need to be explored in bladder cancer. Rho family GTPases, including Rho, Rac, and Cdc42, have been shown to differentially and cooperatively contribute to triggering of invasive behavior in tumor cells (40). More complete understanding of how Rho family GTPases mutually and specifically interact in bladder cancer may throw light on the therapeutic approaches.

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