

# The Activity of RhoA is Correlated with Lymph Node Metastasis in Human Colorectal Cancer

Yoji Takami · Morihiko Higashi · Shinpei Kumagai ·  
Paul C. Kuo · Hidetada Kawana · Keiji Koda ·  
Masaru Miyazaki · Kenichi Harigaya

Received: 31 October 2006 / Accepted: 21 May 2007 / Published online: 28 June 2007  
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**Abstract** Rho family GTPases play a pivotal role in the regulation of numerous cellular functions associated with malignant transformation and metastasis. To evaluate the role of these GTPases in colorectal cancer, the protein expression levels and activities of these proteins in matched sets of tumor and non-tumor tissues of surgical specimens were analyzed. The relationship between the protein levels and activities in tumor tissues to the clinicopathological features was also assessed. The expression levels of RhoA, active RhoA, Rac1, and active Rac1 in tumor tissues were higher than in normal tissues. The amounts of active RhoA protein in primary tumors correlated with lymph nodes metastasis. No relationship was noted between the protein expression levels and other clinicopathological findings. These findings suggest that the Rho family small GTPases are related to malignant transformation and progression of colorectal cancer and the activation of RhoA is associated with the lymph node metastasis.

**Keywords** Colorectal neoplasms · RhoA · Rac1 · Metastasis

## Introduction

Colorectal cancer is one of the most common malignant tumors in the world and the third leading cause of cancer death in Japan. The most important factor that affects the prognosis is the presence and development of metastasis. Cancer metastasis is a complicated multistep cascade that involves cancer-cell detachment from the primary site, invasion, and attachment to the target metastatic organ. In this cascade, Rho family small GTPases are suggested to play important roles [1]. In these Rho-dependent processes, cell motility and invasion are required.

Recently, it has been reported that the Rho family small GTPases act as intracellular molecular switches that regulate cell motility [2]. In malignant tumors, Rho family small GTPases are thought to play important roles in the process of tumor progression and metastasis [3–7]. Small GTPases are known to exist in two states: the inactive-GDP-bound form and the active-GTP-bound form which can perform its functions [8]. Although some clinical reports indicate that levels of Rho family proteins correlate with tumor progression and metastasis, little is known about the activities of Rho family proteins in colorectal cancer [3–7].

In the present study, we investigated the activities and expression levels of Rho family proteins including, RhoA, and Rac1 in advanced colorectal cancer, and evaluated the relationship between Rho family proteins and clinicopathological findings of advanced colorectal cancer.

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Y. Takami · M. Miyazaki  
Department of General Surgery, Chiba University Graduate  
School of Medicine, Chiba, Japan

M. Higashi (✉) · S. Kumagai · H. Kawana ·  
K. Harigaya  
Department of Molecular and Tumor Pathology, Chiba  
University Graduate School of Medicine, 1-8-1 Inohana,  
Chuo-ku, Chiba 260-8670, Japan  
e-mail: higashi@faculty.chiba-u.jp

P. C. Kuo  
Division of General/Transplant Surgery, Duke University  
Medical Center, Durham, NC, USA

K. Koda  
Department of Surgery, Teikyo University Chiba Medical  
Center, Chiba, Japan

## Materials and methods

### Patients and tissue preparation

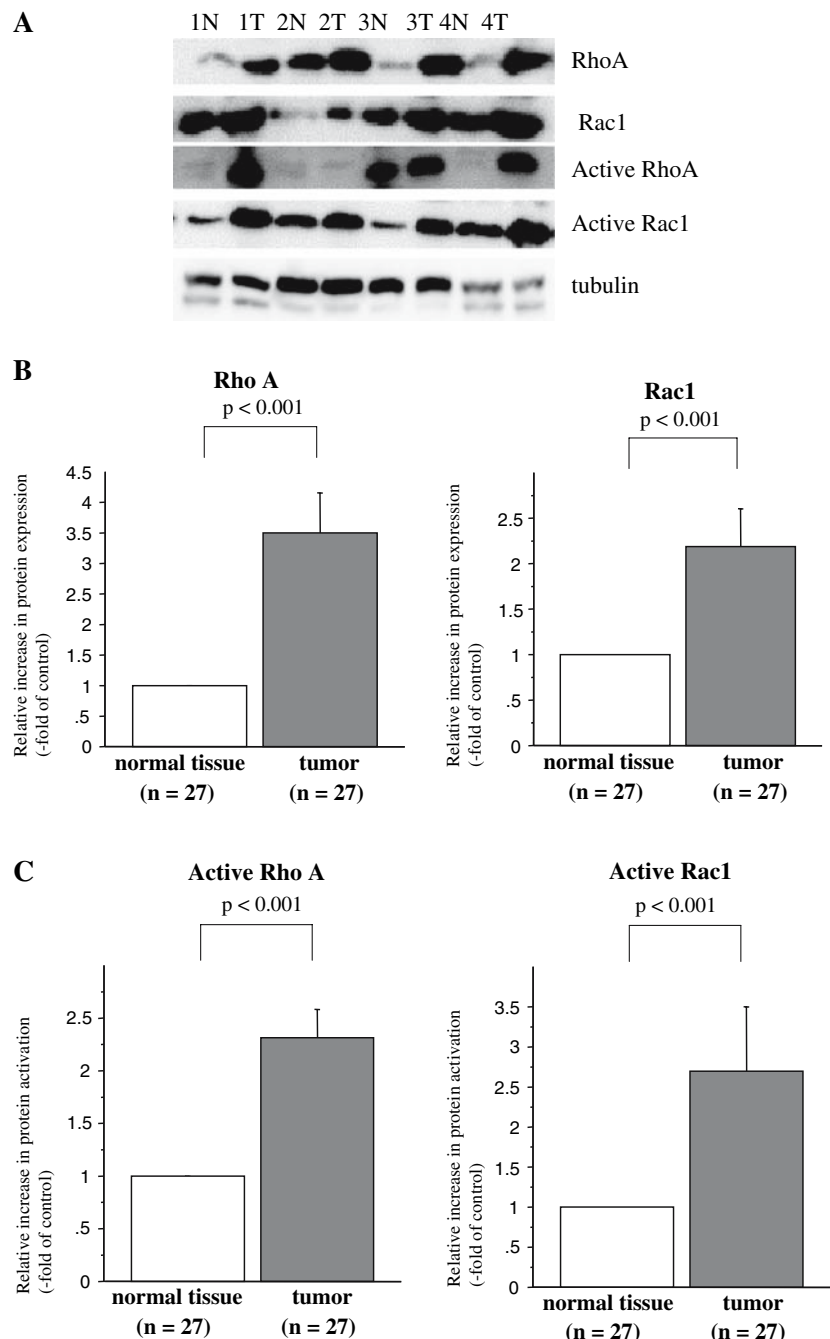
Surgical specimens of advanced colorectal cancer obtained from 27 consecutive Japanese patients with newly diagnosed primary colorectal cancer between October 2001 and April 2005 were examined. The patients consisted of 19 men and 8 women, aged 35–81 years, with a mean age of 64.5 years. Normal tissues were dissected sufficiently away from the tumors and only mucosal tissues were obtained to

avoid the contamination of stromal tissue. The study was conducted in accordance with the Helsinki declaration. The university ethics committee approved this study and all patients provided written informed consent.

### Western blotting

Proteins of tumor and nontumor tissues were extracted in lysis buffer (50 mM Tris-HCl pH 7.5, 25 mM NaCl, 1% Nonidet P-40, and a protease inhibitor mixture) at 4°C for 30 min. The lysate was obtained by centrifugation at

**Fig. 1** Expression and activity levels of RhoA and Rac1 proteins in colorectal cancer. (A) Each number represents a case of colorectal cancer and adjacent nontumor tissue. N, normal tissue; T, tumor. (B) Histogram of relative expression of RhoA and Rac1. Both of the proteins were overexpressed in tumor tissues. Bars: mean  $\pm$  SE. (C) Histogram of relative activity of RhoA and Rac1. Both of the proteins were activated in tumor tissues. Bars: mean  $\pm$  SE



12,000 × *g* for 5 min, and the supernatants were transferred to fresh tubes. Fifty micrograms of cytosolic proteins were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (12.5%). After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, USA). The expression levels of RhoA and Rac1 proteins were analyzed using specific antibodies (RhoA; sc-179, Santa Cruz, Rac1; clone102, BD BioScience,  $\beta$ -tubulin; sc-9104, Santa Cruz). After incubation with peroxidase-conjugated second antibodies, proteins were visualized by chemiluminescence. The blotted membranes were scanned densitometrically using a Fuji LAS1000 Plus charge-coupled device (CCD) camera (Fuji Photo Film Co., Japan) and analyzed with Image Gauge software (Fujifilm). To quantify protein levels, the relative amounts of RhoA and Rac1 were related to those of the corresponding normal tissues, which were set to a value of 1.0, by densitometrical analysis as described previously [4]. The mean values from three sections of tumor and nontumor tissues were calculated.

#### Pull-down assay

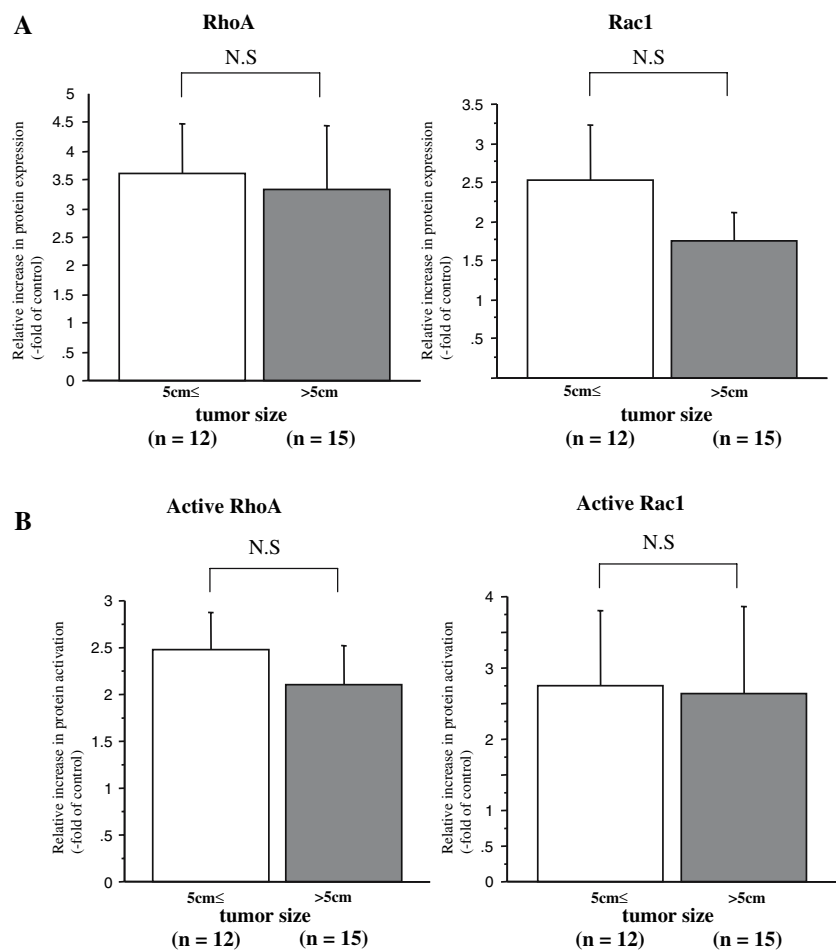
The levels of the active small GTPases RhoA and Rac1 in the GTP-bound forms were measured with pull-down assays. The pull-down assays were carried out as previously described [9]. Briefly, GTP-bound RhoA or GTP-bound Rac1 GTPases were precipitated by mDia or PAK (p21-activated kinase-1), respectively, each fused to glutathione-S transferase precoupled to glutathione-Sepharose beads. These GTP-bound fractions were detected by Western blot analysis, as described above.

#### Statistical analysis

Data are expressed as mean  $\pm$  standard deviation (SD) unless otherwise noted.

Results of Western blotting were statistically analyzed using the Mann–Whitney *U* test. The relationship between Rho protein expression and clinicopathological factors were analyzed using the  $\chi^2$  test and Student's *t* test. Data were analyzed using the Stat View software package

**Fig. 2** Tumor size and RhoA/Rac1 expression. No statistically significant differences were seen between tumor size and the expression of RhoA/Rac1



(Abacus Concepts, Inc.), and the findings were considered significant for  $P$  values  $< 0.05$ .

## Results

### Expression of RhoA and Rac1 is increased in colorectal cancer

To test whether the expression of Rho family proteins were altered in advanced colorectal cancer, we analyzed the expression levels of these small GTPases with Western blot analysis. As shown by the representative cases in Fig. 1A, protein expression of RhoA and Rac1 was detected in both tumors and normal tissues. The amounts of these proteins were significantly higher in cancer tissues,  $3.49 \pm 0.49$  (RhoA) and  $2.18 \pm 0.26$  (Rac1), ompared to normal samples, defined as 1.0 ( $P < 0.001$ ; Fig. 1B).

### RhoA and Rac1 are activated in colorectal cancer

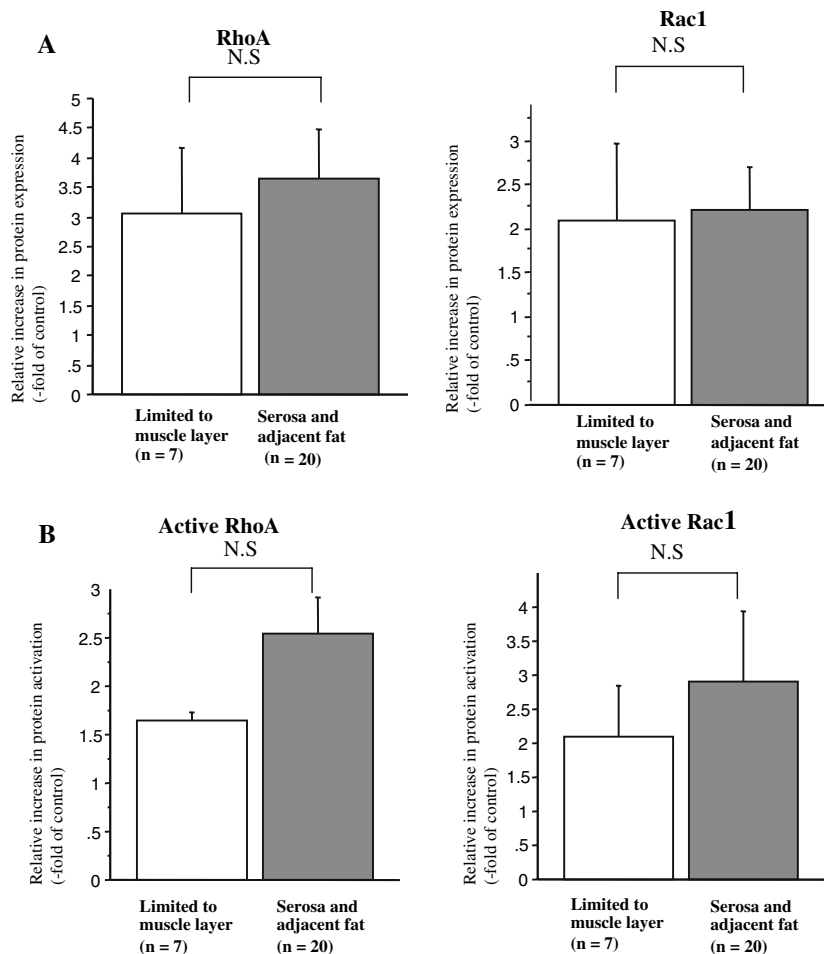
To examine whether the observed increase in protein expressions were accompanied by activities of these pro-

teins, pull-down assays were performed. As shown in Fig. 1, activation of RhoA and Rac1 in cancer tissues was significantly higher than in the normal controls, with means of  $2.37 \pm 0.48$  and  $2.74 \pm 0.49$ , respectively ( $P < 0.001$ ; Fig. 1C).

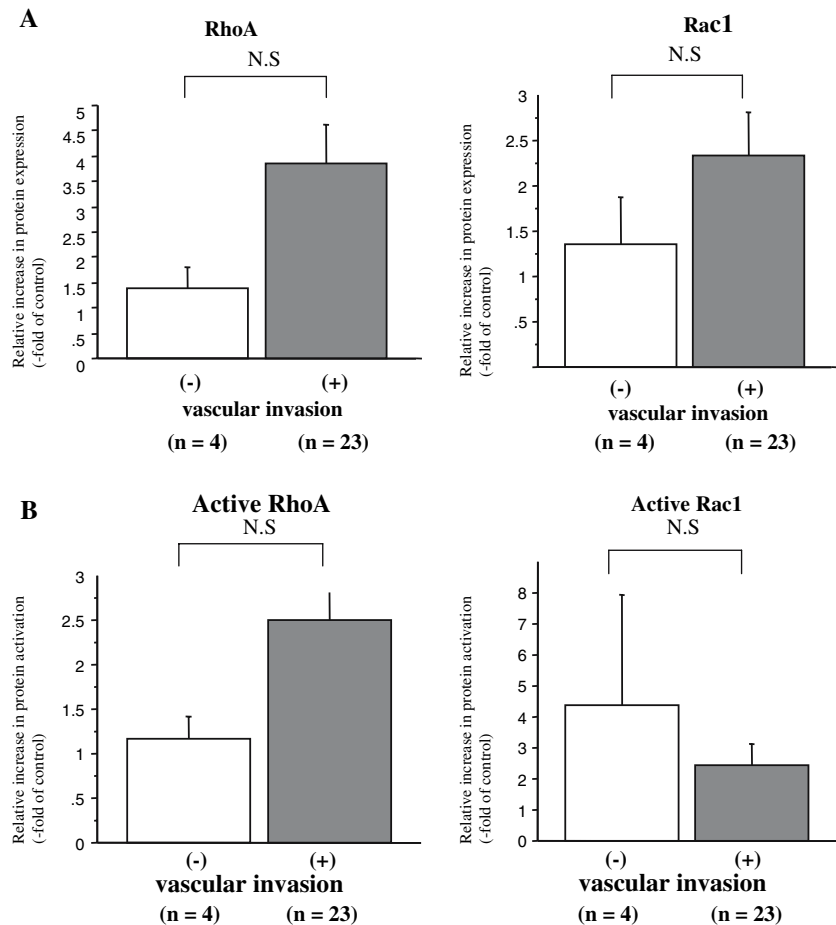
### Activation of RhoA is correlated with lymph node metastasis

To determine the role of Rho family GTPases expression and activation in colorectal cancer, we examined the correlation of RhoA/Rac1 expression and activation with the clinical features. The expression levels of RhoA and Rac1 proteins had no correlation with maximum tumor size (Fig. 2), depth of cancer invasion (Fig. 3), histological grade or blood levels of carcinoembryonic antigen (CEA, data not shown). The expression and activation of RhoA tended to correlate with the presence of vascular invasion (Fig. 4). However, this did not reach statistical significance, possibly as the result of the limited number of patients. The expression of RhoA and the activity of this protein were correlated with the presence of lymph node metastasis (Fig. 5;  $P < 0.001$ ). The mean value for

**Fig. 3** Depth of tumor invasion and RhoA/Rac1 expression. No statistically significant differences were seen between tumor depth and the expression of RhoA/Rac1



**Fig. 4** Vascular invasion and RhoA/Rac1 expression. No statistically significant differences were seen between vascular invasion and the expression of RhoA/Rac1



the active RhoA in the tumor samples was  $2.37 \pm 1.48$ . Cases were divided into two groups, with activity above and below this mean, respectively. As shown in Table 1, the incidence of lymph node metastasis in the high-activity group (9 of 10, 90%) was significantly higher ( $P < 0.001$ ) than that in the low-activity group (2 of 17, 12%).

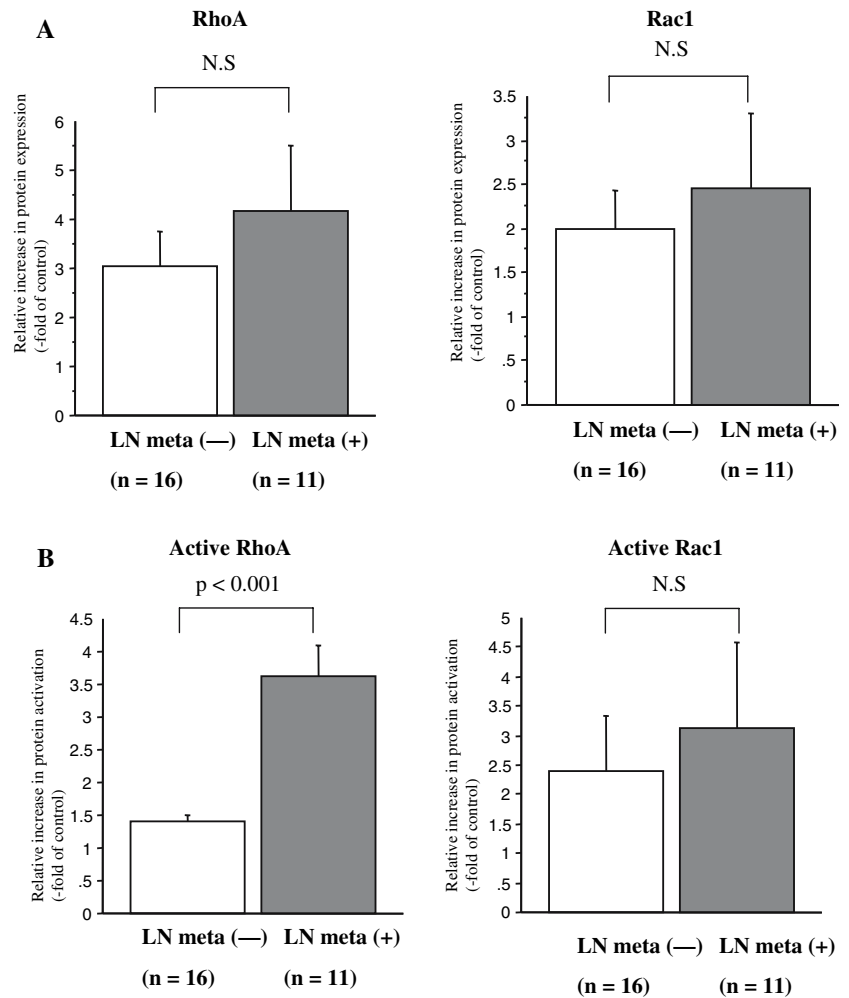
## Discussion

The Rho family small GTPases act as intracellular molecular switches that transduce signals from extracellular stimuli to the actin cytoskeleton and the nucleus, and regulate cell migration and malignant transformation [2]. In the present study, we investigated the expression of Rho family small GTPases in colorectal cancer. The expression of RhoA and Rac1 was increased in cancer tissues when compared with normal mucosa. These results were consistent with the previous reports examining Rho and Rac in various cancers [4–7, 10]. Our results also showed that the activity of RhoA and Rac1 was upregulated in cancer tissues. To our knowledge, this is the first report of the

relationship between the activities of Rho family proteins and colorectal cancer.

Since activation of RhoA and Rac1 was observed in colon cancer, we hypothesized that the activities of these small GTPases might be a marker of malignancy and progression of colorectal cancer. To provide support this hypothesis, we evaluated the relationship between the activities of these proteins and clinicopathological factors. Interestingly, activation of RhoA protein in tumors with lymph node involvement were greater than in those that did not metastasize. These results suggest that the activation of RhoA may play an important role in lymph node metastasis. There are several possible mechanisms. First, since RhoA is a key regulator of cell motility, RhoA might upregulate tumor-cell motility. Second, in gastric cancer, Rho-mediated NF $\kappa$ B activation confers resistance to apoptosis [11]. It is possible that the same mechanism (as in gastric cancer) is acting in colorectal cancer. Third, Vasiliev et al. recently reported that the overexpression of Rho leads to mitosis-associated detachment of cells from epithelial sheets [12]. The activation of RhoA might cause the detachment of primary tumor cells in colorectal cancer.

**Fig. 5** Lymph node (LN) metastasis and RhoA/Rac1 expression. RhoA was activated in the tumor samples with lymph node metastasis



**Table 1** Clinicopathological features and activity of RhoA protein in 27 patients with colorectal carcinoma

Variables	High expression (n = 10)	Low expression <sup>a</sup> (n = 17)	P
Sex (male:female)	7:3	12:5	N.S.
Age	67.6 ± 10.2	62.8 ± 13.9	N.S.
Site			N.S.
Colon	4	9	
Rectum	6	8	
Histological grade <sup>b</sup>			N.S.
G1	0	4	
G2	9	12	
G3	1	1	
Lymph node metastasis			P < 0.001
Absent	1	15	
Present	9	2	
Vascular invasion			N.S.
Absent	5	4	
Present	5	13	
Tumor size			N.S.
≥5 cm	6	6	
<5 cm	4	11	

**Table 1** continued

Variables	High expression ( <i>n</i> = 10)	Low expression <sup>a</sup> ( <i>n</i> = 17)	<i>P</i>
Tumor depth			N.S
Limited to muscular layer	1	5	
Serosa and adjacent fat	9	12	
CEA (Carcinoembryonic antigen)			N.S
≥5	6	8	
<5	4	9	

<sup>a</sup> High- and low-expression groups were determined by a mean value of active RhoA protein (2.37)

<sup>b</sup> G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma

Although many diagnostic advances have occurred, it is still difficult to diagnose lymph nodes metastasis accurately in colorectal cancer [13–15]. The assessment of RhoA activity may be a helpful marker and indication for the presence of lymph nodes metastasis and guidance of subsequent therapy.

**Acknowledgements** We wish to thank S. Narumiya for pGEX-4T-1-mouse mDia RBD; T. Sato for pGEX-PAK-CRIB. We also thank T. Umemiya and M. Azuma for technical assistance.

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