Higher Expression of RhoC Is Related to Invasiveness in Non-Small Cell Lung Carcinoma

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ABSTRACT

Purpose: The activation of Rho proteins has been shown to lead to loss of polarity in cancer cells, as well as reorganization of the cytoskeleton and facilitation of cell motility, possibly resulting in their malignant potential. The clinicopathological significance of RhoC, however, is not yet well known in the case of non-small cell lung cancer (NSCLC).

Experimental Design: The intratumor expression level of RhoC mRNA was determined and compared with that in adjacent nontumorous lung tissue using quantitative reverse transcription-PCR in 49 patients with NSCLC. The relationship between the level of RhoC transcript and clinicopathological factors was examined. RhoC protein expression was confirmed by immunohistochemistry and Western blot analysis in several cases.

Results: Tumor tissue of NSCLC patients demonstrated a copy number of RhoC mRNA that was well correlated with its protein level in each case and was significantly higher than that found in the corresponding nontumorous lung tissue (2.73 × 10^5 versus 1.13 × 10^4 copies/0.08 µg mRNA; P < 0.05). Histopathologically positive cases of lymphatic permeation showed a significantly higher copy number of RhoC than negative cases (4.31 × 10^5 versus 1.93 × 10^5 copies/0.08 µg mRNA; P < 0.05). With regard to venous permeation, the RhoC copy number in positive cases tended to be higher than that seen in negative cases (3.72 × 10^5 versus 2.14 × 10^5 copies/0.08 µg mRNA; P = 0.06).

Conclusions: This is the first demonstration that the expression level of RhoC is correlated to vascular permeation in NSCLC.

INTRODUCTION

Lung cancer is currently a major cause of death due to malignancies in Japan. Among the histological subtypes of pulmonary tumors, NSCLC\(^2\) accounts for a large portion of the patient population, and advanced NSCLC associated with metastasis to the lymph nodes and/or distant organs via vascular permeation remains intractable because adjuvant therapies, including chemoradiotherapy, are less effective. Therefore, more knowledge regarding the genetic and molecular mechanisms of tumor metastasis in NSCLC has been much desired.

To date, a number of small GTP-binding proteins have been identified and are thought to be involved in the signal transduction pathway that controls a diverse set of essential cellular functions such as cell growth, cell differentiation, cytoskeletal organization, intracellular vesicle transport, and secretion (1). The Rho family, a member of the Ras superfamily of small GTP-binding proteins, has specifically been shown to be ADP-ribosylated by exo-enzyme C3 from Clostridium botulinum, resulting in its inactivation (2–4). Activation of Rho proteins leads to the assembly of actin-myosin contractile filaments into focal adhesion complexes that lead to cell polarity and facilitate motility (5–8), as well as to the formation of lamellipodia and adhesion during directed motility (9–11).

Recently, it has been demonstrated that overexpression of RhoC enhanced the metastasis, invasion, and cell morphology of tumor cells and that the dominant-inhibitory Rho mutant was capable of inducing metastasis (12). The relationship between the expression level of RhoC and disease aggressiveness has also been shown in clinical breast cancer (13–15) and pancreatic cancer (16); however, clinical information regarding RhoC expression in NSCLC is sparse.

To determine the possible role of RhoC expression in NSCLC, the present study quantified the RhoC mRNA of NSCLC tissue and examined its significance with respect to clinicopathological factors, especially the invasiveness of tumor cells.

MATERIALS AND METHODS

Patients. Between January 1996 and April 2000, 49 Japanese patients with NSCLC underwent surgical resection at the Department of Surgery II, Kyushu University Hospital (Fukuoka, Japan). No patient had received antitumor treatment before surgery. Tumor and normal lung tissue samples were freshly obtained from resected specimens, frozen immediately in liquid nitrogen, and stored at −80°C until the following experiments.

Immunohistochemistry. Frozen, nonfixed tissue sections (diameter, 5 µm) were fixed with acetone and stored at −20°C. After thawing, the sections were rinsed with 1% hydrogen peroxide for 20 min and subsequently rinsed twice with PBS. After an incubation period of 24 h at 4°C with goat anti-RhoC antibody (diluted 1:400 in PBS; Santa Cruz Biotechnology), the sections were rinsed twice with PBS, and biotiny-
lated secondary antibody (diluted 1:250 in PBS) was added at room temperature for 30 min. After application of avidin-biotin staining at room temperature for 30 min, RhoC protein was visualized using diaminobenzidine (17).

**Western Blot Analysis.** The tissue samples of homogenates derived from normal lung or cancer tissue were treated with detergent solution [62.5 mM EDTA, 50 mM Tris (pH 8.0), 0.4% deoxycholic acid, and 1% NP40] and subjected to SDS-PAGE. Protein on the gel was transformed electrophoretically to a nitrocellulose membrane sheet at 280 mA for 30 min using semi-dry blotting methods. The sheet was then washed intensively with TBS and incubated with the polyclonal antihuman RhoC antibody (1:1000 dilution; Santa Cruz Biotechnology) at 4°C for 8 h. After washing three times with TBS, the membrane was incubated with peroxidase-conjugated goat antibodies against goat IgG at room temperature for 40 min. After the reaction, a semiquantification of RhoC protein was performed by densitometry analysis using the NIH Image program.

**Culture Cell Lines.** The lung cancer cell lines PC-9 (adenocarcinoma), QG95 (squamous cell carcinoma), and A549 (bronchioloalveolar carcinoma) were maintained in continuous culture in RPMI 1640 supplemented with 100 units/ml penicillin-streptomycin and 10% fetal bovine serum. These cell lines were washed, and cell pellets were collected, frozen in liquid nitrogen, and stored at –80°C until RNA extraction.

**RNA Extraction and First-Strand cDNA Synthesis.** Total RNA of cultured cells growing exponentially was extracted using ISOGEN (Nippon Gene, Inc., Tokyo, Japan) according to the manufacturer’s protocol. Total RNA of clinical tissue was isolated using the same method. Synthesis of cDNA was done with 2.0 μg of total RNA using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech).

**Real-Time Quantitative RT-PCR.** Real-time RT-PCR is a sensitive, quantitative, and highly reliable method for RNA quantification. The theoretical basis of the method has been described previously (18–20). Gene expression was measured using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Corp., Foster City, CA). Primers and Taqman Probes (Custam Oligonucleotide Factory, Foster City, CA) were designed to span exon-intron junctions to prevent amplification of genomic DNA and also to result in amplicons of fewer than 200 bp to enhance the efficiency of PCR amplification. Probes were labeled at the 5’-end with the reporter dye molecule 6-carboxyfluorescein (emission $\lambda_{\text{max}} = 582$ nm) and at the 3’-end with the quencher dye molecule 6-carboxytetramethyl-rhodamine (emission $\lambda_{\text{max}} = 582$ nm). Upon amplification, probes annealed to the template are cleaved by the 5’-nuclease activity of the Taq polymerase reaction. This process separates the fluorescent label from the quencher and allows the release of 1 unit of fluorescence for each unit of amplification. By determining the amount

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**Fig. 1** Immunohistochemical analysis for RhoC protein. Each panel shows an immunohistochemical staining reaction using an anti-RhoC protein antibody for the representative cases. The cytoplasm of tumor cells was highly stained for RhoC protein in both squamous cell carcinoma (b, ×20; d, ×50) and adenocarcinoma (c, ×20; e, ×50). RhoC was not detected in the normal lung epithelium (a, ×20).
of fluorescence with each cycle, it is possible to determine the number of cycles necessary to reach a chain amount of fluorescence in a test sample compared with known standard amounts of template provided as a standard curve. DNA strands were generated by PCR amplification of gene products, purification, and quantification by spectrophotometry (absorbance at 260 nm). The number of copies was calculated with the use of the molecular gene’s weight. Real-time PCR of cDNA specimens and DNA strands was conducted in a total volume of 25 μl of with 1× Taqman Master Mix (Perkin-Elmer Corp.) and primers at 15 μM and probes at 10 μM. The primer sequences were as follows: forward primer, 5′-TTTTCATGTTTCGTCAAG; reverse primer, 5′-CAGGATGACATCAGTGTCG; and Taqman probe, CTTTGAGAACTATATTGCGGACATTGAGGTG. Thermal cycler parameters included a 2-min incubation at 50°C, 10 min at 95°C, and 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C at 10 min. Each sample was analyzed in duplicate, and a calibration curve constructed using a 10-fold serial dilution of a total extracted from A549 cells was run parallel with each analysis. The coefficient of correlation was \( r = 0.97 \), and the slope was constant in each experiment. As internal controls, the same samples were tested for \( \beta \)-actin mRNA (Perkin-Elmer Corp.) in the same manner.

**Statistical Analysis.** For statistical analyses, StatView-J 5.0 software (SAS Institute Inc.) was used. The relationship between RhoC mRNA expression and clinicopathological factors was examined using Mann-Whitney \( U \) tests.

**RESULTS**

**Detection of RhoC Protein in NSCLC Tissues.** Cytoplasm of tumor cells was highly stained for RhoC protein in both squamous cell carcinoma (Fig. 1, b and d) and adenocarcinoma (Fig. 1, c and e). RhoC was not detected in the normal lung epithelium (Fig. 1a) or the stromal cells of the tumor tissue.

**Expression of RhoC mRNA in Cell Lines and Clinical NSCLC Tissues.** All lung cancer cell lines tested (PC-9, QG95, and A549) expressed the band corresponding to the RhoC amplicon (Fig. 2). To confirm the genome of RhoC, sequence analysis for the amplicon was performed. This result revealed that lung cancer cells readily express RhoC mRNA. RhoC mRNA expression of tumor and normal tissues from 49 patients was quantified by quantitative RT-PCR. RhoC mRNA expression was found to be significantly higher in tumor tissues than in normal tissues, with mean values of \( 2.73 \times 10^4 \) copies/0.08 μg mRNA in tumor tissues and \( 1.1 \times 10^4 \) copies/0.08 μg mRNA in normal tissues (\( P < 0.05 \)).

**Fig. 2** Expression of RhoC mRNA in lung cancer cell lines. RhoC amplicons of 180 bp were detected in all cell lines (A549, PC-9, and QG56).

**Fig. 3** Detection and quantification of RhoC mRNA in lung cancer tissues. RhoC mRNA expression in tumor and paired normal tissues from 49 patients was quantified by quantitative RT-PCR. RhoC mRNA expression was found to be significantly higher in tumor tissues than in normal tissues, with mean values of \( 2.73 \times 10^4 \) copies/0.08 μg mRNA in tumor tissues and \( 1.1 \times 10^4 \) copies/0.08 μg mRNA in normal tissues (\( P < 0.05 \)).

**Fig. 4** Analysis for detection of RhoC protein in lung cancer tissues. Protein lysates derived from tumor tissues (T) and paired normal lung tissues (N) were subjected to analysis. A band of \( M_r 24,000 \) was observed in each lane. The ratio of RhoC protein [tumor/normal (T/N) ratio of RhoC protein] calculated by densitometry is shown.
patients was quantified by quantitative RT-PCR. RhoC mRNA expression was significantly higher in tumor tissues than in normal tissues (Fig. 3). Data are shown as copy number/0.08 μg total mRNA. The mean values were 27.3 × 10^4 copies/0.08 μg total mRNA in tumor tissues and 1.1 × 10^4 copies/0.08 μg total mRNA in normal tissues (P < 0.05). We confirmed that the level of β-actin expression, which served as the internal control, was almost the same expression in tumor and normal lung tissues (data not shown).

To confirm the expression of RhoC protein, we performed Western blot analysis. As shown for eight pairs of normal versus tumor tissues from the lung, the amount of RhoC protein was determined using densitometry. A band of M_r 24,000 was observed in each lane. Lung cancer tissues expressed RhoC protein at significantly higher levels than normal tissues in all cases tested (Fig. 4). The means of the ratio were 4.05 for protein and 80.2 for mRNA, and those level of tumor tissue was consistently higher than normal tissue in eight cases.

**Relationship between the Expression of RhoC mRNA and Clinicopathological Factors.** We examined the relationship between the expression level of RhoC mRNA and clinicopathological factors (Table 1). There were no significant differences in copy number of RhoC mRNA among subgroups according to gender, pack-year index, histology, and pathological stage. With a special reference to tumor invasiveness, the mean copy number of RhoC mRNA was 4.31 × 10^6 copies/0.08 μg total mRNA for positive lymphatic permeation and 1.93 × 10^5 copies/0.08 μg total mRNA for negative lymphatic permeation (P = 0.002), whereas that of RhoC mRNA was 3.72 × 10^5 copies/0.08 μg total mRNA for positive venous permeation and 2.14 × 10^5 copies/0.08 μg total mRNA for negative venous permeation (P = 0.066). For pleural invasion, the mean copy number of RhoC mRNA was 2.9 × 10^5 copies/0.08 μg total mRNA for positive pleural invasion and 2.4 × 10^5 copies/0.08 μg total mRNA for negative pleural invasion (P = 0.27).

**DISCUSSION**

In the present study, we demonstrated the substantial expression of RhoC protein as well as the objective higher expression of RhoC in NSCLC by quantitative real-time RT-PCR. To the best of our knowledge, this is the first demonstration of the positive correlation of RhoC expression with invasion to lymphatic vessels in tissue samples from patients with NSCLC.

Recent intensive studies have demonstrated several upstream pathways that activate Rho as well as downstream targets of activated Rho (21–25). The activity of Rho forming stress fibers and focal adhesion is mediated by the phosphorylation and activation of two of these targets, Rho-kinase/ROK/ROCK (6, 24, 26) and the myosin-binding subunit of myosin phosphatase (27, 28). Activated Rho kinase directly phosphorylates the myosin light chain, which regulates the formation of stress fibers and focal adhesion contacts and leads to cell membrane ruffling and cell motility (29). Therefore, expression of Rho might be expected to be not only a good candidate marker for invasive and proliferative tumor cells, including those in NSCLC, but also a molecular target of these cells.

Recent studies suggest a possible link between RhoC expression and tumor progression in both breast cancer (13–15) and ductal adenocarcinoma of the pancreas (16). Kleer et al. (13) have demonstrated the significant correlation of RhoC expression, tumor stage, and lymph node metastasis; interestingly, they suggested that RhoC could be a specific molecular indicator for detecting invasive carcinoma with metastatic potential among breast cancers. Additionally, Van Golen et al. (14, 15) also reported that the expression level of the RhoC gene was associated with rapid growth in inflammatory breast cancers; similar findings have also been reported in metastatic adenocarcinomas of the pancreas (16). These studies thus suggest that the expression level of the RhoC gene is associated with the invasive potential of cancer cells, and the present study on NSCLC also supports these findings. Furthermore, all of these studies, including this paper, suggest that RhoC could be a potential therapeutic target for lung cancer.
including ours, also suggest that Rho-related intracellular signals might definitively determine the metastatic potential of malignant neoplasms. Further research is therefore critical.

In the present study, we first demonstrated that the expression level of RhoC mRNA is positively correlated with the invasiveness of NSCLC, which was histologically detected as lymphatic and vascular permeation. Whereas lymphatic permeation and venous permeation are independent prognostic factors in patients with stage I NSCLC (30, 31), overexpression of RhoC may be a significant determinant of the aggressiveness of the disease. The present study is limited in that it includes no assessment of the role of RhoC expression in the long-term prognosis of patients; such an assessment was impossible to carry out due to the limited availability of fresh NSCLC tissue stocks in our laboratory. Our plans for future research therefore include an investigation of the patients’ prognosis at each stage of NSCLC and its subgroups.

In summary, this is, to the best of our knowledge, the first report indicating that RhoC could be both a biological determinant of and a molecular marker for vessel invasiveness of NSCLC. Because a recent experimental study demonstrated that the molecular targeting of RhoC using retroviruses expressing dominant-inhibitory Rho mutant diminished invasive activity in vitro as well as metastatic potentials in vivo (12), a similar strategy, including not only dominant-inhibitory Rho mutant but also RhoC-inhibitory compounds, could provide an efficient anticancer therapy against RhoC-expressing cancer cells by limiting their invasive potential.

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