Cancer Chemother Pharmacol (2012) 69:1545–1555
DOI 10.1007/s00280-012-1862-6

ORIGINAL ARTICLE

The Rho kinase inhibitor fasudil is involved in p53-mediated apoptosis in human hepatocellular carcinoma cells

Yuko Takeba · Naoki Matsumoto · Minoru Watanabe · Sachiko Takenoshita-Nakaya · Yuki Ohta · Toshio Kumai · Masayuki Takagi · Satoshi Koizumi · Takeshi Asakura · Takehito Otsubo

Received: 5 November 2011 / Accepted: 12 March 2012 / Published online: 6 April 2012
© Springer-Verlag 2012

Abstract
Purpose Rho kinase is an important factor in tumor progression. We demonstrated that Rho kinase-associated coil-containing protein kinase (ROCK) is expressed in hepatic tissues in hepatocellular carcinoma (HCC) and confirmed its roles in cell survival in HCC cells using the ROCK inhibitor, fasudil.
Methods ROCK protein levels were estimated in hepatic tissues with HCC compared with healthy liver tissues or hepatic hemangioma tissues using immunohistochemistry. Furthermore, HepG2 and Huh7 cells were cultured with ROCK inhibitor, fasudil for 24 h in vitro. Cell proliferation was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay, and apoptotic cells were detected by cell death ELISA. The expression apoptosis-related proteins were analyzed using Western blotting.
Results Fasudil significantly decreased cell proliferation and induced apoptosis mediated by increases in p53, Bax, caspase-9, and caspase-3 in HepG2 and Huh7 cells. The induction of apoptosis was inhibited in HCC cells precultured with p53 decoy oligodeoxynucleotide.
Conclusion These results suggest that ROCK inhibits the p53-mediated apoptosis pathway in HCC. Fasudil may thus be a beneficial approach to HCC therapy.

Keywords Rho kinase · Fasudil · Apoptosis · p53 · Hepatocellular carcinoma

Y. Takeba (✉) · N. Matsumoto · S. Takenoshita-Nakaya · Y. Ohta
Department of Pharmacology, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan
e-mail: takebay@marianna-u.ac.jp

M. Watanabe
Institute of Experimental Animals, St. Marianna Graduate School of Medicine, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan

T. Kumai
Department of Pharmacogenetics, St. Marianna Graduate School of Medicine, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan

M. Takagi
Department of Pathology, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan

S. Koizumi · T. Asakura · T. Otsubo
Department of Surgery, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer mortality. HCC is primary derived from intrahepatic metastasis and carcinomatous invasion rather than lymph node and distant metastasis [1]. Intrahepatic metastasis of HCC is frequently observed with disease progression, and it is thought to develop through tumor cell dispersal via the portal vein [2]. HCC occurs in chronic liver disease associated with persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) in the majority of cases [3, 4]. Recently, the proportion of Japanese HCC patients negative for the hepatitis B surface antigen and hepatitis C antibody (non-B, non-C) has shown an increasing tend [5]. Although surgery is considered the best treatment option, nonsurgical therapy is also important from the viewpoint of developing effective pharmacologic compounds for the treatment of HCC. It is necessary to

clarify the molecular mechanisms of pathogenesis in non-B, non-C HCC. Exhaustive genetic and protein analyses of HCC demonstrated that several molecular targets contribute to the development of HCC [6, 7]. Preventing the expression of these target proteins may lead to improvements in HCC therapy. Among them, the Rho GTPase family proteins are markedly expressed and play a role in the development of various tumors including HCC [8, 9].

The Rho GTPase family proteins Rho, Rac 1, and Cdc42 control cell proliferation, invasion, migration, cell adhesion, etc. [10]. The GTPases are overexpressed in many types of human cancer, and they contribute to local cancer proliferation and distant metastasis [5, 8]. Rho kinases, termed "Rho-associated coiled coil-containing protein kinase (ROCK)1 and 2", are protein serine/threonine kinases that are activated when bound to the GTP-bound form of the small GTPase Rho A or Rho C. Several studies reported that the Rho and ROCK pathway is associated with intrahepatic metastasis of HCC [11, 12]. The high expression of Rho A protein in HCC plays an important role in intrahepatic recurrence [13]. One Rho kinase inhibitor, fasudil [1-(5-isoquinolinesulfonyl)-homopiperazine: HA-1077], was approved drug in Japan in 1995 for the clinical treatment of vascular cerebral spasm [14, 15]. Rho kinase inhibitors can be used to elucidate the participation of the Rho/Rho kinase pathway in the carcinogenetic processes of tumors. Ying et al. [16] reported that fasudil inhibited tumor progression in human and rat tumor models. Inhibition of the Rho/ROCK signaling pathway suppressed progression and limited intrahepatic metastasis in HCC models [17, 18]. ROCK inhibitors may also be useful as the chemotherapy for the treatment of HCC. However, the mechanism of expression of ROCK in HCC and its downstream signaling mechanisms in the pathogenesis of HCC are still unclear.

In this study, we examined the ROCK protein level in HCC compared that in with healthy liver tissue or benign tumors and the detailed mechanisms of tumor inhibition by fasudil in two HCC cell line.

Materials and methods

Drug and antibodies

The Rho kinase inhibitor fasudil hydrochloride hydrate was obtained from Asahi Kasei Pharma (Tokyo, Japan). The drug was dissolved in RPMI-1640 medium (Invitrogen, Grand Island, NY, USA) and stored at −20 °C until use. Antibodies (Abs) used in the present study were: rabbit anti-ROCK polyclonal Ab, anti-p53 polyclonal Ab, rabbit anti-Bax polyclonal Ab, mouse anti-caspase-3 monoclonal Ab, and rabbit anti-caspase-9 polyclonal Ab (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Human liver tissues

Human liver tissue samples were obtained from patients undergoing partial hepatectomy for HCC ($n = 6$) and hepatic hemangioma ($n = 6$) in our institution. Written informed consent was obtained from each patient prior to surgery. No patient showed evidence of concurrent HBV and HCV infection. In addition, 6 healthy liver tissue samples from the organs of Caucasian and Hispanic transplantation donors were supplied by the National Disease Research Interchange (Philadelphia, PA, USA) through the Biomedical Research Institute, Human and Animal Bridging Research Organization (Chiba, Japan). This study was approved by the St. Marianna University School of Medicine Ethics Committee.

The tissue samples were frozen in liquid nitrogen or fixed in 10 % formalin and embedded in paraffin. The nontumorous specimens taken at least 10 cm distant from the tumorous part of the liver were pathologically demonstrated to be free of tumor cells.

Cell lines and cell cultures

The HCC lines, Huh7, and HepG2 were obtained from the Riken Gene Bank (Tsukuba, Japan). The cells were cultured with RPMI-1640 medium (Invitrogen) containing 10 % fetal bovine serum (Gibco BRL, Grand Island, NY, USA), with penicillin 100 units/ml and streptomycin 100 μg/ml at 37 °C under an atmosphere of 5 % CO_2 and 95 % O_2. The cells were seeded at 5.0×10^5 cells in culture dishes. The cells were incubated for 48 h, and the second day was designated as day 0. From day 0, the cells were incubated with fasudil at a concentration of 50, 100, or 200 ng/ml for 24 h. Plasma concentration of Fasudil will roughly range from 100 to 200 ng/ml in clinical setting for treatment of cerebrovascular accident. The concentration of 100 ng/ml used in this study was determined based on this data.

p53 Decoy oligodeoxynucleotide preincubation

The p53 decoy sequence (5′-AGGCATGCCT-3′) oligodeoxynucleotide (ODN) specific for p53 was designed (Gene Bank No. X02469) and synthesized in phosphorothiolated form. To make double-stranded ODN, p53 decoy ODN was heated at 95 °C for 5 min and then cooled slowly to room temperature to permit annealing. Huh7 cells were preincubated for 3 h in 3 or 5 μM ODN. Thereafter, fasudil was added to each sample and cultured for 24 h [19].

Immunohistochemistry

The hepatic tissue samples fixed in 10 % formalin were embedded in paraffin and then sliced longitudinally into

sections 5 μm thick. Paraffin-embedded samples were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity in the tissue sections was inactivated with 0.1 % hydrogen peroxide. After the sections had been blocked with bovine serum for 30 min, they were incubated with anti-ROCK polyclonal Ab diluted to 1:100. Immunoreactivity in sections was demonstrated using a DAKO Envision system (Carpinteria, CA, USA) according to the manufacturer's instructions. Finally, the tissues were counterstained with hematoxylin. Microscopic observations were performed using a Carl Zeiss Axiophot microscope (Hitschfel Optical Instruments, St. Louis, MO, USA). Rabbit serum was used as a negative control. The samples were analyzed by counting 500 nontumorous hepatocytes or tumor cells in five different areas and determining the mean number of ROCK-positive cells in order to quantify the results. The results were expressed as the percentage of ROCK-positive cells/total number of hepatocytes.

Cell proliferation assay

Cell proliferation was evaluated in the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cell-titer 96 aqueous one-solution cell proliferation assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cells were incubated with fasudil in 96-well plates (Iwaki Glass, Tokyo, Japan) for 24 h. The MTS substrate was added to each well in a ratio of 20 μl of MTS for every 100 μl of culture medium, and the mixture was incubated for 2 h at 37 °C. Absorbance was measured at 490 nm with a microplate reader (Multiskan, Thermo Labsystems, Ventaa, Finland). The cell proliferation rate was expressed as the ratio of optical density of the treated to control cells. At least 8 independent experiments were performed for each experimental condition.

Apoptotic assays

Huh7 and HepG2 cells were cultured with fasudil for 24 h. Furthermore, they were preincubated with p53 decoy ODN (3 and 5 μM each) for 3 h, followed by the addition of fasudil for 24 h. Apoptosis of the cells was evaluated based on the cell death detection enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer's instructions. Briefly, the cells were homogenized in lysis solution and incubated with a mixture of anti-histone biotin and anti-DNA peroxidase for 2 h at room temperature. After washing for removal of unbound component, the ABTS as substrate was reacted for quantitative determination of the amount of nucleosomes. The optical density was measured using a microplate reader (Multiskan, Thermo Labsystems) at 405 nm to quantitate the reactive substrate of the samples.

Western blot analysis

The nuclear proteins in the cells were prepared using a commercial kit (NE-PER nuclear and cytoplasmic extraction kit, Pierce Biotechnology, Rockford, IL, USA), and whole proteins were also extracted with cell lysing buffer (0.1 % NP-40 in PBS). The protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equivalent amounts of proteins (25 μg) were resolved upon 10 % SDS–polyacrylamide gel electrophoresis. Thereafter, proteins were transferred onto nitrocellulose membranes (Habersham Bioscience, Buckinghamshire, UK) and blocked with 5 % skim milk with Tween-Tris-buffered saline (TTBS) (NaCl 1.5 M, Tris 0.1 M, pH 7.5, and 0.5 % Tween 20) overnight at 4 °C. The blots were probed with specific primary Abs for 2 h at the appropriate dilution. After washing in TTBS, the blots were conjugated with a horseradish peroxidase-conjugated second Ab IgG. Reactive proteins were detected in the chemiluminescence assay (ECL plus kit, Habersham Bioscience). The intensity of detected bands was analyzed using the densitograph software CS Analyzer ver. 3.0 (ATTO, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± SD. The statistical analysis was performed using KyPlot software (version 5.0; Kyens Lab, Tokyo, Japan). Differences between groups were analyzed for statistical significance using a Steel multiple-comparison method of the Dwass type. A p value of less than 0.05 was accepted as representing a statistically significant difference.

Results

ROCK production in hepatic tissues of patients with HCC

Immunohistochemical staining for ROCK was performed in HCC tissues corresponding to the nontumorous samples, tumorous samples, and healthy liver samples. The samples were analyzed by counting 500 nontumorous hepatocytes or tumor cells in five different areas and determining the mean number of ROCK-positive cells. The results were expressed as the percentage of ROCK-positive cells/total number of hepatocytes. Many ROCK-positive cells were observed in both hepatocytes of nontumorous hepatocytes (68.75 ± 18.48 %) and tumor cells (47.28 ± 14.86 %) (Fig. 1a, d). Furthermore, ROCK production was also

observed in hepatic tissues of HBV-related HCC (Fig. 1g) and HCV-related HCC (Fig. 1h). There were no ROCK-positive cells in healthy liver samples (Fig. 1i).

Figure 2 shows Western blotting results for tumorous and adjacent nontumorous tissues. The samples were obtained from 6 paired cases of HCC tumorous and adjacent nontumorous tissues. The expressions of ROCK protein in tumor tissues (7,274.44 ± 3,967.05 %) were similar to that in nontumorous tissue (9,220.96 ± 4,077.83 %) samples from all patients. On the other hand, the ROCK protein expression levels both in the tumor tissues (3,626.21 ± 4,819.31 %) and in the nontumorous tissues (1,480.94 ± 2,902.65 %) of benign hepatic hemangioma samples were lower than that in HCC and rarely detected in healthy liver tissue samples (100.00 ± 223.60 %).

Inhibition of ROCK decreased cell proliferation and induced apoptosis in HCC cells

We investigated whether ROCK is associated with pathogenesis such as tumor proliferation in HCC in vitro. We first examined the effects on cell proliferation or apoptosis in Huh7 and HepG2 cells cultured with the ROCK inhibitor fasudil. HepG2 cells incubated with fasudil for 24 h showed a significant decrease in the cell proliferation rate from 79.43 ± 9.52 % at 50 ng/ml to 44.90 ± 11.28 % at 200 ng/ml ($p < 0.01$) (Fig. 3a). Fasudil also inhibited the proliferation of Huh7 cells from 50 ng/ml (78.51 ± 18.30 %) to 200 ng/ml (66.64 ± 14.54 %) in a dose-dependent manner. In addition, when apoptosis in fasudil (50–200 ng/ml)–treated cells was analyzed using cell death

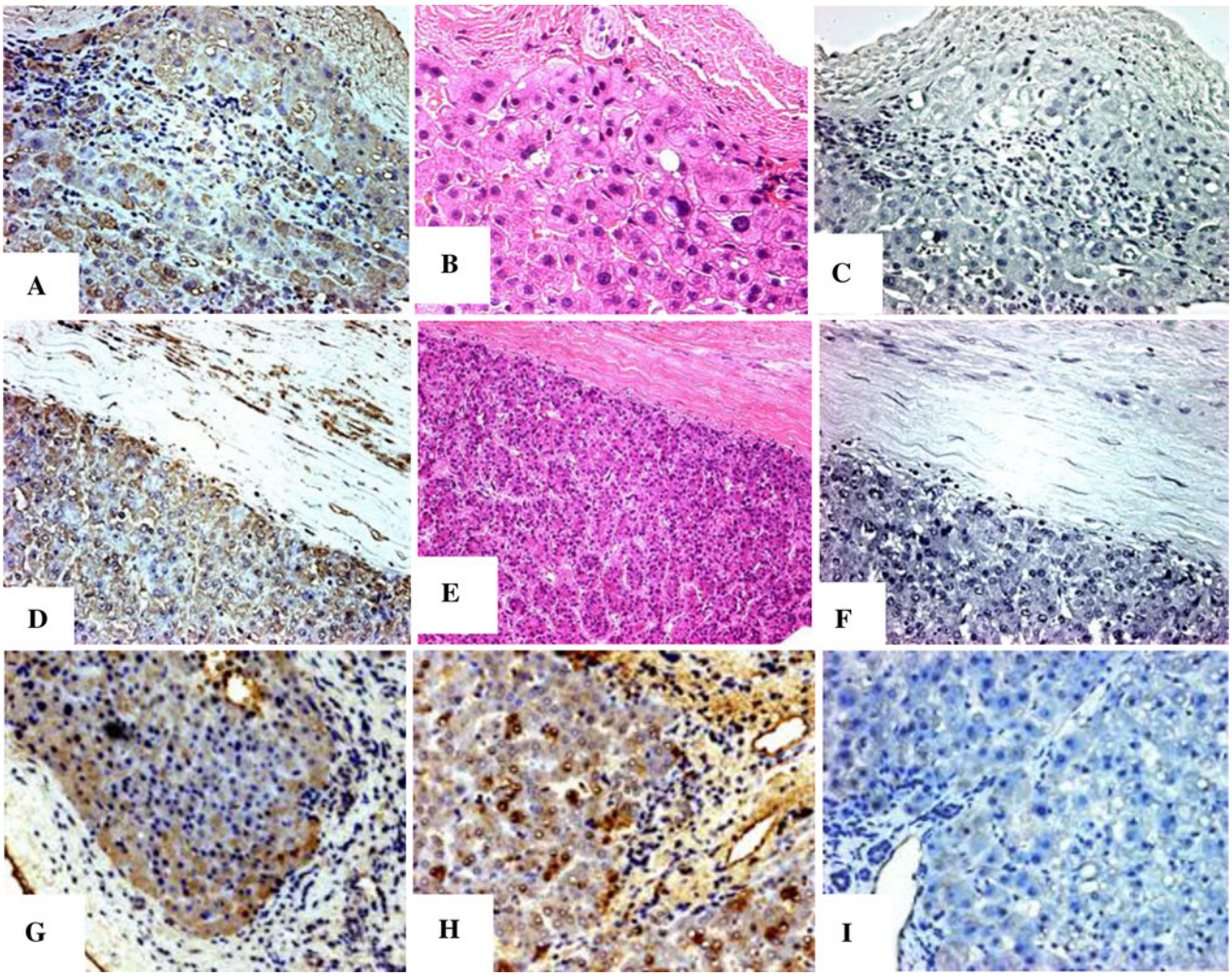

Fig. 1 Immunohistochemical analysis of ROCK in a tumor tissue sample (**d**) and an adjacent nontumorous tissue (**a**). Consecutive sections were stained with hematoxylin and eosin in a tumor tissue sample (**e**) and an adjacent nontumorous tissue (**b**). Anti-goat IgG polyclonal Ab was used as a negative control in a tumor tissue samples (**f**) and an adjacent nontumorous tissue (**c**). The nontumorous specimens taken at least 10 cm distant from the tumorous part of the liver were pathologically demonstrated to be free of tumor cells. All data are representative tissues from of 3 individual patients. Original magnification ×200

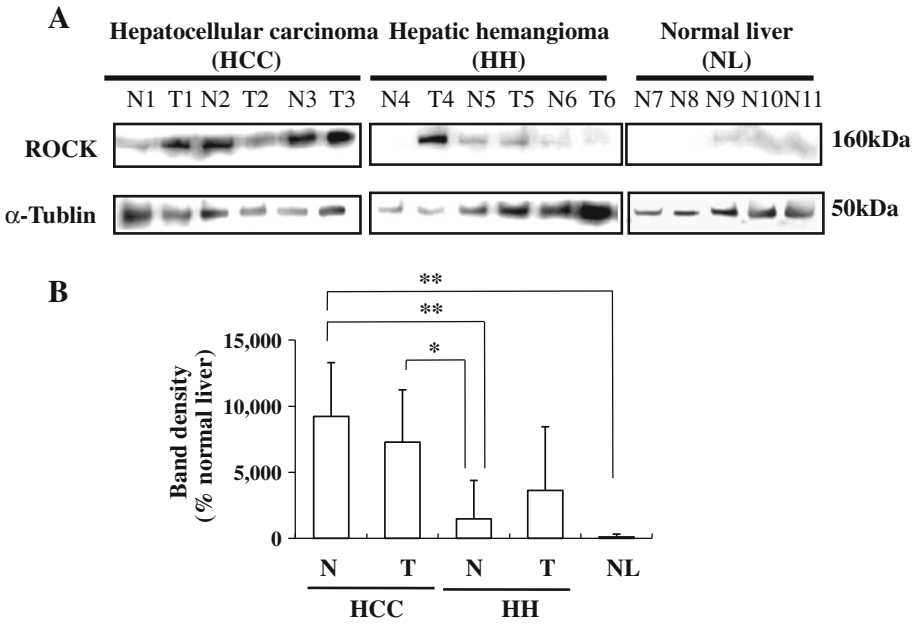

Fig. 2 Western blotting band intensities of ROCK protein expression in HCC. ROCK expression was increased in HCC and adjacent nontumorous tissues. Only low levels of ROCK expression were detected in benign hepatic hemangioma tissues, and none was detected in healthy liver tissue samples. Levels in α-tubulin are shown as a control. *Bar* indicates mean ± SD. The data are representative of 6 independent experiments

ELISA, fasudil increased apoptosis in HepG2 and Huh7 cells in a dose-dependent manner (Fig. 3b).

Effects of fasudil on apoptosis-related protein expression in HCC cells

It was reported that fasudil induces apoptosis through the mitochondrial pathway in cardiomyocytes in cardiac infarction [20]. Western blotting showed the expression of apoptosis-related proteins associated with the mitochondrial pathway in HCC cells cultured with fasudil (50–200 ng/ml). Fasudil significantly increased Bax (control; 100.00 ± 30.97 %, 50 ng/ml, 136.53 ± 85.65 %; 100 ng/ml, 170.43 ± 88.86 %; and 200 ng/ml, 276.43 ± 99.52 %), caspase-9 (100.00 ± 71.39, 235.60 ± 97.49, 284.91 ± 79.34, and 310.55 ± 96.04 %) and caspase-3 (100.00 ± 40.20, 148.73 ± 45.00, 168.05 ± 79.72, and 222.38 ± 86.08 %) expression levels compared with the respective controls in HepG2 cells in a dose-dependent manner (Fig. 3c). Bax (100.00 ± 35.44, 29.52 ± 54.72, 163.19 ± 59.15, and 161.33 ± 47.13 %), caspase-9 (100.00 ± 40.40, 112.45 ± 35.84, 156.50 ± 42.28, and 157.95 ± 74.57 %) and caspase-3 (100.00 ± 40.96, 113.16 ± 49.71, 157.54 ± 42.93, and 194.19 ± 58.67 %) also dose-dependently increased by fasudil treatment in Huh7 cells. These results suggest that fasudil increases the apoptosis through the mitochondrial pathway.

Effects of fasudil on p53 in HCC cells

We investigated the upstream factor in fasudil-induced apoptosis through the mitochondrial pathway in HCC. p53 protein expression increased dose-dependently in the nuclear fraction of HepG2 (100.00 ± 135.26, 439.68 ± 349.67, 560.42 ± 304.63, and 601.57 ± 224.51 %) and Huh7 (100.00 ± 37.60, 154.26 ± 47.09, 177.94 ± 55.32, and 198.61 ± 53.55 %) cells treated with fasudil (Fig. 4).

To examine whether fasudil-induced apoptosis and inhibition of proliferation mediates the p53-dependent pathway, HCC cells were precultured with p53 decoy ODN for 3 h, followed by incubation with fasudil. The p53 decoy ODN (5 μM) prevented the fasudil-induced inhibition of HepG2 and Huh7 cell proliferation (Fig. 5a). Furthermore, apoptosis was significantly inhibited in HepG2 and Huh7 cells incubated with fasudil in the presence of p53 decoy ODN (5 μM) compared with cells incubated with fasudil alone (Fig. 5b). p53 decoy ODN alone statistically did not differ compared with the control or the treatment of fasudil with p53 decoy ODN. Therefore, incubation with p53 decoy ODN alone was confirmed to have no effect on apoptosis or cell proliferation in this experimental system.

Effects of apoptosis on fasudil-treated HCC cells in the presence of p53 decoy ODN

In addition, the increase in Bax (fasudil vs. p53 decoy ODN; 246.99 ± 66.60 vs. 152.36 ± 87.13 %) caspase-9 (169.59 ± 66.06 vs. 78.36 ± 55.80 %) and caspase-3 (195.67 ± 76.61 vs. 112.94 ± 60.96 %) expression in fasudil (100 ng/ml)-treated HCC cells was suppressed after p53 decoy ODN (5 μM) treatment in HepG2 cells (Fig. 6). Pretreatment of p53 decoy ODN also prevented increase in these apoptosis-related proteins in fasudil-treated Huh7 cells (fasudil vs. p53 decoy ODN; Bax, 173.32 ± 61.15 vs. 76.48 ± 37.71 %; caspase-9, 171.65 ± 65.83 vs. 81.80 ±

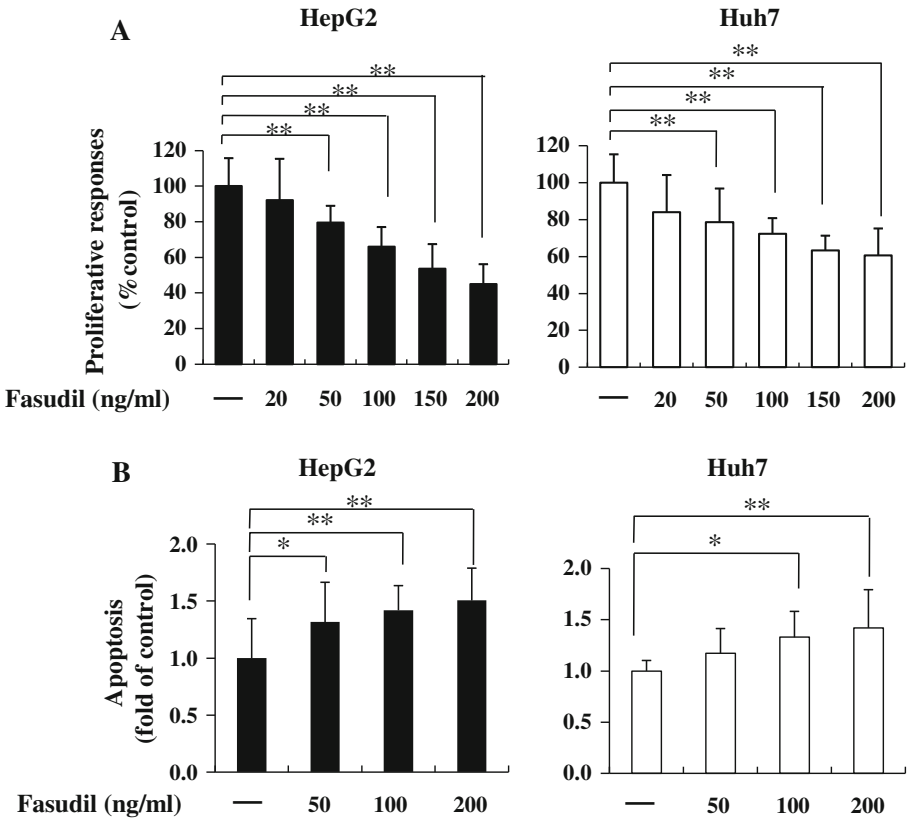

Fig. 3 Cell proliferation and apoptosis in HCC cell lines cultured with fasudil. **a** The cells (5×10^4) were cultured with fasudil in 96-well plates for 24 h. Cell proliferation was detected in the MTS assay. MTS was added to the medium for an additional 2 h. Cell survival was detected by measuring absorbance at 490 nm. Cell proliferation is expressed as the ratio of the optical density of fasudil-treated cells to that of the controls. *Bar* indicates mean ± SD. The data are representative of 10 independent experiments. $^{**}p < 0.01$ versus respective controls. **b** Fasudil-induced apoptosis in HCC cells. Apoptotic cells were measured using cell death detection ELISA and are shown as the percentage of apoptosis in HepG2 and Huh7cells cultured with different doses of fasudil. *Bar* indicates mean ± SD. The data are representative of 10 (HepG2) or8 (Huh7) independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus respective controls. α-Tubulin was used as the loading control. **c** Expression of apoptosis-related proteins in HCC cells after culture with fasudil. Apoptosis-related protein expressions of, Bax, caspase-9, and caspase-3 was analyzed after 24-h culture with fasudil using Western blotting. Fasudil increased the expression of Bax, caspase-9, and caspase-3 proteins in HepG2 and Huh7cells. Details are described in "Materials and Methods". $^{*}p < 0.05$ and $^{**}p < 0.01$ versus respective controls

45.13 %; caspase-3, 343.09 ± 337.88 vs. 141.14 ± 124.90 %) These results suggest that p53 is a key regulator of fasudil-induced apoptosis in HCC.

Discussion

The Rho GTPase family proteins are significantly increased in a variety of tumors, especially during progression to invasive and metastatic forms [21]. The Rho subfamilies Rho A, Rho B, and Rho C share amino acid identity, but their cellular functions are different. Rho A and Rho C are overexpressed in tumors and are involved in carcinogenesis and tumor progression. Several reports demonstrated high levels of the Rho A protein in various cancers, suggesting that Rho A plays an important role in the invasive processes of neoplasia [8, 22]. Fukui et al. [13] reported that Rho A protein in tumorous tissues in patients with HCC was associated with the invasiveness of HCC. They suggested that Rho A and the Rho A signaling pathway may represent targets for HCC therapy. Rho A-driven actin reorganization mainly depends on the serine/threonine protein kinase ROCK [23]. ROCK activation promotes actin-myosin-mediated processes, such as cell motility, cell adhesion, cancer cell proliferation, and phagocytosis in pathophysiological conditions [23]. The Rho/ROCK signaling pathway regulates the cancer cell motility through reorganization of the actin cytoskeleton [24, 25].

In this study, high expression of ROCK protein was observed in tumorous tissues from patients with non-B, non-C HCC as well as in tumorous tissues of HBV- or HCV-related HCC (Fig. 1). In addition, ROCK protein levels in hepatocytes of paired nontumorous HCC tissues were approximately equivalent to those in tumorous tissues. On the other hand, ROCK expression was very low or

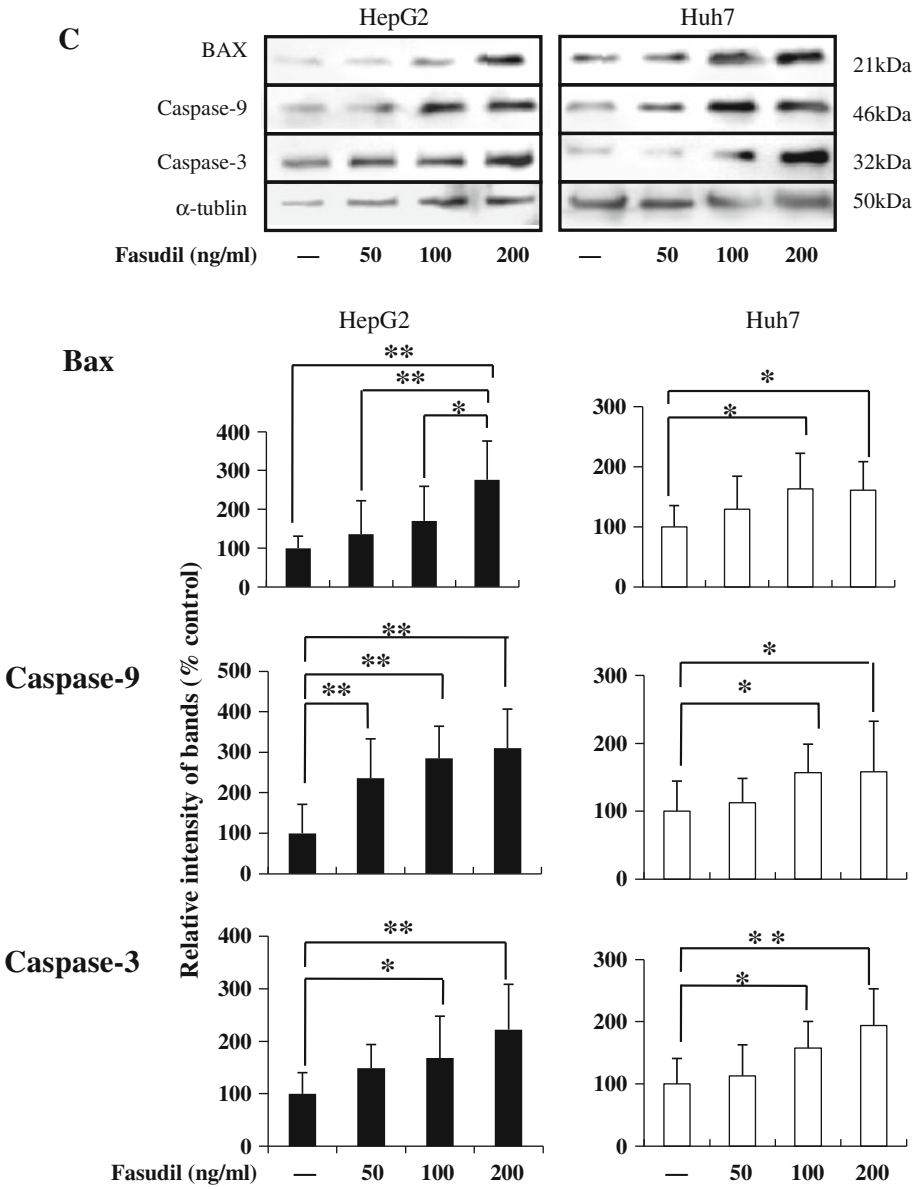

Fig. 3 continued

undetectable in hepatoma and healthy liver tissue samples. Although Rho A protein is mainly expressed in HCC tumor tissues [13], ROCK protein was expressed not only in tumorous tissue but also in nontumorous tissue from HCC patients in the present study. These results suggest that the high expression of ROCK is an important factor in the development of non-B, non-C HCC and may be required to activate downstream signaling factors. The implantation of HCC cells transfected with dominant-negative ROCK was shown to reduce metastasis and motility in mice [24, 26].

Recently, the roles of the Rho/ROCK pathway in tumor development have been explained using specific inhibitors of ROCK, Y-27632, or fasudil. Fasudil is a selective, ATP-competitive inhibitor of ROCK including p160ROCK and ROCK-II. Other target has not been clarified yet and believed to be selective inhibiter [14, 15]. Inhibition of the Rho/ROCK pathway attenuates cyclosporine A-induced nephropathy through the suppression of the induction of inflammatory, apoptotic, and fibrogenic factors, along with inhibition of Smad, mitogen-activated protein kinases, and nitric oxide signaling pathways [27]. Furthermore, ROCK inhibitors induced apoptosis in human cancer cells [28]. Thus, several inhibitory pathways of Rho/ROCK appear to be involved in human tumors. Rho kinase regulates in various cellular functions such as cell proliferation of colon cancer cells or hepatic stellate cells. Furthermore, the knockdown of ROCK reported to inhibit cell proliferations [29, 30]. Prevention of cancer cell proliferation may lead to

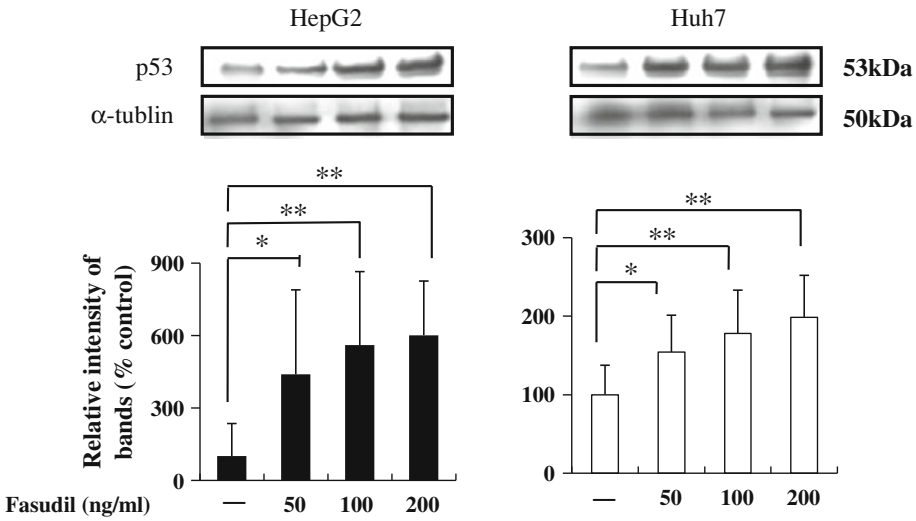

Fig. 4 Fasudil increased expression of p53 protein in the nuclei of HepG2 and Huh7 cells. **a** The cells were cultured for 24 h in the presence of fasudil 50, 100, and 200 ng/ml, respectively. p53 protein expression in the nuclear fraction of HepG2 and Huh7 cells treated with fasudil was determined using Western blotting. α-Tubulin was used as the loading control. $*p < 0.05$ and $**p < 0.01$ versus respective controls

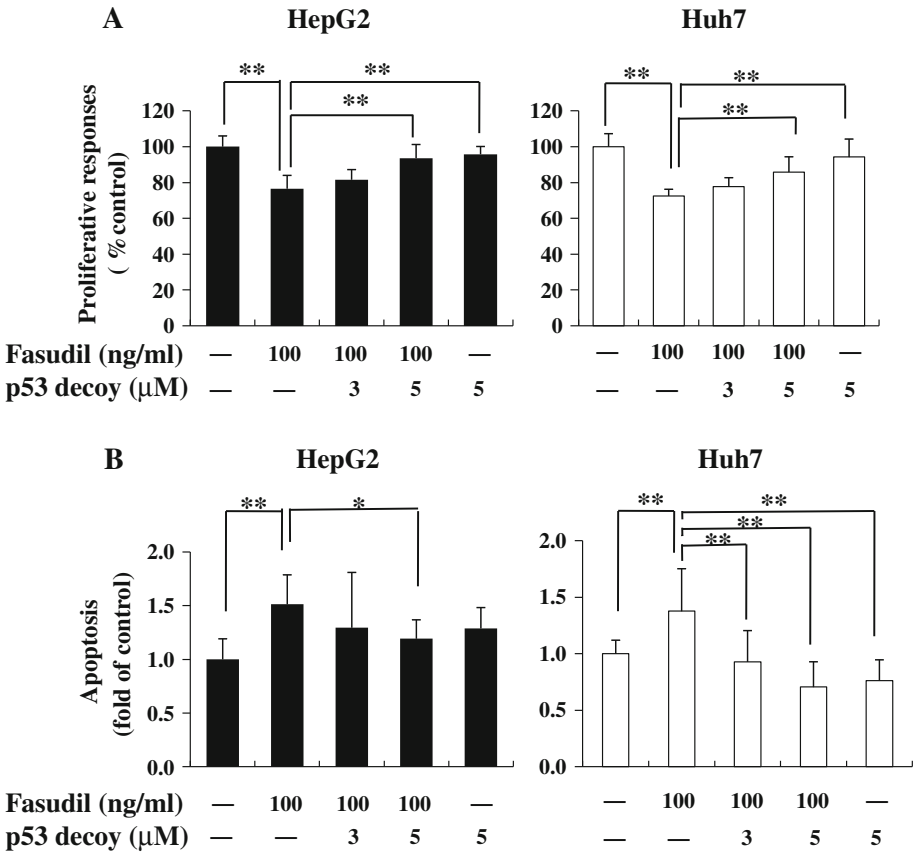

Fig. 5 Effects of p53 decoy ODN on fasudil-induced apoptosis and cell proliferation in HCC cells. Two HCC cells were pretreated with p53 decoy ODN 5 μM followed by 24-h incubation with fasudil. $*p < 0.05$ and $**p < 0.01$ versus fasudil-treated cells. **a** The cell proliferation rate was measured in the MTS assay as described in "Materials and Methods". *Bar* indicates mean ± SD. The data are representative of 9 independent experiments. Details are described in "Materials and Methods". **b** Apoptotic cells were counted using cell death detection ELISA and are shown as the percentage of apoptosis in HepG2 and Huh7 cells pretreated with p53 decoy ODN followed by incubation with fasudil. *Bar* indicates mean ± SD. The data are representative of 9 (HepG2) or 12 (Huh7) independent experiments

suppression in HCC cell inversion and metastasis. However, it is unclear whether ROCK inhibitors can reduce HCC cell survival and their mechanisms of action have not been clarified.

In this study, we used fasudil to examine the detailed mechanisms of apoptosis in non-B, non-C HCC cell lines derived from Japanese patients [31, 32]. Fasudil inhibited the proliferation of Huh7 and HepG2 cells in a dose-dependent manner. In addition, fasudil increased apoptosis in both HCC cells lines (Fig. 3).

Inhibition of the Rho/ROCK pathway has been reported to increase the apoptosis of endothelial cells by activating caspsase-3 [33]. ROCK inhibitor-induced apoptosis was shown to be Bcl-2 sensitive, and Bcl-2 families are known to affect cell apoptosis by regulating the permeability of mitochondria [34, 35]. In addition, fasudil

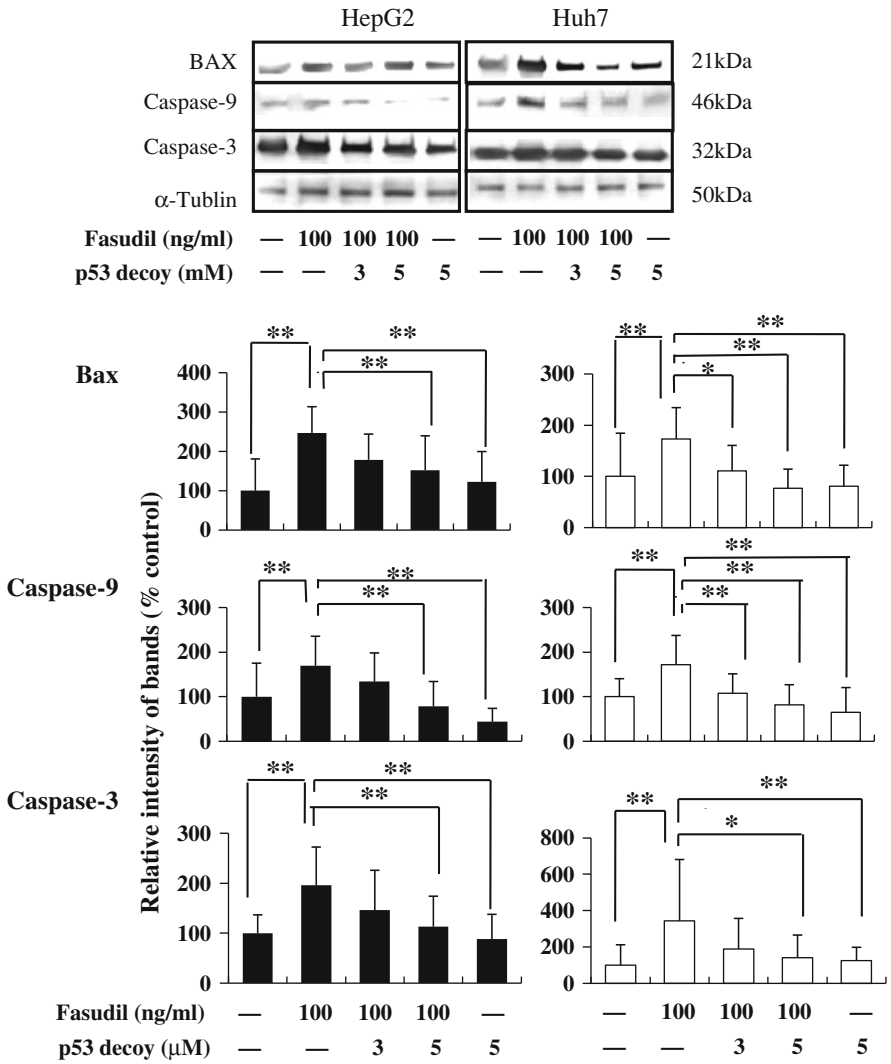

Fig. 6 Effects of p53 decoy ODN on fasudil-induced apoptotic-related proteins in HCC cells. HCC cells were pretreated with p53 decoy ODN 5 μM followed by 24-h incubation with fasudil. The increase in Bax, caspase-9, and caspase-3 protein levels induced by fasudil in HepG2 and Huh 7 cells decreased after pretreatment with p53 decoy ODN. *Bar* indicates mean ± SD. The data are representative of 8 independent experiments. Details are described in "Materials and Methods". $*p < 0.05$ and $**p < 0.01$ versus fasudil-treated cells

attenuated oxidative stress injury via the Bax-mediated pathway in neuroblastoma [20]. We confirmed that fasudil increased Bax, caspase-9, and caspase-3 protein levels in a dose-dependent manner, suggesting that fasudil-induced HCC cell apoptosis is mediated via the mitochondrial pathway.

p53 has a well-documented role in activating gene expression leading to apoptosis. p53 protects against malignant transformation of cells by coordinating cell cycle arrest or by promoting cell death [36, 37]. Mutations of the p53 gene occur in many human tumors [38]. Our previous report demonstrated that the acceleration of p53-mediated apoptosis by anticancer drug agents limited the metastasis of HCC [39]. Bax activation occurs in response to a variety of apoptotic stimuli, and the p53 directly regulates Bax expression and induces apoptosis via the mitochondrial pathway. Rad, a Rho kinase inhibitor, suppressed p53-mediated cell migration in lung cancer [40]. Rho A and ROCK activation is associated with cell migration due to the mutation or loss of p53 [41, 42]. We speculate that p53 signaling may be an important factor in the apoptosis of HCC cells induced by Rho kinase inhibitors. In the present study, a p53 decoy ODN to block p53 function was used in an attempt to clarify the series of signaling pathways in the fasudil-induced apoptosis. Preincubation with p53 decoy ODN followed by the addition of fasudil to the culture medium resulted in the inhibition of apoptosis and HCC cellular responses through the mitochondrial pathway. These findings led to the conclusion that fasudil-induced apoptosis is mediated by p53 and effected through Bax effects on the mitochondria. This newly discovered mechanism of Rho kinase inhibitors may lead to improved treatment of patients with HCC.

In conclusion, HCC cell survival mediated by the Rho/Rho kinase pathway may be associated with the inhibition of p53-mediated mitochondrial apoptosis. Approaches targeting Rho kinase inhibitors may therefore be useful to prevent the progression of HCC.

Acknowledgments This work was supported in the part by grants under the Research on Publicly Essential Drugs and Medical Devices of Health and Labor Sciences Research from the Ministry of Health, Labor and Welfare of Japan. Furthermore, we thank Ms. Shigeko Onuma of the Department of Pathology, St. Marianna University School of Medicine for technical support.

Conflict of interest There is no conflict of interest involved in this manuscript.

References

1. Ikeda K, Saitoh S, Tsubota A, Arase Y, Chayama K, Kumada H, Watanabe G, Tsurumaru M (1993) Risk factors for tumor recurrence and prognosis after curative resection of hepatocellular carcinoma. Cancer 71:19–25
2. Sugihara S, Kojiro M, Nakashima T (1985) Ultrastructural study of hepatocellular carcinoma with replacing growth pattern. Acta Pathol Jpn 35:549–559
3. Okuda K (2000) Hepatocellular carcinoma. J Hepatol 32:225–237
4. Bergsland EK (2001) Molecular mechanisms underlying the development of hepatocellular carcinoma. Semin Oncol 28:521–531
5. Utsunomiya T, Shimada M (2011) Molecular characteristics of non-cancerous liver tissue in non-B non-C hepatocellular carcinoma. Hepatol Res 41:711–721
6. Orimo T, Ojima H, Hiraoka N et al (2008) Proteomic profiling reveals the prognostic value of adenomatous polyposis coli-end-binding protein 1 in hepatocellular carcinoma. Hepatology 48:1851–1863
7. Wang W, Peng JX, Yang JQ, Yang LY (2009) Identification of gene expression profiling in hepatocellular carcinoma using cDNA microarrays. Dig Dis Sci 54:2729–2735
8. Frttz G, Just I, Kanita B (1999) Rho GTPase are over-expressed in human tumors. Int J Cancer 81:682–687
9. Gris F, Bidaud A, Moreau V (2009) Rho GTPase in hepatocellular carcinoma. Biochem Biophys Acta 1795:137–151
10. Amano M, Fukata Y, Kaibuchi K (2000) Regulation and functions of Rho associated kinase. Exp Cell Res 261:44–51
11. Sahai E, Marshall CJ (2002) RHO-GTPases and cancer. Nat Rev Cancer 2:133–142
12. Takamura M, Sakamoto M, Genda T, Ichida T, Asakura H, Hirohashi S (2001) Inhibition of intrahepatic metastasis of human hepatocellular carcinoma by Rho-associated protein kinase inhibitor Y-27632. Hepatology 33:577–581
13. Fukui K, Tamura S, Wada A et al (2006) Expression and prognostic role of Rho A GTPases in hepatocellular carcinoma. J Cancer Res Clin Oncol 132:627–633
14. Wettschureck N, Offermanns S (2002) Rho/Rho-kinase mediated signaling in physiology and pathophysiology. J Mol Med 80:629–638
15. Tamura M, Nakao H, Yoshizaki H et al (2005) Development of specific Rho-kinase inhibitors and their clinical application. Biochim Biophys Acta 1754:245–252
16. Ying H, Biroc SL, Li WW et al (2006) The Rho kinase inhibitor fasudil inhibits tumor progression in human and rat tumor models. Mol Cancer Ther 5:2158–2164
17. Xue F, Takahara T, Yata Y et al (2008) Blockade of Rho/Rho-associated coiled coil-forming kinase signaling can prevent progression of hepatocellular carcinoma in matrix metalloproteinase-dependent manner. Hepatol Res 38:810–817
18. Ogawa T, Tashiro H, Miyata Y et al (2007) Rho-associated kinase inhibitor reduces tumor recurrence after liver transplantation in a rat hepatoma model. Am J Transp 7:347–355
19. Takeba Y, Sekine S, Kumai T et al (2007) Irinotecan-induced apoptosis is inhibited by increased P-glycoprotein expression and decreased p53 in human hepatocellular carcinoma cells. Biol Pharm Bull 30:1400–1406
20. Li Q, Liu D, Huang X, Guo L (2011) Fasudil mesylate protects PC12 cells from oxidative stress injury via the Bax-mediated pathway. Cell Mol Neurobiol 31:243–250
21. Grise F, Bidaud A, Moreau V (2009) Rho GTPases in hepatocellular carcinoma. Biochim Biophys Acta 1795:137–151
22. Kamai T, Tsujii T, Arai K, Takagi K, Asami H, Ito Y, Oshima H (2003) Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer. Clin Cancer Res 9:2632–2641
23. Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, Kaibuchi K (1997) Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 275:1308–1311
24. Shi J, Wei L (2007) Rho kinase in the regulation of cell death and survival. Arch Immunol Ther Exp 55:61–75
25. Itoh K, Yoshioka K, Akedo H, Uehata M, Ishizaki T, Narumiya S (1999) An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nat Med 5:221–225
26. Genda T, Sakamoto M, Ichida T, Asakura H, Kojiro M, Narumiya S, Hirohashi S (1999) Cell motility mediated by rho and Rho-associated protein kinase plays a critical role in intrahepatic metastasis of human hepatocellular carcinoma. Hepatology 30:1027–1036
27. Park JW, Park CH, Kim IJ, Bae EH, Ma SK, Lee JU, Kim SW (2011) Rho kinase inhibition by fasudil attenuates cyclosporine-induced kidney injury. J Pharmacol Exp Ther 338:271–279
28. Rattan R, Giri S, Singh AK, Singh I (2006) Rho/ROCK pathway as a target of tumor therapy. J Neurosci Res 83:243–255
29. Iwamoto H, Nakamuta M, Tada S, Sugimoto R, Enjoji M, Nawata H (2000) A p160ROCK-specific inhibitor, Y-27632, attenuates rat hepatic stellate cell growth. J Hepatol 32:762–770
30. Nakashima M, Adachi S, Yasuda I, Yamauchi T, Kozawa O, Moriwaki H (2010) Rho-kinase regulates negatively the epidermal growth factor-stimulated colon cancer cell proliferation. Int J Oncol 36:585–592
31. Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J (1982) Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res 42:3858–3863
32. Lin Y, Ong LK, Chan SH (1995) Differential in situ hybridization of mutational specific expression of the p53 gene in human hepatoma cell lines. Pathology 27:191–196
33. Hippenstiel S, Chmeck B, N'Guessan PD, Seybold J, Krüll M, Preissner K, Eichel-Streiber CV, Suttorp N (2002) Rho protein inactivation induced apoptosis of cultured human endothelial cells. Am J Physiol Lung Cell Mol Physiol 283:L830–L838
34. Althoefer H, Eversole-Cire P, Simon MI (1997) Constitutively active Galphaq and Galpha13 trigger apoptosis through different pathways. J Biol Chem 272:24380–24386
35. Cho HJ, Baek KE, Park SM et al (2011) RhoGDI2 confers gastric cancer cells resistance against cisplatin-induced apoptosis by upregulation of Bcl-2 expression. Cancer Lett 311:48–56
36. Levine AJ, Momand J, Finlay CA (1991) The p53 tumor suppressor gene. Nature 351:453–456
37. Lakin ND, Jackson SP (1999) Regulation of p53 in response to DNA damage. Oncogene 18:7644–7655
38. Lowe SW, Ruley HE, Jacks T, Housman DE (1993) p53-dependent modulates the cytotoxicity of anticancer agents. Cell 74:957–967
39. Takeba Y, Kumai T, Matsumoto N, Nakaya S, Tsuzuki Y, Yanagida Y, Kobayashi S (2007) Irinotecan activates p53 with its active metabolite, resulting in human hepatocellular carcinoma apoptosis. J Pharmacol Sci 104:232–242

40. Hsiao BY, Chen CC, Hsieh PC et al (2011) Rad is a p53 direct transcriptional target that inhibits cell migration and is frequently silenced in lung carcinoma cells. J Mol Med 89:481–492
41. Guo F, Zheng Y (2004) Rho family GTPases cooperate with p53 deletion to promote primary mouse embryonic fibroblast cell invasion. Oncogene 23:5577–5585
42. Gadea G, de Toledo M, Anguille C, Roux P (2007) Loss of p53 promotes RhoA-ROCK-dependent cell migration and invasion in 3D matrices. J Cell Biol 178:23–30