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RhoE enhances multidrug resistance of gastric cancer cells by suppressing Bax

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ABSTRACT

We have previously reported that RhoE is overexpressed in the SGC7901/VCR cell line. However, the potential role of RhoE in the development of multidrug resistance of gastric cancer is unknown. In the present study, RhoE enhanced the resistance of SGC7901 cells to several kinds of antitumor drugs. RhoE overexpression did not alter the intracellular adriamycin accumulation of SGC7901 cells nor the expression of P-gp and MRP-1, but protected SGC7901 cells from vincristine-induced apoptosis. RhoE was found to downregulate the expression of Bax at a posttranscriptional level. Western blot revealed no effects of RhoE on the activities of the Caspase family of proteins. In brief, our study demonstrated that RhoE may promote the multidrug resistance phenotype of gastric cancer cells by decreasing the expression of Bax at posttranscriptional level, thus inhibiting vincristine-induced apoptosis.

The atypical Rho proteins Rnd1, Rnd2, and RhoE (also known as Rnd3) compose a subgroup of Rho GTPases, which are key regulators in a wide spectrum of cellular biological progresses such as cytoskeleton formation, cell cycle progression, gene transcription and membrane trafficking. Unlike the typical Rho proteins that cycle between two conformations; namely an active, GTP-binding state and an inactive, GDP-binding state; RhoE is present only in a GTP-binding form [1]. Furthermore, RhoE can bind to GTP but can not hydrolyze GTP [1]. These unique features provide RhoE with some unusual functions such as inhibiting cytoskeleton formation by antagonizing the typical Rho protein, RhoA [2]. It was reported that RhoE can interrupt cell cycle progression and cell transformation [3]. Recently, RhoE was found to exert differential effects on apoptosis, which were dependent on the cell background and treatment [4–7].

Chemotherapy is the primary treatment for gastric cancer, but one clinically significant problem that often causes failure of chemotherapy is multiple drug resistance (MDR) [8]. It was recognized that MDR arises from molecular changes, and a variety of mechanisms have been extensively studied in vivo and in vitro [9–12]. However, the complete mechanisms involved in MDR have not been fully characterized, suggesting that there are still some unknown molecules and mechanisms responsible for MDR.

Our laboratory has previously found that RhoE is upregulated in the vincristine resistant gastric adenocarcinoma cell line SGC7901/VCR compared with its parental cell line SGC7901 [13]. Later studies revealed that RhoA and RhoB play specific roles in the sensitivity of gastric cancer cells to antitumor drugs [14,15]. Therefore, we hypothesized that RhoE may affect the MDR phenotype of gastric cancer. In this study, we investigated the potential role of RhoE in MDR of gastric cancer cells and its underlying mechanisms.

Materials and methods

Cell culture. Human gastric adenocarcinoma cell line SGC7901 (obtained from the Academy of Military Medical Science, Beijing, China) and its multidrug-resistant variant SGC7901/VCR (established and maintained in our laboratory) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2 at 37 °C. To maintain the MDR phenotype, vincristine (VCR, 1 μg/ml) was added to the culture media for SGC7901/VCR cells.

Plasmid construction and cell transfection. pSilencer3.1 (Ambion, USA) was used to construct the human RhoE siRNA vectors RhoE-siA and RhoE-siB according to the manufacturer's protocol. Two pairs of specific oligonucleotides (A1, 5′-GATCCGTCTGATCTGCGGACAGATTTCAAGAGAATCTGTCCGCAGATCAGACTTTTTTGGAAA-3′; A2, 5′-AGCTTTTCCAAAAAAGTCTGATCTGCGGACAGATTCTCTTGAAATCTGTCCGCAGATCAGACG-3′; B1, 5′-GATCCGCGGAACAAATCACAGAGATTCAAGAGATCTCTGTGATTTGTTCCGCTTTTTTGGAAA-3′; B2, 5′-AGCTTTTCCAAAAAAGCGGAACAAATCACAGAGATCTCTTGAATCTCTGTGATTTGTTCCGCG-3′) were annealed and then subcloned into the BamHI/HindIII cloning sites of pSilencer3.1. Full-length mouse RhoE vector (pCMV-FLAG-RhoE) was kindly given by Prof. Anne J. Ridley (School of Biomedical and Health Sciences, King's College London, London) [16].

Cell transfection was done with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Briefly, cells

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were plated and grown to 80–90% confluence without antibiotics. Then, SGC7901 cells were transfected with pCMV-FLAG-RhoE (SGC7901-RhoE) and SGC7901/VCR cells were transfected with RhoE-siA or RhoE-siB. SGC7901 cells transfected with pCMV-FLAG vector (SGC7901-FLAG) or SGC7901/VCR cells transfected with pSilencer3.1 vector were used as controls. The expression level of RhoE was evaluated by Western blot analysis.

RNA preparation and semi-quantitative RT-PCR. Total cell RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription was performed on 1 μg of total RNA from each sample using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada). cDNA was then amplified using the followed primer pairs: RhoE: forward, 5′-ACAC ATATGAAGGAGAGAA-3′; reverse, 5′-TAAGGCGGCCGCAACATGA-3′. Bcl-2: forward, 5'-GGTGCCACCTGTGGTCCACCT-3'; 5′-GGTGCCAC CTGTGGTCCACCT-3′; reverse, 5′-CTTCACTTGTGGCCCAGATAGG-3′. Bax: forward, 5′-CTGACATGTTTTCTGACGGC-3′; reverse, 5′-TCAG CCCATCTTCTTCCAGA-3′. β-actin was used as a loading control. All PCR products were then visualized on a 1.0% agarose gel containing 5 μg/ml of ethidium bromide.

Immunocytochemistry assay. SGC7901 and SGC7901/VCR cells were cultured on glass cover slips for 24 h and fixed with 4% paraformaldehyde. The fixed cells were rinsed in PBS and permeabilized with 0.1% TritonX-100 in PBS for 5 min. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 10 min and rinsed in PBS. The slips were incubated with anti-RhoE primary mouse monoclonal antibody (1:50 dilution; Upstate, USA) overnight at 4 °C. Histostain™-Plus kits (Zhongshan, China) were used according to the manufacturer's instructions for detection of RhoE. In the control slips, mouse IgG was used instead of the primary antibody.

Western blot analysis. After the cells were collected from plates, the proteins were extracted using lysis buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8.8), 0.1% SDS, 2 mM EDTA, 1 mM PMSF, 1% NP40, 5 μg/ml aprotinin, 1 μg/ml leupeptin) at 4 °C on ice. The protein samples were then separated by 12% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% fat-free milk at room temperature for 20 min (to detect RhoE) or 2 h (to detect other proteins) and incubated with anti-RhoE antibody (1:500; Upstate, USA), anti-FLAG antibody (1:1000; Beyotime, China), anti-P-gp antibody (1:500; Boster, China), anti-MRP-1 antibody (1:100; Bioss, China), anti-Bcl-2 antibody (1:200; Santa Cruz, USA), anti-Bax antibody (1:200; Santa Cruz, USA), anti-cleavage Caspase antibodies (1:500, respectively; Cell Signaling, USA) and anti-β-actin antibody (1:2000; Sigma–Aldrich, USA) at 4 °C overnight. After being washed twice with water, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:2000; Santa Cruz, USA) for 1.5 h at room temperature. Enhanced chemiluminescence (ECL-kit, Santa Cruz, USA) was used to visualize the antigens. Western blotting for β-actin was used as an internal control.

In vitro drug sensitivity assay. SGC7901, SGC7901/VCR, SGC7901-RhoE, and SGC7901-FLAG cells were seeded into 96-well plates (8×10^3 viable cells/well) and allowed to attach overnight. VCR, adriamycin (ADR), 5-fluorouracil (5-FU), cisplatin (CDDP), mitomycin C (MMC), methotrexate (MTX), cytarabine (Ara) and etoposide (VP-16) were freshly prepared before each experiment. After adhesion, cells were incubated for 24 h in the presence or absence of various concentrations of the antitumor drugs in 200 μl of medium. Then, 50 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added to each well and cells were cultured for another 4 h. The supernatants were then discarded and 150 μl of DMSO (Sigma–Aldrich, USA) was added to each well to dissolve crystals. The absorbance at 490 nm (A) of each well was read on a microplate reader. The relative inhibitory rate of cell growth by drugs was calculated according to the following formula: $R = (A2 - A1)/A2 \times 100\%$ where R is the relative inhibitory rate of cell growth, $A1$ is the absorbance value of cells in the presence of drugs and $A2$ is the absorbance value of control cells without any drug treatment.

Intracellular ADR concentration analysis. The fluorescence intensity of intracellular ADR was determined using flow cytometry as described elsewhere [17]. In brief, SGC7901-RhoE and SGC7901-FLAG cells in the log phase were plated in six-well plates (1×10^6 cells/well). After the addition of ADR to a final concentration of 1 μg/ml, cells were cultured for 1 h. Cells were then either harvested to detect ADR accumulation or culture was continued in a drug-free medium for another 2 h, followed by detection of ADR retention. Finally, the cells were washed twice with cold PBS and the mean fluorescence intensity of intracellular ADR was detected using flow cytometry with an excitation wavelength of 488 nm and emission wavelength of 575 nm. The ADR-releasing index of the gastric cancer cells was calculated using the following formula: releasing index = (accumulation value − retention value)/accumulation value.

Analysis of apoptosis by flow cytometry. VCR was added to SGC7901-RhoE and SGC7901-FLAG cells to a final concentration

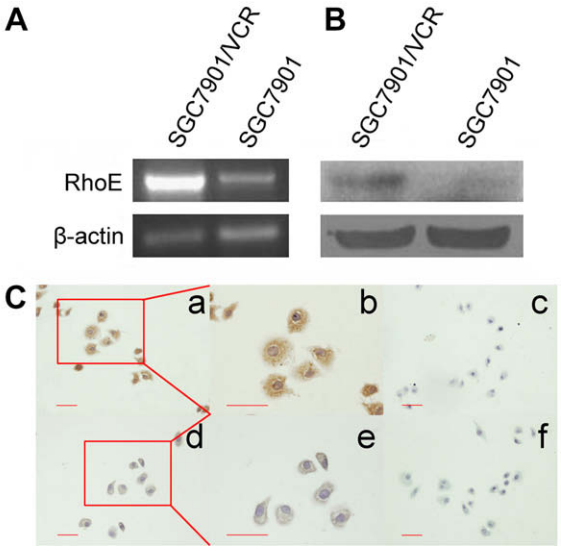

Fig. 1. Expression pattern of RhoE in SGC7901/VCR and SGC7901 cells. Upregulation of RhoE mRNA (A) and protein (B) was observed in SGC7901/VCR cells compared with SGC7901 cells. β-Actin was used as an internal control. Immunocytochemical staining (C) revealed strong staining of RhoE in the cytoplasm and nuclear membrane of SGC7901/VCR cells (a, 200×; b, 400×) compared with that of SGC7901 cells (d, 200×; e, 400×). Mouse IgG was used as a negative control (c, 200×; f, 200×). The magnification bars in each part of Fig. 1C represent 20 μm.

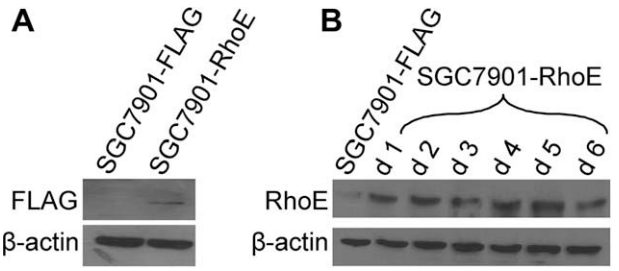

Fig. 2. Induction of RhoE in gastric cancer cells by transfection. SGC7901-RhoE cells showed an exogenous expression of FLAG-tagged RhoE compared with SGC7901-FLAG cells (~30 kDa, A) and this increased expression was observed from day 1 to day 6 after transfection (B). β-Actin was used as an internal control.

Table 1
IC50 values (μg/ml) of gastric cancer cells for antitumor drugs. The sensitivity of gastric cancer cells to antitumor drugs was evaluated using the MTT assay as described in Materials and methods. The concentration of each drug that caused a 50% reduction in number of cells (IC50) was calculated. Values are means ± SD.

	ADR	VCR	CDDP	VP-16	5-FU	MMC	Ara	MTX
SGC7901-RhoE	0.43 ± 0.16*	1.64 ± 0.51*	1.54 ± 0.15*	6.69 ± 1.32*	7.81 ± 0.39*	1.15 ± 0.26*	13.71 ± 2.91	0.47 ± 0.07
SGC7901-FLAG	0.15 ± 0.09	0.61 ± 0.23	0.52 ± 0.17	3.59 ± 1.56	4.54 ± 0.20	0.64 ± 0.27	13.16 ± 4.12	0.48 ± 0.33
SGC7901	0.14 ± 0.02	0.59 ± 0.11	0.52 ± 0.06	3.39 ± 0.97	4.61 ± 0.33	0.61 ± 0.13	13.59 ± 2.66	0.50 ± 0.04
SGC7901/VCR	2.13 ± 0.33	4.37 ± 0.54	2.37 ± 0.18	10.50 ± 1.96	15.97 ± 2.62	4.64 ± 0.88	17.73 ± 2.04	0.97 ± 0.09

* $p < 0.05$ (SGC7901-RhoE vs. SGC7901-FLAG cells).

Table 2
Intracellular ADR accumulation and retention. ADR accumulation and retention were measured by flow cytometry and the intracellular ADR concentration is represented by the average fluorescence intensity. Values are means ± SD. $p < 0.05$ (SGC7901-RhoE vs. SGC7901-FLAG cells).

	Fluorescence intensity		Releasing index
	Accumulation	Retention	
SGC7901-RhoE	287.50 ± 163.27	200.50 ± 110.25	0.275 ± 0.156
SGC7901-FLAG	307.25 ± 190.69	192.75 ± 98.56	0.329 ± 0.140

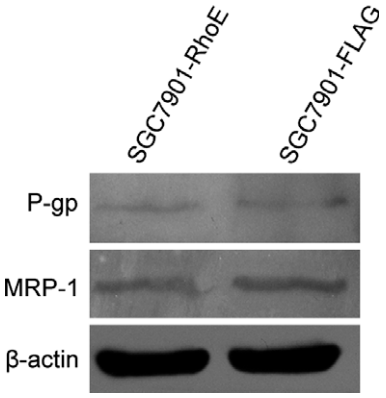

Fig. 3. Detection of P-gp and MRP-1 by Western blot analysis. No differences in the expression of these two proteins were found between SGC7901-RhoE and SGC7901-FLAG cells ($p > 0.05$).

of 0.3 μg/ml to induce apoptosis. Eighteen hours later, cells were harvested and washed twice with cold PBS. After incubation with a mixture containing annexin V and propidium iodide (Roche Diagnostics, USA) in binding buffer for 15 min, the fluorescence of cells was measured by flow cytometry (BD FACSCanto™, USA). Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of cells with a compromised cell membrane [18].

Statistical analysis. Each experiment was repeated at least three times. Numerical data are presented as means ± SD. The difference between means was analyzed with Student's *t* test. All statistical analyses were performed using SPSS11.0 software (Chicago, IL). Differences were considered significant when $p < 0.05$.

Results

The expression of RhoE is higher in SGC7901/VCR cells than in SGC7901 cells at both the mRNA and protein level

RT-PCR (Fig. 1A) revealed a higher level of RhoE mRNA in SGC7901/VCR than in SGC7901 cells ($p < 0.05$). Immunocytochemistry (Fig. 1C) showed that the expression of RhoE was much higher in SGC7901/VCR than in SGC7901 cells, with the latter showing an almost no staining. Of note, RhoE was widely expressed in the cytoplasm and was also localized to the nuclear membrane. Western blot analysis (Fig. 1B) showed a similar result to immunocytochemistry. These results indicate that RhoE is overexpressed in SGC7901/VCR cells compared with SGC7901 cells at both mRNA and protein levels.

Overexpression of RhoE can significantly enhance drug resistance of SGC7901 cells to multiple antitumor drugs

The increased expression of RhoE in SGC7901/VCR might be a secondary effect of MDR phenotype. To investigate whether RhoE can directly regulate MDR of gastric cancer cells, we altered the expression of RhoE in SGC7901 or SGC7901/VCR cells and then examined the changes in sensitivity of gastric cancer cells to multiple antitumor drugs. Because the expression of RhoE was relatively low in SGC7901 and high in SGC7901/VCR, we transfected pCMV-FLAG-RhoE into SGC7901 cells to upregulate RhoE expression, and downregulated it in SGC7901/VCR using siRNA. However, we were unable to detect any endogenous expression of RhoE in SGC7091/VCR cells after siRNA transfection. Therefore, the following experiments were performed based on the induction of RhoE in SGC7901 cells.

As shown in Fig. 2, the intracellular expression of FLAG-tagged RhoE was detected by the anti-RhoE antibody and the anti-FLAG antibody. Furthermore, this upregulation of RhoE could be detected as soon as 24 h after transfection and remained elevated for up to 6 days, which allowed us to continue our experiments.

The IC50 values of SGC7901-RhoE cells for ADR, VCR, CDDP, 5-FU, VP-16, MMC were significantly increased compared with those of SGC7901-FLAG cells (Table 1). However, such results were not found for MTX and Ara. Thus upregulation of RhoE could directly enhance the resistance of gastric cancer cells to multiple antitumor drugs.

Effects of RhoE on intracellular ADR accumulation

The effects of RhoE on intracellular drug accumulation and retention were evaluated using ADR as a probe. The ADR-releasing index of SGC7901-RhoE and SGC7901-FLAG cells were 0.275 ± 0.156 and 0.329 ± 0.140, respectively (Table 2). Statistical analysis revealed no significant difference ($p > 0.05$) for the ADR-releasing index between these two cell lines.

RhoE does not alter the expression of P-gp and MRP-1

Western blotting (Fig. 3) revealed that P-gp and MRP-1 were expressed at a similar level in both cell lines, suggesting that RhoE overexpression could not modulate the expression of these proteins. Taken together, this indicates that RhoE does not modulate the MDR phenotype by decreasing the accumulation of drugs in gastric cancer cells.

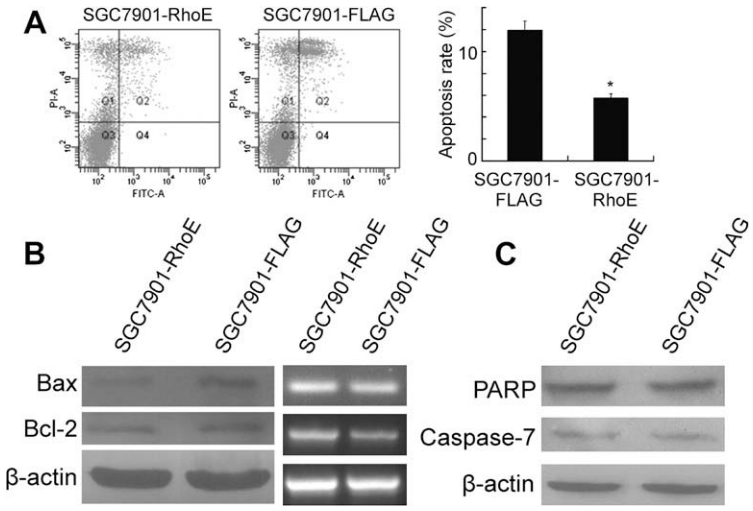

Fig. 4. Observation of the role of RhoE in vincristine-induced apoptosis. (A) Overexpression of RhoE significantly decreased the proportion of apoptotic cells in SGC7901-RhoE cells compared with controls ($p < 0.05$). (B) Western blot analysis revealed that Bax was significantly decreased in SGC7901-RhoE cells ($p < 0.05$), while Bcl-2 was not ($p > 0.05$). RT-PCR showed that mRNA of both Bax and Bcl-2 were expressed at a similar level in SGC7901-RhoE and SGC7901-FLAG cells ($p > 0.05$). (C) Western blot analysis revealed that RhoE overexpression had no effect on the activities of cleaved PARP or Caspase 7 ($p > 0.05$).

Induction of RhoE can inhibit apoptosis of SGC7901 cells induced by VCR by suppressing Bax activity

The development of drug resistance in various cancer cells has been linked to a reduced susceptibility to drug-induced apoptosis, at least in some cases [19,20]. Considering that RhoE has been reported to increase or inhibit cell apoptosis through different pathways depending on different cell backgrounds [4–7], we hypothesized that RhoE could enhance the MDR of gastric cancer cells partly by modulating apoptosis induced by antitumor drugs. In the apoptosis assay, the proportion of apoptotic cells in SGC7901-FLAG cells was significantly greater (>2 folds) than in the SGC7901-RhoE cells (Fig. 4A). This indicates that RhoE inhibits VCR-induced apoptosis of gastric cancer cells. Consequently, we investigated the effects of enforced RhoE expression on the expression of typical apoptotic molecules Bcl-2, Bax, and Caspase family. Western blotting revealed that RhoE overexpression downregulates the expression of Bax protein, but not of Bcl-2, and RT-PCR analysis showed similar expression of Bax in SGC7901-RhoE cells and SGC7901-FLAG cells at the mRNA level (Fig. 4B). The expression of cleaved PARP and Caspase 7 were also similar in SGC7901-RhoE cells and SGC7901-FLAG cells (Fig. 4C); cleaved Caspase 6 and Caspase 9 were not detected. In brief, RhoE appeared to inhibit VCR-induced apoptosis of gastric cancer cells by suppressing the expression of Bax at the posttranscriptional level.

Discussion

RhoE belongs to an atypical subgroup of Rho proteins and has strong homology with RhoA, RhoB, and RhoC, but RhoE only binds with GTP and is unable to hydrolyze GTP, which remains in an active form in cells [1]. The expression of RhoE often changes in many pathological processes, especially in tumorigenesis. Bektic et al. [4] found that RhoE was downregulated in prostate cancer compared with that in normal prostatic cells and re-expression of RhoE resulted in cell cycle arrest at the G_2/M phase and induced apoptosis. A similar result was also found in the U87 human glioblastoma cell line [7]. In contrast, the expression of RhoE was increased in other tumors such as colorectal cancer, pancreatic cancer, lung cancer and melanomas [21–24]. In addition, overexpression of RhoE in non-small cell lung cancer might correlate with an unfavorable outcome [23]. Thus RhoE can not be considered only as an oncogene or tumor-suppressor. Its function appears to vary depending on the organs involved, the stimulating signals, or the way the cancer develops.

RhoE expression was significantly increased in SGC7901/VCR at both the mRNA and protein levels, but the causality between RhoE and MDR has not been elucidated. The MTT assay demonstrated that overexpression of RhoE could enhance the resistance of gastric cancer cells to antitumor drugs, which indicates a direct role of RhoE in the development of MDR. Since VCR and ADR are common substrates for P-gp [25], we investigated the effects of RhoE on intracellular drug accumulation and on the expression of P-gp and MRP-1, two transport related proteins. The results of flow cytometry and Western blot were negative, suggesting that the P-gp pathway does not mediate RhoE-associated MDR. Therefore, we suggest that RhoE regulates MDR by interfering with other biological effects, for example, apoptosis caused by antitumor drugs.

Apoptosis is activated by many chemotherapeutic drugs and mediates killing-cell effects. In our study, the apoptosis assay revealed that increased RhoE significantly inhibits apoptosis induced by VCR, which supports our hypothesis. Considering that antitumor drugs mostly activate the intrinsic pathway of apoptosis, we then analyzed the expression of Bcl-2 and Bax, two key regulators of the intrinsic pathway of apoptosis, after the induction of RhoE. While no marked change in Bcl-2, an antiapoptotic protein, was observed between SGC7901-RhoE and SGC7901-FLAG cells, Bax, which is a pro-apoptosis protein and was shown to promote MDR of gastric cancer [26], was significantly suppressed by RhoE, leading to the survival of gastric cancer cells.

The Caspase family of proteins executes the intrinsic apoptosis pathway. Based on our previous study, we hypothesized that the Caspase family might take part in the inhibition of apoptosis by RhoE. Surprisingly, however, we did not detect any differences in Caspase activities in our cell system, suggesting that other effectors downstream of Bax, rather than inactivation of the Caspase family, are required for RhoE induced cell survival.

In conclusion, we have shown for the first time that RhoE is upregulated in the multidrug-resistant cell line SGC7901/VCR, and that overexpression of RhoE can inhibit apoptosis induced by antitumor drugs by downregulating the proapoptotic protein Bax and significantly promote the MDR phenotype of gastric cancer cells. Further analysis of the biological functions of RhoE in MDR

of gastric cancer will be helpful to further understand the mechanisms of MDR in gastric cancer and to develop new strategies to prevent MDR.'

Acknowledgments

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