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RESEARCH PAPER

RhoGDI2 is associated with HGF-mediated tumor invasion through VEGF in stomach cancer

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Abstract RhoGDP dissociation inhibitor 2 (RhoGDI2) has been identified as a regulator of tumor metastasis; however, its role in cancer remains controversial. The aims of this study were to analyze RhoGDI2 in gastric cancer growth and metastasis, and to determine its possible signaling pathway. The level of expression of RhoGDI2 was further confirmed by real time RT-RCR and Western blot analysis. Transfection of cells with RhoGDI2 shRNA resulted in no effects of cell proliferation, as determined with MTT assays. In an in vitro invasion assay, significantly fewer cells transfected with RhoGDI2 shRNA, compared with control cells, were able to invade across a Matrigel membrane barrier. The role of RhoGDI2 in the level of HGF-induced up-regulation of vascular endothelial growth factor (VEGF) was measured by knockdown of RhoGDI2 with RhoGDI2 shRNA and a chromatic immuno-precipitation assay. The levels of RhoGDI2 and VEGF were up-regulated in cells treated with HGF in a dose-dependent manner. HGF-induced up-regulation of VEGF was repressed by RhoGDI2 knockdown. HGF-induced upregulation of phosphorylated ERK and P38 levels was inhibited in RhoGDI2 knockdown cells. HGF enhanced the binding activity of RhoGDI2 to the VEGF promoter in control cells, but not in RhoGDI2-shRNA cells. Findings of this study also showed a statistically significant difference in the mean RhoGDI2 level before and after surgery ($p < 0.01$) and the mean level of RhoGDI2 before surgery showed a statistically significant difference depending on lymphatic, neural invasion and stage ($p < 0.05$). In conclusion, RhoGDI2 might play an important role in up-regulation of VEGF induced by HGF and contributes to HGF-mediated tumor invasion and metastasis, which may serve as a promising target for gastric cancer therapy.

Keywords RhoGDI2 · VEGF · HGF · Invasion

Introduction

Hepatocyte growth factor (HGF), identified as a mitogen for hepatocytes, was shown to play a role of ligand with a different activity of inducing epithelial cell dissociation. HGF, produced primarily by mesenchymal cells, is a unique growth factor that elicits various cellular responses, such as mitogenesis, motility, and morphogenesis. Multiple roles of HGF in biological events, including scattering, invasion, proliferation, transformation, and angiogenesis through receptor tyrosine kinase c-Met have been widely identified. Although the HGF and c-Met pathway plays an important role in normal cell development, this pathway has been known in pathogenesis of most types of human cancers [1–3].

In the previous report, the author investigated differentially expressed genes in NUGC-3 cells and MKN28 cells

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treated with HGF using 17K human cDNA microarrays. Seven genes, including *Homo sapiens* Bcl2 antagonist of Cell death (BAD), *Homo sapiens* histone deacetylate 5 (HDAC5), Rho GTP dissociation 2 inhibitor (RhoGDI2), Kiss-1, *Homo sapiens* X-ray repair complementing defective repair in Chinese hamster cell 1 (XRCC1), and *Homo sapiens* interleukin 1, beta (IL-1β), HMG1 were upregulated threefold or higher after treatment with HGF. Among these upregulated genes, RhoGDI2 was selected for investigation of its role in HGF-mediated cell invasion and proliferation in gastric cancer cells.

The Rho family, including RhoA, RhoB, RhoC, Rac1, and cdc42 is comprised of small GTPases that control a diverse array of cellular processes, including cell polarity, membrane transport, cytoskeletal dynamics, and gene expression [4–7]. Rho family members are known to be regulated by Rho GDP dissociation inhibitor (RhoGDI). Rho family members, which bind tightly to guanine nucleotide, exist as two forms, a GDP bound and GTP bound form, while the GDP bound conformation is inactive and generally cytosolic, GTP bound conformation is active and mainly membrane bound. Cycling of two forms is regulated by several proteins [3, 8, 9]. Of these proteins, Rho GDP dissociation inhibitors, including RhoGDI1, RhoGDI2, and RhoGDI3, are a small family of chaperone proteins that bind GDP bound conformation and can stabilize this form. For activation, GDP bound conformation should dissociate the Rho GDI and change to the GTP bound form. GTP bound form delivers signal transduction and enables forwarding the signal pathway [10, 11].

Some recent studies have reported a possible association of Rho family with vascular endothelial growth factor (VEGF), which is well known to induce angiogenesis. Bryan et al., suggested a key role of Rho signaling in multiple aspects of VEGF-mediated angiogenesis [12]. Some evidence also suggests that Rho GDIs, as regulators of Rho family, also are associated with a VEGF signaling pathway and play an important role in regulation of cancer growth and metastasis [5, 13]. Among these RhoGDIs, the role of pathogenesis of RhoGID2 in cancer remains especially controversial [14–17]. In this study, the role of HGF-mediated upregulation of RhoGDI2 in gastric cancer growth and metastasis was investigated.

Materials and methods

Cell culture

Two human gastric cancer cell lines, poorly differentiated adenocarcinoma, NUGC-3, and the moderately differentiated tubular adenocarcinoma, MKN-28, were obtained from the Korea Cell Line Bank (Seoul, Republic of Korea). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, twofold vitamin solution, and 50 U/ml penicillin/streptomycin (Life Technologies, Inc., Gaithersburg, MD, USA). Unless otherwise noted, cells underwent passage and were removed from flasks when 70–80 % confluent.

Reagents and antibodies

Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Bio-Rad Laboratories (Philadelphia, PA, USA). Recombinant human HGF (R&D Systems Inc., Minneapolis, MN, USA) and a rabbit polyclonal antibody against human RhoGDI2 were purchased from Abnova (Jhouzih St, Taipei, Taiwan). Antibodies against ERK, p38, phospho-p38, and phospho-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). Recombinant VEGF protein was purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA).

RNA extraction and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Cell were lysed with 700 µl of TRI reagent (Molecular Research Center, Ohio) by vortexing and incubated on ice for 30 s. Chloroform (140 µl) was added to the lysates and then vortexed for 30 s. The mixture was incubated for 15 min on ice and centrifuged at 15,000 rpm for 20 min at 4 °C. The aqueous phase was transferred into a new incubator and an equal volume of isopropanol was added. The mixture was incubated for 15 min on ice and centrifuged at 15,000 rpm for 20 min at 4 °C. After discarding supernatants, pellets were washed with 70 % cold ethanol and air dried. The pellets were dissolved in DEPC treated distilled water. The concentration of RNA was determined by measuring absorbance at 260 nm using a UV spectrophotometer (Shimadzu, Japan).

Complementary DNA (cDNA) was synthesized from total RNA using MMLV reverse transcriptase (Promega Corp., Madison, WI, USA), using the oligo (dT) priming method in a 10 µl reaction mixture. PCR was performed in a 10 µl reaction volume containing 10 mM Tris–HCl pH 8.5, 50 mM KCl, 1 µl cDNA, 200 µM dNTPs, 1 mM $MgSO_4$, 1U platinum pfxTaq polymerase, and 2 µM primers. The reactions included the initial denaturation at 95 °C for 4 min; 27 cycles at 94 °C for 15 s and 60 °C for 15 s and 72 °C for 30 s; and the final extension at 72 °C for 10 min. PCR products were separated on a 1.5 % agarose gel containing ethidium bromide and visualized on a UV transilluminator.

Western blot analysis

Cells were harvested and incubated with a lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 % Trion X-100, 10 % glycerol, 1 mM PMSF, 1 mM sodium vanadate, and 5 mM NaF) with protease inhibitors and centrifuged at 15,000 rpm, 4 °C for 10 min. Proteins (50 μg) were separated on 10 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were soaked with 5 % non-fat dried milk in TTBS (10 mM Tris–HCl pH 7.5, 150 mM NaCl, and 0.05 % Tween-20) for 30 min and then incubated overnight with a primary antibody at 4 °C. After washing six times with TTBS for 5 min, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1.5 h at 4 °C. The membranes were rinsed three times with TTBS for 30 min and antigen–antibody complex was detected using the enhanced chemiluminescence detection system.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cells (1,500/well) were seeded in 96-well plates in DMEM supplemented with 5 % FBS and incubated for 24 h. Cells were then serum-starved for 24 h and treated for 72 h with or without HGF (10 ng/ml). At the end of this incubation period, 50 μl of 2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added and the cells were allowed to incubate for 3 h at 37 °C. The supernatant was carefully removed by aspiration, and convert dye was dissolved with 100 μl DMSO. The plates were placed in a microplate shaker for 5 min and the absorbance was measured at 570 nm using a Biorad multiscan plate reader.

RhoGDI2 knockdown with short hairpin RNA (shRNA)

The human RhoGDI2-specific shRNA expression vector (RhoGDI2-shRNA, RHS4533-TCN0000047413, RHS4533-TCN0000047417) containing RhoGDI2-targeted shRNA sequence (AAACCCAGGGCTGCCTTGGAAAAG) was purchased from Open Biosystems (Huntsville, AL, USA). Cells were transfected with RhoGDI2-shRNA using Lipofectamine (Life Technologies Inc., Gaithersburg, MD, USA). Clonal selection was performed by culturing with puromycin (10 μg/ml) followed by serial dilution of the cells. Stable transfectant clones with low expression of the target genes were identified by Western blot analysis.

RhoGDI2 ELISA

Levels of abundance of RhoGDI2 in patient sera were measured using ELISA (BioVendor, Candler, NC, USA) according to the manufacturer's instructions. Serum samples from patients with stomach cancer were collected and were assayed simultaneously and in duplicate. Serial dilutions of RhoGDI2 standard were assayed in parallel with serum samples. The optical density was plotted against standard RhoGDI2 concentrations for generation of the standard curve according to the manufacturer's protocol.

Standard two chamber invasion assay

Control cells and transfected cells (1×10^4) were placed in the upper chamber of a Matrigel migration chamber with 0.8-micron pores (Fisher Scientific, Houston, TX, USA) in media containing 5 % FBS with/without HGF (10 ng/ml). After incubation for 48 h, cells were fixed and stained using a HEMA 3 stain set (Curtis Matheson Scientific, Houston, TX, USA) according to the manufacturer's instructions. The stained filter membrane was cut and placed on a glass slide. Migrated cells were counted under light microscopy (10 fields at 200× power).

Chromatin immunoprecipitation (CHIP) assay

The chromatin immunoprecipitation (CHIP) assay was performed using the chips assay kit (Upstate Biotechnology, Waltham, MA, USA) following the manufacturer's directions. Briefly, cells were fixed with 1 % formaldehyde at 37 °C for 10 min. Cells were washed twice with ice-cold PBS with protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 1 mg/ml aprotinin, and 1 mg/ml pepstatin A), scraped and pelleted by centrifugation at 4 °C. Cells were re-suspended in a lysis buffer (1 % SDS, 10 mM EDTA, and 50 mM Tris–HCl, pH 8.1), incubated for 10 min on ice, and sonicated for shearing of DNA. After sonication, lysate was centrifuged for 10 min at 13,000 rpm at 4 °C. The supernatant was diluted in CHIP dilution buffer (0.01 % SDS, 1 % Triton X-100, 2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl, and protease inhibitors). Primary antibodies were added, followed by overnight incubation at 4 °C with rotation. The immunocomplex was collected by protein A/G agarose beads and washed with low salt washing buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 200 mM Tris–HCl, pH 8.1, and 150 mM NaCl), high-salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 200 mM Tris–HCl, pH 8.1, and 500 mM NaCl), A LiCl washing buffer (0.25 M LiCl, 1 % NP40, 1 % deoxycolate, 1 mM EDTA, and 10 mM Tris–HCl, pH 8.1), and, finally, 1TE buffer (10 mM Tris–HCl, and 1 mM EDTA, pH 8.0). The immunocomplex was then eluted using the elution buffer (1 % SDS, 0.1 M $NaHCO_3$, and 200 mM NaCl) and the cross-links were reversed by heating at 65 °C for 4 h. After reaction, the samples were adjusted to 10 mM EDTA, 20 mM Tris–HCl, pH 6.5, and 40 mg/ml proteinase K, and incubated at 45 °C for 1 h. DNA was recovered and was

subjected to PCR amplification of the VEGF promoter region (+3 to −224) were 5′-ttttcaggctgtgaaccttg-3′(forward) and 5′-gatcctccccgctaccag-3′(reverse).

Luciferase assay

The transcriptional regulation of VEGF by HGF, RhoGDI2 was examined using transient transfection with an VEGF promoter luciferase reporter construct (VEGF-pMetluc reporter). Cell transfection was performed using Lipofectamin^{TM} 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For the luciferase reporter gene assay, control cells and shRhoGDI2 expression cells were cotransfectioned with 1 μg VEGF-pMetluc-reporter plasmids and 0.05 μg of pHYK plasmids, which was used as an internal transfection-efficiency control. Transfected cells were stimulated with/without 10 ng/ml HGF for 1 h. The promoter activity was analyzed in each well of the cultured medium using a Dual Glo^{TM} luciferase assay system with a Turner Designs instrument luminometer (Turner Designs, Sunnyvale, CA, USA). The measured luminescence of firefly luciferase was divided by renilla luciferase and the resulting quotient corresponded to the relative amounts of luciferase.

Patient's sample

We obtained the blood samples of stomach cancer patients who underwent a surgery in our hospital from January to December of 2013. We obtained 10 cc of blood sample pre and post operatively and a written informed consent was taken from the patients. The study protocol was approved by Institutional Review Board of our hospital.

Statistical analysis

Values are expressed as mean ± SD. The Student's *t* test was used for the analyses. A *p* value of less than 0.05 was considered statistically significant.

The difference between RhoGDI2 levels preoperatively and RhoGDI2 levels postoperatively was compared using paired *t* test. Mean value of pre-operation RhoGDI2 level was analyzed using two sample *t* test and one way ANOVA according to character of endpoints.

Results

Up-regulation of RhoGDI2 level after treatment with HGF

To validate upregulation of the RhoGDI2 gene, RT-PCR and Western blot analysis were performed. RT-PCR showed that the expression level of RhoGDI2 mRNA was increased by treatment with HGF (Fig. 1a). The RhoGDI2 protein level was increased after treatment with HGF, confirmed by Western blot analysis (Fig. 1b). The expression level of RhoGDI2 protein was also increased with increasing concentration of HGF (0, 10, and 40 ng/ml) (Fig. 1c).

Effects of RhoGDI2 knockdown on HGF-mediated cell proliferation

To examine the effects of RhoGDI2 knockdown on HGF-mediated cell proliferation in gastric cancer cells, stable RhoGDI2-shRNA cells were prepared by transfection of RhoGDI2 shRNA into NUGC-3 and MKN-28 cells. The control cells and RhoGDI2-shRNA cells (shRhoGDI2(1), shRhoGDI2(2)) were treated with or without 10 ng/ml HGF for 72 h and cell proliferation was measured using the MTT assay. HGF induced an increase in cell proliferation in control cells and RhoGDI2-shRNA cells (shRhoGDI2(1), shRhoGDI2(2)) ($p < 0.01$). However, no significant difference of HGF-mediated cell proliferation was observed between control cells and RhoGDI2-shRNA cells (shRhoGDI2(1), shRhoGDI2(2)) ($p > 0.05$) (Fig. 2), suggesting that HGF-mediated upregulation of RhoGDI2 has no effect on cell proliferation induced by treatment with HGF.

Effect of RhoGDI2 knockdown on HGF-mediated cell invasion

To determine whether RhoGDI2 plays a role in HGF-mediated cell invasion, an in vitro invasion assay was performed using a Matrigel migration chamber. While treatment with HGF resulted in increased cell invasion in control cells, HGF-mediated cell invasion was significantly decreased in RhoGDI2-shRNA cells (shRhoGDI2(1), shRhoGDI2(2)) compared to control cells (Fig. 3). This result suggests that HGF-mediated upregulation of RhoGDI2 might be involved in regulation of cell invasion.

Effect of RhoGDI2 knockdown on HGF-mediated phosphorylation of ERK and p38

Because RhoGDI2 knockdown inhibits HGF-mediated cell invasion in NUGC-3 and MKN-28 cells and HGF is known to exert its activity through ERK and p38 phosphorylation [18], the effect of RhoGDI2 knockdown on HGF-mediated phosphorylation of ERK and p38 was measured. We identified that phosphorylation of p38 and ERK was increased by HGF. And then we checked the effect the RhoGDI2 knockdown of phosphorylation of p38 and ERK with HGF treatment by western blotting. RhoGDI2-shRNA

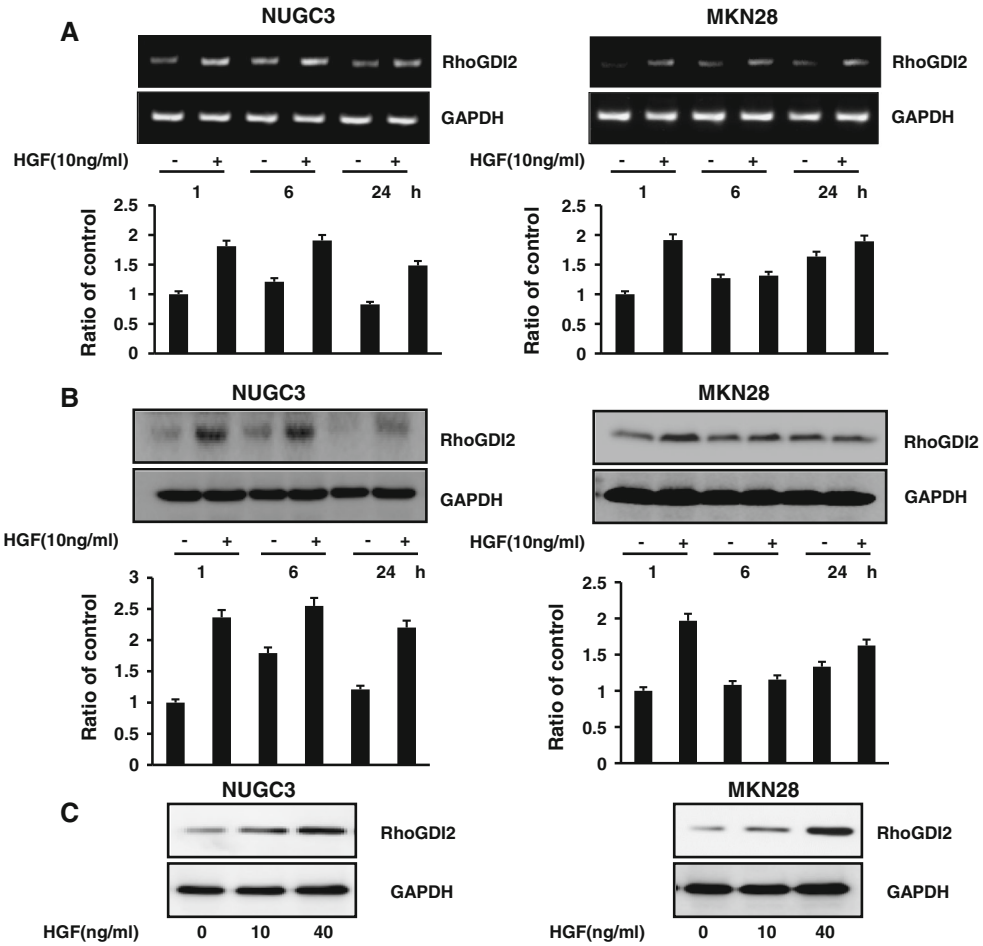

Fig. 1 Effects of HGF on the expression level of RhoGDI2 in NGUC-3 and MKN-28 cells. Cells were serum-starved for 24 h, treated with/without HGF 10 ng/ml for the indicated times, and harvested. The expression levels of RhoGDI2 RNA and protein were confirmed by reverse transcription-polymerase chain reaction (**a**) and Western blot analysis (**b**). Also, the density of the bar was measured compared with control. The graph of each bar was represented. Serum-starved cells were treated with increasing concentrations of HGF (0, 10, and 40 ng/ml) for 1 hand harvested. Expression levels of RhoGDI2 were confirmed by Western blotting (**c**). Representative data from three independent experiments are illustrated

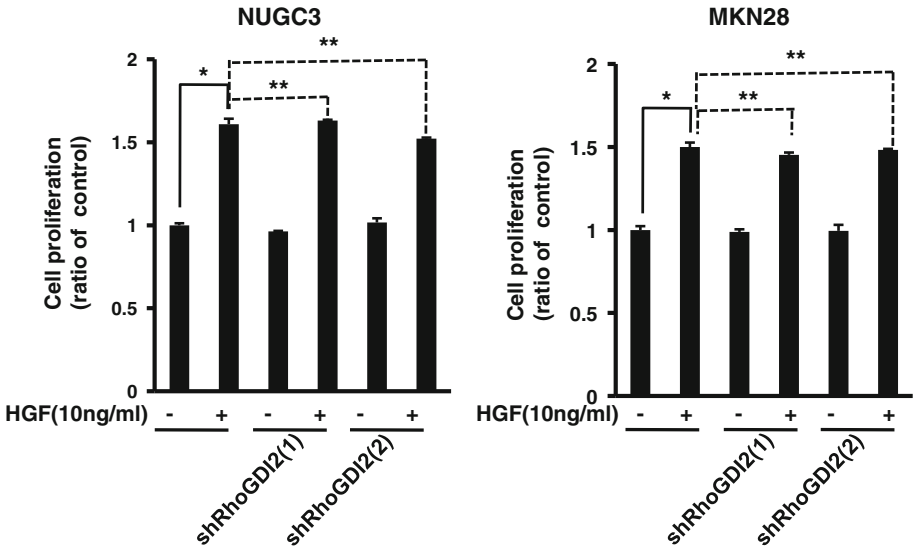

Fig. 2 Effects of RhoGDI2 knockdown on cell proliferation. Control cells and stable RhoGDI2-shRNA cells (shRhoGDI2(1), shRhoGDI2(2)) (1,000/well) were seeded in 96-well plates with DMEM media supplemented with 5 % FBS and incubated for 24 h. After serum-starvation for 24 h, cells were treated with or without 10 ng/ml HGF for 72 h. Cell proliferation was measured by MTT assays and expressed as a percentage of HGF-untreated control cells. Values are expressed as mean ± SD of three independent experiments performed in triplicate. *$p < 0.01$, **$p > 0.05$

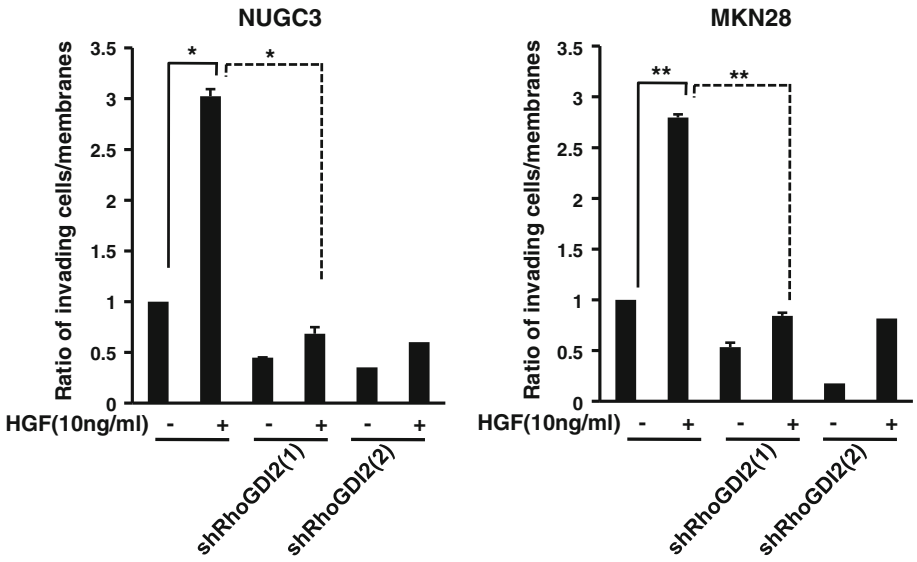

Fig. 3 Effect of RhoGDI2 knockdown on HGF-mediated cell invasion. Stable RhoGDI2-shRNA cells and control cells (shRhoGDI2(1), shRhoGDI2(2)) were treated with or without 10 ng/ml HGF for 48 h. Cell invasion was measured using the standard two chamber invasion assay with Matrigel migration chambers. Values are expressed as mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$

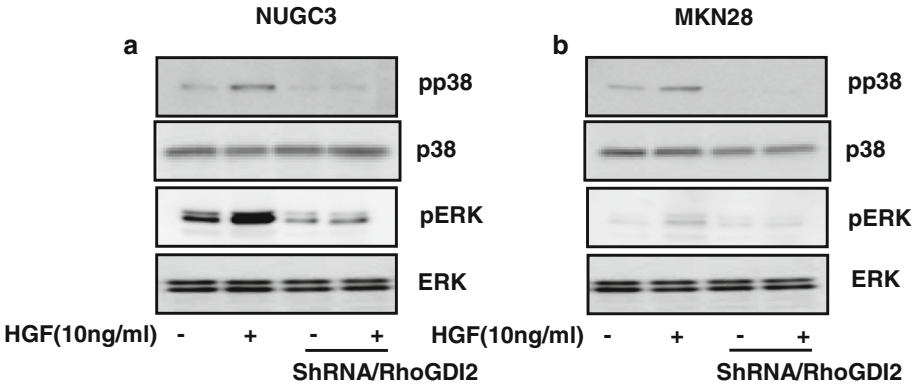

Fig. 4 Effects of RhoGDI2 knockdown on p38 and ERK phosphorylation. **a** Control cells and stable RhoGDI2-shRNA cells (1×10^6/well) were plated overnight in complete medium, serum-starved for 24 h, treated with or without 10 ng/ml HGF for 1 h, and harvested. **b** The expression levels of p38 and ERK phosphorylation were analyzed by Western blotting. Representative data from three independent experiments are illustrated

cells showed a decreased in HGF-mediated phosphorylation of ERK and p38 compared to control cells (Fig. 4). This result suggests that upregulation of RhoGDI2 by HGF might be involved in regulation of ERK and p38 phosphorylation induced by HGF.

Up-regulation of VEGF after treatment with HGF

VEGF is a well-known angiogenic factor and angiogenesis is a key factor in regulation of cancer cell invasion. Therefore, in order to investigate an important molecular link among HGF, cell invasion, and HGF-mediated upregulation of RhoGDI2, the expression level of VEGF protein was measured in NUGC-3 and MKN-28 cells treated with HGF. The VEGF protein level was increased after treatment with HGF, confirmed by Western blot analysis (Fig. 5a). Treatment with HGF resulted in a dose-dependent increase in the levels of VEGF protein (0, 10, and 40 ng/ml) (Fig. 5b).

Effect of RhoGDI2 knockdown on the expression level of VEGF protein

To reveal that HGF-mediated upregulation of RhoGDI2 might be involved in regulation of HGF-mediated expression of VEGF, control cells and RhoGDI2-shRNA cells were treated with or without HGF and the level of VEGF protein was measured by Western blotting. Although treatment with HGF resulted an increase in the expression level of VEGF protein in control cells, HGF-induced upregulation of VEGF was not observed in RhoGDI2-shRNA cells (Fig. 6). This result suggests that RhoGDI2 might play an important role in regulation of HGF-mediated upregulation of VEGF.

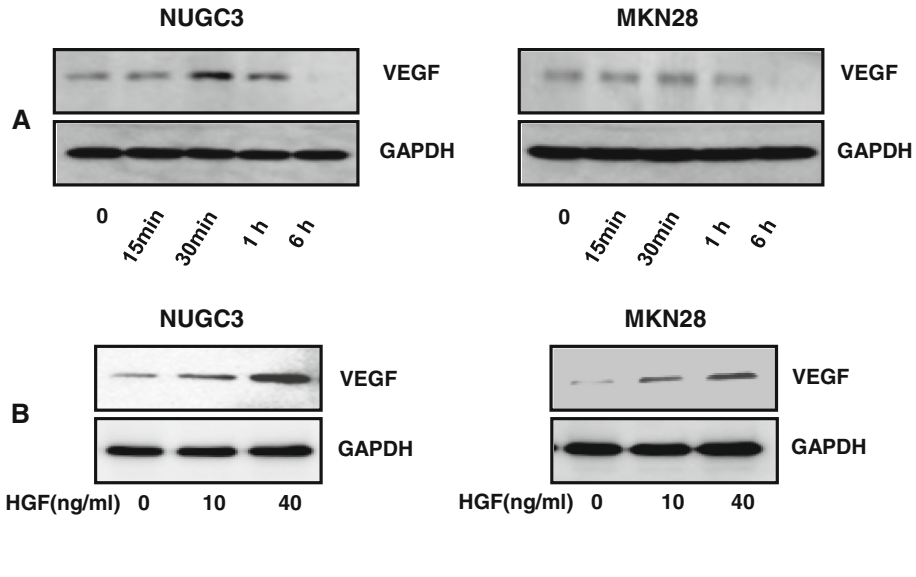

Fig. 5 Effect of HGF on VEGF expression. Cells were serum-starved for 6 h, treated with/without HGF 10 ng/ml for the indicated times, and harvested (**a**). Serum-starved cells were treated with increasing concentrations of HGF (0, 10, and 40 ng/ml) for 1 hand harvested (**b**). Expression levels of VEGF were confirmed by Western blotting. Representative data from three independent experiments are illustrated

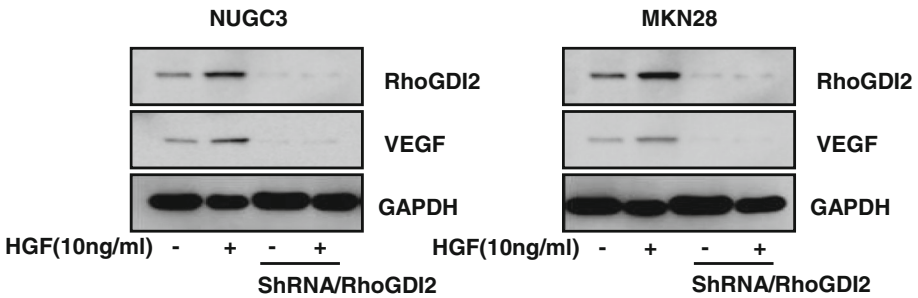

Fig. 6 Effects of RhoGDI2 knockdown on HGF-mediated upregulation of VEGF. Control cells and stable RhoGDI2-shRNA cells (1×10^6/well) were plated overnight in complete medium, serum-starved for 24 h, treated with or without 10 ng/ml HGF for 1 h, and harvested. Expression levels of RhoGDI2 and VEGF were analyzed by Western blotting. Representative data from three independent experiments are illustrated

Binding of RhoGDI2 to the VEGF promoter

Because RhoGDI2 knockdown blocked VEGF upregulation induced by HGF, the question of whether RhoGDI2 might regulate transcriptional activity of VEGF mRNA by binding to the VEGF promoter was investigated. Two putative RhoGDI2 binding sites were found in the VEGF promoter through computer-based analysis for the promoter sequence (Fig. 7a). To examine the question of whether the presumed RhoGDI2 binding sites in the VEGF promoter regulates VEGF transcription, binding of RhoGDI2 protein to the VEGF promoter in control cells and RhoGDI2-shRNA cells treated with or without HGF was analyzed using the CHIP assay. HGF enhanced RhoGDI2 binding activity to the VEGF promoter in control cells, but not in RhoGDI2-shRNA cells (Fig. 7b). These results imply that RhoGDI2 might be involved in HGF-mediated up regulation of VEGF in gastric cancer cells.

To identify the functional activity of binding between RhoGDI2 and VEGF promoter, the plasmid containing VEGF promoters sequence was transfected RhoGID2-shRNA cells and control cells with or without HGF. The promoter activity by luciferase assay was analyzed. HGF enhanced the promoter activity to the VEGF promoter in control cells, but not in RhoGDI2-shRNA cells (Fig. 8).

Analysis of RhoGDI2 levels in sera of gastric patients before and after surgery

After all analyses, it could be presumed that RhoGDI2 mediates cancer cell invasion and metastasis through the VEGF pathway. The author hypothesized that after surgery, RhoGDI2 level would be decreased in patients' sera, compared to the level before surgery. Pre and post operation sera were obtained from 50 patients with gastric cancer and evaluated for RhoGDI2 levels by ELISA.

Of these patients, 31 patients were male (62 %) and 19 were female (38 %). Median age was 61.2 (range 39–84) years old, and 23 patients had stage Ia (43 %), the highest fraction in 50 patients. The number of patients of early stage and advanced stage was 25 patients, respectively. The mean preoperative RhoGDI2 level of 50 patients was

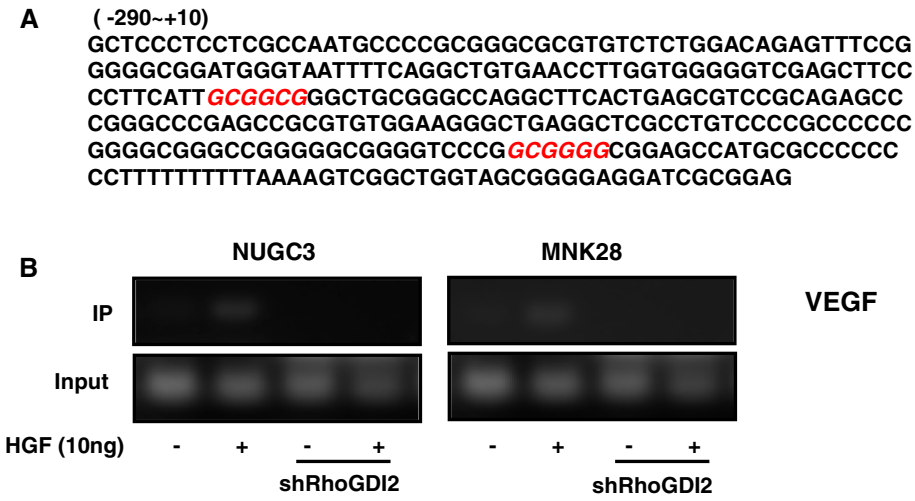

Fig. 7 Binding of RhoGDI2 to the VEGF promoter. Two putative RhoGDI2 binding sites in the VEGF promoter. *Red line* marks the location of the RhoGDI2 binding site (**a**). The CHIP assay for binding of RhoGDI2 to the VEGF promoter. Immunoprecipitation was performed using an anti-RhoGDI2 antibody. Amplification of a fragment of the proximal VEGF promoter containing the RhoGDI2 binding site was confirmed (**b**). Representative data from three independent experiments are shown. (Color figure online)

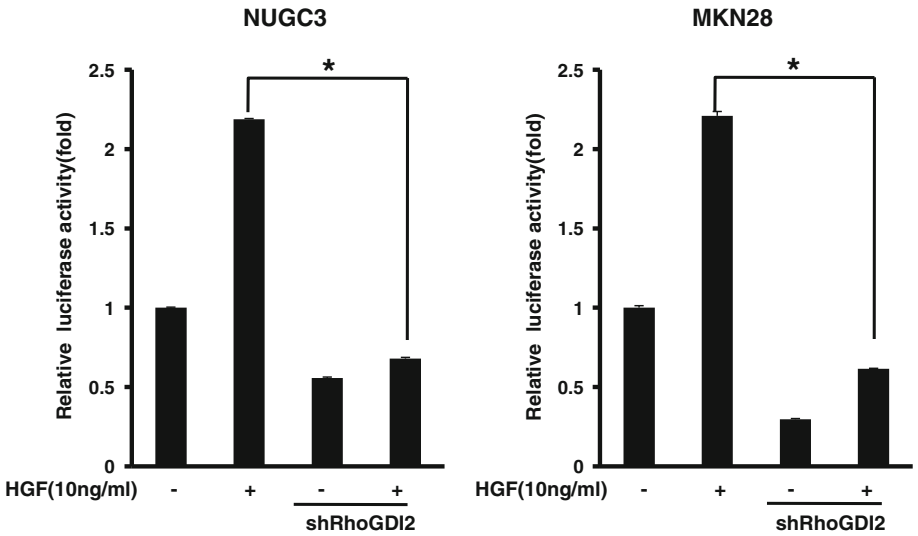

Fig. 8 HGF and RhoGDI2 induced VEGF promoter activity. Stable RhoGDI2-shRNA cells and control cells were cotransfected with the plasmid containing VEGF promoters sequence and stimulated with/without 10 ng/ml HGF for 1 h. The promoter activity was analyzed in each well of the cultured medium using a Dual Glo^{TM} luciferase assay system with a luminometer (Turner Designs, Sunnyvale, CA, USA). The measured luminescence of firefly luciferase was divided by renilla luciferase and the resulting quotient corresponded to the relative amounts of luciferase. *$p < 0.01$

0.892 ng/ml and that of postoperative RhoGDI2 level was 0.679 ng/ml. A statistically significant difference in mean RhoGDI2 levels was observed between the two groups ($p < 0.01$) (Fig. 9). In addition, the mean pre and post operation RhoGDI2 levels according to patients' characteristics, namely tumor size, histologic type, lymph node metastasis, lymphatic, and neural invasion and staging was analyzed. Of these characteristics, statistically significant difference was observed in lymphatic invasion ($p = 0.024$) and neural invasion ($p = 0.016$) and in staging ($p = 0.014$). No significant difference was observed in other characteristics (Table 1).

Discussion

Tumor invasion and metastasis are fundamental factors in determining the aggressiveness of many human cancers. To disseminate from primary tumor, cancer cells need to attain the ability of degradation and migration through

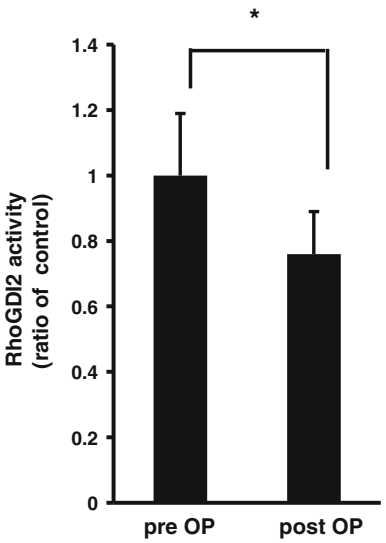

Fig. 9 RhoGDI2 levels in pre or post operation sera of gastric patients. Pre or post operation sera of 50 patients diagnosed with gastric cancer were obtained and RhoGDI2 levels in the sera were measured by ELISA. $*p < 0.01$

Table 1 Clinicopathologic features and pre operation of RhoGDI2 level of 50 patients

Clinicopathologic features	No.	Mean of preop RhoGDI2 ± SD (ng/ml)	*p* value*
Tumor size			
≤5 cm	33	0.860939 ± 0.17	0.275
>5 cm	17	0.916559 ± 0.16	
Histologic type			
Well diff	7	0.819071 ± 0.20	0.485
Mod diff	4	0.942500 ± 0.05	
Poor diff	27	0.865981 ± 0.17	
Other	12	0.925625 ± 0.18	
Lauren classification			
Intestinal	14	0.883607 ± 0.16	0.784
Diffuse	32	0.871375 ± 0.18	
Mixed	4	0.934500 ± 0.17	
Serosa invasion			
Negative	46	0.872337 ± 0.17	0.292
Positive	4	0.966250 ± 0.11	
L/N metastasis			
Negative	33	0.849545 ± 0.16	0.077
Positive	17	0.938676 ± 0.17	
Lymphatic invasion			
Negative	29	0.834517 ± 0.16	0.024
Positive	21	0.942452 ± 0.17	
Neural invasion			
Negative	35	0.842714 ± 0.16	0.016
Positive	15	0.966500 ± 0.16	
Stage			
Early	25	0.821800 ± 0.16	0.014
Advanced	25	0.937900 ± 0.16	

SD standard deviation

p value * < 0.05 were considered to be statistically significant

extracellular matrix [19, 20]. Since VEGF is one of the factors in migration of endothelial cells and formation of new vessels during angiogenesis [21, 22], multiple downstream signaling pathways have been implicated in control of VEGF-dependent effects. Of these downstream signaling [23, 24], Rho signaling associated with Rho family GTPases is reportedly principal for VEGF dependent angiogenesis and capillary formation [25–27].

RhoGDIs are regulators of small GTPase that stabilize GDP-bound Rho Protein in the cytoplasm in order to prevent activation [28]. Among these RhoGDIs, RhoGDI2 is found primarily in hematopoietic tissues [29, 30]. Recent studies have reported that RhoGDI2 is also expressed in non-hematopoietic tumor cells, including bladder, ovarian, and colorectal cancer. Reduced expression of RhoGDI2 was reported to show an association with poor prognosis in patients with advanced bladder cancer and breast cancer [16, 17, 31, 32]. In contrast, an association of increased expression of RhoGDI2 with up-regulation of tumorigenesis and metastasis in ovarian cancer and colorectal cancer has been suggested [14, 15]. After analysis of all of these findings, it appears that RhoGDI2 has an opposed role in cancer progression according to tumor type.

RhoGDI2 function in gastric cancer has also been reported. According to a report by Cho et al., the expression level of RhoGDI2 is increased at higher gastric tumor stage and there is a significant relationship between RhoGDI2 expression and lymph node metastasis. They suggested that RhoGDI2 can function as a positive regulator of tumor progression in gastric cancer [33].

The present study clearly demonstrated that RhoGDI2 is upregulated in NUGC-3 and MKN-28 cells treated with HGF (Fig. 1) and HGF-mediated upregulation of RhoGDI2 is involved in regulation of cell invasion induced by HGF through the VEGF pathway (Figs. 5, 6). In addition, it was shown that RhoGDI2 exerts it activity through binding to the VEGF promoter (Fig. 7). Although some studies have suggested that Rho family is essential for multiple aspects of VEGF-mediated angiogenesis [12] and that RhoGDI2 may be associated with angiogenesis [33–35], RhoGDI2 was found to control the VEGF pathway directly so that cells showing RhoGDI2 expression might have an ability to metastasize and promote growth through angiogenesis. These results are supported by our finding that HGF-mediated cell invasion was decreased in RhoGDI2 knockdown cells compared to control cells (Fig. 3).

On the basis of these findings, it was hypothesized that the level of RhoGDI2 would be decreased after gastric cancer surgery by removal of gastric tumor and lymph node and the level of pre operation RhoGDI2 would be higher in patients with advanced gastric cancer. ELISA was used for quantification of the serum level of RhoGDI2. Analysis of serum RhoGDI2 in 50 gastric cancer patients

showed a statistically significant difference in the mean RhoGDI2 level before and after surgery ($p < 0.01$) (Fig. 9) and the mean level of RhoGDI2 before surgery showed a statistically significant difference depending on lymphatic and neural invasion ($p < 0.05$). In addition, significant differences were observed in RhoGDI2 level before surgery according to cancer staging and macroscopic type ($p < 0.05$) (Table 1). Therefore, findings of this study showed that cancer patients with lymphatic invasion, neural invasion, or advanced staging have higher expression of RhoGDI2, suggesting that a high level of RhoGDI2 might affect an increase in progression and metastasis of gastric cancer.

What is important is that in the first study, quantitative analysis of RhoGDI2 level was performed using ELISA and provided evidence showing that the clinical data supported the findings in vivo. But, RhoGDI2 secretion in blood stream may not be always induced by HGF. To be a promising target in prevention of metastasis in gastric, further studies of association of HGF and RhoGDI2 is needed. Based on these results, we want to study actively the role of stomach cancer.

In conclusion, findings of this study show that RhoGDI2 plays an important role in up-regulation of VEGF and contributes to HGF-mediated tumor invasion and metastasis. RhoGDI2 may be an important factor in understanding of biology in tumorigenesis and metastasis in gastric cancer, which might be a promising target for gastric cancer therapy.

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