Chemotherapy Resistance in Diffuse-Type Gastric Adenocarcinoma Is Mediated by RhoA Activation in Cancer Stem-Like Cells

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Abstract

Purpose: The Lauren diffuse type of gastric adenocarcinoma (DGA), as opposed to the intestinal type (IGA), often harbors mutations in RHOA, but little is known about the role of RhoA in DGA.

Experimental Design: We examined RhoA activity and RhoA pathway inhibition in DGA cell lines and in two mouse xenograft models. RhoA activity was also assessed in patient tumor samples.

Results: RhoA activity was higher in DGA compared with IGA cell lines and was further increased when grown as spheroids to enrich for cancer stem-like cells (CSCs) or when sorted using the gastric CSC marker CD44. RhoA shRNA or the RhoA inhibitor Rhosin decreased expression of the stem cell transcription factor, Sox2, and decreased spheroid formation by 78% to 81%. DGA spheroid cells had 3- to 5-fold greater migration and invasion than monolayer cells, and this activity was Rho-dependent. Diffuse GA spheroid cells were resistant in a cytotoxicity assay to 5-fluorouracil and cisplatin chemotherapy, and this resistance could be reversed with RhoA pathway inhibition. In two xenograft models, cisplatin inhibited tumor growth by 40% to 50%, RhoA inhibition by 32% to 60%, and the combination by 77% to 83%. In 288 patient tumors, increased RhoA activity correlated with worse overall survival in DGA patients (P = 0.017) but not in IGA patients (P = 0.612).

Conclusions: RhoA signaling promotes CSC phenotypes in DGA cells. Increased RhoA activity is correlated with worse overall survival in DGA patients, and RhoA inhibition can reverse chemotherapy resistance in DGA CSC and in tumor xenografts. Thus, the RhoA pathway is a promising new target in DGA patients.

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Introduction

Gastric cancer accounts for nearly 1 million new cancer cases worldwide per year and nearly 700,000 deaths, thus accounting for almost 10% of all cancer-related deaths (1). Except in a few Asian countries such as Japan and South Korea where there is endoscopic screening for gastric cancer, the majority of patients with gastric cancer present with advanced disease. Overall survival for patients with metastatic disease is 3 to 5 months with best supportive care (2). The response rate to multiagent chemotherapy is 50% or greater, but nearly all patients develop chemotherapy resistance, and median survival is extended only to 9 to 11 months (3).

In 1965, Lauren described two distinct histologic types of gastric adenocarcinomas: intestinal and diffuse (4). The intestinal type exhibits components of glandular, solid, or intestinal architecture as well as tubular structures. The diffuse type demonstrates single cells or poorly cohesive cells infiltrating the gastric wall, and progressive disease can ultimately lead to limitis plastica (a.k.a. leather bottle stomach). The two Lauren types of gastric adenocarcinoma have distinct clinical profiles (5). The intestinal type is more common in men and older patients, and is associated with environmental exposures such as Helicobacter pylori (H. pylori) infection. The diffuse type is more common in women and in younger patients and more associated with familial occurrence.

Recently, two studies in Nature Genetics found mutations in RHOA in 14.3% to 25.3% of diffuse gastric cancers (6, 7). This high rate of RHOA mutation in diffuse gastric cancers was confirmed by The Cancer Genome Atlas (TCGA), and the TCGA also found additional fusions in GTPase-activating proteins (GAP), which regulate RhoA activity (8).

RhoA is the founding member of the Rho GTPase family, which also includes Cdc42 and Rac1 (9). These proteins serve as intracellular molecular switches cycling between a GTP-bound active form and a GDP-bound inactive form. RhoA acts through a variety of effectors, including Rho-associated, coiled-coil–containing protein kinase (ROCK), to control processes such as actin–myosin-dependent cell contractility, cell motility, and cell cycle. Currently, very few studies have examined the role of RhoA in diffuse gastric cancer development and progression.

The cancer stem cell theory postulates that cancers harbor a subset of cells that share characteristics of normal stem cells, with a capacity for self-renewal and differentiation (10). Numerous...
Translational Relevance

Gastric cancer accounts for nearly 700,000 cancer-related deaths worldwide per year. The majority of patients with gastric cancer present with advanced disease. The response rate to multagent chemotherapy is 50% or greater, but nearly all patients develop chemotherapy resistance, and median survival is extended only to 9 to 11 months. Recently, genomic studies have found a high rate of mutations in RHOA for the Lauren diffuse type of gastric cancer. Here, we show that the RhoA pathway maintains diffuse gastric cancer stem cell phenotypes such as spheroid formation and promotes epithelial-to-mesenchymal transition. Inhibition of the RhoA pathway can reverse chemotherapy resistance in vitro and in xenograft models. In 136 patients with diffuse gastric cancer, high RhoA activity correlates with significantly worse overall survival. Thus, the combination of RhoA inhibition and chemotherapy may be an effective therapeutic strategy in patients with diffuse gastric cancer.

Materials and Methods

Cell lines and reagents

SNU-638, SNU-719, AGS, and NCI-N87 (subsequently referred to as N87) are Lauren intestinal-type gastric adenocarcinoma cell lines, and KATOIII, SNU-668, SNU-601, and MKN-45 are Lauren diffuse-type gastric adenocarcinoma cell lines. AGS, N87, SNU-601, and MKN-45 cells were obtained from the ATCC. SNU-638, SNU-719, KATOIII, and SNU-601 are from the Korean Cell Line Bank (KCLB). Both ATCC and KCLB perform cell line characterization using short tandem repeat DNA profiling. Cell lines were actively passaged for less than 6 months from the time that they were received from ATCC or KCLB, and UKCCCR guidelines were followed (13). KATOIII cells were maintained in DMEM. All other gastric cancer cell lines were maintained in RPMI-1640. All media were supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin, and L-glutamine 2 mmol/L (“regular media”). Cells were grown and monolayers or spheroids as previously described (14). 5-fluorouracil was purchased from US Biological, and cisplatin was purchased from Enzo Life Sciences. RhoA inhibitor (Rhosin), PI3K inhibitor (LY294002), JNK inhibitor (SP600125), MEK I and II inhibitor (U0126), and p38 MAP kinase inhibitor (SB202190) were purchased from Calbiochem. ROCK inhibitor (Fasudil) was purchased from Abcam.

Western blot analysis

Samples were collected in RIPA buffer (Sigma) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics), and protein concentration was determined by the Bio-Rad Protein Assay. Western blot analysis was performed using the following antibodies: Akt (sc-8312), ERK1 (sc-271270), p38 (sc-81621), JNK2 (sc-827), and c-Myc (sc-40) purchased from Santa Cruz Biotechnology; Sox2 (2748, 3579), Oct-4 (2750), Nanog (4893), Cdc42 (2466), MYPT1 (2634), phospho-MYPT1 (4563), Slug (9585), Snail (3879), MMP-2 (4022), MMP-9 (3852), phospho-Akt (ser473) (9271), phospho-p38 (9211), phospho-ERK1/2 (9101), phospho-JNK1/2 (#9251), CD44 (3578), and cleaved caspase-3 (9661) from Cell Signaling Technology; RhoA (ab54835) from Abcam; Rac1 (61065) and N-cadherin (610920) from BD Biosciences; Zeb1 (NB-1-05987) from Novus Biologicals; and β-actin from Sigma.

Rho GTPase activity assays

RhoA activity assays were performed using Rho activation assay beads (GST-Rhotein-RBD on glutathione beads) according to the manufacturer's instructions (Active Rho Detection Kit; Cell Signaling Technology). Rac1 and Cdc42 activity assays were performed using Rac1/Cdc42 assay reagent (GST-PAK1-RBD on glutathione beads) according to the manufacturer's instructions (Active Rac1 Activation Assay Kit; EMD Millipore and Active Cdc42 Detection kit; Cell Signaling Technology).

Fluorescence activated cell sorting and magnetic cell sorting

For FACS, cells were dissociated using Accutase (Innovative Cell Research) and resuspended in PBS containing 0.5% BSA. The cells were stained with FITC-conjugated CD44 (BD555478) or isotype control antibody (BD555742) from BD Biosciences and analyzed on a BD FACS Calibur (BD Biosciences) using Cell Quest software.

CD44-positive cells were sorted by a magnetics cell sorting system (Miltenyi Biotech). Cells were dissociated using Accutase and stained with CD44-Micro Beads. Cells were then passed through a LS magnetic column where CD44-positive cells were retained. The CD44-positive cells were then eluted from the column after removal from the magnet. Quantitative analysis of CD44-positive cells was performed by immunofluorescence using FITC-conjugated CD44 antibody (555478; BD Biosciences).

shRNA

Silencing of RhoA was achieved via lentiviral transduction of human RhoA shRNA (sc-29471-V; Santa Cruz Biotechnology). A scramble shRNA control (SC-108080) and a GFP control (sc-29471-V; Santa Cruz Biotechnology; Sox2 (2748, 3579), Oct-4 (2750), Nanog (4893), JNK2 (sc-827), and c-Myc (sc-40) purchased from Santa Cruz Biotechnology; Sox2 (2748, 3579), Oct-4 (2750), Nanog (4893), Cdc42 (2466), MYPT1 (2634), phospho-MYPT1 (4563), Slug (9585), Snail (3879), MMP-2 (4022), MMP-9 (3852), phospho-Akt (ser473) (9271), phospho-p38 (9211), phospho-ERK1/2 (9101), phospho-JNK1/2 (#9251), CD44 (3578), and cleaved caspase-3 (9661) from Cell Signaling Technology; RhoA (ab54835) from Abcam; Rac1 (61065) and N-cadherin (610920) from BD Biosciences; Zeb1 (NB-1-05987) from Novus Biologicals; and β-actin from Sigma.

Gelatin zymography

Production of MMP-2 and MMP-9 was analyzed by gelatin zymography as previously described (15). Cells were dissociated using Accutase and stained with CD44-Micro Beads. Cells were then passed through a LS magnetic column where CD44-positive cells were retained. The CD44-positive cells were then eluted from the column after removal from the magnet. Quantitative analysis of CD44-positive cells was performed by immunofluorescence using FITC-conjugated CD44 antibody (555478; BD Biosciences).

Cancer cell proliferation, migration, and invasion assays

To assay for proliferation, spheroid cells were dissociated with Accutase and monolayer cells were collected with trypsin. Proliferation, migration, and invasion assays were performed as previously described (16, 17).
For the 3D invasion assay, cells were stably transduced with a lentiviral vector expressing GFP. Each well of a μ-Plate 24-well (ibidi) was coated with 200 μl Matrigel (Becton Dickinson) and spheroid media (2:1) at 37°C for 30 minutes. One thousand cells in 200 μl spheroid media were added to each well and incubated for 3 to 5 days. Cells were visualized with an inverted confocal microscope (Leica Microsystems). Image processing was performed using Imaris 7.6 (Bitplane).

Mouse studies

All mouse protocols were approved by the Institutional Animal Care and Use Committee. To generate subcutaneous flank tumor, 5 × 10⁶ MKN-45 or SNU-668 cells were resuspended in 100 μl of Hank’s balanced salt solution (HBSS) and injected s.c. into the right flank of athymic, nude, 6- to 8-week-old male BALB/c nu/nu mice following isoflurane anesthesia. Mice were assigned into treatment groups (6 mice/group) when tumors reached 50 mm³ in volume, designated as day 0. Cisplatin 2 mg/kg or carrier (PBS) was injected i.p. one time a week. Fusudil 100 mg/kg or carrier (PBS) was injected i.p. daily. Tumors were measured and processed as previously described (17).

Patients

Patients with adenocarcinomas arising in the stomach or gastroesophageal junction Siewert type II or III who underwent radical gastrectomy or esophagectomy with potentially curative intent (R0 and R1) from May 2006 to March 2012 at Seoul National University Bundang Hospital (SNUBH; South Korea) were included. The end of the follow-up period was March 5, 2012. The median follow-up time was 60 months. The SNUBH Institutional Review Boards approved this study, and informed consents for study of tumor tissue were obtained from each patient's tumor was based on the 7th edition of American Joint Committee on Cancer TNM staging system (18).

Immunocytochemistry

A portion of each patient's tumor was obtained from the surgical specimen and was embedded in a paraffin block of 289 tumor samples using a tissue array device (Beecher Instruments Inc.). A representative core biopsy (2 mm in diameter) was obtained from each case of tumor and embedded in a TMA block. Formalin-fixed, paraffin-embedded sections were processed as previously described (17). For RhoA analysis, antibodies used were anti-RhoA (ab54835; Abcam) and anti-phospho-RhoA (ab125275; Abcam). Phospho-RhoA stain was predominantly nuclear. Phospho-RhoA scores (0–300) were calculated by multiplying the staining intensity (0, 1, 2, or 3) by the staining extent (0%–100%). For detection of apoptosis, stemness, and metastasis, paraffin-embedded sections were deparaffinized, and sections were incubated with anti-human CD44 (#3570; Cell Signaling Technology), anti-Sox2 (#3579; Cell Signaling Technology), and anti-cleaved caspase-3 in a solution of PBS with 1% BSA and 0.1% Triton X-100 at 4°C overnight. Staining was visualized using anti-mouse Alexa Flour 594 and anti-rabbit Alexa Flour 594. Nuclei were counterstained using DAPI. Stained cells were visualized with an inverted confocal microscope. Image processing was performed using Imaris 7.6.

Immunohistochemistry and immunofluorescence

Spheroids were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Following cell fixation, cells were incubated with CD44 FITC conjugate, Sox2, Slug, and/or RhoA antibody in a solution of PBS with 1% BSA and 0.1% Triton X-100 at 4°C overnight. Staining was visualized using mouse Alexa Flour 594 (A11005; Life Technologies) and anti-rabbit Alexa Flour 594 (A11012; Life Technologies). Nuclei were counterstained using 40, 6-diamidino-2-phenylindole (DAPI; Sigma). Stained cells were visualized with an inverted confocal microscope. Image processing was performed using Imaris 7.6.

RhoA activity is increased in diffuse gastric cancer spheroid cells and in CD44(+) cells

We first examined levels of the Rho GTPases RhoA, Rac1, and Cdc42 in four Lauren intestinal type and four Lauren diffuse-type gastric adenocarcinoma cell lines. Levels of total RhoA, Rac1, and Cdc42 were similar in all eight cell lines grown as monolayers or as spheroids. Growth in spheroid formation conditions enriched for CSCs (19). In the MKN-45 and SNU-668 diffuse gastric cancer cell lines grown as monolayers or as spheroids, levels of active Rac1 and active Cdc42 were variable among the cell lines. We next examined RhoA, Rac1, and Cdc42 activity in the MKN-45 and SNU-668 diffuse gastric cancer cell lines grown as monolayers or as spheroids. Growth in spheroid formation conditions enriches for CSCs (19). In the MKN-45 and SNU-668 diffuse gastric cancer cell lines, levels of total RhoA and Rac1 were similar in cells grown as monolayers or as spheroids, but levels of GTP-bound active RhoA and active Rac1 increased significantly in spheroid cells compared with monolayer cells (Fig. 1B). Levels of total and active Cdc42 remained similar in monolayer and spheroid cells.

Our group and others have shown that CD44 expression delineates a subpopulation of gastric cancer cells with CSC properties (12, 17). Following separation of MKN-45 and SNU-668 cells grown as spheroids into CD44(+) and CD44(-) cells using a magnetic column, we found that active RhoA and active Rac1, but not active Cdc42, increased in CD44(+) cells compared with CD44(-) cells (Fig. 1C). Immunofluorescence of CD44(+) and CD44(-) cells grown in spheroid formation conditions confirmed colocalization of CD44 and RhoA (Fig. 1D). We also found increases in the self-renewal proteins Sox2 and Nanog but not Oct-4 or c-Myc in the diffuse
gastric cancer cell lines when grown as spheroids (Supplementary Fig. S1A). Following separation of spheroids into CD44(+) and CD44(-) cells, all four self-renewal proteins were increased in CD44(+) cells compared with CD44(-) cells (Supplementary Fig. S1B).

We next grew MKN-45 and SNU-668 cells in spheroid formation conditions and examined the effects of the RhoA shRNA or the RhoA inhibitor Rhosin. Rho shRNA effectively knocked down RhoA expression and decreased Sox2 expression (Supplementary Fig. S1C). The RhoA inhibitor Rhosin did not affect levels of total RhoA but decreased RhoA activity and Sox2 expression (Supplementary Fig. S1D). RhoA inhibition with RhoA shRNA or Rhosin decreased spheroid formation by 78% to 84% (Fig. 1E) and decreased expression of CD44 and RhoA in spheroids (Supplementary Fig. S1E). Thus, RhoA activity is increased in diffuse-type gastric adenocarcinoma cell lines when grown as spheroids and in CD44(+) spheroid cells, and RhoA inhibition blocks spheroid formation.

RhoA promotes diffuse gastric cancer migration and invasion

RhoA is known to act through a variety of effectors to control actin–myosin-dependent cell contractility and cellular motility (20). We next examined the role of RhoA in the migration and invasion of diffuse gastric cancer cells. Both MKN-45 and...
SNU-668 cells after being grown as spheroids had increases in expression of N-cadherin, which is a cell surface marker of epithelial-to-mesenchymal transition (EMT), and increases in Snail and Slug, which are transcription factors associated with EMT (Fig. 2A; ref. 21). These differences were more pronounced when spheroid cells were separated into CD44(+) and CD44(-) fractions (Fig. 2B). EMT is also associated with upregulation of matrix metalloproteinases (MMP), including MMP-2 and MMP-9 (22). CD44(+) spheroid cells showed increased expression of MMP-2 but not MMP-9 as determined by Western blot analysis and increased secretion of MMP-2 but not MMP-9 as determined by zymography (Fig. 2C). Cells grown as spheroids had 3.0- to 4.4-fold greater migration and 4.2- to 5.0-fold greater invasion compared with monolayer cells (Fig. 2D). We also performed a 3D invasion assay with CD44(+) and CD44(-) spheroid cells and found significantly more invasion with CD44(+) cells compared with CD44(-) cells (Fig. 2E). Spheroid cells transduced with RhoA shRNA had drastically diminished migration and invasion (Supplementary Fig. S2A). RhoA shRNA also reduced expression of N-cadherin, MMP-2, Snail, and Slug in MKN-45 and SNU-668 spheroid cells (Supplementary Fig. S2B). When spheroid cells were separated into the CD44(+) and CD44(-) fractions, CD44(+) cells had 13.9- to 19-fold more migration and 9.2- to 12.3-fold more invasion compared with CD44(-) cells, and the RhoA inhibitor Rhosin reduced the ability of CD44(+) spheroid cells to migrate and invade to that of CD44(-) cells (Fig. 2F). Rhosin also reduced N-cadherin, Snail, Slug, and MMP-2 expression in CD44(+) cells (Supplementary Fig. S2C). Thus, diffuse-type gastric
adenocarcinoma cells grown as spheroids or selected for CD44 expression show an increase in expression of EMT markers as well as an increase in migration and invasion, and inhibition of RhoA activity blocks these EMT phenotypes.

**P13K/Akt is upstream and activates RhoA, whereas JNK is downstream**

Several lines of evidence indicate that the P13K–Akt pathway plays a key role in cancer stem cell biology (23), and some studies suggest that the P13K–Akt pathway may regulate RhoA activity in various solid tumors (24, 25). We thus examined this signaling pathway in diffuse gastric cancer spheroid cells. Compared with gastric cancer cells grown as monolayers, gastric cancer spheroid cells had increased phosphorylation of Akt (Supplementary Fig. S3A). We treated spheroid cells with the P13K inhibitor LY294002 and confirmed decreased phosphorylation of Akt and JNK1/2 (Fig. 3A). In addition, LY294002 significantly reduced levels of active RhoA and Slug. When LY294002 was added to cells in spheroid formation conditions, it dramatically inhibited the formation of spheroids along with expression of CD44 and Slug (Fig. 3B). When LY294002 was added to spheroid cells in migration and invasion assays, the P13K inhibitor blocked migration by 88% to 92% and blocked invasion by 90% to 95% (Fig. 3C).

Spheroid cells, compared with monolayer cells, had increased levels of phosphorylated ERK1/2 and phosphorylated JNK1/2 but not phosphorylated p38 (Supplementary Fig. S3B). MKN-45 and SNU-668 spheroid cells were treated with RhoA shRNA or a control scrambled shRNA and examined by Western blot analysis for activity of these MAPK proteins. Rho shRNA led to significant reduction in JNK1/2 phosphorylation but no reduction in ERK1/2 or p38 phosphorylation (Fig. 3D). Spheroid cells were then treated with the JNK inhibitor SP600125, and this resulted in decreased phosphorylation of JNK1/2 and decreased levels of Slug, but there was no change in levels of total or active RhoA (Fig. 3E). JNK inhibition also resulted in reduced formation of spheroids (Supplementary Fig. S3C), and reduced spheroid cell migration and invasion (Supplementary Fig. S3D). Inhibition of ERK1/2 using the inhibitor U0126 and inhibition of p38 using the inhibitor SB202190 had little or no effect on the migration or invasion of spheroid cells (Supplementary Fig. S4A and S4B). Thus, the P13K–Akt pathway lies upstream of RhoA, whereas JNK lies downstream of RhoA, and pharmacologic blockade of P13K and JNK mimics the effects of RhoA inhibition on spheroid formation, migration, and invasion.

**RhoA inhibition reverses chemotherapy resistance**

Numerous studies have demonstrated that CSCs are more resistant to chemotherapy (11). We thus examined the sensitivity of MKN-45 and SNU-668 monolayer and spheroid cells to two commonly used chemotherapies for gastric cancer, 5-fluorouracil, and cisplatin. For monolayer cells, cell viability was reduced by 54% to 58% when exposed to 5-fluorouracil at 5 μmol/L and 56% to 58% when exposed to cisplatin at 5 μmol/L (Fig. 4A and B). 5-fluorouracil decreased cell viability in spheroid cells by only 13% to 20%, and cisplatin decreased cell viability by only 16% to 18%. RhoA shRNA alone reduced proliferation in monolayer and spheroid cells by only 18% to 30%, and the RhoA inhibitor Rhosin alone reduced proliferation by only 13% to 23%. The combination of RhoA shRNA or Rhosin and chemotherapy had little or no additive effect over chemotherapy alone on monolayer cells. However, there was a more than additive effect when RhoA inhibition and chemotherapy were added to spheroid cells, with decreases in cell viability ranging from 60% to 69%. Similar results were obtained when the P13K inhibitor LY294002 or the JNK inhibitor SP600125 were used in place of RhoA inhibition (Fig. 4C and D). In comparing the effects of P13K, RhoA, and JNK inhibition on spheroid formation of CD44(+) spheroid cells, P13K inhibition with LY294002 was slightly more efficacious that RhoA inhibition with Rhosin, which in turn was slightly more efficacious than the JNK inhibition with SP600125 (Fig. 4E). Thus diffuse gastric cancer spheroid cells are relatively resistant to chemotherapy, and this resistance can be overcome with RhoA pathway inhibition.

The effects of RhoA pathway inhibition and cisplatin chemotherapy were next examined on gastric cancer xenografts in mice. MKN-45 cells were stably transduced with control shRNA or RhoA shRNA. These cell lines were then grown as xenografts in athymic nude mice. After tumors reached 50 to 100 mm³ in size, mice were randomized to treatment with cisplatin or PBS. Control tumors treated with PBS grew to over 800 mm³ in just 15 days following randomization. Tumors transduced with RhoA shRNA or treated with cisplatin grew to an average size of 503 and 420 mm³, respectively (Fig. 5A). The combination of RhoA shRNA and cisplatin dramatically inhibited tumor growth, with tumors growing to an average of only 144 mm³ (83% less of control tumors) during the treatment period. After 15 days, xenografts were harvested and analyzed (Fig. 5B). The combination of RhoA shRNA and cisplatin had minor effects on cell proliferation as measured by Ki-67 expression, but did cause more than additive increases in overall apoptosis as measured by cleaved caspase-3 expression (Fig. 5C). Furthermore, there were significant decreases in cells expressing both CD44 and Slug and cells expressing both CD44 and Sox2. Decreases in CD44, Slug, and Sox2 were confirmed by Western blot analysis (Fig. 5D).

**Potential therapeutic use of RhoA pathway inhibition in diffuse gastric cancer patients**

There are currently no direct inhibitors of RhoA in clinical use. ROCK is a major downstream effector of RhoA (9), and the ROCK inhibitor fasudil is approved in Japan for the use in cerebral vasospasm secondary to subarachnoid hemorrhage (26). ROCK phosphorylates myosin phosphatase target subunit 1 (MYPT1), which is a regulatory subunit of protein phosphatase 1 and involved in a pathway for smooth muscle contraction. We examined total and phosphorylated MYPT1 levels in MKN-45 and SNU-668 monolayer and spheroid cells and found increased phosphorylated MYPT1 levels in spheroid cells. When fasudil was applied to diffuse gastric cancer cells grown as spheroids, it decreased phosphorylated MYPT1 and Sox2 levels (Supplementary Fig. S5A). The addition of fasudil to spheroid formation media significantly reduced the number of spheroids formed and also significantly reduced levels of CD44 and Sox2-positive cells (Supplementary Fig. S5B and S5C). Fasudil also reduced expression of EMT markers N-cadherin and Slug in spheroid cells (Supplementary Fig. S5D), and reduced migration and invasion of spheroid cells by 81% to 83% and 83% to 86%, respectively (Supplementary Fig. S5E).

SNU-668 xenografts were used to examine the efficacy of fasudil with or without chemotherapy in blocking tumor growth. Fasudil alone inhibited tumor growth by 32%, cisplatin alone inhibited tumor growth by 40%, and the combination of fasudil and chemotherapy inhibited tumor growth by 77% (Fig. 6A).
Figure 3.
The PI3K-Akt pathway activates RhoA. A, Western blot analysis of MKN-45 and SNU-668 spheroid cells for Akt, RhoA, JNK, and Slug following treatment with the PI3K inhibitor LY294002 or carrier (DMSO). B, immunofluorescence images of MKN-45 and SNU-668 spheroids treated with the LY294002 or carrier (DMSO). C, photos and graphs of migration and invasion assays for MKN-45 and SNU-668 cells grown as spheroids and treated with the Akt inhibitor LY294002 or carrier (DMSO). D, Western blot analysis for JNK, ERK, p38, and Slug for MKN-45 and SNU-668 cells grown as spheroids and transduced with RhoA shRNA (sh.RhoA) or scrambled shRNA (sh.Scr). E, Western blot analysis for RhoA, JNK, and Slug after MKN-45 and SNU-668 cells were grown as spheroids and treated with the JNK inhibitor SP600125 or carrier (DMSO); bars, SD; *, P < 0.05.
Thus, fasudil was as effective as RhoA shRNA when combined with chemotherapy in blocking the growth of tumor xenografts. Tumors were harvested at the end of the treatment period and analyzed (Fig. 6B). The combination of fasudil and cisplatin resulted in a modest decrease in tumor cell proliferation as measured by Ki-67 expression (19% less than control) but a 31.8-fold increase in apoptosis as measured by cleaved caspase-3 expression and a 74% to 89% decrease in expression of CD44, Slug, and Sox2 (Fig. 6C).

To determine whether RhoA activity in human gastric adenocarcinoma tumors correlated with outcomes, we examined 288 tumor samples from gastric adenocarcinoma patients who underwent surgical resection of their gastric tumors (Supplementary Table S1). One hundred thirty-six patients had diffuse tumors, 129 had intestinal tumors, and 23 had mixed tumors. Given there is no reliable antibody to detect active RhoA in human tissues, we used an antibody for phosphorylated RhoA. Phosphorylation of RhoA significantly increases its interaction with Rho GDP.
Figure 5.
Cisplatin chemotherapy combined with RhoA inhibition in MKN-45 diffuse gastric cancer xenografts. MKN-45 cells were transduced with RhoA shRNA (Smo.shRNA) or control (Scr.shRNA) and grown and xenografted. A, tumor growth curves for MKN-45 xenografts treatment with Scr.shRNA or RhoA.shRNA and PBS or cisplatin. B, photos of representative tumor from each treatment group. C, photos and graphs following immunohistochemical analysis of tumors for proliferation using Ki-67 (green), apoptosis using cleaved caspase-3 (red), CD44 (green), Slug (red), and Sox2 (white); scale bar, 20 μm. D, Western blot analysis of tumor lysates for CD44, cleaved caspase-3, Slug, and Sox2; bars, SD; *, P < 0.05 compared with control; **, P < 0.05 compared with all other groups.
Figure 6.
ROCK inhibition in SNU-668 xenografts; RhoA activity and survival in diffuse gastric cancer patients. 
A, tumor growth curves for SNU-668 xenografts treatment with cisplatin and/or fasudil. B, photos of representative tumor from each treatment group. C, graphs following immunohistochemical analysis of tumors for proliferation using Ki-67 and apoptosis using cleaved caspase-3 and immunofluorescence analysis for CD44 and Slug and CD44 and Sox-2. Cisplatin (Cis), Fasudil (Fas).
D, Kaplan–Meier overall survival curves for patients undergoing surgical resection for diffuse gastric cancer stratified by low versus high RhoA activity in the primary tumor. E, diagram of the RhoA pathway in diffuse gastric cancer CSCs; bars, SD; *, \( P < 0.05 \) compared to control; **, \( P < 0.05 \) compared to all other groups.

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dissociation inhibitor (RhoGDI), thus keeping RhoA in its inactive state (27). Thus, phosphorylated RhoA is inversely associated with RhoA activity. When RhoA activity was examined in all patients’ tumor samples, there was no significant difference in overall survival based on high versus low RhoA activity (Supplementary Fig. S6A). However, when patients with diffuse tumors and intestinal tumors were examined separately, patients with diffuse tumors having high RhoA activity had significantly worse overall survival than patients having tumors with low RhoA activity (Fig. 6D). In patients with diffuse tumors, 5-year actuarial survivals for patients with high RhoA and low RhoA activity were 52% and 81%, respectively. There was no significant difference in survival of patients with intestinal-type tumors based on RhoA activity (Supplementary Fig. S6B). Thus, high RhoA activity is independently associated with worse overall survival in patients with diffuse gastric cancer.

**Discussion**

The Lauren diffuse type of gastric adenocarcinoma is frequently highly infiltrative and resistant to chemotherapy. This study is the first to demonstrate a vital role of the RhoA pathway in diffuse gastric cancer CSCs for the maintenance of chemotherapy resistance. Diffuse gastric cancer cell lines grown as spheroids have enrichment of the gastric CSC marker CD44 as well as high levels of RhoA activity. Inhibition of RhoA in diffuse gastric CSCs using shRNA knockdown or pharmacologic inhibition blocked spheroid formation, migration, and invasion. Diffuse gastric CSCs are resistant *in vitro* to 5-fluorouracil or cisplatin chemotherapy, and this chemoresistance resistance could be reversed with RhoA pathway inhibition. In gastric CSCs, the PI3K–Akt pathway was found to be upstream of RhoA, and JNK was found to be downstream of RhoA and ROCK (Fig. 6E). Inhibitors of PI3K, JNK, and the RhoA effector ROCK-blocked diffuse gastric CSC phenotypes similarly to direct RhoA inhibition. RhoA inhibition in diffuse gastric cancer xenografts acted with chemotherapy to block tumor growth, and histologic examination of treated tumors showed dramatic increases in tumor cell apoptosis and depletion of CD44 (+) cells. The ROCK inhibitor fasudil, which is clinically available for use in patients, showed similar results to RhoA shRNA in an alternative diffuse gastric cancer xenograft model. Finally, examination of tumor specimens from patients with gastric cancer patients who underwent surgical resection of their gastric tumors revealed, for those patients with diffuse but not intestinal gastric cancer, that high RhoA activity in tumors is an independent predictor of worse overall survival. These results collectively suggest that inhibition of the RhoA pathway may provide a novel therapeutic target for combating the invasiveness and chemoresistance of diffuse gastric cancers.

The TCGA Stomach–Esophageal Analysis Working Group examined a total of 295 human gastric adenocarcinomas and associated germline DNA for (i) gene mutations using whole-exome sequencing, (ii) copy-number changes using Affymetrix SNP 6.0 arrays, and (iii) methylation using Illumina Human MethylChip450 array (8). On the basis of these analyses, four molecular classifications of gastric cancer adenocarcinoma were identified: Epstein-Barr virus (EBV)–positive, microsatellite instability (MSI)/hypermutated, genomically stable (GS), and chromosomal instability (CIN). The vast majority of diffuse-type gastric cancers fell into the GS subtype, and in this subtype RhoA was one of the most commonly mutated genes. RhoA overexpression has been identified in a variety of other cancers, and RhoA activity has been linked to tumorigenesis and invasion (28, 29). For example, in colorectal cancer, increased RhoA expression in primary tumors correlates with lymph node metastasis and liver metastasis (30, 31). Fairly little is known about the role of RhoA in gastric cancer. Pan and colleagues (32) systematically examined mRNA levels of seven Rho GTPases in 53 tumors from gastric cancer patients and in 7 gastric cancer cell lines. Increased expression of RhoA correlated with higher TNM stage and a poorly differentiated histologic subtype. However, the mechanism by which RhoA activity may promote diffuse gastric cancer tumorigenesis and metastasis has been poorly understood.

Cytotoxic chemotherapy is a rather blunt instrument in the treatment of cancer, and targeted therapies either alone or in combination with chemotherapy have demonstrated improved efficacy in a variety of solid tumors including gastric cancer. Deng and colleagues (33) performed a comprehensive survey of genomic alterations in gastric cancer and found the existence of five distinct subgroups defined by signature genomic alterations in FGFR2, V-Ki-ras Kirsten rat sarcoma viral oncogene homolog (KRAS), EGFR, HER2, and c-MET. In addition to these five pathways for gastric cancer tumor growth, the VEGF-A pathway plays an important role in driving tumor angiogenesis in gastric cancers (34). It remains unclear whether gastric CSCs may also be resistant to targeted therapies. A small percentage of gastric adenocarcinomas overexpress HER2, and the addition of trastuzumab to chemotherapy prolonged survival in these patients from 11 to 14 months in a randomized trial (35). However, combining cytotoxic chemotherapy with agents targeting the VEGF-A or EGFR pathways has not been demonstrated to increase survival in unselected gastric cancer patients (36–38). Clearly targeted agents must be matched with the correct patient population, and it is quite possible that RhoA pathway inhibition may only be effective for diffuse gastric cancers or even just a subset of diffuse gastric cancers with high RhoA activity.

Fasudil is an orally available ROCK inhibitor, which has been shown to modify myosin light-chain phosphorylation in smooth muscle cells leading to vasodilation (39). It is approved in Japan for the treatment of cerebral vasospasm following surgery for subarachnoid hemorrhage and associated cerebral ischemic symptoms (26). One study found that fasudil alone inhibited tumor growth in three animal models of orthotopic tumor growth or metastasis (40). More recent studies suggest fasudil, in combination with other agents, may be effective in blocking tumor growth. Kumar and colleagues (41) found in a *Kras*-driven model of non-squamous cell lung cancer that fasudil combined with the proteasome inhibitor bortezomib had a substantial effect in *Kras* mutant but not in *Kras* wild-type tumors. Here, we show that fasudil works similarly to direct RhoA inhibition in blocking CSC phenotypes and works effectively with chemotherapy in blocking growth of diffuse gastric cancer xenografts. We further demonstrate that increased RhoA activity in the tumors of patients with diffuse gastric cancer undergoing potentially curative surgery correlates with worse overall survival. Thus, the results of this study may form the basis of a clinical trial of fasudil and chemotherapy for metastatic diffuse gastric cancer.

In conclusion, we have entered an era of precision medicine where we consider one cancer-type like gastric cancer to be composed of multiple different subtypes. The pathways driving tumor progression and metastasis can be quite different among these subtypes, and thus response to targeted treatment can be variable. It
is essential to identify key pathways that drive tumorigenesis and progression of gastric cancer subtypes. In this study, we define the role of the RhoA pathway in the diffuse type of gastric cancer, and specifically find that RhoA activity plays a major role in maintaining CSC phenotypes. We see from our correlative studies in patients tumor samples that RhoA activity is a marker of poor overall survival, and targeting this pathway is promising new strategy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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