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Aberrant alternative splicing of *RHOA* is associated with loss of its expression and activity in diffuse-type gastric carcinoma cells

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ABSTRACT

RhoA is a member of Rho family small GTPases that regulates diverse cellular functions. Recent large-scale sequencing studies have identified recurrent somatic mutations of *RHOA* in diffuse-type gastric carcinoma (DGC), indicating that *RHOA* is a driver of DGC. In this study, we investigated the possible abnormalities of *RHOA* in a panel of gastric carcinoma (GC) cell lines. Pulldown assay and immunoblot analysis showed that the activity and expression of RhoA were detectable in all GC cell lines tested, except for two DGC cell lines, HSC-59 and GSU. *RHOA* coding region sequencing revealed that aberrant alternative splicing of *RHOA* occurred in these cell lines. Quantitative real-time PCR analysis showed that the expression of wild-type *RHOA* was nearly undetectable, whereas splicing variants were almost exclusively expressed in HSC-59 and GSU cell lines. However, the expression levels of *RHOA* splicing variants were very low and the corresponding proteins were not detected by immunoblotting. Moreover, the splicing isoforms of RhoA protein were neither efficiently expressed nor activated even if ectopically expressed in cells. These results indicate that aberrant alternative splicing of *RHOA* results in the loss of its activity and expression in DGC cells.

1. Introduction

Gastric carcinoma (GC) is one of the leading causes of cancer-associated mortality in the world. There are two major histological subtypes of gastric adenocarcinomas according to the Laurén classification: intestinal and diffuse [1]. Diffuse-type gastric carcinoma (DGC) primarily consists of poorly differentiated carcinoma cells and often exhibits aggressive progression, including rapid infiltrative growth and frequent metastasis to lymph nodes and the peritoneum [2]. These aggressive phenotypes lead to the poor prognosis of patients with DGC [3,4].

RhoA is a member of Rho family small GTPases that regulates diverse cellular processes, such as cell growth, survival, polarity, adhesion, migration, cytokinesis, and differentiation [5,6]. RhoA shuttles between inactive GDP-bound and active GTP-bound states, and the shuttling is strictly regulated by several GTPase-activating proteins (GAPs), guanine nucleotide-exchanging factors (GEFs), and guanine nucleotide-dissociation inhibitors (GDIs). Active RhoA transmits signals through binding and activating downstream effectors, such as Rho-associated protein kinase (ROCK). In GC cells, RhoA and ROCK have been implicated in cell migration, invasion, and metastasis via regulation of the cytoskeletal dynamics [7]. Recent large-scale genome sequencing projects of GC discovered that highly recurrent mutations of *RHOA* occur almost exclusively in DGC [8–10]. Additionally, recurrent fusions between CLDN18, a tight junction gene, and ARHGAP26 or ARHGAP6, both encode for GAP for RhoA, occur in DGC in a mutually exclusive manner with *RHOA* mutations [9,11]. Recurrent *RHOA* mutations have also been found in some lymphomas and head neck cancer [12–19]. Although in vitro results have indicated that *RHOA* mutations support malignant phenotypes of DGC cells, it remains controversial whether the mutations are gain-of-function or loss-of-function [20,21]. Moreover, it is still unclear whether other genetic abnormalities of *RHOA* exist in DGC or not.

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Abbreviations

CA	constitutively active
DGC	diffuse-type gastric carcinoma
DN	dominant-negative
GAP	GTPase-activating protein
GC	gastric carcinoma
GDI	guanine nucleotide-dissociation inhibitor
GEF	guanine nucleotide-exchanging factor
LOH	loss of heterozygosity
RBD	Rho-binding domain
ROCK	Rho-associated protein kinase
WT	wild-type

In this study, we found that expression and activity of RhoA are abrogated in two DGC cell lines in which aberrant alternative splicing of *RHOA* occurs. This finding indicated that downregulation of *RHOA* due to aberrant alternative splicing may be involved in oncogenesis of DGC.

2. Materials and methods

2.1. Cell culture

Human GC cell lines HSC-59, HSC-60, HSC-64, HSC-44PE, 58As9, and 58As1 have been described previously [22]. MKN7, MKN74, NUGC-4, KATO-III, and IM95 were obtained from the Health Science Research Resources Bank. NCI-N87 and SNU-5 were obtained from the ATCC. GCIY, H-111-TC, GSU, and KE-97 were provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. These cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 10 units/mL of penicillin, and 10 μg/mL of streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. 293FT cells were purchased from Thermo Fisher Scientific and cultured according to the manufacturer's instructions.

2.2. Antibodies, reagents, and constructs

Anti-DDDDK (Flag) tag antibody was purchased from MBL. Antibodies against the C-terminal region of RhoA (#2117), RhoB (#2098), RhoC (#3430), and β-actin (#5125) were purchased from Cell Signaling Technologies. Antibody against Rho (ab40673) was purchased from Abcam. Secondary antibodies were purchased from GE Healthcare. Ponceau S was purchased from Beacle. For ectopic expression of Flag-tagged RhoA proteins, cDNAs encoding the human wild-type (WT), constitutively-active form (CA), dominant-negative form (DN), and splicing isoforms of RhoA were subcloned into p3XFlag-CMV14 vector (Sigma Aldrich) with In-Fusion HD Cloning Kit (Takara). All cDNAs were verified by sequencing.

2.3. Plasmid transfection

Cells were transfected with the indicated plasmids using Polyethylenimine Max (Polysciences) according to the manufacturer's instructions. At day 1 post transfection, the cells were subjected to pulldown assay and immunoblotting.

2.4. PCR

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). Template cDNAs were synthesized with ReverTra Ace (TOYOBO). Quantitative RT-PCR was performed using Thunderbird qPCR Mix (TOYOBO) in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). For standard PCR amplification, PrimeSTAR Max DNA Polymerase (Takara) was used. The sequences of primer pairs used are shown in Table 1.

2.5. Immunoblotting

Cells were lysed in a lysis buffer containing 50 mM HEPES, pH 7.0, 1% Triton-X 100, 1.5 mM $MgCl_2$, 1 mM EGTA, 150 mM NaCl, 10% glycerol, and protease and phosphatase inhibitor cocktails. The lysates were subjected to immunoblot analysis as previously described [23].

2.6. RhoA pulldown assay

The amount of active GTP-bound RhoA was determined using the RhoA Pull-down Activation Assay Biochem Kit (Cytoskeleton) and Rho Assay Reagent (Merck).

3. Results

3.1. RhoA expression and activity in GC cell lines

We first examined RhoA expression and activation status, i.e., the amount of GTP-bound protein, in a panel of GC cell lines by the RBD pulldown assay (Fig. 1). As a result, variable amounts of active and total RhoA were detected in the GC cell lines. There were no apparent tendencies in the levels of total and active RhoA between intestinal- and diffuse-type GC cell lines. Of note, a slightly lower molecular weight band of RhoA was detected in IM95, an intestinal-type moderately differentiated GC cell line. Additionally, GTP-bound active and total RhoA were both undetectable in two DGC cell lines, HSC-59 and GSU, suggesting the existence of *RHOA* abnormality in these cell lines.

3.2. Identification of aberrant RHOA splicing

We next explored the possible *RHOA* mutations in GC cell lines. cDNA containing the *RHOA* coding region was amplified by PCR and directly sequenced (Fig. 2A). We identified a missense mutation (c.331C>T, p.P111S) in IM95 cells, which has already been annotated in the database Cancer Cell Line Encyclopedia. Thus, it is likely that this mutation caused the mobility shift of RhoA in the immunoblot.

Although there were no *RHOA* point mutations in other cell lines, aberrant *RHOA* sequence was found in two DGC cell lines, HSC-59 and GSU, and the sequence accuracy was compromised right after exon 2 (Fig. 2B). Consistent with this observation, PCR products of the *RHOA* coding region yielded multiple bands for both cell lines by agarose gel electrophoresis (Fig. 2C). Subcloning and sequencing revealed that these bands corresponded to different splicing variants of *RHOA*. HSC-59 and GSU cells each expressed at least three *RHOA* splicing isoforms, of which two were common (Fig. 2D). The isoform found only in HSC-59 was named H-1 and that in GSU was named G-1. The two common isoforms were named HG-2 and HG-3. WT *RHOA* coding sequence comprised exons 2, 3, 4, and 5. However, H-1 contained intron 3 instead of exon 3. G-1 contained intron 2 in place of exon 3 and HG-2 lacked exon 3, and both these splicing variants lead to frameshift and premature termination of the protein. HG-3 lacked both exons 3 and 4, resulting in the shortest variant. HG-2 and HG-3 have been annotated in the NCBI database as transcript variant 7 (NM_001313947) and transcript variant 6 (NM_001313946),