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RASSF10 is epigenetically silenced and functions as a tumor suppressor in gastric cancer

Ziran Wei [a,1], Xia Chen [b,1], Ji Chen [a,1], Weimin Wang [a], Xudong Xu [b], Qingping Cai [a,*]

[a] Department of General Surgery, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai, China
[b] Urology Department, Minhang District Central Hospital, Shanghai, China

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ABSTRACT

Ras association domain family (RASSF) proteins are encoded by several tumor suppressor genes that are frequently silenced in human cancers. In this study, we investigated *RASSF10* as a target of epigenetic inactivation and examined its functions as a tumor suppressor in gastric cancer. *RASSF10* was silenced in six out of eight gastric cancer cell lines. Loss or downregulation of *RASSF10* expression was associated with promoter hypermethylation, and could be restored by a demethylating agent. Overexpression of *RASSF10* in gastric cancer cell lines (JRST, BGC823) suppressed cell growth and colony formation, and induced apoptosis, whereas RASSF10 depletion promoted cell growth. In xenograft animal experiments, *RASSF10* overexpression effectively repressed tumor growth. Mechanistic investigations revealed that RASSF10 inhibited tumor growth by blocking activation of β-catenin and its downstream targets including c-Myc, cyclinD1, cyclinE1, peroxisome proliferator-activated receptor δ, transcription factor 4, transcription factor 1 and CD44. In conclusion, the results of this study provide insight into the role of RASSF10 as a novel functional tumor suppressor in gastric cancer through inhibition of the Wnt/β-catenin signaling pathway.

1. Introduction

Gastric cancer (GC) remains the second leading cause of cancer mortality worldwide [1]. In addition to multiple genetic alterations, recent studies indicate that epigenetic mechanisms, especially DNA methylation, are also associated with the development and progression of GC [2,3]. Promoter CpG methylation alters cancer-related gene expression and thereby modulates cancer pathways involved in cell proliferation, apoptosis and metastasis [4]. Several tumor suppressor genes associated with epigenetic alterations have been identified in GC, such as *RASSF1A*, *CHFR*, *CDH4*, and *CMTM3* [5–8], and genome-wide methylation screening has identified many genes in GC that are inactivated by DNA methylation [9,10]. However, the precise role of DNA methylation in GC remains unclear.

The RASSF (Ras association domain family) proteins comprise 10 members (RASSF1–10) [11–14], all of which share a conserved Ras association domain in the C-terminal (RASSF1–6) or N-terminal (RASSF7–10) region. All demonstrate biological properties compatible with a tumor suppressor function, and some are frequently inactivated or downregulated by promoter methylation during tumorigenesis [15–18]. RASSF10 is a recently identified member of the RASSF family, the gene for which is located on chromosome 11p15.2 [13]. Previous studies have revealed that *RASSF10* is frequently hypermethylated in leukemia, glioblastoma, thyroid and prostate carcinomas [19–22]. However, the expression of *RASSF10* and its role in GC remains unknown.

In the present study, we investigated *RASSF10* silencing by promoter hypermethylation in GC cells, and examined its role as a tumor suppressor, as well as the mechanisms responsible for its actions. The findings of our study suggest that epigenetic inactivation of RASSF10 plays a key role in gastric carcinogenesis.

2. Materials and methods

2.1. Cell lines

Eight GC cell lines (AGS, JRST, BGC823, MGC803, SNU1, KatoIII, MKN7, and MKN28) were obtained from the American Type Culture Collection (Manassas, VA, USA). The immortalized human gastric epithelial cell line GES1 was obtained from the Cancer Research Institute of Beijing, Beijing University, China. All cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum. Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere.

* Corresponding author.
E-mail address: qingping_caicz@163.com (Q. Cai).
[1] These authors contributed equally to this work.

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2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was generated from 1 μg total RNA using SuperScript III (Invitrogen) and polyN primers. The PCR primers for *RASSF10* were 5′-CGCCATGGATCCTTCGGA-3′ (forward) and 5′-GCAGCCGTCCTCCAAAAGC-3′ (reverse). After PCR, the amplified products were electrophoresed in 2.5% Nuseive gels. The β-actin gene was used as an endogenous control. To analyze the restoration of *RASSF10* expression, AGS, JRST, KatoIII, and SNU1 cells were incubated for 96 h with the demethylating agent 2 μM 5-aza-2′-deoxycytidine (Sigma–Aldrich, St Louis, MO, USA) and then harvested for RT-PCR analysis.

2.3. Methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS)

Genomic DNA was extracted from GC cell lines using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Bisulfite modification of DNA, and MSP were performed as described previously [23]. The MSP primer sequences for the *RASSF10* promoter were: 5′-GGGTATTTTGGGTAGAGTTAGAGTG-3′ (forward) and 5′-AAACAAACTAAAAAACAACTACAAC-3′ (reverse) for unmethylated reactions (126 bp); and 5′-GGGTATTTTGGGTAGAGTTAGAGC-3′ (forward) and 5′-AAACAAACTAAAAAACGACTACGAC-3′ (reverse) for methylated reactions (126 bp). BSG was performed to characterize the methylation density in the *RASSF10* promoter using the BigDye Terminator Cycle Sequencing kit version 1.0 (Applied Biosystems, Foster City, CA, USA). The primers were: 5′-TTTGGGTTTTGAGTTTTTTGTATTT-3′ (forward) and 5′-ACTACACTAACCCTATTCCCCC-3′ (reverse). Twenty-three CpG sites spanning the −331 and −74 bp regions were evaluated. Sequences were analyzed using SeqScape software (Applied Biosystems).

2.4. Lentivirus infection and oligonucleotide transfection

The *RASSF10* sequence was purchased from Origene Technologies (Rockville, MD, USA). *RASSF10* siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Constructs containing the *RASSF10* or *RASSF10* siRNA sequence were cloned into the lentivirus-based expression plasmid pCDH-CMV-MCS-EF1-coGFP constructs (System Biosciences, CA, USA). Virus particles were harvested 48 h after pCDH-CMV-*RASSF10* or pCDH-CMV-*RASSF10* siRNA transfection with the packaging plasmids pRSV/pREV, pCMV/pVSVG and pMDLG/pRRE into 293T cells using Lipofectamine 2000 reagent (Life Technologies, Inc., Grand Island, NY, USA). JRST and BGC823 cells were infected with recombinant lentivirus-transducing units plus 10 μg/ml Polybrene (Sigma, St Louis, MI, USA).

2.5. Cell proliferation and apoptosis assays

Cells were plated in 24-well plates at 5×10^3 cells per well and cultured for 1–4 days, respectively. Cells were trypsinized and counted with a Coulter counter (Beckman Coulter, Fullerton, CA, USA). For analysis of apoptosis, cells were stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) and analyzed with a flow cytometer (FACScan®; BD Biosciences, Mountain View, CA, USA) equipped with CellQuest software (BD Biosciences). All assays were conducted in triplicate.

2.6. Colony-formation assay

Cells (1×10^3) were plated into 10-cm dishes and cultured for 2 weeks to allow colony formation. After 2 weeks, the cells were washed twice with phosphate-buffered saline (PBS), fixed with methanol/acetic acid (3:1, v/v), and stained with 0.5% crystal violet. The number of colonies was counted under the microscope. All experiments were performed in triplicate wells in three independent experiments.

2.7. Western blotting

Proteins from the cells were extracted in radio-immunoprecipitation assay buffer (Beyotime, China) and protein concentrations were determined using a BCA assay kit (Beyotime). Cell extracts (20–30 μg) were boiled with equal amounts of loading dye for 10 min and separated by 10% polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Hybond-ECL, GE HealthCare, UK). Membranes were blocked in PBS with 0.1% Tween 20 (PBS-T) containing 5% non-fat milk for 1 h, and then incubated with primary and secondary antibodies in PBS-T containing 5% non-fat milk. The following primary antibodies were used at the indicated dilutions: RASSF10, β-catenin, cyclinD1, PPARδ, cyclinE1, β-actin (1:1000; Abcam, UK); c-Myc, CD44, TCF-1, and TCF-4 (1:500; Santa Cruz Biotechnology). Primary antibody incubations were carried out overnight at 4 °C. The membranes

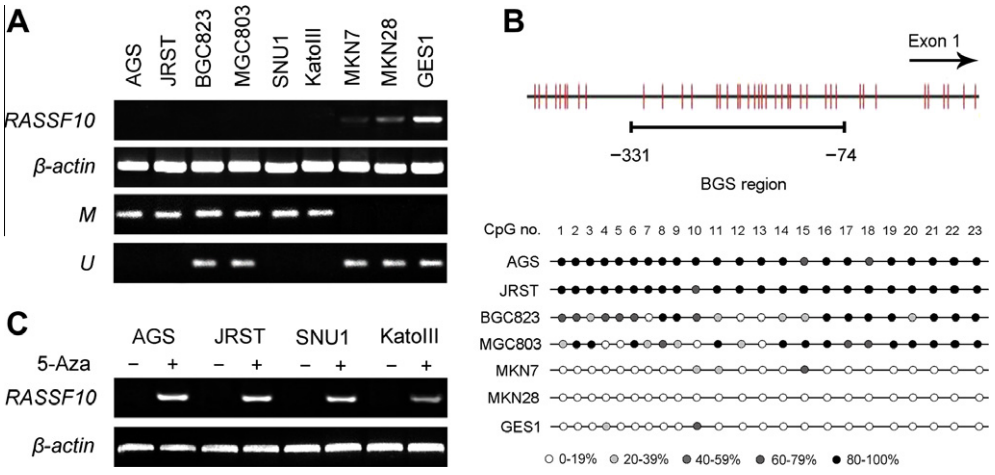

Fig. 1. Expression and methylation status of *RASSF10* in GC cell lines. (A) *RASSF10* was frequently silenced and methylated in GC cell lines, but was expressed and unmethylated in GES1 immortalized gastric epithelial cells. (B) The methylation status of the *RASSF10* promoter was confirmed by BGS. (C) Pharmacological demethylation with 2 μM 5-aza-2′-deoxycytidine restored *RASSF10* expression.