CpG island promoter hypermethylation of Ras association domain family 1A gene contributes to gastric carcinogenesis

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Received February 12, 2014; Accepted November 20, 2014

DOI: 10.3892/mmr.2014.3055

Abstract. Methylation rates of the Ras association domain family 1A gene (RASSF1A) have been variously reported as between 7.5 and 66.7% in gastric carcinoma tissues. The role of this gene in gastric cancer also remains to be fully elucidated. The present study aimed to investigate whether promoter hypermethylation of RASSF1A occurs in gastric adenocarcinoma tissues and gastric cancer cell lines, and to determine the effects of RASSF1A in gastric carcinoma cell lines. The results showed a methylation-specific band only in SNU-719, MKN28 and AGS human gastric cancer cells (indicating full methylation), none of which exhibited RASSF1A expression. By contrast, SNU-16, MKN-45 and KATO-III human gastric carcinoma cells exhibited methylation as well as unmethylation-specific bands (indicating partial methylation), and all displayed positive or weakly positive expression of RASSF1A. Bisulfite sequencing in AGS and SNU-719 cells revealed that virtually all CpG sites were densely methylated. When SNU-719, MKN-28 and AGS cells were treated with the demethylating agent 5-aza-2'-deoxycytidine, RASSF1A gene expression was restored and the methylation-specific polymerase chain reaction pattern was altered in all three cell lines. Transfection of a plasmid expressing RASSF1A into AGS and SNU-719 cells significantly inhibited cell proliferation. Exogenous RASSF1A also reduced the expression of cyclin D1 and phospho-retinoblastoma protein, and increased that of p27 as demonstrated by western blot analysis. Furthermore, RASSF1A expression was significantly reduced (P=0.048) and the methylation rate was elevated in gastric adenocarcinoma tissues, compared with those in adjacent healthy intestinal metaplasia (34.6 vs. 66.7%, P=0.029). The present study indicated that epigenetic silencing of RASSF1A is frequently caused by promoter hypermethylation in gastric cancer cell lines as well as in gastric adenocarcinoma tissues, which may contribute to gastric carcinogenesis.

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Abbreviations: RASSF1A, Ras association domain family 1A gene; MSP, methylation-specific polymerase chain reaction; 5-Aza-dc, 5-Aza-2'-deoxycytidine; qRT-PCR, quantitative reverse transcription polymerase chain reaction; WST, water-soluble tetrazolium salt

Key words: Ras association domain family 1A, epigenetics, hypermethylation

Introduction

Gastric cancer is one of the most frequent types of malignancy in Korea as well as in East Asia, and a number of factors contribute to its development, including genetic or epigenetic events. To date, numerous regulatory genes, including adenomatous polyposis coli gene (APC), P14, P16, death-associated protein (DAP)-kinase (DAPK), human mutL homolog 1 (hMlH1) and runt-related transcription factor 3 (RUNX3), have been shown to be frequently inactivated by promoter hypermethylation in gastric adenocarcinoma tissues (1-3). Previous studies using allelic loss mapping have identified a homozygous deletion in the 3p21.3 region of lung, breast or kidney tumor cells (4,5). Furthermore, certain tumor suppressor genes in this locus are known to be commonly inactivated by promoter hypermethylation, which results in transcription inhibition (6).

Among them, Ras-associated factor 1A (RASSF1A) has been the most extensively investigated, and is known to be frequently methylated in numerous types of cancer (7). Exogenous RASSF1A in RASSF1A expression-negative cancer cell lines may promote apoptosis and cell cycle arrest, and inhibit cell proliferation and tumorigenicity. The RASSF1A protein forms a complex with protein modulator of apoptosis-1 (MAP-1), which induces a conformational change in proapoptotic proteins, such as B-cell lymphoma-associated X, as well as mitochondrial membrane perforation and cytochrome c release (8). Transfection of clones stably expressing RASSF1A was shown to increase the binding capacity of $p120^{E4F}$, a transcriptional repressor, to the cyclin A2 promoter, which in turn regulates cell cycle progression (9). Numerous other studies have demonstrated the molecular mechanisms underlying the regulation of multiple biological processes by RASSF1A (8,10,11).

A number of methylation studies have demonstrated aberrant CpG island promoter hypermethylation of RASSF1A in gastric adenocarcinoma tissues. These studies were based on the use of conventional methylation-specific polymerase chain reaction (MSP) analysis. However, the methylation rate among gastric cancer tissue samples was variously reported as low (0-7.5%) (12,13) or moderate to high (58.7-66.7%) (3,14-16). Furthermore, significant differences in the methylation ratio of the RASSF1A promoter between gastric cancer and pre-cancerous tissues adjacent to cancerous tissues (intestinal metaplasia) have not been consistently demonstrated. An *in vitro* study described an alteration of tumorigenicity, including changes in cell proliferation, cell cycle progression and apoptosis, following introduction of RASSF1A into expression-negative gastric cancer cell lines (17).

The present study aimed to investigate whether promoter methylation of RASSF1A occurred frequently in various gastric cancer cell lines, using conventional MSP as well as bisulfite sequencing. In addition, the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-dc) was used to examine the restoration of gene expression through alteration of methylation status. Furthermore, the effect of transfection of RASSF1A expression-negative gastric cancer cell lines with an RASSF1A-expressing plasmid on cell growth and cell cycle machinery was investigated. Finally, the difference in expression and methylation levels of RASSF1A in pre-cancerous tissues adjacent to gastric cancer and gastric cancer tissues was examined using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and MSP methods.

Materials and methods

Human gastric tissues and gastric cancer cell lines. A total of 14 samples of gastric mucosa tissue from patients with chronic gastritis or dyspepsia (NL group), 32 pre-cancerous gastric tissue samples adjacent to cancerous tissues from patients with gastric cancer (NT group) and 21 gastric adenocarcinoma tissue samples (GC group) were obtained using forceps biopsy during esophagogastroduodenal endoscopic examination. Written informed consent was obtained from all patients and healthy volunteers. In addition, the clinical characteristics of gastric cancer patients were investigated, including T stage (T1 vs. T2~4), differentiation (differentiated vs. undifferentiated) and Lauren's classification (intestinal vs. diffuse). Tissue specimens were maintained in 1 ml RNA-stabilizing reagent (RNAlater; Qiagen, Valencia, CA, USA) at room temperature overnight and then stored at -70˚C prior to use. The human gastric cancer cell lines SNU-16, SNU-638, SNU-719, MKN-28, MKN-45, KATO-III and AGS as well as the cervical cancer cell line Hela were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). All cell lines were cultured in RPMI (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL) and penicillin/streptomycin (1.0%; Gibco-BRL). The use of human gastric mucosal tissue in this study was approved by the Ethics Committee of Korea University College of Medicine, Guro Hospital (Seoul, Korea).

RT-PCR and qRT-PCR. Total RNA was extracted from each cell line or tissue using Trizol™ (Invitrogen Life Technologies) following the manufacturer's instructions. cDNA was subsequently produced using a high capacity cDNA RT kit (Applied Biosystems, Foster City, CA, USA) and treatment with 1 U DNAse (Promega Corp., Madison, WI, USA). In order to analyze the expression of RASSF1A in gastric cancer cell lines, RT-PCR was conducted by modifying a previously described method (18). Briefly, 20 ng prepared cDNA was used to generate 25 μl PCR product using Econo Taq® PLUS Green Master Mix (Lucigen Co., Middleton, WI, USA). PCR was conducted using the following conditions: Initial denaturation at 94˚C for 2 min, followed by 40 cycles of denaturation at 94˚C for 15 sec, annealing at 55˚C for 15 sec, extension at 72˚C for 15 sec and a final extension at 72˚C for 10 min. In order to detect specific gene expression of the A isoform among the RASSF1 splicing variants (A-H), the following primers were used: Forward, 5'-AGTGCGCGCATTGCAAGTT-3', which crossed the 1st and 2nd exon boundary, and reverse, 5'-GCTCGTCCACGTTCGTGTC-3', which crossed the 2nd and 3nd exon boundary (GenBank accession no. NM_007182). These primers were specific to isoform A (19) and produced products of 123 bp. GAPDH was used as a reference gene for each sample and the primer sequences used were as follows: Forward, 5'-GGTCTCCTCTGACTTCAACA-3' and reverse, 5'-AGCCAAATTCGTTGTCATAC-3' (19). All primers were designed by ourselves, with the exception of the primers for GAPDH, MSP-2 and bisulfite sequencing, and all primers were supplied by Macrogen, Seoul, Korea. Five microliters of PCR products were loaded on a 2% agarose gel, and positive bands were obtained by staining with ethidium bromide (Amresco, Solon, OH, USA).

In order to compare the expression levels of the RASSF1A gene in gastric tissues samples from the NL, NT and GC groups, qRT-PCR was conducted as described previously (11). Briefly, following RT as described above, 100 ng cDNA was used as a DNA template and qRT-PCR was performed using a Takara SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan). Primer sequences were the same as those used for RT-PCR and the PCR condition was as follows: 30 sec of initial denaturation at 95˚C, followed by 40 cycles of denaturation (95˚C for 5 sec) and annealing (60˚C for 30 sec). Ct values were obtained following PCR and normalized to those of GAPDH for quantitative analysis.

Conventional MSP. Genomic DNA was extracted using QIAamp genomic DNA kit (Qiagen). Bisulfite-modified genomic DNA was subsequently produced using commercial bisulfite kit (EZ DNA methylation kit; Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Forward and reverse primers for MSP were designed to partially cover the CpG island of RASSF1A, which is located from +91 to +300 bp, based on the transcription initiation site (GenBank accession number AC_002481; Fig. 1A, MSP-1). The following primers were used: Forward, 5'-TAGCGTTTAAAGTTAGCGAAGTAC-3' and reverse, 5'-GAACTAAAAACGATAACCACGAC-3' for methylation-specific sequences and forward, 5'-AGTGTTTAAAGTTAGTGAAGTATGG-3' and reverse, 5'-CAAACTAAAAACAATAACCACAAC-3' for

unmethylation-specific sequences. The following PCR conditions were used: Initial denaturation at 94˚C for 2 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 60˚C for 30 sec, extension at 72˚C for 30 sec and a final extension at 72˚C for 10 min. Each primer set was able to amplify 207 bp of PCR product. Samples were loaded on a 2% agarose gel, and visualized as described above for RT-PCR.

In order to perform conventional MSP using gastric tissues, primers used previously were modified (Fig. 1A, MSP-2) (14,16). The following primers were used: Forward, 5'-GGGTTTTGCGAGAGCGCG-3' and reverse, 5'-GCTAACAAACGCGAACCG-3' for methylation-specific sequences and forward, 5'-GGGGTTTTGTGAGAGTGTG-3' and reverse, 5'-CACTAACTTTAAACACTAAC-3' for unmethylation-specific sequences. The following PCR conditions were used: Initial denaturation at 94˚C for 2 min, followed by 45 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec, extension at 72˚C for 30 sec and a final extension at 72˚C for 10 min. The primers were able to amplify 169 and 183 bp of PCR products, respectively. Samples were reported as exhibiting positive methylation if a clear bands of the correct size was produced by the methylation-specific primers. Peripheral blood mononuclear cells for methylated and unmethylated control genomic DNA were obtained from the healthy volunteers in the present study.

Demethylation with 5-Aza-dc. SNU-719, MKN-28 and AGS cells were seeded at a density of $1x10^6$ cells/ml in a 100-mm diameter dish for 24 h. The following day, cells were treated with 5 μmol/l of the DNA demethylating agent, 5-Aza-dc (Sigma-Aldrich, St. Louis, MO, USA) and this was continued for four consecutive days. Cells were then harvested in order to extract total RNA, genomic DNA and protein for RT-PCR, MSP and western blotting, respectively.

Bisulfite sequencing. Bisulfite-modified DNA was amplified by primers anchoring from +137 to +443 bp relative to the transcription initiation site (Fig. 1A; BS). The following primer sequence were used: Forward, 5'-GGGGAGTTTGAGTTTATTGAGTT-3' and reverse, 5'-CTACCCCTTAACTACCCCTTCC-3'. The resulting 297 bp PCR products were cloned into a pCR2.1-TOPO vector (Invitrogen Life Technologies, Carsbad, CA, USA) and three to five clones were randomly obtained for the subsequent sequencing analysis. Sequencing reactions were performed in an MJ Research PTC-225 Peltier Thermal Cycler using an ABI PRISM® BigDye™ Terminator Cycle Sequencing kit with AmpliTaq® DNA polymerase (FS enzyme; Applied Biosystems) according to the manufacturer's instructions.

Transfection. The pCIneoFLAG-His6-RASSF1A plasmid was purchase from Adgene (#37016; Cambridge, MA, USA). The backbone of the plasmid vector pCIneoFLAG-His6 was modified by digesting the pCIneoFLAG-His6-RASSF1A plasmid with EcoRI (5) and SaI (3), and then ligating with DNA polymerase, which were all purchase from Promega Corp. For the transfection, AGS and SNU-719 cells were cultured for 24 h until they reached 70-80% confluence and transfected with 2 μg plasmid using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Cells were collected at the indicated time-points for further functional analyses.

Water-soluble tetrazolium salt-1 (WST-1) cell proliferation assay. In order to quantify the inhibitory effect of RASSF1A expression on cell proliferation, a commercial WST-1 assay kit (EZ-CYTOX, Dogen, Seoul, Korea) was used according to the manufacturer's instructions (20). Briefly, $1x10^4$ AGS and SNU-719 cells per well were cultured in 96 wells at 37˚C for 24 h, prior to transfection with RASSF1A-plasmid or empty plasmid for 24, 48 or 72 h. Untransfected cells were also cultured for the same time period as a control. Following transfection, each well was treated with 10 μl WST for 4 h and absorbance at 450 nm was measured using an ELISA reader (Epoch, serial no. 1212265; BioTek Instruments, Seoul, Korea). All experiments were performed in triplicate.

Western blot analysis. A mouse monoclonal antibody against human RASSF1A (NBP2-03644) was obtained from Novus Biologicals (Littleton, CO, USA), and rabbit polyclonal antibodies against cyclin D1 (sc-718), p27 (sc-527), p-Rb (Ser 608; sc-56174) and β-actin (sc-47778) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). A total of 80-100 μg cytoplasmic proteins were extracted using CelLytic-M (C2978; Sigma-Aldrich) with a protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Manneheim, Germany). Primary antibodies were diluted at 1:1,000 in Tris-buffered saline with Tween20 (Biosesang Inc., Seongnam, Korea) containing 5% non-fat milk (BD Difco™, Sparks, MD, USA). Probed membranes were incubated overnight at 4˚C with the above primary antibodies, each at a dilution of 1:1,000. The membranes were then incubated with 1:1,000 goat anti-mouse or anti-rabbit IgG secondary antibodies (170-6516 and 170-6515; Bio-Rad, Hercules, CA, USA) for 1 h at room temperature. Protein bands were detected by exposing the membrane to enhanced chemiluminescence (Western Lightning Plus-ECL; PerkinElmer, Inc., Waltham, MA, USA) for 1 min.

Statistical analysis. Relative mRNA (mRNA) expression levels of RASSF1A were normalized to those of GAPDH. Continuous data are presented as the median ± interquartile range (log2 ratio) and categorical data as the percentage (frequency) of methylation. Stayistical analysis was performed using SPSS 19.0 software (International Business Machines, Armonk, NY, USA). Analysis of variance was performed for continuous data and Pearson's χ^2 method was used for categorical data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression and methylation status of RASSF1A in gastric carcinoma cell lines. Expression of the RASSF1A gene in seven gastric carcinoma cell lines was investigated using RT-PCR. Among the cell lines examined, four (SNU-638, SNU-719, MKN-28 and AGS) had no detectable expression of RASSF1A. By contrast, SNU-16 and KATO-III cells exhibited positive expression and MKN-45 cells exhibited

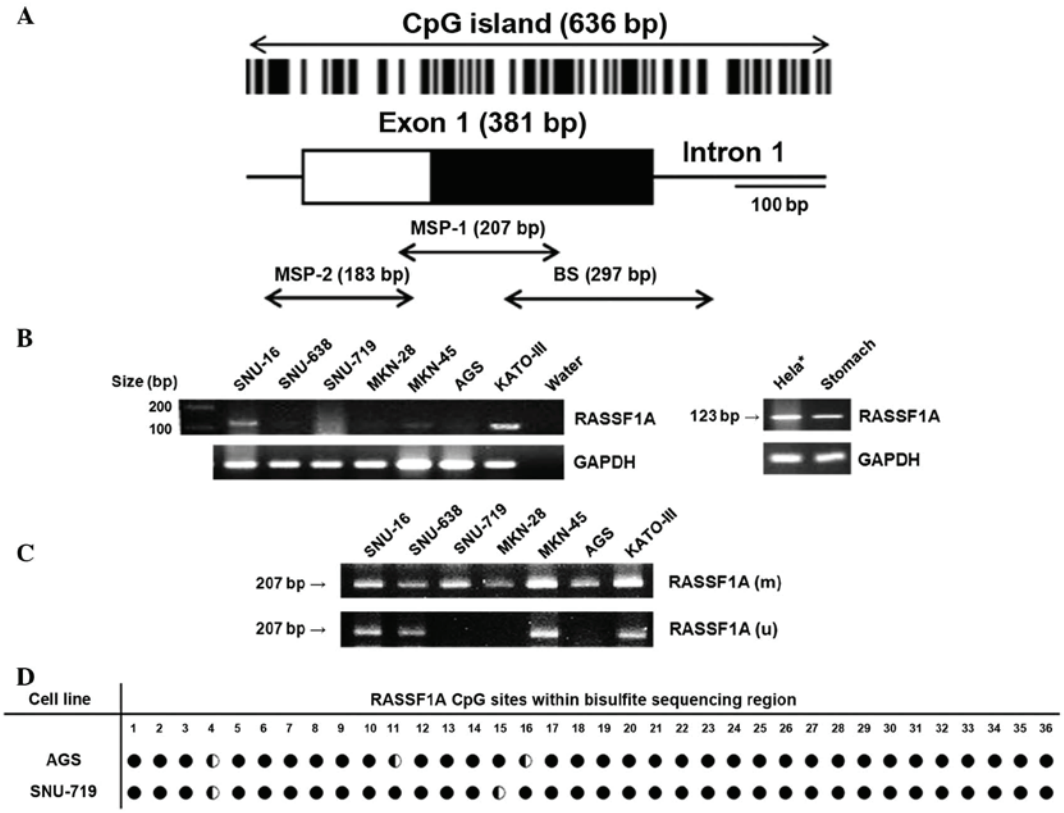

Figure 1. Gene expression and methylation of RASSF1A in gastric carcinoma cell lines. (A) Schematic representation of the 5'-region of human RASSF1A gene. Box indicates exon, including coding (white) and non-coding (black) regions. Vertical bars show CpG sites and arrows indicate regions subject to MSP-1 (+91-+300, based on transcription initiation site), MSP-2 (-73-+109) and bisulfite sequencing (+137 -+443). (B) Reverse transcription polymerase chain reaction showed RASSF1A gene expression to be negative in SNU-638, SNU-719, MKN-28 and AGS cells and positive or weakly positive in SNU-16, MKN-45 and KATO-III cells. (C) Conventional MSP demonstrated that all gastric carcinoma cell lines were partially (SNU-16, SNU-638, MKN-45, KATO-III) or fully (SNU-719, MKN-28, AGS) methylated. (D) Methylation status of RASSF1A in AGS and SNU-719 cells. Numbers show CpG sites within bisulfite sequencing region, and • indicates fully methylated cytosine, whereas ◐ indicates partially methylated cytosine. *Hela was used as a positive control for RASSF1A expression. M, methylation-specific band; U, unmethylation-specific band; RASSF1A, Ras association domain family 1A; MSP, methylation-specific polymerase chain reaction.

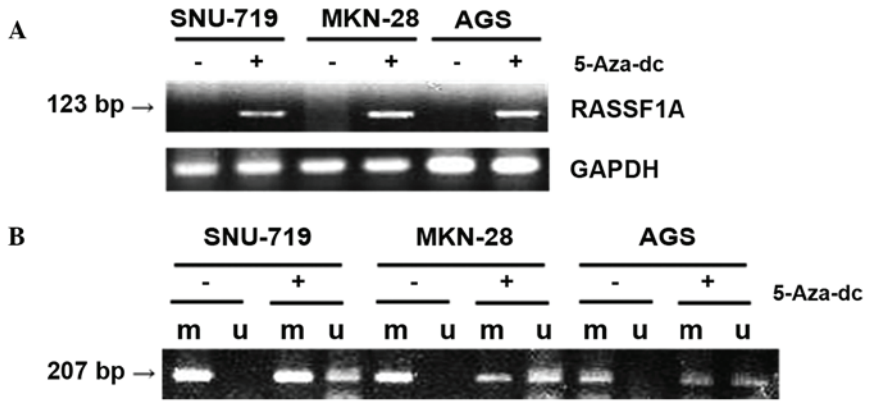

Figure 2. Expression and MSP pattern of RASSF1A following treatment with demethylating agent 5-Aza-dc. (A) Renewed expression of RASSF1A. RASSF1A expression-negative and fully methylated cell lines (SNU-719, MKN-28, AGS) treated with 5 μmol/l of 5-Aza-dc for four days. Reverse transcription polymerase chain reaction showed that RASSF1A expression was restored by treatment with 5-Aza-dc. (B) Alteration of MSP pattern. SNU-719, MKN-28 and AGS were only methylation specific-positive by MSP. Following four days of Aza-dc treatment, all three cell lines exhibited methylation- and unmethylation-specific bands. M, methylation-specific band; U, unmethylation-specific band; RASSF1A, Ras association domain family 1A; MSP, methylation-specific polymerase chain reaction; Ada-dc, 5-aza-2'-deoxycytidine.

weakly positive expression (Fig. 1B). The methylation status in the seven gastric carcinoma cell lines was then investigated using a conventional MSP method. Only methylation-specific bands were detected in SNU-719, MKN28 and AGS cells (indicating full methylation of the promoter). These cell lines did not express RASSF1A. By contrast, SNU-16, MKN-45 and KATO-III cells exhibited methylation- and unmethylation-specific bands (indicating partial methylation). These

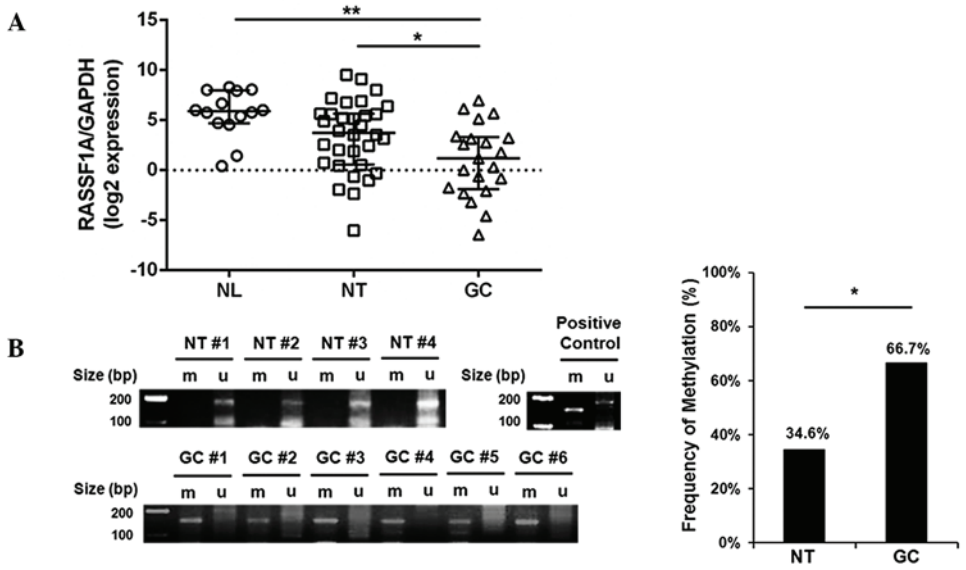

Figure 3. Gene expression and MSP in gastric carcinoma tissue. (A) Quantitative reverse transcription polymerase chain reaction analysis. (B) MSP. Representative MSP in NT and GC groups (upper panel). Positive and negative control genomic DNA was obtained from the peripheral blood mononuclear cells of healthy volunteers, which were bisulfite-modified and methylated by the CpG methyltransferase M. SssI (m) or not (u). NL indicates normal gastric mucosa which exhibited chronic gastritis only, NT indicates non-tumorous tissues adjacent to gastric carcinoma which exhibited intestinal metaplasia and GC indicates gastric carcinoma tissues. *P<0.05 and **P<0.01 vs. NL group. M, methylation-specific band; U, unmethylation-specific band; RASSF1A, Ras association domain family 1A; MSP, methylation-specific polymerase chain reaction.

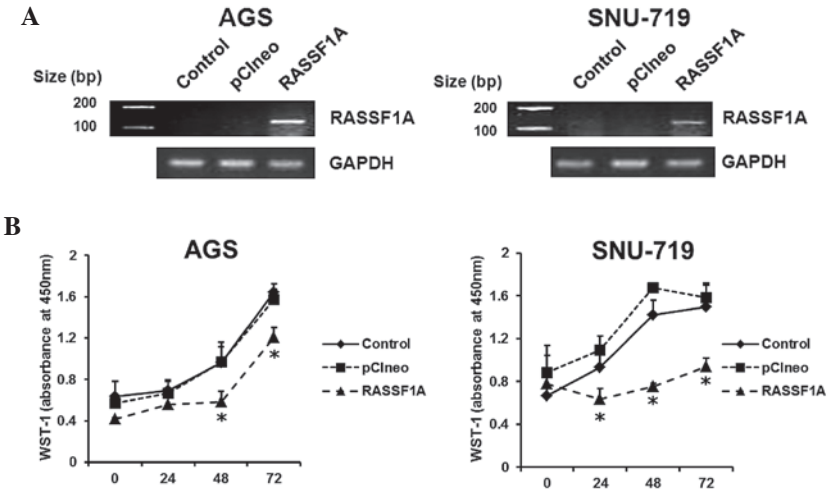

Figure 4. Plasmid transfection and cell proliferation assay. (A) Following two days of transfection, reverse transcription polymerase chain reaction analysis demonstrated that only RASSF1A plasmid-transfected cells (AGS, SNU-719) exhibited positive RASSF1A expression. (B) WST-1 assay showed that exogenous RASSF1A expression significantly inhibited cell proliferation from two days (AGS) and one day (SNU-719) following transfection. *P<0.05 vs. control and pCIneo groups. RASSF1A, Ras association domain family 1A; WST-1, water-soluble tetrazolium salt 1; pCIneo, backbone-modified plasmid.

cell lines all exhibited positive or weakly positive expression of RASSF1A. SNU-638 cells also exhibited methylation- and unmethylation-specific bands. However, this cell line did not express detectable levels of RASSF1A (Fig. 1C). These results indicated that the methylation status of the CpG island promoter is associated with undetectable or reduced expression of RASSF1A.

In order to validate the promoter hypermethylation of RASSF1A in gastric cancer cell lines, AGS and SNU-719 cells, which did not express detectable levels of RASSF1A and exhibited only methylation-specific band in the MSP experiments, were selected and used to conduct bisulfite sequencing, using primers targeting regions adjacent to MSP-1 primers. Bisulfite sequencing demonstrated that the majority of CpG sites were densely methylated in AGS and SNU-719 cell lines (Fig. 1D).

Altered expression and MSP pattern of RASSF1A following treatment with 5-Aza-dc in gastric carcinoma cell lines. Three gastric cancer cell lines (SNU-719, MKN-28 and AGS) were selected, which were negative for RASSF1A expression, as detected using RT-PCR, and only exhibited methylation-specific bands according to MSP analysis (Fig. 1). These cells were treated with 5 μmol/l 5-Aza-dc for four days. Following treatment, RT-PCR was used to demonstrate that RASSF1A was now expressed in these cells (Fig. 2A). MSP

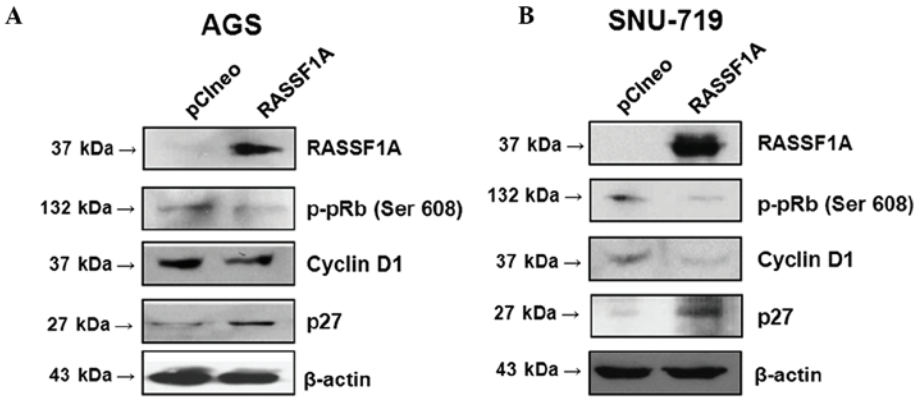

Figure 5. Plasmid transfection and western blotting. RASSF1A expression plasmid and backbone plasmid were transfected into AGS and SNU-719 cells for two days. (A and B) Representative western blots in AGS and SNU-719 cells, respectively. RASSF1A protein expression was only demonstrated in RASSF1A plasmid-transfected cells, in which upregulation of p27 and downregulation of cyclin D1 was observed. RASSF1A, Ras association domain family 1A; p, phosphorylated; pRb, retinoblastoma protein; pCIneo, backbone-modified plasmid.

performed following treatment with 5-Aza-dc demonstrated unmethylation-specific bands in all three cell lines and also thinner methylation-specific bands in the MKN-28 and AGS cell lines, compared with the untreated control (Fig. 2B). These findings also supported the hypothesis that aberrant methylation of RASSF1A induced transcription inhibition in gastric carcinoma cell lines.

Reduced gene expression and increased methylation rate in gastric carcinomas. In order to investigate RASSF1A expression and methylation patterns in gastric tissues, the mRNA levels of RASSF1A in the NL, NT and GC groups was compared using qRT-PCR. All tissues in the NL group displayed evidence of chronic gastritis only; however, those in the NT group had intestinal metaplasia, a pre-cancerous stage of gastric adenocarcinoma. As hypothesized, RASSF1A expression was significantly reduced in the GC group compared with that in the NL and NT groups (P=0.001 and 0.032, respectively; Fig. 3A). Furthermore, 14 out of 21 gastric carcinoma tissue samples (66.7%) in the GC group were methylation-positive, whereas only 9 out of 26 non-tumorous tissue samples (34.6%) were positive in the NT group (P=0.029; Fig. 3B). These results indicated that RASSF1A expression was significantly reduced in association with promoter hypermethylation in gastric carcinoma compared with that in pre-cancerous adjacent tissues. This process may contribute to gastric tumorigenesis.

Exogenous RASSF1A expression inhibits proliferation of gastric carcinoma cell lines. Two RASSF1A expression-negative cell lines (AGS and SNU-719) were selected in order to investigate the anti-proliferative effects of RASSF1A in gastric carcinoma cells. Following transfection of the RASSF1A expression plasmid for two days, a renewed expression of RASSF1A was demonstrated in AGS and SNU-719 cells by RT-PCR. This effect was not observed in untreated cells or cells transfected with the backbone-modified plasmid (Fig. 4A). RASSF1A expression plasmids were then transfected into AGS and SNU-719 cells for 24, 48 or 72 h. A WST-1 assay was then conducted , which demonstrated significant inhibition of cell proliferation at two days (for AGS) or one day (for SNU-719) following transfection, compared with that of untreated cells or cells transfected with the backbone plasmid (Fig. 4B). These findings indicated that epigenetic silencing of RASSF1A may induce cell proliferation in gastric carcinoma cell lines.

Exogenous RASSF1A expression modulates cell cycle machinery proteins in gastric carcinoma cell lines. A western blot assay was performed in order to investigate the effect of RASSF1A on the regulation of cell cycle machinery proteins. AGS and SNU-719 cell lines, which were transfected with RASSF1A expression plasmid, upregulated the expression of p27, an inhibitory regulator of the G_1/S transition, and downregulated that of cyclin D1, an inducer of G_1/S cell cycle progression, as well as that of phosphorylated retinoblastoma protein (p-pRb), which is phosphorylated and inactivated by the cyclin D1-cyclin-dependent kinase 4 (CDK4) complex (Fig. 5A and B) (21). These findings suggested that RASSF1A may inhibit the Ras/Raf kinase pathway, in turn promoting cell cycle arrest in G_1 phase and inhibiting cell cycle progression in gastric carcinoma cell lines.

Discussion

In the present study, ~2/3 of the samples in the GC group exhibited methylation, which was significantly higher than the percentage in the NT group. This was correlated with the downregulation of RASSF1A expression, which was significantly reduced in the GC group compared with that in the NL or NT group. A number of studies investigating the hypermethylation of RASSF1A in gastric carcinoma reported a higher frequency of promotor methylation in gastric cancer tissues than that in the surrounding non-cancerous tissues (3,16). However, other studies have failed to reproduce these (12,13,22). The latter studies reported a relatively low methylation rate in the gastric carcinoma tissues which were examined (0-7.5%) as well as in adjacent non-cancerous tissues. The results of the present study support the hypothesis that promoter methylation frequently occurs in gastric carcinoma tissues and that levels of methylation are higher than those in non-cancerous tissues. The present study also examined differences in various characteristics of gastric carcinoma, including tumor invasion (T1 vs. T2-4), differentiation (differentiated vs. undifferentiated)

or Lauren's classification (intestinal or diffuse) (23), between methylated and unmethylated samples. However, no significant differences were detected (data not shown), which may have partly been a result of the small sample size. A previous study demonstrated significantly reduced expression of the RASSF1A gene in advanced stage and undifferentiated gastric carcinomas (24). However, a separate study of methylation using surgical specimens of gastric carcinoma did not detect any significant differences in the frequency of methylation according to tumor invasion, Lauren's classification or distant metastasis (3). Further research is required to assess the association between clinico-pathological characteristics and RASSF1A promoter hypermethylation in gastric carcinoma.

At the *in vitro* level, a number of gastric carcinoma cell lines were shown to be RASSF1A expression-negative. These lines all exhibited a methylation-specific band only in the MSP analysis. Gastric carcinoma cell lines that exhibited partial methylation of the RASSF1A promoter were undifferentiated and derived from metastatic carcinoma (SNU-16, SNU-638, MKN-45 and KATO-III), whereas cells that exhibited full methylation were predominantly well-differentiated (SNU-719 and MKN-28) or from a primary carcinoma that was not known to have metastasized (SNU-719 and AGS). The role of RASSF1A in the differentiation or metastasis of gastric carcinoma requires further investigation. The advantages of the present study were that it was able to demonstrate renewed expression in association with an altered methylation pattern of RASSF1A in gastric carcinoma cell lines by utilizing a demethylating agent. Numerous *in vitro* studies have reported an altered MSP pattern of RASSF1A following treatment with 5-Aza-dc in other cancer cell lines (25,26). However, few studies have demonstrated this finding in various gastric carcinoma cell lines, and, to the best of our knowledge, only one previous study has shown the effect of 5-Aza-dc and sodium butyrate on the expression of RASSF1A in gastric cancer cell lines (27). Following treatment with the demethylating agent, a change in the MSP pattern in all three fully methylated gastric carcinoma cell lines (SNU-719, MKN-28, AGS) was observed, and this was correlated with gene expression. This suggested that transcription inhibition of RASSF1A is closely associated with CpG island promoter hypermethylation in gastric carcinoma cells.

Numerous studies have investigated the function of RASSF1A in cancer cell lines, and it has been shown that RASSF1A inhibited cell proliferation and tumor growth (28). The two common pathways which RASSF1A is hypothesized to modulate in cancer cells are apoptosis and cell cycle progression. Within these pathways, cyclin D1 has been identified as a cell cycle activator, which is regulated by RASSF1A (29,30). It has been suggested that the amplification of cyclin D1 may remove p27 from CDK2 and contribute to cell cycle progression. pRb is also linked to, and inactivated by cyclin D1 (31). The present study demonstrated that exogenous RASSF1A significantly inhibited cell proliferation in AGS and SNU-719 cells, as expected. It was also shown to upregulate p27 and downregulate cyclin D1 and p-pRb. To the best of our knowledge, this was a novel finding, which indicated that cell cycle machinery proteins, in particular p27, may be modulated by RASSF1A in gastric cancer cell lines. Given that p27 is neither inactivated nor hypermethylated in gastrointestinal cancer cell lines (32,33), unlike inhibitor of CDK4 family proteins, such as p16 (34,35), it is likely that p27 expression was restored by the exogenous introduction of RASSF1A into expression-negative gastric carcinoma cell lines.

In conclusion, the results of the present study indicated that the CpG island promoter hypermethylation of RASSF1A commonly occurs in gastric carcinoma cell lines as well as in gastric carcinoma tissues. This was supported by the observation that treatment with the demethylating agent 5-Aza-dc altered the expression and methylation pattern of RASSF1A in RASSF1A-negative cell lines. Exogenous expression of RASSF1A by plasmid transfection significantly inhibited cell proliferation and modulated the expression of a cell cycle activator (cyclin D1) and an inhibitor (p27). Future studies are required to elucidate the function of RASSF1A in gastric carcinogenesis by investigating clinicopathological variables influenced by RASSF1A expression and hypermethylation in gastric carcinoma tissues, as well as by identifying target genes through establishing stably RASSF1A-expressing gastric cell lines.

Acknowledgements

This study was supported by grants from Korea University (grant no. R1111971) and PacificPharma Corp. (grant no. Q1307141; Seoul, Korea).

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