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Cytoplasmic RASSF2A is a proapoptotic mediator whose expression is epigenetically silenced in gastric cancer

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Gastric cancer cells often show altered Ras signaling, though the underlying molecular mechanism is not fully understood. We examined the expression profile of eight ras-association domain family (*RASSF*) genes plus MST1/2 and found that *RASSF2A* is the most frequently downregulated in gastric cancer. *RASSF2A* was completely silenced in 6 of 10 gastric cancer cell lines as a result of promoter methylation, and expression was restored by treating the cells with 5-aza-2′-deoxycytidine. Introduction of RASSF2A into non-expressing cell lines suppressed colony formation and induced apoptosis. These effects were associated with the cytoplasmic localization of RASSF2A and morphological changes to the cells. Complementary DNA microarray analysis revealed that RASSF2A suppresses the expression of inflammatory cytokines, which may in turn suppress angiogenesis and invasion. In primary gastric cancers, aberrant methylation of *RASSF2A* was detected in 23 of 78 (29.5%) cases, and methylation correlated significantly with an absence of the lymphatic invasion, absence of venous invasion, absence of lymph node metastasis, less advanced stages, Epstein–Barr virus, absence of p53 mutations and the presence of the CpG island methylator phenotype-high. These results suggest that epigenetic inactivation of *RASSF2A* is required for tumorigenesis in a subset of gastric cancers.

Introduction

Gastric cancer is one of the most common of human neoplasias (1). It often arises through the accumulation of multiple genetic changes, including mutation of *APC*, *K-ras* and *p53*, but the frequencies of oncogene and tumor suppressor gene mutation in gastric cancer are relatively low, as compared those seen in colorectal cancer (2,3). On the other hand, recent studies have shown that epigenetic alterations (e.g. DNA methylation) play a key role in silencing such cancer-related genes such as *p16INK4A*, *CHFR*, *E-cadherin*, *14-3-3σ* and *DAP-kinase* (4–7), and genome-wide methylation screening has identified a number of genes inactivated by DNA methylation in gastric cancer (8,9). In that regard, *Helicobacter pylori* infection, a potent gastric carcinogenic factor, reportedly induces methylation of various genes in the gastric mucosa (10). Still, the precise role of DNA methylation in gastric cancer remains unclear.

Ras proteins play essential roles in controlling the activity of several important signaling pathways that regulate cellular proliferation, migration and apoptosis (11), and alteration of Ras signaling can lead to tumorigenesis. In fact, mutations of *K-ras* and *BRAF* have been observed in a number of human neoplasias (12,13), though their mutation rarely occurs in gastric cancer (14). Notably, activated forms of Ras also can induce senescence and apoptosis, indicating the presence of negative effectors regulated by Ras. Such effectors are commonly the products of RASSF family genes, and contain a Ras association (RA) domain. Alterations of these negative effectors of *Ras* also can play a role in tumorigenesis. For instance, epigenetic inactivation of *RASSF1*, a candidate tumor suppressor gene on chromosome 3p21, has been well characterized in a wide variety of tumors (15,16). So far, six members of the *RASSF* gene family, *RASSF1A*, *RASSF2A*, *RASSF4*, *RASSF5/NORE1*, *RASSF6* and *RASSF8*, have been shown to be inactivated or downregulated in human neoplasias (15–25). Of those, *RASSF2A* is frequently inactivated in colorectal, gastric, lung and nasopharyngeal cancers, although the molecular mechanisms by which RASSF2A functions as a tumor suppressor remains unknown (17,20,22,23,25).

In addition, *MST1* and *MST2* encode serine/threonine kinases that associate with RASSF family proteins (e.g. RASSF1 and RASSF5/*NORE1*) (26,27), and they are epigenetically inactivated in soft tissue sarcoma (28). The role of the RASSF/MST pathway in gastric cancer is not known, however.

In the present study, we examined the epigenetic alteration of *RASSF* family genes together with *MST1/2* in a panel of gastric cancer cell lines. We found that, of those, *RASSF2A* is the most frequently downregulated in gastric cancer. Introduction of RASSF2A into gastric cancer cells that do not otherwise express the gene significantly diminished colony formation and induced apoptosis. Induction of apoptosis by RASSF2A is associated with morphological changes and cytoplasmic localization of RASSF2A. Taken together, these findings suggest that epigenetic inactivation of *RASSF2A* plays a key role in tumorigenesis in gastric cancer.

Abbreviations: 5-aza-dC, 5-aza-2′-deoxycytidine; CIMP, CpG island methylator phenotype; NLS, nuclear localization signal; PCR, polymerase chain reaction; RA, Ras association.

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Materials and methods

Cell lines and specimens

Twelve gastric cancer cell lines and 78 primary gastric cancer specimens were used in this study. Among the cell lines, eight were obtained from American Type Culture Collection, Tokyo, Japan or Japanese Collection of Research Bioresources Tokyo, Japan, whereas two (HSC44 and SH101) were kindly provided by Dr K.Yanagihara at the National Cancer Center Research Institute and were described previously (29,30). The 78 gastric cancer specimens were described previously (8,31). Written informed consent was obtained from every patient and approved by the Institutional Review Board. DNA was prepared using the standard phenol–chloroform method. Total RNA was isolated using Trizol (Invitrogen).

Reverse transcription–polymerase chain reaction

Five micrograms of total RNA were reverse transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) was carried out using primers specific for *RASSF2* exon 1A and exon 1B (supplementary Table 1 is available at *Carcinogenesis* Online). To analyze

restoration of RASSF2A, JRST, KatoIII, SNU1, SNU638 and HSC44 cells were incubated for 72 h with 2 μM 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma, St Louis, MO). For quantitative real-time PCR, reactions were carried out using a 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers and probes for each gene are shown in supplementary Materials and Methods (available at *Carcinogenesis* Online). The relative levels of *RASSF2A* expression were quantified using the ΔCt value, which yields a ratio of the expression of a target gene to that of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Bisulfite sequencing and pyrosequencing

Sodium bisulfite treatment was performed as described previously (32). The primer sequences and PCR parameters used are shown in supplementary Table 1 (available at *Carcinogenesis* Online). PCR products were cloned into pCR4.0 vector using a TOPO-TA Cloning Kit (Invitrogen). The sequencing reaction was carried out using a BigDye terminator cycle sequencing kit (PerkinElmer Biosystems, Foster city, CA), and sequencing was carried out using an ABI PRISM 3100 sequencer according to the manufacturer's guidelines (Applied Biosystems). Pyrosequencing was carried out as described previously (33,34), and the details are shown in supplementary Materials and Methods (available at *Carcinogenesis* Online).

Colony formation assay

An expression plasmid, pcDNA-*RASSF2A*, containing the entire coding region of human *RASSF2A* was constructed using the expression vector pcDNA3.1(+), which harbors a gene conferring geneticin resistance. Cells at 25% confluence in 10 cm dishes were transfected with 5 μg of one of three expression plasmids (pcDNA-*RASSF2A*, pcDNA-*RASSF2*-ΔRA or pcDNA3.1 control vector) using Nucleofector (Amaxa, Berlin, Germany) as described previously (17). Twenty-four hours after transfection, the cells were split 1:10 and grown for 14 days in the presence of G418 (Gibco BRL, Grand Island, NY) (0.3–0.6 mg/ml). The cells were then fixed and stained with Giemsa, and the number of colonies was scored. Experiments were performed in triplicate and repeated on two independent occasions.

Construction of adenoviral vectors and flow cytometry analysis

The generation, purification and infection procedures used with the replication-deficient recombinant adenovirus were described previously (35,36). For flow cytometric analysis, 1×10^6 cells were infected with an adenoviral vector containing RASSF2A (aa 1–326), RASSF2A-ΔN (aa 164–326), RASSF2A-ΔRA (aa 1–163), RASSF2A-ΔNLS (aa 1–149/168–326) or green fluorescent protein. The cells were then incubated for 24, 48 or 72 h, trypsinized, fixed with methanol, rehydrated with phosphate-buffered saline, treated with 2 mg/ml RNase for 30 min at 37°C and stained in 50 μg/ml propidium iodide solution. Fluorescence-activated cell sorting analysis was carried out using a Becton Dickinson FACScan flow cytometer (Braintree, MA).

Immunocytochemistry

Cells (1×10^4) were seeded on glass coverslips, infected with an adenoviral vector and fixed with 4% paraformaldehyde. Immunofluorescence analysis was carried out using mouse anti-Flag antibody and Alexa 488. For paxillin staining, the cells were incubated for 1 h with 0.1 μg/ml rhodamine-conjugated phalloidin (BD Bioscience, San Jose, CA). For Flag-RASSF2A staining, cells were incubated first with mouse anti-Flag monoclonal antibody (Sigma) and then with Alexa 488 goat anti-mouse antibody (Molecular Probes, Eugene, OR). Once labeled, the cells were examined under an Olympus IX71 fluorescence microscope. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole.

Results

Epigenetic inactivation of RASSF family genes in gastric cancer cell lines

To determine the expression profile of negative effectors of Ras in gastric cancer, we examined the expression status of eight *RASSF* family genes plus *MST1* and *MST2* in a panel of gastric cancer cell lines. Of the 10 genes analyzed, the expression of RASSF2 was the most frequently downregulated in gastric cancer (Figure 1A). As a result of different transcription start sites, *RASSF2* has four major transcription variants: *RASSF2A* variant 1, *RASSF2A* variant 2, *RASSF2B* and *RASSF2C*. Analysis of the expression of each of these four isoforms revealed that both RASSF2A variants are expressed in all the normal tissues we tested (Figure 1B, upper panel). In contrast, their expression was lost in six gastric cancer cell lines (Figure 1B, lower panel) and, in MKN28 cells, expression of variant 1 was readily detected, but expression of variant 2 was very weak. Treating the gastric cancer cell lines with a methyltransferase inhibitor restored expression of the *RASSF2A* variants, indicating a role for DNA methylation in the silencing of *RASSF2A* expression (Figure 1C). Moreover, when we next examined *RASSF2* expression after treating cells with a low dose of 5-aza-dC and/or Trichostatin A(TSA), we found that treating HCC44 cells with 300 nM TSA or 0.2 μM 5-aza-dC had little effect on gene expression, but TSA plus 5-aza-dC acted synergistically to restore *RASSF2A* expression, which suggests the involvement of histone deacetylation in the gene's silencing (Figure 1D). Very little, if any, expression of *RASSF2B* and *RASSF2C* was detected (data not shown).

Aberrant methylation of RASSF2A in gastric cancer cell lines

To assess the role of DNA methylation in the silencing of *RASSF2A* in more detail, we used bisulfite sequencing to examine 37 CpG sites located around exons 1A and 1B of *RASSF2* in our panel of gastric cancer cell lines (Figure 1D). We detected high levels of methylation of exon 1A in JRST and KatoIII cells, which were consistent with the expression analysis. MKN28 cells showed high levels of methylation of exon 1B but not 1A, which are consistent with their expression of *RASSF2A* variant 1 and only a minimal amount of *RASSF2A* variant 2. Only sparse methylation was detected in MKN7, MKN74 and SH101 cells, which expressed the gene.

Effect of RASSF2A on cell growth and apoptosis in gastric cancer cells

To determine whether RASSF2A exerts an antiproliferative effect, we carried out a set of colony formation assays. Schematic representations of the various RASSF2 deletion constructs are shown in supplementary Figure 1 (available at *Carcinogenesis* Online). We found that introduction of RASSF2A into JRST and HSC44 cells resulted in a significant reduction of colony formation (Figure 2A and B). In contrast, a mutant form of RASSF2A that lacked the RA domain (ΔRA) was less able to suppress growth, indicating a role for the RA domain in suppression of cell growth. To investigate the role of RASSF2A-mediated inhibition of growth in more detail, we introduced Ad-*RASSF2A* into gastric cancer cell lines in which the gene was silenced. Transformation by Ad-*RASSF2A* induced apoptosis in JRST and SNU1 cells, which otherwise did not express *RASSF2A*, but did not induce apoptosis in cells that did express *RASSF2A*.

During the course of the experiments, we repeatedly noticed that RASSF2A transformants exhibited a round morphology, suggesting that *RASSF2A* expression leads to the inhibition Ras and, in turn, inhibition of focal adhesion and stress fiber formation and activation of paxillin. To test that idea, we used immunocytochemistry to examine paxillin expression (Figure 3A) and confirmed that the RASSF2A-induced round morphology was caused by the absence of stress fibers. We then examined whether these morphological changes induce apoptosis or whether it is apoptosis that induces the observed morphological changes. After transformation with Ad-*RASSF2A*, the numbers of floating cells increased, as did the incidence of apoptosis (Figure 3B). When we treated the cells with a non-specific caspase inhibitor, the number of floating cells increased, but the number of apoptotic cells decreased. This suggests that *RASSF2A* expression alters the morphology of cells, which leads first to a loss of adhesion and then to caspase-dependent apoptosis.

We next analyzed its protein motifs and found that RASSF2A carries three putative nuclear localization signals (NLSs) in the N-terminal region of its RA domain (NLS, ***RR***RGNVRTPSDQ***RR***I***RR***; start position, 150). Although RASSF2A expressed in JRST cells was localized in both the nucleus and cytoplasm, RASSF2A lacking the NLS (RASSF2A-ΔNLS) was found only in the cytoplasm, indicating its NLS is a key determinant of the intracellular distribution of RASSF2A (Figure 4A). Interestingly, when introduced into cells, RASSF2A-ΔNLS exerted a strong proapoptotic effect (Figure 4B).

Identification of genes induced by RASSF2A

To learn more about the molecular pathway via which RASSF2A suppresses cell growth and induces apoptosis, we used

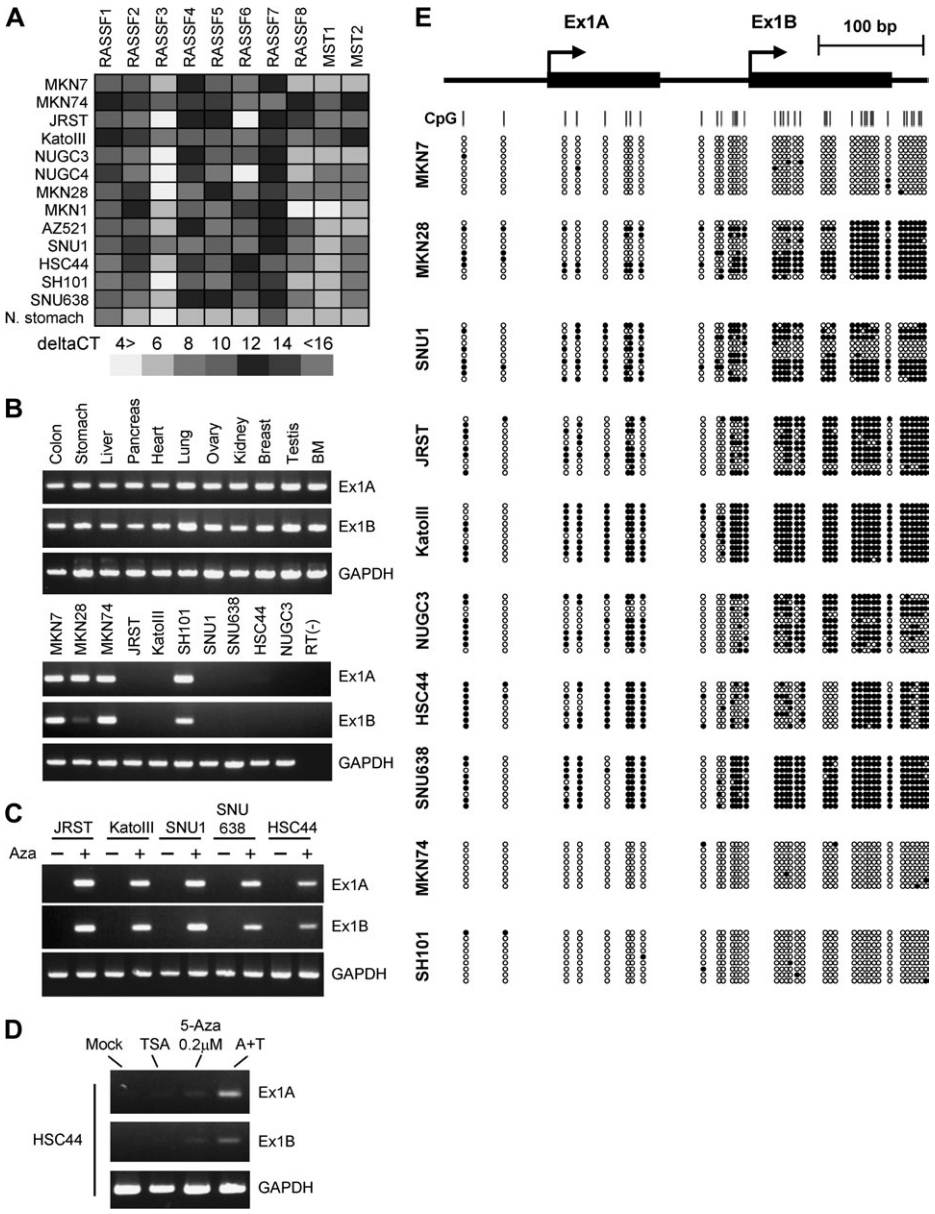

Fig. 1. Epigenetic inactivation of *RASSF2A* in gastric cancer cell lines. (**A**) Expression profile of *RASSF1–8*, *MST1* and *MST2* in gastric cancer cell lines. The heat map shows the expression profiles in normal colon, normal stomach and three cancer cell lines. Levels of expression are normalized to *GAPDH*. (**B**) Expression of *RASSF2A* variant 1 (Ex1A) and variant 2 (Ex1B) in gastric cancer cell lines. Reverse transcription–PCR analysis was performed using primers that specifically amplify two isoforms of *RASSF2*. The cell lines examined were shown on the top. Expression of *GAPDH* was examined to confirm the integrity of RNA. (**C**) Restoration of *RASSF2* expression by treating cells with 1.0 μM 5-aza-dC. (**D**) Restoration of *RASSF2* expression using 5-aza-dC and TSA. HSC44 cells were treated with mock, 300 nM TSA for 18 h, 0.2 μM 5-aza-dC for 72 h or 0.2 μM 5-aza-dC for 72 h followed by 300 nM of TSA for 18 h (A + T). (**E**) Analysis of *RASSF2A* methylation. Schematic representation of the *RASSF2A* CpG island is shown on the top. Arrows indicate the transcription start sites. Bisulfite sequencing analysis of *RASSF2A* was carried out using DNA from three gastric cancer cell lines that express both variant 1 and variant 2 (MKN7, MKN74 and SH101), six gastric cancer cell lines that do not express either variant (JRST, KatoIII, SNU1, SNU638, NUGC3 and HSC44) and MKN28 cells, which express variant 1 but only a low level of variant 2. Open circles indicate the unmethylated CpG sites; filled circles indicate the methylated CpG sites.

a complementary DNA microarray to determine the genes induced or suppressed by RASSF2A. We found that, in JRST cells, overexpression of RASSF2A led to upregulation of 39 genes and downregulation of 31 genes (supplementary Table 2 is available at *Carcinogenesis* Online). Gene ontology analysis revealed that genes involved in immune responses, viral function, cell-to-cell signaling and infection were all significantly enriched among RASSF2A inducible/downregulated genes (supplementary Table 3 is available at *Carcinogenesis* Online). Ingenuity pathway analysis also revealed that genes regulated via the nuclear factor-κB pathway were frequently downregulated by RASSF2A (supplementary Figure 2 is available at *Carcinogenesis* Online). To confirm the results of the microarray analysis, we performed real-time PCR to examine the expression of several putative RASSF2A target genes (supplementary Figure 3A is available at *Carcinogenesis* Online). The levels of IL-8, LCN2, CXCL2, CXCL3, CXCL5 and CCL20 expression were all reduced in cells transformed by Ad-RASSF2A. Conversely, expression of CXCL2 and CXCL5 was upregulated in cells where *RASSF2A* expression was knocked down using specific small interfering RNA (si*RASSF2*) (supplementary Figure 3B is available at *Carcinogenesis* Online).

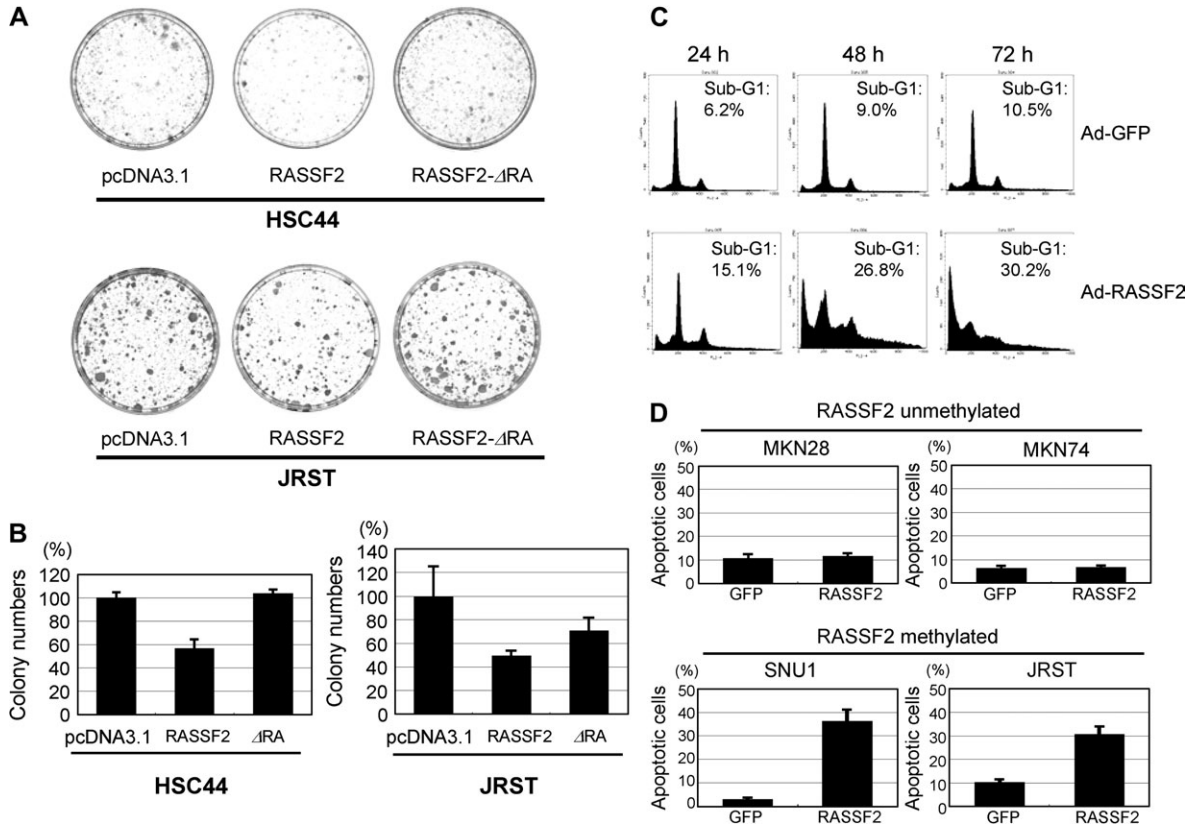

Fig. 2. RASSF2A-mediated growth suppression and apoptosis in gastric cancer cell lines. (**A** and **B**) Suppression of cell growth by RASSF2A. Growth suppression was evaluated by assaying geneticin-resistant colony formation. JRST and HSC44 cells were transfected with either pCDNA3.1 (control plasmid), RASSF2A or RASSF2A-ΔRA and incubated in RPMI 1640 medium containing 0.6 mg/ml G418. After 14 days, the plates were stained with Giemsa solution (A), and the colonies were counted (B). The bars indicate means ± SD of three independent experiments. (**C** and **D**) Flow cytometric analysis. (C) Representative flow cytometry after introduction of Ad-RASSF2A. The incidence of sub-G_1 cells was determined 72 h after infection. (D) Quantitative analysis of sub-G_1 cells. Percentages of sub-G_1 cells are shown on the *y*-axis. Error bars indicate standard error.

Clinicopathological features of gastric cancers with or without methylation of RASSF2A

Finally, we used pyrosequencing to examine the methylation status of *RASSF2A* in a set of primary gastric cancer specimens (Figure 5A). Primers and probe sets were designed to detect methylation in the region around the transcription start sites in exons 1A and 1B. We used minimum of 10% methylation as a cutoff for methylation positivity, as methylation in normal tissue is always <10%. The degree of methylation in 78 primary gastric cancers, including 23 cases in which samples of adjacent normal tissue were also collected, are shown in supplementary Figure 4 (available at *Carcinogenesis* Online). Methylation of exon 1A was detected in 23 of the 78 (29.5%) tumors, whereas methylation of exon 1B was detected in 20 (25.6%) of the tumors. Good quality RNA was obtained from 18 specimens (10 with no methylation of *RASSF2*-Ex1A or *RASSF2*-Ex1B; 8 with methylation of both *RASSF2*-Ex1A and *RASSF2*-Ex1B), which we used to examine the correlation between DNA methylation and *RASSF2A* expression. We found that the level of *RASSF2A* expression was significantly lower in tumors with methylation than in tumors without methylation ($P < 0.01$, Fisher's exact test, two sided, Figure 5B). The methylation profiles, mutations of *p53/K-ras* and the epstein-barr virus (EBV) status of the 78 gastric tumors studied are summarized in Figure 5C (31). There was significant association between methylation of RASSF2A and an absence of lymphatic invasion ($P = 0.007$), absence of venous invasion ($P = 0.008$), absence of lymph node metastasis ($P = 0.029$), less advanced stage ($P = 0.005$), EBV ($P < 0.001$), absence of p53 mutations ($P = 0.002$) and the presence of CpG island methylator phenotype (CIMP)-high ($P < 0.001$). There were significant correlations between methylation of *RASSF1* and *RASSF2A*-Ex1A ($P < 0.001$), *RASSF1* and *RASSF2A*-Ex1B ($P < 0.001$) and *RASSF2A*-Ex1A and *RASSF2A*-Ex1B ($P < 0.001$) (supplementary Table 4 is available at *Carcinogenesis* Online). Moreover, when we then examined the correlation between methylation of *RASSF1/RASSF2A* and 12 other cancer-related genes previously examined for methylation (31), we found significant correlations between methylation of *RASSF1* and eight genes, between methylation of *RASSF2A*-Ex1A and nine genes and between methylation of *RASSF2A*-Ex1B and nine genes. These tumors thus appear to have a defect in their machinery regulating methylation (supplementary Table 4 is available at *Carcinogenesis* Online). The correlations between *RASSF2A* methylation and the clinicopathological features are summarized in supplementary Table 5 (available at *Carcinogenesis* Online).

We also detected methylation of *RASSF1A* in 12 of 78 (15.3%) primary gastric cancers, where it was significantly associated with diffuse type ($P = 0.004$), EBV ($P < 0.001$), absence of p53 mutation ($P = 0.032$) and the presence of CIMP-high ($P < 0.001$).

Discussion

Among the eight *RASSF* family genes and *MST1/2*, we found that *RASSF2A* is the most frequently downregulated in gastric cancer cell lines. Although *RASSF2A* has two non-coding exons, 1A and 1B, previous studies examined the methylation of *RASSF2A* using methylation specific PCR, which would detect methylation downstream of the CpG islands (22). In the present study, we showed that the majority of cancer cells are methylated in the regions around both exons 1A and 1B. In MKN28 cells, however, transcripts from exons 1A and 1B were differentially expressed due to the low level of methylation of exon 1B. Similarly, three primary gastric cancers showed methylation of exon 1A but not exon 1B. All 20 primary gastric cancers that

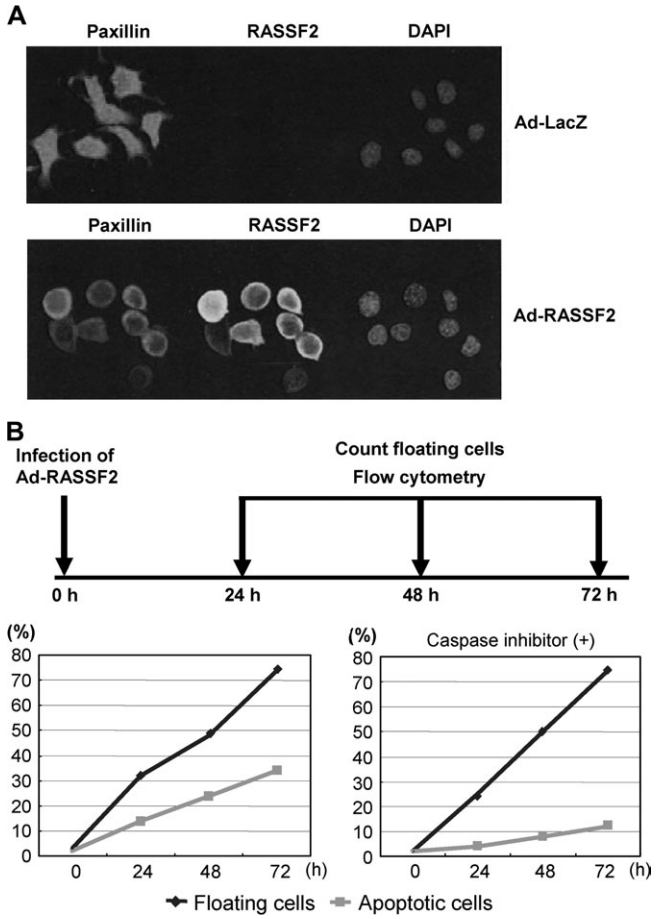

Fig. 3. (**A**) Morphological change induced by RASSF2A. JRST cells were infected with Ad-RASSF2A and incubated for 24 h, after which they were stained with rhodamine-conjugated phalloidin and anti-RASSF2 antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole. (**B**) Effect of caspase inhibition on floatation-dependent cell death. After infection with Ad-RASSF2A, the numbers of floating cells were counted. Percentages of apoptotic cells were determined by flow cytometry.

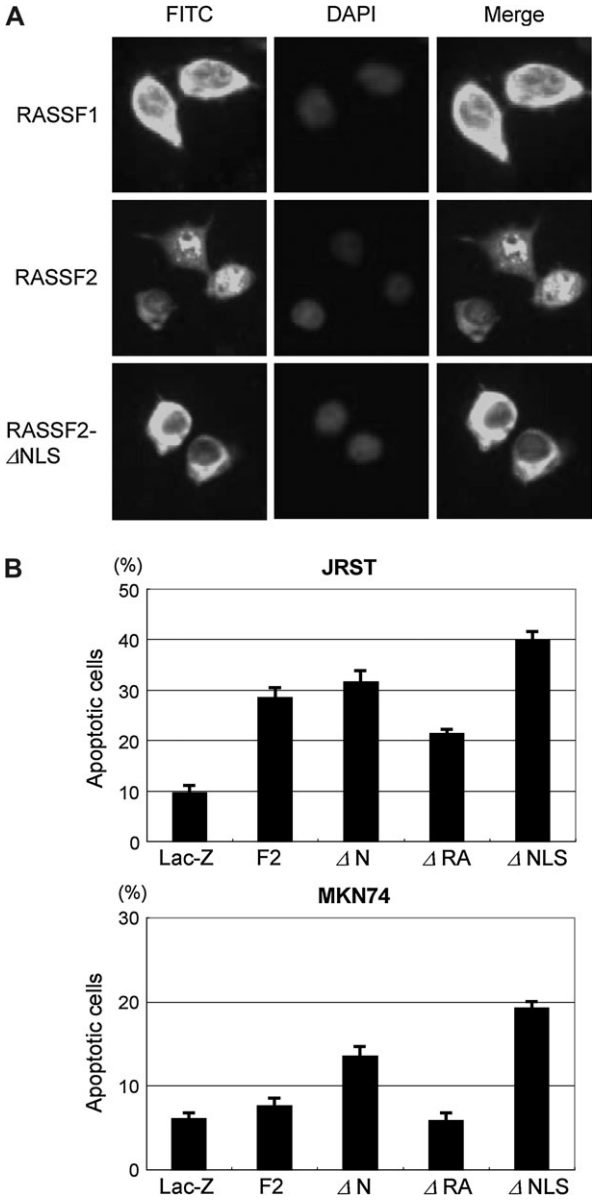

Fig. 4. Role of a NLS in determining the cellular distribution of RASSF2A and apoptosis. (**A**) Cellular distribution of RASSF1 and RASSF2A. (**B**) Apoptosis induced by the indicated RASSF2A mutants. Cells were harvested, fixed in methanol and stained with propidium iodide 72 h after infection with the respective adenoviral vector. Percentages of apoptotic cells are shown on the *y*-axis. Various adenoviral vectors were infected at a multiplicity of infection of 100. Lac-Z: Ad-LacZ; F2: Ad-*RASSF2;* ΔN: Ad-*RASSF2*-ΔN; ΔRA: Ad-*RASSF2*-ΔRA; ΔNLS: Ad-*RASSF2*-ΔNLS.

showed methylation of exon 1B also showed methylation of exon 1A. Normal tissues express both *RASSF2A* variants, and the functional consequences of expressing only one remain unknown.

Methylation of *RASSF2A* was somewhat heterogeneous in several cell lines (e.g. HSC44 and NUGC3). In particular, the region around the transcription start site in exon 1A has few CpG sites, and methylation of low-density CpG islands reportedly does not mediate stable gene silencing (37). Thus, other mechanisms such as aberrant histone modification may also be involved in silencing *RASSF2A*. That idea was confirmed by our finding that, in HSC44 cells, low doses of 5-aza-dC and TSA, which had no effect alone, acted synergistically to induce *RASSF2A*. Further studies will be needed to fully characterize the way in which histone modification is involved in silencing *RASSF2A*.

The role of epigenetic inactivation of *RASSF2A* in gastric cancer is not fully understood. Although alterations of *K-ras/BRAF* are rare in gastric cancer, extracellular stimulation by growth factors, cytokines and reactive oxygen species caused by *H.pylori* is known to activate the Ras pathway (38,39). Likewise, genetic alterations of *erbB-2* (e.g. point mutations and gene amplification) also can lead to Ras activation (40,41). In addition to direct activation of the Ras pathway, inactivation of negative regulators of Ras also appears to contribute to tumorigenesis (42). In that regard, *RASSF* family genes have been identified as potential-negative regulators of Ras-induced growth that are silenced by DNA methylation in various human neoplasias, including gastric cancer. Furthermore, we have shown here that restoration of *RASSF2A* expression suppresses growth of gastric cancer cells in which *RASSF2A* expression had been silenced. RASSF2A's ability to suppress cell growth is abrogated when its RA domain is deleted, indicating that interaction with Ras is critical to the tumor suppressor function of RASSF2A.

The growth suppression induced by RASSF2A reflects an increased incidence of apoptosis. The molecular mechanism by which RASSF2A induces cell death remains unclear, though we observed that gastric cancer cells exhibit a round phenotype after infection with Ad-*RASSF2A*. Such rounded cells were rarely detected among cells infected with Ad-LacZ. The increase in rounded cells appears to be unrelated to mitotic morphological changes, as accumulation of cells at G_2/M phase of the cell cycle was not detected (Figure 2C). Paxillin staining indicated that restoration of *RASSF2A* expression leads to a loss of stress fibers, which would inhibit cell spreading. This is noteworthy in

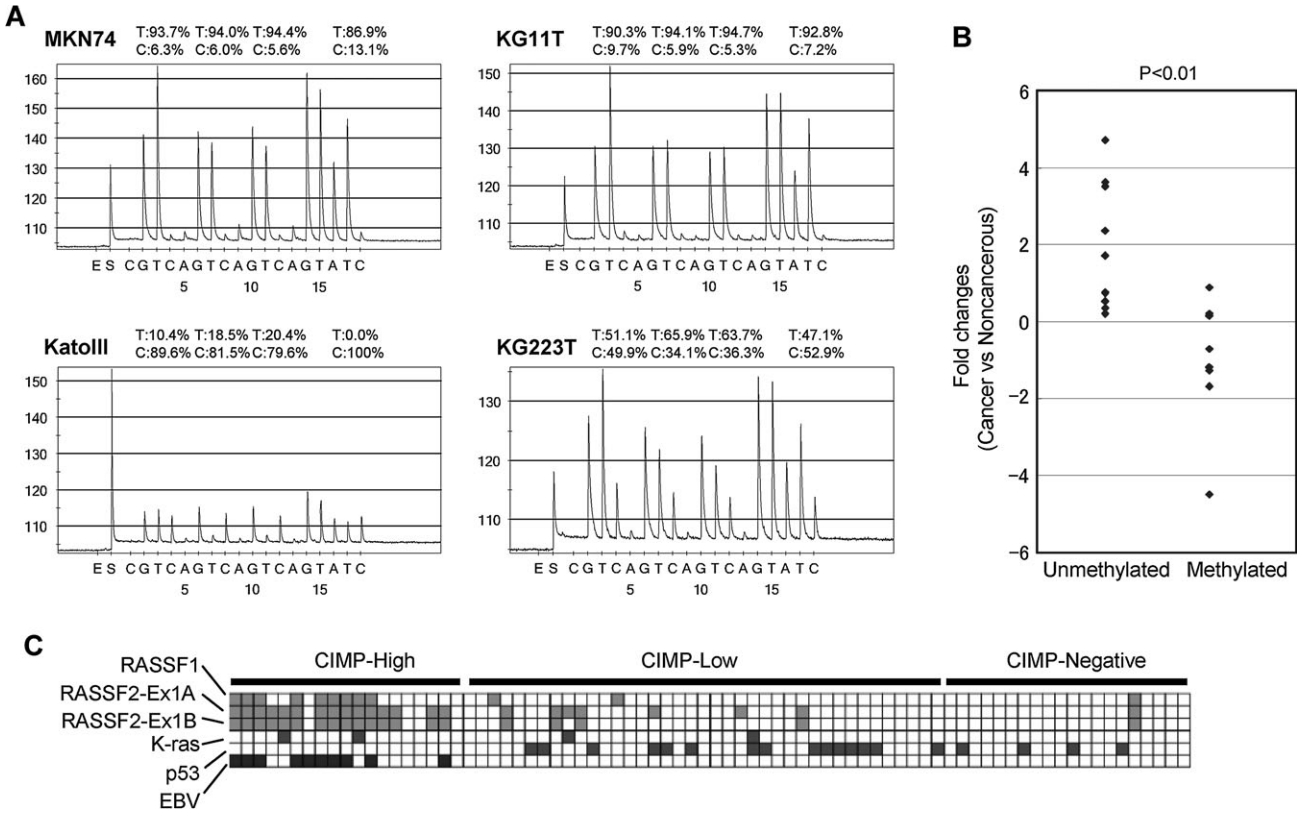

Fig. 5. Methylation of *RASSF2A* in primary gastric cancers. (**A**) Representative pyrogram traces for *RASSF2A*. Cell lines and specimens are shown below the columns. Percentages of methylated CpG alleles are shown above the columns. (**B**) Expression of *RASSF2A* in primary gastric cancers with or without DNA methylation. Real-time PCR was carried out using complementary DNA from primary gastric cancers. Levels of *RASSF2A* expression relative to those in normal tissues are shown on the *y*-axis. (**C**) Profiles characterizing the methylation of *RASSF1A*, *RASSF2A* variant 1 (*RASSF2*-Ex1A) and RASSF2A variant 2 (*RASSF2A*-Ex1B); mutation of *K-ras* or *p53* and the presence of EBV in 78 gastric cancers. Positive status is indicated by a solid box. CIMP status is shown above the column.

that mediators downstream of Ras (e.g. Rho) are involved in maintaining the integrity of the actin cytoskeleton (43); moreover, RASSF2A suppresses the activation of Rho (17), which would be expected to induce morphological changes reflecting a loss of stress fibers. We also found that the percentage of apoptotic cells, but not the percentage of floating cells, was reduced by a non-specific caspase inhibitor, indicating that death occurred while the cells were floating—i.e. cell death was not the reason the cells were floating.

Microarray screening to identify RASSF2A target genes failed to detect induction of proapoptotic genes. Our finding that RASSF2A-induced apoptosis was suppressed by a caspase inhibitor suggests that RASSF2A acts via a caspase-dependent pathway and is consistent with the microarray data, in that caspase-dependent apoptosis is often independent of gene transcription (44). Apparently, RASSF2A induces apoptosis through mechanisms other than inducing proapoptotic genes.

We detected RASSF2A in both the cytoplasm and nucleus. Protein motif analysis revealed that RASSF2A contains NLS in the region adjacent to the RA domain, and deletion of the NLS led to accumulation of RASSF2A in the cytoplasm and induction of apoptosis. Apparently, RASSF2A's proapoptotic activity is mediated by the cytoplasmic protein, and because RASSF2A interacts with Ras, which is localized in the cytoplasm, it would seem reasonable that cytoplasmic RASSF2A possesses the tumor suppressor activity. The role of nuclear RASSF2A remains to be determined.

Little is known about the downstream effectors of RASSF2A. Our microarray analysis revealed that genes involved in inflammatory responses were downregulated by RASSF2A. It was recently shown that activation of Ras leads to upregulation of IL-8, which promotes tumor vasculogenesis and growth (45). It also has been reported that CXCL5, CXCL2 and CXCL3 play important roles in regulating angiogenic factors in various human tumors (46), and high levels of expression of CCL20 and its receptor, CCR6, are involved in colorectal cancer metastasis (47). LCN2, also known as neutrophil gelatinase-associated lipocalin, was shown previously to be overexpressed in a variety of tumors. LCN2 interacts with MMP9 and is involved in epidermal growth factor-induced epithelial–mesenchymal transition (48). Taken together, these findings suggest that RASSF2A may not only suppress tumor growth directly but also inhibit inflammation and angiogenesis through inhibition of Ras-signaling pathways.

We found that *RASSF2A* is frequently methylated in primary gastric cancers that show no lymphatic invasion, venous invasion or lymph node metastasis, less advanced stages and CIMP-high, which means these phenotypes may simply represent the characteristics of CIMP-high gastric cancers. We also found that methylation of RASSF2A was strongly correlated with the presence of EBV. Aberrant methylation caused by EBV is reportedly associated with induction of DNMT1 (49), which is activated by EBV via the c-Jun-NH_2-kinase/activator protein-1-signaling pathway (50). Elucidation of the mechanism by which EBV regulates DNA methylation in gastric cancer could be crucial for the development of new therapeutic agents.

In conclusion, we propose that RASSF2A is a key tumor suppressor in gastric cancer that acts by suppressing cell growth and inducing apoptosis. Our results also suggest that the antitumor activity of RASSF2A is associated with its cytoplasmic localization and suppression of inflammatory cytokine expression. Thus, manipulation of the cellular localization of RASSF2A may be an important target for cancer therapy.

Supplementary material

Supplementary Tables 1–5, Figures 1–4 and Materials and Methods can be found at http://carcin.oxfordjournals.org/

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