Frequent Epigenetic Inactivation of RASSF1A by Aberrant Promoter Hypermethylation in Human Gastric Adenocarcinoma

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Abstract

Methylation associated inactivation of RASSF1, a putative tumor suppressor identified at 3p21.3, has been frequently observed in several human malignancies, including lung and breast cancers. To explore the penetrance of RASSF1 in gastric carcinogenesis, we performed expression and mutation analyses of 3 isoforms of RASSF1 (A, B, and C) in 150 gastric specimens, including 15 carcinoma cell lines. RASSF1A and RASSF1B transcripts were not expressed in 60% (9 of 15) and 33% (5 of 15) of gastric carcinoma cell lines, respectively, whereas RASSF1C was detectable in all cell lines. Bisulfite DNA sequencing analysis revealed that the CpG island in the RASSF1A promoter is hypermethylated in all RASSF1A-nonexpressing cell lines. In addition, both RASSF1A and RASSF1B were re-expressed by treatment with the demethylating agent 5-aza-2'-deoxycytidine. Among 90 primary gastric adenocarcinomas examined, 41 (46%) and 19 (21%) expressed no or abnormally low levels of RASSF1A and RASSF1B, respectively, and 12 (13%) tumors showed no expression of both isoforms. Loss or abnormal down-regulation of RASSF1A correlated with tumor stage and grade but not with histological types of tumors. Methylation-specific PCR analysis demonstrated that 95% (39 of 41) of RASSF1A-nonexpressing primary tumors are methylated at the CpG sites in the promoter, whereas none of the adjacent noncancerous or normal tissues are methylated. No somatic mutations were detected in RASSF1 transcripts expressed in unmethylated tumors. However, 10 methylated tumors, including 4 cell lines, showed low genomic levels of RASSF1 and expressed no RASSF1 transcripts, suggesting that RASSF1A inactivation might be caused by both epigenetic and genetic mechanisms in a subset of gastric adenocarcinomas. In conclusion, our data indicate that epigenetic transcriptional silencing of RASSF1, especially RASSF1A isoform, is a frequent event in gastric tumorigenesis and might play an important role in the malignant progression of gastric adenocarcinomas.

Introduction

Gastric adenocarcinoma is one of the most commonly diagnosed malignancies worldwide and a leading cause of cancer mortality in certain areas such as Korea, Japan, South America, and Eastern Europe (1). Although evidence has accumulated indicating the involvement of the alterations of multiple genes such as p53, K-ras, c-erbB2, K-sam, and E-Cadherin, the underlying molecular events that drive the neoplastic process in gastric cancer are largely undefined (2). LOH is one of the most frequent genetic alterations in solid tumors, and the characterization of chromosomal regions with a high rate of LOH leads to the identification of putative tumor suppressor genes. Previous cytogenetic and LOH studies of gastric adenocarcinomas have shown the significant allelic loss on several chromosomal arms, including 3p, 4p, 5q, 12q, 17p, and 18q (3–5).

The short arm of chromosome 3 is one of the most frequently deleted regions in gastric cancers, and many gastric cancer cell lines have homozygous deletions of 3p (4, 6). Recent allelotyping analyses detected deletion of 3p in 25–81% of gastric adenocarcinomas, and several genomic regions, including 3p14, 3p21, and 3p25–26, were revealed to undergo frequent allelic loss in gastric cancers, suggesting the presence of multiple candidate tumor suppressor gene(s) on 3p (4, 5). Loss of expression or mutational alterations of fragile histidine triad and von Hippel-Lindau, which are known to reside at 3p14 and 3p25–26, respectively, have been observed in many gastric cancers, implicating a role in gastric tumorigenesis (7, 8).

Recently, RASSF1 was suggested as the major target tumor suppressor at 3p21.3 based on its frequent epigenetic silencing and LOH in lung cancers (9). RASSF1 encodes more than seven isoforms, including RASSF1A, RASSF1B, and RASSF1C, which are derived from alternative mRNA splicing and promoter usage. Transcriptional silencing of RASSF1A was observed in a considerable proportion of lung, breast, ovarian, and nasopharyngeal cancers by de novo methylation at the CpG island in the promoter (10–13). In small cell lung cancers, allelic loss of 3p21.3 was associated with RASSF1A methylation, suggesting that both genetic and epigenetic mechanisms are implicated in RASSF1A inactivation in some tumor types (11).

The three major isoforms of RASSF1 have four common exons (exons 3–6), which encode a Ras association domain (14). RASSF1A has two 5' exons (1α and 2αβ) and encodes a 39 kDa peptide (9). RASSF1A contains an NH2-terminal cysteine-rich diacylglycerol/ phorbol ester binding domain, and its COOH terminus shows high homology to Ras effector Nore1/Maxp1 (15). RASSF1B contains exons 1β and 2αβ and most likely encodes only the Ras association domain. Transcription of RASSF1C initiates in exon 2γ, and its protein product lacks similarity to Nore1 or any other known protein. In addition to containing the predicted Ras association domain, RASSF1A and RASSF1C have PEST sequences, and a serine residue within this region was identified as a putative phosphorylation target in vitro for ataxia-telangiectasia-mutated (16).

It is well documented that Ras proteins bind a diverse array of effector molecules and mediate tumor suppressive effects, such as terminal differentiation and apoptosis, as well as oncogenic effects (17, 18). Recent studies demonstrated that exogenous expression of RASSF1A decreases in in vitro colony formation, suppresses anchorage-independent growth, and dramatically reduces tumorigenicity in vivo (9, 10). In this context, RASSF1A may play a role as an effector molecule in the Ras-activated growth inhibition signaling pathways. It was also hypothesized that RASSF1A inactivation may shift the balance of Ras activities toward a growth-promoting effect and thus be a tumorigenic mechanism that is distinct from the oncogenic activation of Ras signaling in tumors (10, 19). Interestingly, loss of RASSF1C has been detected in six of nine ovarian cell lines, and RASSF1C protein was found to bind Ras in a GTP-dependent manner both in vitro and in vivo and mediate the apoptotic effects of oncogenic Ras.
suggesting that RASSF1 isoforms might have tissue-specific roles (19).

In the present study, we investigated the expression and mutation status of RASSF1 located at 3p21.3 in a series of primary gastric adenocarcinomas and cell lines to explore the candidacy of RASSF1 as a suppressor in gastric carcinogenesis. Our data demonstrate that RASSF1A and RASSF1B expression is lost or down-regulated in a majority of gastric cell lines and primary tumors by aberrant promoter hypermethylation. Moreover, altered expression of RASSF1A correlated with tumor stage and grade, suggesting that inactivation of RASSF1A may play a critical role in the malignant progression of gastric cancers.

Materials and Methods

Tissue Specimens and Human Cell Lines. Total 135 gastric tissues, including 90 primary adenocarcinomas and 15 normal gastric tissues, were obtained from 90 gastric cancer patients and 15 noncancer patients by surgical resection in the Kyung Hee University Medical Center (Seoul, Korea). Tissue specimens were snap-frozen in liquid N2 and stored at −70°C until used. Fifteen human gastric cancer cell lines (SNU1, SNU5, SNU16, SNU216, SNU484, SNU601, SNU620, SNU638, SNU719, MKN1, MKN28, MKN45, MKN74, AGS, and KATO-III) were obtained from Korea Cell Line Bank (Seoul National University, Seoul, Korea) or American Type Culture Collection (Rockville, MD). Extraction of total cellular RNA and synthesis of cDNA were performed as described previously (20). Genomic DNA was extracted from the same cells of the tissues from the DNA phase after RNA was extracted.

Quantitative PCR Analysis. Our PCR-based strategies used for quantitative analysis of expression and genomic levels of RASSF1 were described previously (21). Briefly, 1:4 diluted cDNA (12.5 ng/50 µl PCR reaction) undergoing 24–36 cycles was observed to be within the logarithmic phase of amplification and yielded reproducible results with primers RSF-3 (sense; 5'-TCTGGGCGGCTGTCGGCACC–3') and RSF-4 (antisense; 5'-GAACTTGTGATGACCTGGTCTG–3') for RASSF1A, 1B (sense; 5'-CCGTTTCCAGACGGCCCAAGT–3') and RSF-4 (antisense; see above) for RASSF1, 1C (sense; 5'-GGAGGGCCTTCCTTCGAAA–3') and RSF-4 (antisense; see above) for RASSFIC, and an endogenous expression standard gene GAPDH (22, 23). PCR was done for 34 cycles at 95°C (1 min), 58°C–62°C (0.5 min), and 72°C (1 min) in 1.5 mM MgCl2-containing reaction buffer (PCR buffer H; Perkin-Elmer). RT-PCR products (10 µl) were resolved on 2% agarose gels. Quantitation of RASSF1 expression levels was achieved by densitometric scanning of the ethidium bromide-stained gels. Absolute area integrations of the curves representing each specimen were then compared after adjustment for GAPDH expression. For genomic PCR analysis, 200 ng of genomic DNA were used for amplification of the exon 3 region of RASSF1 with intron-specific primers RSF3 (sense; 5'-ATGTATATGTACATCAGGGA–3') and RSF4 (antisense; 5'-CAGGACATAGCCGTTACC–3'). Integration and analysis were performed using Molecular Analyst software program (Bio-Rad, Hercules, CA).

5-aza-2'-deoxycytidine Treatment. To assess reactivation of RASSF1 expression, 15 gastric cancer cell lines were plated in six-well tissue plates 24 h before treatment. 5-aza-2'-deoxycytidine (Sigma Chemical Co., St. Louis, MO) was added to the fresh medium at concentrations of 5 µM in duplicate, and cells were harvested after 4 days.

Bisulfite DNA Sequencing. Genomic DNA (1 µg) in a volume of 50 µl was denatured by NaOH (final concentration 0.3 M). Hydroquinone (30 µl of 10 mM) and 520 µl of 3 M sodium bisulfite (pH 5.0) were added and incubated at 55°C for 16–20 h. DNA samples were purified using Wizard DNA clean-up system (Promega Corp., Madison, WI), again treated with NaOH at 37°C for 15 min, precipitated with ethanol, and resuspended in distilled water. Bisulfite-modified DNA (50 ng) was subjected to PCR amplification of the CpG island in the RASSF1A promoter using primers PS (5'-CATCCCTCTCAGCCATTGTCG-3') and PAS (5'-TCTTATTTATCACTTCTTCATTTT-3'). The PCR products were cloned into pCR® vectors (Invitrogen, Carlsbad, CA), and 10 clones of each specimen were sequenced by automated fluorescence-based DNA sequencing to determine the methylation status.

Methylation-specific PCR. PCR was performed with methylation-specific primers MS-1 (sense; 5'-TTTTTTCCATTTTGGTCTCCT-3') and MS-2 (antisense; 5'-CGTCTTGCATTGGCTGCG-3') and unmethylation-specific primers UMS-1 (sense; 5'-TCACCTTTTTCCTTTTCTCT-3') and UMS-4 (antisense; 5'-CTTTTTTTCTCTCTTTTTTCTC-3') using 200 ng of the bisulfite-modified genomic DNA as templates for 38 cycles at 95°C for 1 min, at 60°C–63°C for 1 min, and 72°C for 1 min. The PCR products (15 µl) were resolved on a 2% agarose gel.

Nonisotopic RT-PCR-SSCP Analysis. Nonisotopic RT-PCR-SSCP analysis was performed as described previously (20). The isoform-specific regions (1α, 2α, 2b, and 2γ) and exons 3–6 of RASSF1 transcripts were amplified with 10 sets of primers. Sequences of the primers used for our PCR-SSCP analysis will be obtained on request. The PCR products (20 µl) were denatured with loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol), and 15 µl of double-distilled water. After heating at 95°C for 5 min, samples were loaded in wells precooled to 4°C. SSCP was performed using 8% non-denaturating acrylamide gels containing 10% glycerol at 4°C–8°C or 18°C–22°C.

Results and Discussion

Expression Status of RASSF1 in Normal Gastric Tissues. To elucidate the status of RASSF1 during gastric cancer pathogenesis, we initially evaluated mRNA expression of three RASSF1 isoforms (A, B, and C) in 15 normal gastric tissues obtained from noncancer patients by quantitative RT-PCR. The three RASSF1 isoform transcripts were detected in normal tissues analyzed (Figs. 1 and 2). Consistent with a previous report, RASSF1B levels were significantly low compared with RASSF1A and RASSF1C, and we used quantitative nest-PCR approach for analysis of RASSF1B expression (9). Quantitative PCR was repeated at least three times for each specimen, and no significant variations were observed in expression levels of the three isoforms among normal tissue specimens (RASSF1A, 1.19–1.45; RASSF1B, 0.60–0.82; RASSF1C, 0.98–1.38; Fig. 2).
Loss of RASSF1 Expression in Gastric Carcinoma Cell Lines. We next characterized the expression status of RASSF1 in 15 gastric carcinoma cell lines. As shown in Fig. 1A, RASSF1A and RASSF1B were not expressed in nine (60%) and five (33.3%) cell lines, respectively. Whereas two cell lines (SNU5 and SNU620) showed no expression of both isoforms, seven (SNU1, SNU484, SNU601, SNU719, MKN28, MKN74, and AGS) and three (SNU216, MKN1, and MKN45) showed RASSF1A- and RASSF1B-specific loss, respectively. Thus, 80% (12 of 15) of gastric cell lines examined showed no expression of RASSF1A and/or RASSF1B mRNA. RASSF1C mRNA was detectable in all cells. However, four (SNU484, SNU620, SNU719, and MKN28) cell lines expressed abnormally low levels of RASSF1C. Interestingly, these cell lines also showed low genomic levels of the RASSF1 gene, suggesting allelic deletion of the gene in these cell lines.

Frequent Alteration of RASSF1 Expression in Primary Gastric Carcinomas. Next we analyzed RASSF1 expression in 90 primary gastric carcinomas, including 30 matched sets. Expression levels of RASSF1A, RASSF1B, and RASSF1C were observed in the ranges of 0.02–1.47, 0.05–0.86, and 0.52–1.42, respectively, and RASSF1A and RASSF1B levels in tumors showed a significant difference compared with those in normal tissues (Figs. 1A and 2). On the basis of the RASSF1 expression in normal gastric tissues, we arbitrarily set expression levels less than a half (RASSF1A < 0.67, RASSF1B < 0.36, and RASSF1C < 0.61) of normal means (RASSF1A, 1.34; RASSF1B, 0.72; and RASSF1C, 1.22) as abnormally low. No or abnormally low expression of RASSF1A and RASSF1B was found in 45.6% (41 of 90) and 21.1% (19 of 90) of primary carcinomas, respectively, and 13.3% (12 of 90) of tumors showed altered expression for both isoforms (Fig. 2). Thus, 53.3% (48 of 90) of primary gastric carcinomas were identified to have loss or abnormal reduction of RASSF1A and/or RASSF1B. Among 30 matched sets from the same patients, tumor-specific reduction of RASSF1A and RASSF1B was found in 22 (73.3%) and 9 (30%) cases, respectively (Fig. 1A). Additionally, loss or abnormal reduction of RASSF1A was significantly high in advanced tumors (30 of 48, 62.5%) compared with early stage tumors (11 of 42, 26.2%; P < 0.0001) and more frequent in poorly differentiated tumors (26 of 42, 61.9%) than well or moderately differentiated tumors [33.3% (3 of 9) and 30.8% (12 of 39), respectively] (P = 0.01). However, RASSF1A alteration showed no association with histological types of tumor [diffused, 44.8% (13 of 29); intestinal, 45.9% (28 of 61)]. In contrast to RASSF1A, loss or abnormal reduction RASSF1B expression showed no correlation with histopathological characteristics of tumors (Fig. 2). Expression of RASSF1C isoform was observed in all tumors examined, but abnormally low expression was identified in six (6.7%) tumors (Fig. 1). As for four cell lines, these tumors showed low levels of the gene in quantitative genomic PCR and also expressed no or low RASSF1A and/or RASSF1B, suggesting that altered expression of RASSF1 mRNA might be associated with allelic deletion of the gene in a subset of gastric cancers. Collectively, our results indicate that loss or abnormal reduction of RASSF1, especially RASSF1A isoform, is a frequent event in gastric tumorigenesis and may contribute to the malignant progression of human gastric cancers.

RASSF1B is expressed mainly in hematopoietic cells, whereas RASSF1A and RASSF1C are expressed ubiquitously (9). Although its levels were significantly low, we could detect RASSF1B expression in normal and cancerous gastric tissues. Some RASSF1B transcripts detected in tissue specimens might be derived from the infiltrating lymphocytes, but expression of RASSF1B in gastric carcinoma cell lines and its tumor-specific alteration raises the possibility that
was subjected to PCR amplification of the primer sets. The PCR products (20 nM sequences using unmethylation-specific (U) and methylation-specific (M) primer sets. The PCR products were cloned, and 10 clones of each specimen were sequenced. The 15 CpG sites analyzed were identified to be completely methylated in all RASSF1A-nonexpressing cell lines, whereas none of the RASSF1A-expressing cells showed methylation in this region (Fig. 4). Additionally, in five primary tumors examined, complete or high rate of methylation was identified in four tumors expressing no or low levels of RASSF1A, whereas no methylation was found in the adjacent noncancerous tissues and a RASSF1A-expressing tumor.

To determine the overall frequency of RASSF1A methylation in gastric cancers, the 3p21 region, where the RASSF1 gene is located, undergoes frequent allelic losses in a variety of human malignancies, including gastric cancer (4, 5, 24, 25). Recently, RASSF1A inactivation by two hits (allelic loss at 3p21.3 and promoter methylation) was demonstrated in small cell lung cancers (11). Although we did not perform the comprehensive LOH study for RASSF1 locus, quantitative genomic PCR analysis revealed that 26.7% (4 of 15) of gastric cell lines and 14.6% (6 of 41) of primary tumors have low RASSF1 gene levels (Fig. 1). These observations suggest that abnormal expression of RASSF1 might be associated with the allelic deletion of the gene in a subset of tumors, and somatic mutations might exist in the RASSF1A transcripts expressed from the remaining allele. For screening of RASSF1 mutations, RT-PCR-SSCP analysis was performed for the entire coding region of three isoform transcripts. However, we failed to detect any types of mutation leading to amino acid substitutions or frameshifts except for previously described polymorphisms at codons 53 (CGC to CGT) and 56 (CCC to CCT), whereas 34.4% (31 of 90) of the same set of tumors were found to carry p53 mutations, indicating that somatic mutation is not a main mechanism for RASSF1A inactivation in gastric cancers (13).

Hypermethylation of the CpG Island in the RASSF1A Promoter. To investigate whether aberrant DNA methylation might be involved in the loss of RASSF1A expression, the 15 gastric cancer cell lines were treated with the demethylating agent 5-aza-2'-deoxycytidine. As shown in Fig. 3, RASSF1A and RASSF1B transcripts were re-expressed in all nonexpressing cell lines, including the A549 lung cancer cell line used as a control, indicating that RASSF1A and RASSF1B are transcriptionally silenced in these cells by aberrant DNA methylation, and the promoters of these two isoforms are concomitantly hypermethylated in some cancers (9). No detectable changes in RASSF1C expression were observed, which is consistent with the previous report showing that CpG sites in the presumed RASSF1C promoter region were not methylated in human cancer cell lines (9–12).

To additionally define the methylation status of the gene, we performed bisulfite DNA sequencing analysis of the CpG island in the RASSF1A promoter. The promoter region (nucleotide –139 to +3) spanning 15 CpG sites was amplified by PCR using sodium bisulfite-modified genomic DNA as templates, and 10 PCR clones of each specimen were sequenced (9). The 15 CpG sites analyzed were identified to be completely methylated in all RASSF1A-nonexpressing cell lines, whereas none of the RASSF1A-expressing cells showed methylation in this region (Fig. 4). Additionally, in five primary tumors examined, complete or high rate of methylation was identified in four tumors expressing no or low levels of RASSF1A, whereas no methylation was found in the adjacent noncancerous tissues and a RASSF1A-expressing tumor.

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Fig. 4. Methylation status of the CpG island in the RASSF1A promoter. The RASSF1A promoter region comprised of 15 CpG sites (nucleotide –139 to +3) was amplified by PCR with bisulfite-modified genomic DNA as templates. The PCR products were cloned, and 10 clones of each specimen were sequenced. The 15 CpG sites are numbered according to those listed in Ref. 9. Black, gray, and white squares indicate complete methylation (70–100%), partial methylation (10–60%), and unmethylation, respectively. N1-N5, normal tissues; T1-T5, tumor tissues.
gastric tumors, we performed methylation-specific PCR analysis for 15 cell lines and 90 primary tumors. All of nine cell lines and 39 (95.1%) of 41 primary carcinomas with loss or abnormal reduction of RASSF1A expression were found to be methylated, whereas none of RASSF1A-expressing tumors and 15 normal tissues showed methylation (Fig. 5). BstUI digestion assay for the CpG island region that comprises two BstUI recognition sites (CCGG) was also performed as described previously, and the identical results were obtained (data not shown). These results indicate that hypermethylation at the CpG island in the RASSF1A promoter is strongly associated with loss or abnormal reduction of RASSF1A expression.

In conclusion, our data presented here clearly demonstrate that RASSF1, particularly RASSF1A, undergoes epigenetic silencing in a considerable proportion of gastric adenocarcinomas by aberrant promoter methylation. Additionally, loss or abnormal reduction of RASSF1A expression showed a strong correlation with tumor stage and grade, indicating the implication of RASSF1A inactivation in the malignant progression of gastric cancer. Although additional studies are required to characterize the biological significance of RASSF1A inactivation in gastric tumorigenesis, our study suggests that RASSF1A methylation could be a useful molecular marker for detection and prognosis of gastric cancers.

References
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