RASSF2, a potential tumour suppressor, is silenced by CpG island hypermethylation in gastric cancer

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RASSF2, a member of the RASSF1 family, has recently been identified as a potential tumour suppressor. We examined methylation status in multiple regions which included the CpG island and spanned the transcription start site of RASSF2 in 10 gastric cancer cell lines, as well as 78 primary gastric cancers and corresponding non-neoplastic gastric epithelia. Hypermethylation of RASSF2 in at least one of the regions examined was detected in seven (70%) of the 10 cell lines; two (20%) exhibited hypermethylation in all the regions examined including the transcription start site and lost expression of RASSF2 mRNA, which could, however, be restored by 5-aza-2’-deoxycytidine treatment, while the other five (50%) cell lines exhibited hypermethylation at the 5’- and/or 3’- edge, with four of them expressing RASSF2 mRNA. In primary gastric cancers and corresponding non-neoplastic gastric epithelia, frequencies of RASSF2 methylation ranged from 29% (23 out of 78) to 79% (62 out of 78) and 3% (two out of 78) to 60% (47 out of 78), respectively, at different CpG sites examined. Methylation was frequently observed at the 5’- and 3’- edges, and became less frequent near the transcription start site in both the primary gastric cancers and corresponding non-neoplastic gastric epithelia. Hypermethylation near the transcription start site was mostly cancer-specific. We thus showed that RASSF2 is silenced by hypermethylation near the transcription start site in gastric cancer. Hypermethylation was found initially to occur at the 5’- and 3’- furthest regions of the CpG island in non-neoplastic gastric epithelia, to gradually spread near the transcription start site to shut down RASSF2 expression, and ultimately to constitute a field-defect placing tissue increased risk for development of gastric cancer.

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Human cancers develop and progress by accumulation of both genetic and epigenetic alterations. Mutations of p53 are frequent genetic (structural) alterations in gastric cancer (Tamura, 2002). E-cadherin is inactivated through any combination of gene mutation, loss of heterozygosity (LOH), and promoter hypermethylation in gastric cancer, especially that of undifferentiated histological type (Tamura et al., 2001). Recent evidences of frequent silencing of hMLH1, p16, RUNX3, and other tumour suppressor and tumour-related genes by promoter hypermethylation suggests that epigenetic alterations play the most important role in gastric carcinogenesis (Tamura, 2004). Furthermore, promoter hypermethylation initially occurs in non-neoplastic gastric epithelia, increases with age, and ultimately silences function to constitute a field-defect that may predispose tissue to development of gastric cancer (Waki et al., 2002, 2003b). Many genes become methylated in gastric epithelia during aging (Waki et al., 2003a), although frequencies of methylation depends on the sites of CpGs examined within a gene promoter (Satoh et al., 2002).

Ras family genes are associated with the signal transduction from G-protein-coupled receptors and activation of the ras-signal transduction pathway frequently observed in human tumours (Shih et al., 1982; Malumbres and Pellicer, 1998; Ayllon and Rebollo, 2000). Ras proteins interact with a wide spectrum of regulators and downstream effectors to produce various cellular responses, including cell proliferation, differentiation, and apoptosis (Malumbres and Pellicer, 1998). Recently, the ras effectors/tumour suppressors RASSF1 and NOR1 were found to be inactivated by promoter hypermethylation in a variety of human tumours (Vavvas et al., 1998; Dammann et al., 2000; Pfeifer et al., 2002; Hesson et al., 2003; Vos et al., 2003a). RASSF2 was identified as a third member of the RASSF1 family and a ras effector/tumour suppressor (Vos et al., 2003a). RASSF2 binds to K-ras in a GPT-dependent manner via the Ras effector domain, but interacts with H-ras weakly (Vos et al., 2003a). RASSF2 inhibits the growth of tumour cells, promotes both cell cycle arrest and apoptosis, and is frequently downregulated in lung tumour cell lines (Vos et al., 2003a). The RASSF2-methylated RKO, colorectal cell line, without RASSF2 RNA expression showed increase of apoptosis and cell growth inhibition (Akino et al., 2005). These observations indicate that RASSF2 shows tumour suppressor activity in various human cancers.

In the present study, we examined the methylation status of RASSF2 at multiple regions which included the CpG island and
spanned the transcription start site in gastric cancer cell lines, as well as primary gastric cancers and corresponding non-neoplastic gastric epithelia. The regions critical for methylation silencing of RASSF2 mRNA expression were examined by analyses of cell lines, while methylation spreading near the RASSF2 transcription start site was examined by analyses of primary gastric cancers and corresponding non-neoplastic gastric epithelia.

MATERIALS AND METHODS

Samples

A total of 10 gastric cancer cell lines with various histologies were cultured under appropriate conditions in our laboratory: MKN1, an adenosquamous cell carcinoma; MKN7, a well-differentiated adenocarcinoma; MKN28 and MKN74, moderately differentiated adenocarcinomas; MKN45 and KWS-I, poorly differentiated adenocarcinomas; KATO-III, a signet ring cell carcinoma; ECC10 and ECC12, endocrine cell carcinomas; and TSG 1, a hepatoid carcinoma. In total, 78 gastric cancer samples and matching non-neoplastic gastric tissues were obtained at surgery from 78 patients (Table 1). The patients ranged in age from 43 to 89 years (mean, 67.6 years). All the patients received follow-up care, for a median of 36.1 months (range, 1–77 months). All samples were stored at –80°C until processed. DNA was extracted from the 10 gastric cancer cell lines, 78 primary gastric cancers and corresponding non-neoplastic gastric epithelia using SepaGene (Sankyo-Junyaku, Tokyo, Japan). Total RNA was isolated from the 10 gastric cancer cell lines with the TRIZOL reagent (Gibco BRL, Life Technologies, Gaithersburg, MD, USA).

Methylation-specific PCR (MSP)

Treatment of DNA samples with sodium bisulphite converts all unmethylated cytosines to uracils but does not affect methylated cytosines. Briefly, 2 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulphite. The samples were then purified using Wizard DNA purification resin (Promega, Madison, WI, USA), treated with NaOH, recovered in ethanol and resuspended in 30 μl of distilled water. Amplification was performed in a 20 μl reaction volume containing 2 μl of GeneAmp PCR Gold Buffer (PE Applied Biosystems, Foster City, CA, USA), 1.0 mM MgCl2, 1 μl of each primer, 0.2 μM dNTPs, and 1 U of Taq polymerase (AmpliTaq Gold DNA Polymerase, PE Applied Biosystems). Hot start PCR was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles, each of which consisted of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s, followed by a final 7-min extension at 72°C. A positive control and a negative control (distilled water without DNA) were included for each amplification. The PCR products were separated on a 6% non-denaturing polyacrylamide gel. To detect regions playing critical roles in regulating the expression of RASSF2 mRNA, we designed six primer sets spanning the RASSF2 transcription start site and including the CpG island (GenBank accession number AL 133354) (Figure 1). The following primer sets were used: 5′-GTT TAT TCG GTT TAT TC-3′ and 5′-CAC GTC TAA CCG ACC CGC CA A ATC G-3′ for the methylated RASSF2-U2 sequence (upstream 2; the upstream sequence furthest from the transcription start site) (212 bp); 5′-GTT TAT TGA TTT TGT TAT TGT GA-3′ and 5′-TCA CAT CTA ACC AAC CCA CAA AT C-3′ for the unmethylated RASSF2-U2 sequence (213 bp); 5′-TGG TTT ATC GGA TTT GTT GGT GTC TAT C-3′ and 5′-CCA ACC CGA AAA AAT CGC TAA CGA CG-3′ for the methylated RASSF2-U2 sequence (upstream 1; the upstream sequence nearest the transcription start site) (237 bp); 5′-TTG GTT ATT GGA TTT GTT GTT TG T-3′ and 5′-CACC ACC CAA AAA AAT CAC TAA CAA CAA AT C-3′ for the unmethylated RASSF2-U1 sequence (237 bp); 5′-GTA TTT GCG GGT AGT GTT GTT TC-3′ and 5′-TTA ACG CCG ACC CGC CGA TCG-3′ for the methylated RASSF2-D1 sequence (downstream 1; the downstream sequence nearest the transcription start site in CpG island) (183 bp); 5′-TTG GGT TTA TAA AAA AAG AAG GAT GAT-3′ and 5′-CAG ACA AAC CCC CAC CAA AAA AAG CAA AAC ACC CG-3′ for the methylated RASSF2-D2 sequence (135 bp); 5′-TTG GGT TTA AAA AAG AAG GAT GAT-3′ and 5′-CAG ACA AAC CCC CAC CAA AAA AAG CAA AAC ACC CG-3′ for the unmethylated RASSF2-D2 sequence (137 bp); 5′-TTG GGT TTA AAA AAG AAG GAT GAT-3′ and 5′-CAG ACA AAC CCC CAC CAA AAA AAG CAA AAC ACC CGC C-3′ for the unmethylated RASSF2-D2 sequence.

Table 1 Correlation between RASSF2 CpG island hypermethylation and clinicopathological characteristics in gastric cancer

| Characteristics         | Methylated at U1 and D1 | Others | P-value |
|-------------------------|-------------------------|--------|---------|
| Number of patients      | 21                      | 50     | NS      |
| Age (mean)              | 69.4                    | 66.9   | NS      |
| Gender                  |                         |        |         |
| M                       | 15                      | 36     |         |
| F                       | 6                       | 14     |         |
| Stage                   |                         |        |         |
| Early                   | 7                       | 8      | NS      |
| Advanced                | 14                      | 42     |         |
| Histological differentiation |                   |        | NS      |
| Differentiated          | 12                      | 27     |         |
| Undifferentiated        | 9                       | 23     |         |
| Location                |                         |        | NS      |
| Upper                   | 4                       | 9      |         |
| Middle                  | 6                       | 18     |         |
| Lower                   | 10                      | 19     |         |
| Unknown                 | 1                       | 4      |         |
| Lymph node metastasis  |                         |        | NS      |
| Present                 | 11                      | 31     |         |
| Absent                  | 10                      | 19     |         |
| Lymphatic permeation    |                         |        | 0.033   |
| Ly (−)                  | 7                       | 6      |         |
| Ly (+)                  | 14                      | 44     |         |
| Venous permeation       |                         |        | NS      |
| v (−)                   | 8                       | 10     |         |
| v (+)                   | 13                      | 40     |         |

NS = not significant by Fisher’s exact probability test and Mann–Whitney’s U-test.

Figure 1 Location of methylation-specific PCR (MSP) near the transcription start site including (A) CpG island of RASSF2. Vertical lines indicate individual CpG sites. TS, transcription start site; (B) exon 1 and intron 1 boundary.

The expression of RASSF2 was examined by analyses of cell lines, as well as primary gastric cancers and corresponding non-neoplastic gastric epithelia using SepaGene.
RASSF2-D3 sequence (166 bp); 5’-AAT TTG TTT AGA AGA TGG TGG T-3’ and 5’-ATC CTT CCT TCT TCT TTA ACC CA-3’ for the unmethylated RASSF2-D3 sequence (170 bp); 5’-CGA AGG AGG GGG AGA TC-3’ and 5’-TCC GCC GCC GTC TCC TTA AGC-3’ for the methylated RASSF2-D4 sequence (downstream 4; the downstream sequence of furthest from the transcription start site) (148 bp); and 5’-GTG TTG AAG GAG GGT GGG AGAT-3’ and 5’-AAT CCA CCA CCA TCT TCT AAA CA-3’ for the unmethylated RASSF2-D4 sequence (154 bp). The positions of amplified regions are shown in Figure 1. As a positive control, Sss I methylase (New England BioLabs, Inc., Beverly, MA, USA) was used to methylate 100 mg of liver tissue-derived DNA obtained from an autopsy sample, and was modified by sodium bisulphite as described above.

Reverse transcription–PCR (RT–PCR)

Isolated RNA was reverse-transcribed and amplified using a one-step RT–PCR System (Gibco BRL). Primer sequences used were 5’-AAG ACA TCC GTG TTC ACA CC-3’ and 5’-TGG TCT TCA TGG CTC AGA TT-3’ for RASSF2A mRNA (462 bp); and sense, 5’-AAA TCT GCC ACC ACA CCT T-3’ and antisense, 5’-AGG ACT TGG TGG TCT AGG AG-3’ for 5-aza-beta (646 bp). Primers for RASSF2A mRNA can also amplify RASSF2B mRNA product, but product size of RASSF2B mRNA (409 bp) is smaller than that of RASSF2A mRNA. Thus, they could be differentiable.

5-aza-2’-deoxycytidine (5-aza-dC) treatment

To examine restoration of RASSF2 mRNA expression, two cell lines, KATO-III and KWS-I, were incubated for 96 h with 5 μM 5-aza-dC (Sigma, St Louis, MO, USA), and then harvested for RNA extraction and RT–PCR. MKN74, which expresses RASSF2 mRNA with an unmethylated CpG island, was used as a control.

Statistical analysis

Statistical comparisons were performed using Fisher’s exact probability test and Mann–Whitney’s U-test. Values of P<0.05 were considered significant.

RESULTS

Methylation status and mRNA expression of RASSF2 in gastric cancer cell lines

Hypermethylation of RASSF2 in at least one of the regions examined was detected in seven of the 10 cell lines, MKN1, MKN7, MKN28, MKN45, KATO-III, KWS-I, and ECC10 (Figure 2A). Among these, KATO-III and KWS-I exhibited hypermethylation in all the regions examined, and lost RASSF2 mRNA expression (Figure 2A), which, however, was restored by 5-aza-dC treatment (Figure 2B). The other five of these seven cell lines, MKN1, MKN7, MKN28, MKN45, and ECC10 exhibited methylation only in outskirt regions, and four of them expressed RASSF2 mRNA (Figure 2A). Three cell lines, MKN74, TSG11, and ECC12, did not exhibit hypermethylation in any region. TSG11 and ECC12 lost RASSF2 mRNA expression despite being unmethylated.

Hypermethylation RASSF2 CpG island in primary gastric cancer and corresponding non-neoplastic gastric epithelia

Methylation frequencies of RASSF2 varied in the regions upstream and downstream of the transcription start site. Of primary gastric cancers and corresponding non-neoplastic epithelia, hypermethylation was observed in 55% (43 out of 78) and 36% (28 out of 78) at U2, 29% (23 out of 78) and 3% (two out of 78) at U1, 42% (33 out of 78) and 6% (five out of 78) at D1, 45% (35 out of 78) and 13% (10 out of 78) at D2, 54% (42 out of 78) and 28% (22 out of 78) at D3, 79% (62 out of 78) and 60% (47 out of 78) at D4, respectively (Figures 3 and 4). Unmethylated RASSF2 sequence was present in all the samples (data not shown). Gastric cancers with methylation at U1 and D1 exhibited significantly less frequent lymphatic permeation than unmethylated gastric cancers (Table 1) No significant correlation was observed between methylation status of RASSF2 and other clinicopathological factors. Methylation status of RASSF2 did not significantly influence event-free survival rate, as determined by Kaplan–Meier curve analysis with the log-rank test and Breslow–Gehan–Wilcoxon test (data not shown).
DISCUSSION

Results in gastric cancer cell lines suggest that hypermethylation near the transcription start site was associated with loss of RASSF2 mRNA expression, although other mechanisms might also contribute to RASSF2 silencing. While by analyses of samples from primary gastric cancer patients, frequencies of methylation at each region investigated were higher in primary gastric cancers than in corresponding non-neoplastic gastric epithelia. Very recently, Akino et al reported the significant association between methylation and expression status of RASSF2 in colon cancer cell lines and primary colorectal cancers. They identified the RASSF2 promoter region and showed that absence of RASSF2 transcription was caused by DNA methylation, not by alteration of transcription factors, and also showed histone acetylation at the 5’ region of RASSF2 in colorectal cancer cell lines with DNA methylation (Akino et al, 2005). It has also been reported that inactivation of RASSF2 with siRNA increased phosphorylation of MAP kinase, and that RASSF2 suppressed downstream signalling in the RAS pathway (Akino et al, 2005). Colorectal tumours with RASSF2 methylation showed K-ras/BRAF mutations significantly more frequently than those without RASSF2 methylation (Akino et al, 2005), although earlier studies have shown that K-ras mutation and RASSF2 methylation are mutually exclusive (Hesson et al, 2005). In the present study, we have showed that RASSF2 methylation is frequent in gastric cancer, despite the rarity of K-ras/BRAF mutations in this tumour (Kim IJ et al, 2003).

Methylation in the 5’- and 3’- furthest regions within the CpG island was very frequent in both primary gastric cancers and corresponding non-neoplastic gastric epithelia, but became less frequent near the transcription start site. Methylation near the transcription start sites at U1 and D1 appeared to be mostly cancer-specific. These findings are essentially the same as those for DAP-kinase hypermethylation (Satoh et al, 2002). Therefore, the pattern of spread of methylation, initially at the outskirts of CpG islands and then progressing to regions critical for gene silencing, might be common to various types of methylation-related gene silencing. Since frequencies of methylation are influenced by the location of MSP, as noted above, the significance of detection of DNA methylation in a giver tumour suppressor or tumour-related gene varied significantly. For example, hypermethylation near the transcription start site, which is cancer-specific and results in gene silencing, can be used as a diagnostic marker of malignancy in tissues or other samples, such as serum or ascites, and hypermethylation at a region next to such a critical region might be an early signal of carcinogenesis.

Gastric cancers with methylation at U1 and D1, a change critical for RASSF2 silencing, exhibited significantly less frequent lymphatic permeation than unmethylated gastric cancers. Gastric cancers with a high frequency of microsatellite instability (MSI-H), which is the result of hMLH1 silencing by hypermethylation, exhibited less frequent lymph node metastasis (dos Santos et al, 1996; Wu et al, 2000), and several genes are simultaneously methylated together with hMLH1 (Kim H et al, 2003; Homma et al, 2005).

In conclusion, hypermethylation of RASSF2 initially occurs in the 5’- and 3’- outskirt regions of the CpG island in non-neoplastic

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**Figure 3** Examples of methylation-specific PCR (MSP) in primary gastric cancers (T) and corresponding non-neoplastic gastric epithelia (N). Methylation is frequent in outskirt regions (U2 and D4), but becomes less frequent near the transcription start site (U1 and D1) in both gastric cancers and non-neoplastic gastric epithelia. DW, distilled water; P, positive control; and SM, size marker.

| Region | Gastric cancer (N=78) | Non-neoplastic gastric epithelia (N=78) |
|--------|-----------------------|---------------------------------------|
| U2     | 55% (43)              | 36% (28)                              |
| U1     | 29% (23)              | 3% (2)                                |
| D1     | 42% (33)              | 6% (5)                                |
| D2     | 45% (35)              | 13% (10)                              |
| D3     | 54% (42)              | 28% (22)                              |
| D4     | 79% (62)              | 60% (47)                              |

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**Figure 4** Percentages of hypermethylation in various regions (U2–D4) of RASSF2.
gastrointestinal epithelia, spreads near the transcription start site to shut down RASSF2 expression, and ultimately constitutes a field-defect which places tissue at increased risk for the development of gastric cancer.

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