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Distinct methylation patterns of two *APC* gene promoters in normal and cancerous gastric epithelia

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The adenomatous polyposis coli (*APC*) tumor suppressor gene is mutationally inactivated in both familial and sporadic forms of colorectal cancers. In addition, hypermethylation of CpG islands in the upstream portion of *APC*, a potential alternative mechanism of tumor suppressor gene inactivation, has been described in colorectal cancer. Because a subset of both gastric and colorectal cancers display the CpG island methylator phenotype, we hypothesized that epigenetic inactivation of *APC* was likely to occur in at least some gastric cancers. *APC* exhibits two forms of transcripts from exons 1A and 1B in the stomach. Therefore, we investigated CpG island methylation in the sequences upstream of exons 1A and 1B, i.e., promoters 1A and 1B, respectively. We evaluated DNAs from 10 gastric cancer cell lines, 40 primary gastric cancers, and 40 matching non-cancerous gastric mucosae. Methylated alleles of promoter 1A were present in 10 (100%) of 10 gastric cancer cell lines, 33 (82.5%) of 40 primary gastric cancers, and 39 (97.5%) of 40 non-cancerous gastric mucosae. In contrast, promoter 1B was unmethylated in all of these same samples. *APC* transcripts from exon 1A were not expressed in nine of the 10 methylated gastric cancer cell lines, whereas APC transcripts were expressed from exon 1B. Thus, expression from a given promoter correlated well with its methylation status. We conclude that in contrast to the colon, methylation of promoter 1A is a normal event in the stomach; moreover, promoter 1B is protected from methylation in the stomach and thus probably does not participate in this form of epigenetic *APC* inactivation.
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Keywords: hypermethylation; *APC*; gastric cancer

Introduction

The adenomatous polyposis coli (*APC*) tumor suppressor gene, isolated and mapped to chromosomal band 5q21 (Joslyn *et al*., 1991; Kinzler *et al*., 1991), is responsible not only for the hereditary cancer syndrome, familial adenomatous polyposis, but also for sporadic colorectal cancer development due to mutations within its coding sequence (Nishisho *et al*., 1991; Miyoshi *et al*., 1992a,b). When the APC protein binds to β-catenin, it promotes the phosphorylation of highly conserved serine and threonine residues in β-catenin's NH2 terminus by GSK-3, thereby targeting *β-catenin* for degradation via the proteosome system (Papkoff *et al*., 1996; Orford *et al*., 1997). Loss of APC function results in nuclear accumulation of *β-catenin*, which acts as a transcriptional activator by binding to the Tcf–Lef (T cell factor/lymphoid enhancer factor) family of transcription factors, ultimately leading to loss of cellular growth control (Morin *et al*., 1997; Sparks *et al*., 1998). Truncating mutations in *APC*, with concordant loss of the wild-type allele, occur frequently and early in colorectal tumorigenesis (Miyoshi *et al*., 1992b). Despite frequent heterozygous deletion involving the *APC* locus on chromosome 5q21 in gastric cancers (Rhyu *et al*., 1994; Tamura *et al*., 1996; Nishizuka *et al*., 1998), *APC* mutations have been reported infrequently in gastric and other extracolonic gastrointestinal cancers (Ogasawara *et al*., 1994; Powell *et al*., 1994, 1996).

Recently, it was reported that aberrant DNA methylation of promoter region CpG islands can serve as an alternative mechanism to coding region mutations for the inactivation of tumor suppressor or tumor-related genes, including retinoblastoma (*Rb*), von Hippel-Lindau (*VHL*), *p16, p15, hMLH1*, and *E-cadherin* (Graff *et al*., 1997; Herman *et al*., 1998; Tamura *et al*., 2000). In addition, *APC 5′* region hypermethylation has been reported in colorectal cancers (Hiltunen *et al*., 1997). In gastric cancers, promoter CpG island hypermethylation of *p16, hMLH1*, and *E-cadherin* has been reported (Fleisher *et al*., 1999; Suzuki *et al*., 1999; Tamura *et al*., 2000). Because a subset of gastric and colorectal cancers display a CpG island methylator phenotype (Toyota *et al*., 1999a,b), we hypothesized that *APC* is inactivated through promoter hypermethylation in both of these cancer types.

In order to test this hypothesis, we studied *APC* gene promoter methylation status in DNAs from 10 gastric cancer cell lines and 40 primary gastric cancers, as well as in 40 matching non-cancerous gastric mucosae by using the methylation-specific PCR (MSP) technique. Because *APC* expresses multiple transcripts in a tissue-specific manner, and because two forms of transcripts

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originating from exons 1A and 1B have been identified in the stomach (Horii *et al*., 1993), we investigated CpG island methylation in the regions upstream of exons 1A and 1B. We studied promoter 1A, deposited at Genbank as accession No. U02509; and promoter 1B (accession No. D13981), respectively.

Results

Methylation status of promoters 1A and 1B, and expression of APC mRNA in gastric cancer cell lines

The methylation status of promoters 1A and 1B was determined by MSP (Herman *et al*., 1996) using methylated and unmethylated sequence-specific primers (Figure 1). Methylated *APC* alleles of promoter 1A were present in all 10 gastric cancer cell lines examined (Figure 2). In eight cell lines (MKN1, MKN7, MKN28, MKN45, MKN74, KATO-III, ECC10 and ECC12), only the methylated allele was present, suggesting biallelic or complete hypermethylation (i.e., occurring in all cells). It is also possible that some of these cell lines had lost one of two parental alleles by loss of heterozygosity and that methylation affected the remaining allele. However, two cell lines, KWS-I and TSG11 (derived from a poorly differentiated adenocarcinoma and a hepatoid carcinoma cell line, respectively), showed the presence of both methylated and unmethylated alleles, suggesting monoallelic or heterogenous hypermethylation (e.g., occurring in a subpopulation of cells). Methylation status at 1AM1 agreed with that at 1AM2 (Figure 1) in all cell lines tested (Figure 2). Direct sequencing of the methylated DNA PCR product from KATO-III cells confirmed the retention of cytosines at all CpGs within the PCR product (Figure 3), whereas cytosines were all converted to thymines in normal mucosal DNA after bisulfite treatment (data not shown). No methylated *APC* alleles of promoter 1B were present in any of the 10 gastric cancer cell lines examined (Figure 4). Transcripts from exon 1A were detected only in KWS-I cells by RT–PCR, and those from exon 1B were found in 9 of 10 cell lines except for KATO-III (Figure 5). Results of RT–PCR of exons 6–10 to test mRNA expression levels were concordant with those of exon 1B (Figure 5). Levels of *APC* expression were nearly concordant with the methylation status of promoters 1A and 1B as detected by MSP (Table 1), except that TSG11 cells had lost expression from exon 1A despite the fact that both methylated and unmethylated alleles were present, and KATO-III had lost expression from exon 1B despite the unmethylated status of promoter 1B.

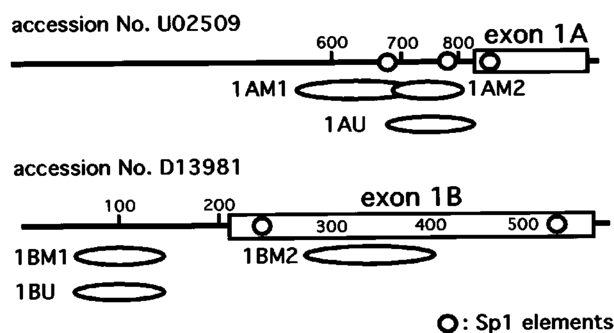

Figure 1 Locations of MSP primers used. 1AM1 and 1AM2 are PCR-amplified regions for methylated sequences, and 1AU for unmethylated sequences, respectively, of promoter 1A (accession No. U02509). 1BM1 and 1BM2 are PCR-amplified regions for methylated sequences, upstream of or within exon 1B, and 1BU represents unmethylated sequences of promoter 1B (accession No. D13981)

Methylation status of promoters 1A and 1B in 40 primary gastric cancers and their corresponding non-cancerous gastric mucosae

Promoter 1A was methylated in 33 (82.5%) of 40 primary gastric cancers and 39 (97.5%) of 40 matching non-cancerous gastric mucosae. Methylated alleles of promoter 1A in both cancerous and matching non-cancerous DNAs were present in 32 (80%) of 40 cases. Relative hypermethylation in tumor DNA, i.e., methylation in cancer DNA but lack of methylation

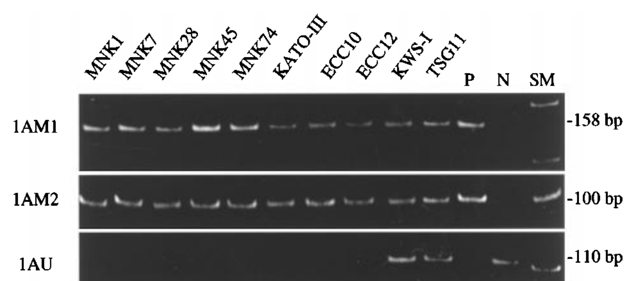

Figure 2 Results of MSP at promoter 1A in 10 gastric cancer cell lines. Methylated *APC* alleles of 1AM1 and 1AM2 are present in all 10 gastric cancer cell lines. Unmethylated alleles of 1AU are seen only in KWS-I and TSG11 cells. P, Sss I-treated DNA as a positive control; N, peripheral blood DNA as a negative control; SM, size marker

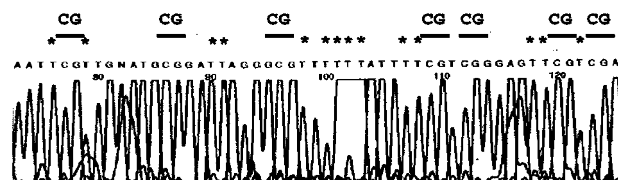

Figure 3 Sequencing histogram of methylated PCR product amplified from KATO-III cells. Cytosines at CpG sites remain as cytosines, whereas other cytosines are converted to thymines (asterisks) after bisulfite modification

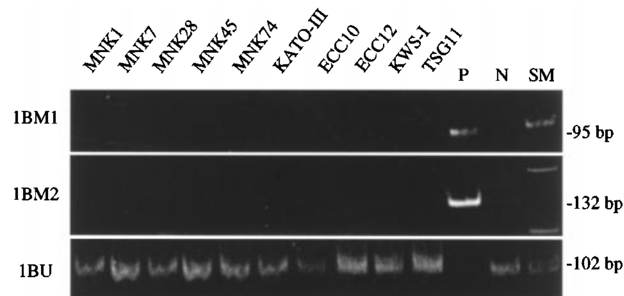

Figure 4 Results of MSP at promoter 1B in 10 gastric cancer cell lines. Methylated alleles of 1BM1 and 1BM2 are absent, whereas unmethylated alleles of 1BU are present in all the cell lines. P, Sss I-treated DNA as a positive control; N, peripheral blood DNA as a negative control; SM, size marker

in matching non-cancerous DNA, was observed in only one (2.5%) of 40 cases. Hypomethylation in cancer DNA, i.e. lack of methylation in cancer DNA and methylation in matching non-cancerous DNA, was observed in seven (17.5%) of 40 cases. In contrast, no methylated alleles of promoter 1B were detected in any cancerous or non-cancerous DNAs.

Unmethylated alleles of promoters 1A and 1B (1AU and 1UB) (Figure 1) were present in all the cancerous and non-cancerous DNAs. Methylation status at 1AM1 was completely in agreement with that of 1AM2 (Figure 1). One exceptional tumor showed hypermethylation at 1AM1, but was unmethylated at 1AM2. The methylation status of promoter 1A did not correlate with histological type, stage, age or sex in primary gastric cancers.

Discussion

Hypermethylation in only one of the two described *APC* promoters, promoter 1A, was studied in a report on colorectal cancers (Hiltunen *et al*., 1997), although two forms of transcripts from exons 1A exon 1B were also expressed in the normal colon, in agreement with our findings in the normal stomach (Horii *et al*., 1993). We found a significant difference between non-cancerous colorectal and gastric mucosae in the methylation status of promoter 1A. Promoter 1A was methylated in 97.5% of non-cancerous gastric mucosae in our study. However, heavy CpG methylation was found in colorectal cancers, but not in adenomas or normal colonic mucosae, suggesting the involvement of *APC* gene promoter hypermethylation in the progression of colorectal cancers (Hiltunen *et al*., 1997). To further investigate the methylation status of promoter 1A in non-cancerous tissues, we studied non-cancerous DNAs from five samples of peripheral blood, five colonic samples, and 10 breast samples from cancer-bearing and non-bearing individuals. No methylated alleles of promoter 1A were present in any of the DNA samples listed above, using an identical MSP procedure (data not shown). Therefore, this hypermethylation can be considered tissue-specific. We also studied DNAs of the esophagus, stomach, small intestine, and colon from autopsies of a 28-week stillborn infant and a 16-year-old female, and found an absence of methylated alleles of promoter 1A (data not shown). These results, coupled with the high prevalence of methylation of promoter 1A in non-cancerous gastric mucosae, suggest that methylation of this promoter in the normal stomach may constitute an age-related event.

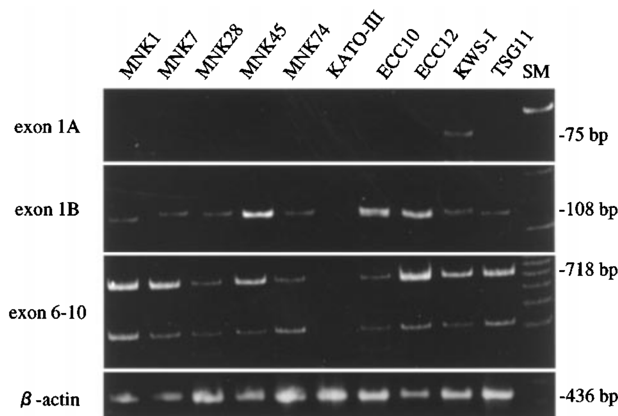

Figure 5 RT–PCR in 10 gastric cancer cell lines. Transcripts from exon 1A are present only in KWS-11 cells. Transcripts from exon 1B are visible in all cell lines except KATO-III. Full-length (718 bp; exon 6–10) and alternatively spliced *APC* mRNA (see Oshima *et al*., 1993) are consistently present in all cell lines except KATO-III. SM, size marker

Although promoter 1A was frequently methylated in the normal stomach, *APC* was still expressed from exon 1B because promoter 1B was never methylated. Thus, methylation of promoter 1A alone does not appear to be oncogenic in the stomach. Sp1 elements, which can protect promoter CpG islands from methylation, exist in both promoters 1A and 1B, and hypermethylation probably originates within the flanking regions of the CpG island (Graff *et al*., 1997). The distinct methylation patterns observed in normal stomach may be the result of positional differences between these two promoters, or of other mechanisms for CpG island protection.

We detected methylated alleles of promoter 1A in both cancerous and non-cancerous gastric tissues in the majority of cases (80%, or 32/40). Hypomethylation of cancer DNA relative to matching normal control DNA was observed in seven (17.5%) of 40 cases in our study. It is noteworthy that hypomethylation of the *hMLH1* promoter has recently been described in colorectal cancers (Kuismanen *et al*., 1999). However, it is possible that hypomethylation of promoter 1A observed in the present study might simply represent a passenger event during the malignant transformation of unmethylated cells.

Six gastric cancer cell lines, namely KATO-III, MKN1, MKN7, MKN28, MKN45 and MKN74,

Table 1 Methylation status and expression of APC in 10 gastric cancer cell lines

	Methylation		*Expression*			
Cell line	*1A*	*1B*	*1A*	*1B*	*6–10*	*Histological type*
MKN1	M	U	–	+	+	Adenosquamous carcinoma
MKN7	M	U	–	+	+	Well differentiated adenocarcinoma
MKN28	M	U	–	+	+	Moderately differentiated adenocarcinoma
MKN45	M	U	–	+	+	Poorly differentiated adenocarcinoma
MKN74	M	U	–	+	+	Moderately differentiated adenocarcinoma
KATO-III	M	U	–	–	–	Signet-ring cell carcinoma
ECC10	M	U	–	+	+	Endocrine cell carcinoma
ECC12	M	U	–	+	+	Endocrine cell carcinoma
KWS-I	M/U	U	+	+	+	Poorly differentiated adenocarcinoma
TSG11	M/U	U	–	+	+	Hepatoid carcinoma

M, methylated allele; U, unmethylated allele

showed an identical methylation pattern, i.e. methylation of promoter 1A and nonmethylation of promoter 1B. Transcripts from exon 1A were absent and those from exon 1B and exons 6–10 were present in all of these cell lines, except for KATO-III. The absence of *APC* transcripts in KATO-III is concordant with loss of APC protein reported in a previous study (Nishimura *et al*., 1995). KWS-I and TSG11 cell lines exhibited both methylated and unmethylated alleles of promoter 1A; however, transcripts from exon 1A were observed only in KWS-I. These results raise the possibility that mechanisms other than promoter methylation resulted in the absence of *APC* expression.

In conclusion, promoter 1A of the *APC* gene frequently undergoes methylation in non-cancerous and malignant gastric mucosae in a tissue-specific manner, while promoter 1B is protected from changes in methylation in the stomach. Abnormal methylation involving *APC*, including both hyper- and hypomethylation of *APC* promoters in other tissue types, appears to merit further investigation.

Materials and methods

Cell lines and tissues

Ten gastric cancer cell lines with differing 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 TSG11, a hepatoid carcinoma (Motoyama *et al*., 1986). Forty gastric cancers and their matching normal gastric mucosae were obtained surgically from 40 Japanese patients. The tumors included 13 differentiated and 27 undifferentiated carcinomas histologically, and constituted 19 early and 21 advanced cancers. As controls, we investigated samples from autopsies of a 28-week stillborn infant, a 16-year-old female, as well as five normal colonic mucosae, 10 normal breast tissues and five peripheral blood samples. The samples were immediately frozen in liquid nitrogen after resection and stored at $-80^\circ C$ until processing. Genomic DNA and mRNA were extracted using standard procedures.

Methylation-specific PCR

DNA methylation patterns in two *APC* promoters, namely promoter 1A and promoter 1B, were determined by MSP (Herman *et al*., 1996). MSP distinguishes unmethylated from methylated alleles of a given gene based on sequence changes produced following bisulfite treatment of DNA, which converts unmethylated cytosines to uracils, while leaving methylated cytosines unaffected. Subsequent PCR using primers specific to sequences that correspond to either methylated or unmethylated DNA sequences was then performed. Primer sequences used were 1AM1f (5′-TGTTTTGCGGATTTTTTTC-3′; nt 551–569) and 1AM1r (5′-GCAATAAAACACAAAACCCCG-3′; nt 686–706) (158 bp), 1AM2f (5′-TATTGCGGAGTGCGGGTC-3′; nt 701–718) and 1AM2r (5′-TCGACGAACTCCCGACGA-3′; nt 781–798) (100 bp) for methylated DNA sequences of promoter 1A, 1BM1f (5′-TGTTTAGGTAGTAATGGTTTAC-3′; nt 51–72) and 1BM1r (5′-TAAAACCTATTATACGCAAACG-3′; nt 122–143) (95 bp), 1BM2f (5′-GGATTTGCGGGTTTAGGTTC-3′; nt 279–298) and 1BM2r (5′-CATAAAAAACGCCGAAACCCG-3′; nt 388–408) (132 bp) for methylated DNA sequences of promoter 1B and exon 1B, respectively and 1AUf (5′-GTGTTTTATTGTGGAGTGTGGGTT-3′; nt 695–718) and 1AUr (5′-CCAATCAACAAACTCCCAACAA-3′; nt 781–802) (110 bp) for unmethylated DNA sequences of promoter 1A, 1BUf (5′-GGTTGTTTAGGTAGTAATGGTTTAT-3′; nt 48–72) and 1BUr (5′-AAACTAAAACCTATTATACACAAACA-3′; nt 122–147) (102 bp) for unmethylated DNA sequences of promoter 1B (Genbank Accession No. U02509 and D13981). These regions are shown in Figure 1. Briefly, 2 microgram of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, WI, USA), treated with NaOH, precipitated with ethanol, and resuspended in 30 μl water. Modified DNAs were amplified in a total volume of 20 μl $1\times$GeneAmp PCR Gold Buffer (PE Applied Biosystems, Foster City, CA, USA) containing 1.0 mM $MgCl_2$, 1 μM each primer, 0.2 mM dNTPs, and one unit Taq polymerase (AmpliTaq Gold DNA Polymerase, PE Applied Biosystems). After activation of the Taq polymerase at 95°C for 10 min, PCR was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles consisting of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s, followed by a final 7-min extension at 72°C for all primer-sets. PCR products were then loaded onto non-denaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Preparation of positive control

100 μg of peripheral blood DNA was treated according to the manufacturer's protocol (New England BioLabs, Inc., Beverly, MA, USA) by Sss I Methylase. Sss I-treated DNA was modified by sodium bisulfite as described above.

Reverse-transcription PCR

APC transcripts from exon 1A and exon 1B as well as of exon 6–10 were analysed by RT–PCR in the 10 gastric cancer cell lines. One microgram of total RNA isolated from the cell lines was reverse-transcribed by AMV RTase XL (Takara, Kyoto) at 42°C for 30 min, and then amplified by PCR as described above. Primer sequences were: NA (5′-GGAGACAGAATGGAGGTGC-3′) and mdf-APC5 (5′-CAACTGATCATATGAAGCTGCAGCCAT-3′) (75 bp), NB (5′-GCGAGCAGGAGCTGCGT-3′) and mdf-APC5 (5′-CAACTGATCATATGAAGCTGCAGCCAT-3′) (108 bp) (Horii *et al*., 1993), mdf-exon6 (5′-AGAATAGCCAGAATTCAGCAAATCG-3′) and mdf-exon10 (5′-GAAAGTTTCATTAGAACACACACAG-3′) (718 bp) for *APC* (Oshima *et al*., 1993), and β-actin-sense (5′-CCAGAGCAAGAGAGGTATCC-3′) and β-actin-antisense (5′-CTGTGGTGGTGAAGCTGTAG-3′) (436 bp).

Sequencing

The methylated DNA-derived PCR product from KATO-III and unmethylated PCR product from normal DNA (extracted from the peripheral blood of a healthy volunteer) were directly sequenced using the dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (PE Applied Biosystems) and an automated DNA sequencer (ABI PRISM 310, PE Applied Biosystems).

Acknowledgments
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