Monoallelic Methylation of the APC Promoter Is Altered in Normal Gastric Mucosa Associated with Neoplastic Lesions

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ABSTRACT

Adenomatous polyposis coli (APC) promoter hypermethylation has been reported frequently in normal gastric mucosa, but it remained to be clarified whether this occurs in every individual. In this study, methylation of the APC promoter was analyzed in histologically normal-appearing gastric mucosa samples by methylation-sensitive single-strand conformation analysis and by a methylation-sensitive dot blot assay. Epithelial cell samples were collected by microdissection from tissue sections. Equal amounts of methylated and unmethylated APC alleles were found in all gastric mucosa samples from patients without any gastric lesions (20 samples). Allele-specific methylation analysis showed that the methylation of the APC promoter was monoallelic; however, which allele was methylated depended on the cell type. Increased or decreased methylation was found in 10 of 36 (28%) normal gastric mucosa samples adjacent to a gastric or esophageal adenocarcinoma. No allelic loss was found at the APC locus. Modification of the methylation status was also found in 3 of 21 (14%) normal-appearing gastric mucosa samples adjacent to intestinal metaplasia. In contrast, all normal mucosa samples in cases with chronic gastritis but without metaplasia or dysplasia showed a monoallelic methylation pattern. Our results indicate the following: (a) In normal gastric mucosa, the APC promoter shows monoallelic methylation, which is not due to imprinting but most likely due to allelic exclusion; (b) the excluded allele differs between foveolar and glandular epithelial cells; (c) the APC methylation pattern is frequently altered in normal-appearing gastric mucosa of gastric or esophageal adenocarcinoma patients; and (d) such alterations also occur in normal gastric mucosa adjacent to intestinal metaplasia.

INTRODUCTION

The adenomatous polyposis coli (APC) gene is a tumor suppressor gene involved in the Wnt/β-catenin signaling pathway (1). Loss of APC function can result in nuclear accumulation of β-catenin, which leads to expression of genes such as c-Myc, via transcriptional activation through the β-catenin/T-cell factor transcription complex (2).

Promoter hypermethylation of tumor suppressor or tumor-related genes has been reported in human tumor cell lines (3) and human cancer (4). Methylation of the APC gene promoter was observed not only in breast, lung, prostate, and bladder cancer but also in gastrointestinal cancer, including colon, gastric, pancreatic, liver, and esophageal carcinoma (5–8). The methylation status of the APC gene promoter has already been investigated in gastric mucosa. However, discordant results were obtained. Some studies found APC gene promoter methylation in normal gastric mucosa (9–12), and others did not (5), which leaves APC gene promoter methylation status in normal gastric mucosa open to question. A possible explanation for the discrepancies in the literature might be the nature of the tissue samples: normal tissues were obtained from cancer patients and not (5), which leaves questions regarding the definition of “histologically normal.” Microdissection is probably the only reliable approach to separate preneoplastic or neoplastic cells and morphologically normal cells in human tissue sections and thus ensure the specificity of the molecular events encountered.

Although several methods for analyzing DNA methylation status have been reported (16–18), we prefer the methylation-sensitive single-strand conformation analysis (MS-SSCA) for reasons of specificity and sensitivity (19, 20). The MS-SSCA is semi-quantitative and uses primers without CPG sites to avoid selective amplification of either methylated or unmethylated DNA. This method also allows establishment of clonal variations in the DNA methylation status for clones comprising as few as 5–10% of the total cell population (20).

In addition, we established a methylation-sensitive dot blot assay (MS-DBA), which uses two internal oligoprobe complementary to the amplified sequence, to reveal the proportion of cytosine and 5-methyl-cytosine for two CPG sites in the DNA sample.

In this study, we analyzed the APC promoter 1A methylation status and APC protein expression in histologically normal gastric mucosa in cases without any gastric disease and in histologically normal gastric mucosa in cases with histologic abnormalities: chronic gastritis without intestinal metaplasia or dysplasia, intestinal metaplasia, and adenocarcinoma of the stomach or the esophagus. In addition, topographic heterogeneity of mucosal APC promoter hypermethylation was examined in normal gastric mucosa of patients with esophageal adenocarcinoma.

MATERIALS AND METHODS

Tissue Samples. The cases were selected from the files of our institute and included surgical specimens and endoscopic biopsies. All tissue samples, except those used for the allele-specific methylation analysis, had been fixed in buffered formalin and embedded in paraffin. All of the available histologic sections were reexamined by a pathologist (C.F.). The biopsies were selected from cases with evidence of gastric disease, as determined by endoscopy with biopsies, undertaken because of nonspecific symptoms or for anemia of unknown etiology. Gastritis was classified according to the Sydney system.

The tissue samples included 98 histologically normal gastric mucosal biopsies: 15 biopsies of normal fundus mucosa (patient age range, 60–65 years); 5 biopsies of normal gastric (antrum and/or fundus) mucosa (patient age range, 17–20 years); 21 biopsies of fundus mucosa with chronic gastritis, mostly due to Helicobacter pylori infection; 21 biopsies of normal fundus mucosa from cases with intestinal metaplasia but without dysplasia in other biopsies from the same endoscopy session; 22 samples of normal gastric (antrum or fundus) mucosa from surgical specimens with an adenocarcinoma in the antrum or cardia; and 14 samples of gastric type (antrum and/or fundus) mucosa surrounding an adenocarcinoma which had developed in Barrett’s esophagus.

In addition, multiple (four to five) histologically normal gastric mucosa samples were analyzed in three cases of adenocarcinoma in Barrett’s esophagus.

The allele-specific methylation analysis of the APC promoter was performed on 16 normal frozen gastric mucosa samples selected from the tissue bank of our institute.

Microdissection, DNA Extraction, and Sodium Bisulfite Conversion. Manual microdissection was performed on tissue sections under microscopic control as described previously (21). Final histologic control before collection of the epithelial cells for molecular analysis confirmed that contamination with stromal tissues was negligible. The selected cells were suspended in 50 μL of Tris-EDTA buffer and digested with proteinase K. The DNA was extracted...
with phenol, followed by phenol/chloroform, and coprecipitated in ethanol with 20 μg of glycerol. The pellet was resuspended in 30 μL of 10 mmol/L Tris-HCl (pH 8.5), and the DNA was kept at −20°C until use. DNA extracted from microdissected tissues was modified in 40 μL of water with sodium bisulfite as described previously (20).

Methylation-Sensitive Single-Strand Conformation Analysis of the APC Promoter. A 159-bp fragment of the APC gene promoter (GenBank accession number U02509) was amplified by nested polymerase chain reaction (PCR) using the following primers specific to the upper modified strand: 5′-GGGTTAGGTTAGGTTAG-3′ (sense) and 5′-AACATACCAATAACACCATA-3′ (antisense) for the outer PCR amplification, and 5′-GGGTTAGGTTAGGTTAGGTT-3′ (sense) and 5′-CCACACACACACACACACAC-3′ (antisense) for the inner PCR amplification. Our amplified nested product includes 13 CpG sites between −210 and −52 relative to the APC major transcription start site (22). Both PCRs were carried out in the presence of 5% dimethyl sulfoxide (Merck, Dietikon, Switzerland) with the same amplification profile: 94°C for 30 seconds, 53°C for 45 seconds, and 72°C for 75 seconds. For the outer PCR, 2 μL of modified DNA were used in a total volume of 20 μL; the inner PCR was done with 1 μL of the first PCR product in a total volume of 20 μL. Forty and 20 cycles were performed for the outer PCR and inner PCR, respectively. All of the PCRs were repeated at least twice. Amplification was confirmed by analysis on a 2% agarose gel. Single-strand conformation analysis was performed with 5 μL of PCR products as described previously (20).

Methylation-Sensitive Dot Blot Assay of the APC Promoter. A MS-DBA was performed using two different commercially synthesized oligo-probes specific for the amplified sequence. The probes were labeled using a digoxigenin oligonucleotide 3′-End Labeling Kit (Roche, Rotkreuz, Switzerland) following the manufacturer’s instructions. One probe was designed to hybridize to methylated DNA containing two CG dinucleotides, another one contained two TG dinucleotides to recognize the unmethylated DNA. The sequences were 5′-GATGCGGATTAGGGCTTTT-3′ and 5′-GATGCGGGATTAGGGCTTTT-3′, respectively. In brief, 1.5 μL of NaOH-denatured nested PCR products and positive controls were immobilized in duplicate on two NytranN membranes (Schleicher and Schuell, Dassel, Germany). The hybridization was performed with 5 to 10 pmoL of the respective probes for 2 to 3 hours at 50°C. The membranes were washed twice with 2× SSC containing 0.1% SDS at room temperature for 5 minutes and twice with 0.5× SSC at 53.5°C for 15 minutes. After incubation with anti-digoxigenin-AP Fab fragments (Roche) and addition of CDP-Star ready-to-use solution (Roche), the membranes were exposed to a X-OMAT film (Eastman Kodak Co., Rochester, NY). The results were read by comparison of the intensity of the spots on both membranes using Scion Image (Scion Corp., Frederick, MD).

Preparation of Positive Control. Ssxl methylase (New England Biolabs, Beverly, MA) was used to methylate 15 to 20 μg of normal colon DNA obtained from frozen tissues. A full methylation was confirmed by digestion with MspI (Promega, Madison, WI) and HpaII (Amersham Biosciences, Buckinghamshire, United Kingdom) restriction enzymes. Ssxl methylated and unmethylated DNA were mixed at different ratios to obtain a methylation scale of the 3′-untranslated region and in exon 11 of the APC gene. The amplification and the enzymatic digestion of the fragments were done as described previously (23). Electrophoretic separation was performed on an 8% polyacrylamide gel. The gel was stained in SYBR Gold gel stain (Molecular Probes, Eugene, OR), diluted 1:10,000 in 1× Tris-borate EDTA buffer, and visualized with ultraviolet light.

Immunohistochemistry. APC immunohistochemistry was performed using a mouse monoclonal antibody that recognizes the COOH terminus of human APC (ab120; Abcam, Cambridge, United Kingdom). Four-micrometer paraffin sections were cut and mounted on coated slides. Slides were dewaxed and rehydrated in a xylene-ethanol series. Endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide solution for 20 minutes, and antigen retrieval was performed in a microwave oven (750 W) for 5 to 10 minutes in 10 mmol/L sodium citrate buffer. The slides were washed with PBS and, to prevent nonspecific binding, incubated for 30 minutes with normal goat immunoglobulin (Dako, Glostrup, Denmark) diluted 1:30 in PBS and containing 0.2% bovine serum albumin (Sigma, St. Louis, MO). Sections were then incubated for 2 hours at room temperature with the anti-APC antibody diluted 1:500 in PBS containing 0.2% bovine serum albumin. The ENVISION+ System/horseradish peroxidase kit (Dako) was used according to the manufacturer’s instructions, and the results were visualized with diaminobenzidine.

RESULTS

The APC Promoter 1A Is Methylated in Normal Gastric Mucosa. In the 20 antrum or fundus samples from patients without any histopathological lesions of the gastric mucosa, methylation analysis of the APC promoter region by MS-SSCA showed two approximately equidense patterns of band mobility, one corresponding to unmethylated CpG sites, and the other corresponding to completely methylated CpG sites. Furthermore, the APC promoter methylation pattern was found in the specimens irrespective of age (Fig. 1A). Similar results were obtained when the MS-DBA was applied (Fig. 2A). These results suggest a monoallelic methylation pattern of the APC promoter, but random methylation cannot be excluded.

Methylation of the APC Promoter Is Monoallelic. Allele-specific methylation analysis of the APC promoter was performed on 16 frozen normal gastric mucosa samples. Single-strand conformation analysis and sequencing showed that seven of these were heterozygous for the SNP (rs2019720). MS-SSCA confirmed an equal amount of methylated and unmethylated alleles. To specifically analyze each allele, we amplified a 907-bp sequence that spans both the APC promoter and the SNP. Only five of the seven heterozygous samples could be reproducibly amplified (in at least three independent experiments). The seminested MSP amplified only the methylated DNA strand (Fig. 3A). Sequence analysis at the SNP locus showed a mixture of the polymorphic bases. The
ratio between the two bases differed from sample to sample (Fig. 3B). We hypothesized that the allele methylated might be different for different cell types in the gastric glands. To verify this hypothesis, we microdissected the foveolar epithelium layer from the glandular layer in five samples. Within microdissected samples, the methylation was allele specific. The methylated alleles in foveolar cells were consistently different from those in the glandular cells (Fig. 3C and D; Table 1).

The Monoallelic Methylation Pattern of the APC Promoter Is Modified in Normal Gastric Mucosa of Patients with Gastric or Esophageal Adenocarcinoma. In histologically normal gastric mucosa samples, taken at 1 to 5 cm from a gastric or esophageal adenocarcinoma, changes in the APC promoter monoallelic methylation pattern were observed in 28% of the samples (10 of 36 samples). Of the samples taken from 22 patients with a gastric cancer, 4 showed changes in the methylation pattern (Table 2): in 3 samples, decreased
methylation was found; and in 1 sample, complete methylation of both alleles was found (Figs. 1C and 2C). In normal gastric mucosa of 43% of cases (6 of 14) of esophageal adenocarcinoma in Barrett’s esophagus, changes in the APC promoter monoallelic methylation pattern were found (Figs. 1C and 2C). In five samples, low or no methylation was found, whereas in one case, complete methylation of the APC promoter alleles had occurred (Table 2).

Multiple normal gastric mucosa samples were studied in three cases of esophageal adenocarcinoma. One case showed reduced methylation in all five samples (Fig. 4A), one case showed reduced methylation in four of five samples (Fig. 4B), and in the third case, the methylation status varied between samples. A modified methylation pattern was found even at a distance of >10 cm from the tumor (Fig. 4C).

Modification of the APC Promoter Monoallelic Methylation Pattern Is Not due to Allelic Loss. LOH in the gastric mucosa samples was determined by comparing gastric mucosa with other tissues from the same patient. Only 14 of 36 samples were informative for the APC exon 11 and/or the 3′-untranslated region loci, respectively, and allelic loss for the APC locus was found in none of these samples.

The Monoallelic Methylation Pattern of the APC Promoter Is Modified in Normal Gastric Mucosa Close to Intestinal Metaplasia. A total of 21 histologically normal-appearing gastric mucosa samples from cases with chronic gastritis without intestinal metaplasia, mostly due to H. pylori, were studied. All samples showed equidense methylated and unmethylated bands, a result similar to that

Table 1  Patient characteristics, stomach localization, and A to G polymorphic bases

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y)/Sex</th>
<th>Anatomical zone</th>
<th>SNP base within the methylated allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Foveolar epithelium</td>
</tr>
<tr>
<td>1</td>
<td>59/M</td>
<td>Fundus</td>
<td>G</td>
</tr>
<tr>
<td>2</td>
<td>70/M</td>
<td>Antrum</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>53/M</td>
<td>Fundus</td>
<td>G</td>
</tr>
<tr>
<td>4</td>
<td>72/M</td>
<td>Fundus</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>49/M</td>
<td>Fundus</td>
<td>A</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.

Table 2  Methylation status of the APC promoter in histologically normal-appearing gastric mucosa

<table>
<thead>
<tr>
<th>Without gastric disease (60–65 y old)</th>
<th>Hypomethylation</th>
<th>Monomethylation</th>
<th>Biallelic methylation</th>
<th>Cases without monomethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/15 (0)</td>
<td>15/15 (100)</td>
<td>0/15 (0)</td>
<td>0/15 (0)</td>
<td>0/15 (0)</td>
</tr>
<tr>
<td>Without gastric disease (17–20 y old)</td>
<td>0/5 (0)</td>
<td>5/5 (100)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>With chronic gastritis</td>
<td>0/21 (0)</td>
<td>21/21 (100)</td>
<td>0/21 (0)</td>
<td>0/21 (0)</td>
</tr>
<tr>
<td>Near intestinal metaplasia</td>
<td>2/21 (9)</td>
<td>18/21 (86)</td>
<td>1/21 (5)</td>
<td>3/21 (14)</td>
</tr>
<tr>
<td>Near gastric cancer</td>
<td>3/22 (14)</td>
<td>18/22 (82)</td>
<td>1/22 (4)</td>
<td>4/22 (18)</td>
</tr>
<tr>
<td>Near esophageal cancer</td>
<td>5/14 (36)</td>
<td>8/14 (57)</td>
<td>1/14 (7)</td>
<td>6/14 (43)</td>
</tr>
</tbody>
</table>

NOTE. Values represent n (%).
observed in gastric mucosa without evidence of gastric disease (Figs. 1B and 2B).

In contrast, 3 of the 21 histologically normal gastric mucosa samples taken from cases with chronic gastritis with intestinal metaplasia showed modified APC promoter methylation: 2 were hypomethylated, and 1 showed biallelic methylation (Figs. 1B and 2B; Table 2).

Expression of APC Protein Correlates with APC Gene Promoter Methylation Status. APC protein expression was analyzed by immunohistochemistry in sections consecutive to those used for microdissection. Only parietal cells stained. In all but one of the samples with either hypomethylation or monoallelic methylation of the APC promoter, expression of the APC protein was found (Figs. 1A and 1B). In contrast, in the samples with a biallelic methylation pattern, the APC protein was not expressed (Fig. 5C).

DISCUSSION

APC promoter methylation status was studied in normal mucosa from the gastric antrum and fundus of patients without histologic evidence of gastric disease. Equidense bands representing fully methylated and fully unmethylated APC promoter alleles were observed by MS-SSCA, indicating that APC promoter methylation occurs in normal gastric mucosa. All of the normal gastric mucosa samples showed the same methylation pattern, independent of their localization in the stomach.

The existence of clonal cell populations with partial methylation in this region of the genome can be excluded for two reasons. Firstly, no additional bands corresponding to clones with partially methylated alleles were observed by MS-SSCA (see Fig. 1). Secondly, the results obtained by MS-SSCA were confirmed by MS-DGA. Consequently, the equidense bands, implying that 90% of the DNA is methylated, can be explained by either methylation of one allele in all cells (monoallelic methylation) or the existence of quantitatively equivalent subpopulations of cells: one with methylation of both alleles, one without methylation, and one other with methylation of only one allele (stochastic methylation; ref. 24).

Three main categories of monoallelic methylation have been described: random methylation, allelic exclusion, and genomic imprinting (24–28). To determine whether the APC promoter methylation observed in gastric cells was a monoallelic or stochastic event, allele-specific methylation analysis within the APC promoter was performed. We used a long-range seminested MSP strategy to span both the methylated APC promoter and the A to G SNP at position +684. Sequencing revealed that the methylation of the APC promoter occurred in both alleles. This observation clearly indicates that the APC gene is not imprinted in the normal gastric mucosa. Nevertheless, the two polymorphic bases A and G were not observed at a 1:1 ratio within the APC methylated alleles. This led us to hypothesize that methylation of the APC promoter was not random but might be dependent on the gastric cell type. To test this hypothesis, we separated the normal gastric mucosa by microdissection into two distinct areas: the foveolar epithelium, which is composed of only one cell type (surface mucous cells); and the gastric glands, which are composed of three different cell types (parietal, chief, and enteroendocrine cells). The mucous neck cells, which are located between the foveolar epithelium and glands, were not included in the analysis. Seminested MSP sequence analysis of the two areas revealed that APC promoter methylation was allele specific. Furthermore, the two tissue compartments had APC promoter methylation of consistently different alleles, which explains why a mixture of both alleles was found when the compartments were not separated. These observations allow us to confirm that APC promoter methylation is monoallelic. Because either allele was methylated in a cell-specific manner, the methylation of the APC promoter most likely corresponds to allelic exclusion. Allelic exclusion, as described for the immunoglobulin and T-cell receptor genes, is defined as a stable inactivation by methylation of one allele, independent of the parental origin (28).

Tissue-specific monoallelic expression has been reported previously. For example, the WT1 gene showed either paternal, biallelic, or maternal expression in specific tissue types from a single individual (29, 30). Our results indicate that allelic exclusion, as we observed for the APC gene, can also be cell type specific within a heterogeneous tissue. In gastric mucosa, epithelial cells differentiate in several di-
resections, with cells migrating upward toward the luminal surface to create the foveolar epithelium and downward to form the glandular cells (31). Depending on the direction of differentiation, either one or the other APC allele is excluded.

DNA methylation is involved in tumor suppressor gene inactivation in neoplastic cells and may also function as a regulator of age-related changes in normal aging cells. For the estrogen receptor gene, IGF2 and MysD age-related promoter methylation has been reported (32–34). Methylation of the APC gene promoter was found to be age dependent in the stomach and lung (11, 35, 36). For example, Waki et al. (36) noticed APC methylation in almost all of the gastric mucosa samples from patients over the age of 40 years, but in <50% of younger patients. In our series, we found no difference between young and aged individuals.

We also studied methylation of the APC promoter in normal mucosa from patients with gastric or esophageal adenocarcinoma. In 28% of these samples, decreased or increased methylation was observed. We found expression of APC protein in all normal gastric mucosa samples without or with monoallelic methylation of the APC promoter. APC protein, however, was not expressed in samples with biallelic APC promoter methylation. Alteration in the methylation pattern of normal tissues associated with a tumor was first observed for the IGF2 gene in colorectal cancer patients (37). More recently, hypermethylation of the APC, p16, and E-cadherin promoters was reported in normal mucosa adjacent to gastric cancer (13–15) and in epithelia bordering breast, lung, and prostate cancer (6, 8, 38). Pathological epigenetic fixation can be propagated by either complete absence of expression or biallelic expression. The biological significance of this phenomenon is currently unclear. It might represent a field change in an epithelium exposed to carcinogenic conditions. This suggests that, in normal tissues, changes in the pattern of promoter methylation, as we observed for APC in normal gastric mucosa, might precede the development of neoplasia, without accompanying morphologic manifestations (39). A practical implication of this notion is that the definition of “normal control” tissue adjacent to a cancer might need to be revisited.

To determine whether APC promoter 1A is altered only in the context of neoplasia or is already altered under conditions that precede neoplasia we studied normal gastric mucosa samples adjacent to areas of intestinal metaplasia in the stomach and to chronic gastritis without intestinal metaplasia. Of the intestinal metaplasia samples, 14% showed altered methylation, whereas this was not observed in uncomplicated chronic gastritis. This observation indicates that altered promoter methylation occurs in normal gastric mucosa early on in the process of carcinogenesis.

Apparent hypermethylation of the APC promoter 1A might be observed due to loss of the normal unmethylated allele. In none of our samples, however, was LOH of the APC locus observed. This also excludes apparent hypomethylation due to the loss of the methylated allele. Loss of monoallelic methylation can be disrupted, which includes gain or loss of methylation (40). The most commonly involved gene is the IGF2 gene, located on chromosome 11p15.5. Along with IGF2, other genes in the 11p15.5 locus were hypomethylated, suggesting a hypomethylation mechanism affecting a large subchromosomal domain (41). We are currently studying the methylation pattern of other genes located in the APC locus to verify this hypothesis.

In conclusion, we detected monoallelic APC promoter methylation in normal gastric mucosa. This monoallelic methylation is not due to imprinting but is most likely due to allelic exclusion, which is specific for different cell types in the gastric mucosa. APC promoter methylation is frequently altered in histologically normal-appearing gastric mucosa from patients with gastric or esophageal adenocarcinoma. These alterations were seen in the context of intestinal metaplasia, but not in uncomplicated chronic gastritis. Altered gene promoter methylation might represent a field change in cell systems exposed to a carcinogenic environment, preceding morphologic manifestations of (pre)neoplasia.

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