CpG Island Methylation in Premalignant Stages of Gastric Carcinoma

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Abstract

There are limited reports on methylation analysis of the premalignant lesions of gastric carcinoma thus far. This is despite the fact that gastric carcinoma is one of the tumors with a high frequency of CpG island hypermethylation. To determine the frequency and timing of hypermethylation during multistep gastric carcinogenesis, non-neoplastic gastric mucosa (n = 118), adenomas (n = 61), and carcinomas (n = 64) were analyzed for their p16, human Mut L homologue 1 (hMLH1), death-associated protein (DAP)-kinase, thrombospondin-1 (THBS1), and tissue inhibitor of metalloproteinase 3 (TIMP-3) methylation status using methylation-specific PCR. Three different classes of methylation behaviors were found in the five tested genes. DAP-kinase was methylated at a similar frequency in all four stages, whereas hMLH1 and p16 were methylated in cancer samples (20.3% and 42.2%, respectively) more frequently than in intestinal metaplasia (6.3% and 2.1%, respectively) or adenomas (9.8% and 11.5%, respectively). However, hMLH1 and p16 were not methylated in chronic gastritis. THBS-1 and TIMP-3 were methylated in all stages but showed a marked increase in hypermethylation frequency from chronic gastritis (10.1% and 14.5%, respectively) to intestinal metaplasia (34.7% and 36.7%, respectively; P < 0.05) and from adenomas (28.3% and 26.7%, respectively) to carcinomas (48.4% and 57.4%, respectively; P < 0.05). The hMLH1, THBS1, and TIMP-3 hypermethylation frequencies were similar in both intestinal metaplasia and adenomas, but the p16 hypermethylation frequency tended to be higher in adenomas (11.5%) than in intestinal metaplasia (2.1%; P = 0.073). The average number of methylated genes was 0.6, 1.1, 1.1, and 2.0 per five genes per sample in chronic gastritis, intestinal metaplasia, adenomas, and carcinomas, respectively. This shows a marked increase in methylated genes from non-metaplastic mucosa to intestinal metaplasia (P = 0.001) as well as from premalignant lesions to carcinomas (P = 0.002). These results suggest that CpG island hypermethylation occur early in multistep gastric carcinogenesis and tend to accumulate along the multistep carcinogenesis.

Introduction

Gastric carcinogenesis is a multistep process composed of genetic and epigenetic alterations involving protooncogenes, tumor suppressor genes, cell-cycle regulator genes, tissue-invasion-related genes, or mismatch repair genes (1). Methylation of gene regulatory elements, sor genes, cell-cycle regulator genes, tissue-invasion-related genes, or hypermethylation. To determine the frequency and timing of hypermethylation during multistep gastric carcinogenesis, non-neoplastic gastric mucosa (n = 118), adenomas (n = 61), and carcinomas (n = 64) were analyzed for their p16, human Mut L homologue 1 (hMLH1), death-associated protein (DAP)-kinase, thrombospondin-1 (THBS1), and tissue inhibitor of metalloproteinase 3 (TIMP-3) methylation status using methylation-specific PCR. Three different classes of methylation behaviors were found in the five tested genes. DAP-kinase was methylated at a similar frequency in all four stages, whereas hMLH1 and p16 were methylated in cancer samples (20.3% and 42.2%, respectively) more frequently than in intestinal metaplasia (6.3% and 2.1%, respectively) or adenomas (9.8% and 11.5%, respectively). However, hMLH1 and p16 were not methylated in chronic gastritis. THBS-1 and TIMP-3 were methylated in all stages but showed a marked increase in hypermethylation frequency from chronic gastritis (10.1% and 14.5%, respectively) to intestinal metaplasia (34.7% and 36.7%, respectively; P < 0.05) and from adenomas (28.3% and 26.7%, respectively) to carcinomas (48.4% and 57.4%, respectively; P < 0.05). The hMLH1, THBS1, and TIMP-3 hypermethylation frequencies were similar in both intestinal metaplasia and adenomas, but the p16 hypermethylation frequency tended to be higher in adenomas (11.5%) than in intestinal metaplasia (2.1%; P = 0.073). The average number of methylated genes was 0.6, 1.1, 1.1, and 2.0 per five genes per sample in chronic gastritis, intestinal metaplasia, adenomas, and carcinomas, respectively. This shows a marked increase in methylated genes from non-metaplastic mucosa to intestinal metaplasia (P = 0.001) as well as from premalignant lesions to carcinomas (P = 0.002). These results suggest that CpG island hypermethylation occur early in multistep gastric carcinogenesis and tend to accumulate along the multistep carcinogenesis.

On the basis of the similarities in both the morphological and genetic aspects between colorectal and gastric cancer, it has been postulated that gastric carcinomas may arise from gastric adenomas, similarly to the colorectal adenoma-carcinoma sequence. Gastric adenoma is an (endoscopically) distinct and (histologically) circumscribed benign neoplasm composed of tubular or villous structures lined by dysplastic epithelium (18). The incidence of malignant transformations of gastric adenomas has been reported to be ~10% on long-term follow-up studies (19, 20). Although there is some controversy, the precancerous nature of intestinal metaplasia is suggested by the observation that gastric carcinoma often occurs in the background of intestinal metaplasia, and that the risk of gastric carcinoma is proportional to the extent of metaplasia (21). Furthermore, the genetic alterations frequently observed in gastric carcinoma also have been recorded in intestinal metaplasia (22, 23).

Although epigenetic change has been recognized as an important mechanism underlying gastric carcinogenesis progression, there has been limited data regarding the epigenetic abnormalities in both gastric adenoma and intestinal metaplasia. To determine the chronology of hypermethylation during multistep gastric carcinogenesis, promoter hypermethylation of p16, hMLH1, DAP-kinase, THBS1, and TIMP-3 in intestinal metaplasia, gastric adenomas, and gastric carcinomas was analyzed.

Materials and Methods

Sixty-four archival samples of surgically resected gastric carcinomas, 61 archival samples of endoscopically resected gastric adenomas, and 118 archival samples of endoscopically obtained non-neoplastic gastric mucosae (49 intestinal metaplasia and 69 non-metaplastic mucosae) were studied. Among these samples, the gastric carcinoma samples were characterized previously for 12/11/00; accepted 2/9/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This work was supported by the Korea Science and Engineering Foundation (Grant No. 1999-2-208-004-5).

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2 The abbreviations used are: hMLH1, human Mut L homologue 1; TIMP-3, tissue inhibitor of metalloproteinase 3; DAP-kinase, death-associated protein kinase; THBS1, thrombospondin-1; MSP, methylation-specific PCR.
expression. Through our previous studies (5, 24), it was confirmed that both p16 and hMLH1 promoter hypermethylation strongly correlated with the absence of both p16 and hMLH1 proteins, respectively.

**DNA Preparation.** After identifying carcinoma, adenoma, or intestinal metaplasia on H&E-stained slides, portions of carcinoma, adenoma, or metaplastic mucosa were scraped from 20-μm-thick paraffin sections. The materials collected were dewaxed by washing in xylene and then by rinsing in ethanol. The dried tissues or fresh frozen samples were digested with proteinase K and subjected to sodium bisulfite modification as described previously (25). MSP was performed to examine methylation frequency from premalignant lesions to gastric carcinomas.

**MSP.** Both normal and tumors DNAs were subjected to sodium bisulfite modification as described previously (25). MSP was performed to examine methylation at promoter regions of p16, hMLH1, DAP-kinase, THBS1, and TIMP-3. The primer sequences for each gene, for both methylated and unmethylated reactions, are described in Table 1. To amplify the bisulfite-modified promoter sequence of methylated reactions, a PCR mixture containing 20 μM Tris (pH 8.3), 50 mM KCl, and 1.5 mM MgCl2, deoxynucleotide triphosphates (each at 0.2 mM), primers (10 pmol each), and bisulfite-modified DNA (30–50 ng) in a final volume of 25 μL was used. For the amplification of DAP-kinase, THBS1, and TIMP-3, a PCR mixture containing 1× PCR buffer [10 mM Tris (pH 8.3), 50 mM KCl, and 1.5 mM MgCl2], deoxynucleotide triphosphates (each at 0.2 mM), primers (10 pmol each), and bisulfite-modified DNA (30–50 ng) in a final volume of 25 μL was used. The reactions were hot-started at 97°C for 1 min before the addition of 0.75 units of Taq polymerase (Takara Shuzo Co., Kyoto, Japan). The amplifications were carried out in a Thermal cycler (Perkin-Elmer, Foster City, CA) for 35 cycles (1 min at 95°C, variable temperatures according to the primer, and 1 min at 72°C) and a final 10-min extension. The PCR products were electrophoresed on a 2.5% agarose gel and visualized under UV illumination using an ethidium bromide stain.

**Sequencing Analysis.** The PCR products were purified using the JET-SORB gel extraction kit (Genomed, Bad Oeynhausen, Germany) and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted from individual clones by alkaline lysis plasmid minipreparation. The inserted PCR fragments of four individual clones obtained from each sample were sequenced with both M13 reverse and M13 (−20) forward primer using the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin-Elmer) and an ABI Prism 377 DNA Sequencer (Perkin-Elmer).

**Results**

Table 1 summarizes the promoter hypermethylation frequency of each gene in non-neoplastic and neoplastic gastric samples, and Fig. 1 shows a representative example of MSP analysis. The timing of hypermethylation during multistep gastric carcinogenesis from gastritis to carcinoma samples varied according to the gene. On the basis of the timing, these five genes were able to be classified into three groups. p16 and hMLH1 were not methylated in chronic gastritis samples and showed a 2-fold or greater increase in the hypermethylation frequency from premalignant lesions to gastric carcinomas. DAP-kinase was hypermethylated at a similar frequency in chronic gastritis, intestinal metaplasia, and gastric adenoma, and gastric carcinoma samples. THBS1 and TIMP-3 were hypermethylated both in non-neoplastic and neoplastic samples, with a marked increase in the methylation frequency from chronic gastritis (10.1% and 14.5%, respectively) to intestinal metaplasia (34.7% and 36.7%, respectively; P < 0.05) and from intestinal metaplasia or adenomas (28.3% and 26.7%, respectively) to carcinomas (48.4% and 57.4%, respectively; P < 0.05) and from intestinal metaplasia or adenomas (28.3% and 26.7%, respectively) to carcinomas (48.4% and 57.4%, respectively; P < 0.05).

### Table 1. Primer sequences and PCR conditions for MSP analysis

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′–3′)</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16 mf</td>
<td>TTATTTAGGGTGTGGGCGATGCG</td>
<td>150</td>
<td>65</td>
</tr>
<tr>
<td>mR</td>
<td>GACCCCCGAACCCGACCCCTAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uF</td>
<td>TTTTTGTTGGAGTTGATACATGTT</td>
<td>151</td>
<td>60</td>
</tr>
<tr>
<td>uR</td>
<td>CAACCCCAACCACACCATATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hMLH1 mR</td>
<td>TATATGCTGTGATGCATTTG</td>
<td>153</td>
<td>60</td>
</tr>
<tr>
<td>uF</td>
<td>TGGGTTGTTGTGTTATGTTG</td>
<td>124</td>
<td>60</td>
</tr>
<tr>
<td>uR</td>
<td>ACCACCATCCTGATACTCCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAP-kinase mR</td>
<td>TGGTAGCGGAGGAGTCGAGT</td>
<td>98</td>
<td>60</td>
</tr>
<tr>
<td>uF</td>
<td>GGGAGTATGGTTGATTGAATGT</td>
<td>106</td>
<td>60</td>
</tr>
<tr>
<td>uR</td>
<td>CAATAACCTCAAAAAACACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>THBS1 mR</td>
<td>TGCGGCGGGATTTAAAATGCC</td>
<td>74</td>
<td>62</td>
</tr>
<tr>
<td>uF</td>
<td>GATCCGTGTTGTTGTGTTGTT</td>
<td>115</td>
<td>62</td>
</tr>
<tr>
<td>uR</td>
<td>CCTAAAGCTCAAAAAACACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-3 mF</td>
<td>CTGTGCTATTTTTTTTGTATTTT</td>
<td>116</td>
<td>59</td>
</tr>
<tr>
<td>mR</td>
<td>CCGAAAAACCCGGCTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uF</td>
<td>TTTTTGTTGTGATTTTTTTGTATTTT</td>
<td>122</td>
<td>59</td>
</tr>
<tr>
<td>uR</td>
<td>CCCCCAAAACCCGCCCTCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. The frequency of promoter hypermethylation of p16, hMLH1, DAP-kinase, THBS1, and TIMP-3 in chronic gastritis, intestinal metaplasia, adenomas, and carcinomas

<table>
<thead>
<tr>
<th>Chronic gastritis (n = 69)</th>
<th>Intestinal metaplasia (n = 49)</th>
<th>Gastric adenoma (n = 61)</th>
<th>Gastric carcinoma (n = 61)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>0</td>
<td>2.1%^a</td>
<td>11.5%^ab</td>
</tr>
<tr>
<td>hMLH1</td>
<td>0%</td>
<td>6.3%^ab</td>
<td>9.8%</td>
</tr>
<tr>
<td>DAP-kinase</td>
<td>25%</td>
<td>36.7%</td>
<td>33.9%</td>
</tr>
<tr>
<td>THBS1</td>
<td>10.1%^d</td>
<td>34.7%^e</td>
<td>28.3%^f</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>14.5%^d</td>
<td>36.7%^e</td>
<td>26.7%^g</td>
</tr>
</tbody>
</table>

* Intestinal metaplasia versus gastric adenoma; P > 0.05 (two-tailed Fisher’s exact test).
  
* Intestinal metaplasia versus gastric carcinoma; P < 0.001 (two-tailed Fisher’s exact test).
  
* Gastric adenoma versus intestinal metaplasia, > 0.05, (two-tailed Fisher’s exact test).
  
* Intestinal metaplasia versus carcinoma, P = 0.054, (two-tailed Fisher’s exact test).
  
* NS, not significant.
  
* Chronic gastritis versus intestinal metaplasia, P = 0.002, (two-tailed Fisher’s exact test).
  
* Intestinal metaplasia versus carcinoma, P > 0.05, (two-tailed Fisher’s exact test).
  
* Chronic gastritis versus intestinal metaplasia, P = 0.027, (two-tailed Fisher’s exact test).
  
* Gastric adenoma versus carcinoma, P = 0.008, (two-tailed Fisher’s exact test).
  
* Intestinal metaplasia versus carcinoma, P = 0.036, (two-tailed Fisher’s exact test).
  
* Gastric adenoma versus carcinoma, P = 0.001, (two-tailed Fisher’s exact test).

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In terms of the hypermethylation frequency of the five genes tested, there was no statistically significant difference observed between intestinal metaplasia and gastric adenomas.

Bisulfite genomic sequencing of the representative PCR products of each gene showed that all cytosines at non-CpG sites were converted to thymine. This excluded the possibility that successful amplification could be attributable to incomplete bisulfite conversion. All PCR products of each gene showed extensive methylation of CpG sites that are located inside the amplified genomic fragments. The results of both the MSP and bisulfite sequencing analyses were consistent, indicating that it is appropriate to draw inferences from the results of a methylation-specific PCR assay regarding the methylation status of the gene promoters.

With the results of bisulfite genomic sequencing, we determined the methylation profiles of CpG sites in the MSP products of each gene promoter and compared the methylation density between each step lesion. There was no difference between each step lesion in either the number of methylated CpG sites or the methylation frequency of each CpG site. In the five genes, the vast majority of CpG sites exhibited methylation at a frequency of ≥75% (Fig. 2).

The number of methylated promoters was determined in each sample and defined as the methylation index. The average methylation index for each stage is shown in Table 3. Gastric carcinoma averaged 2.0 per five methylation events per tumor, which is much higher than those of chronic gastritis, intestinal metaplasia, or adenoma (0.5, 1.1, 1.1, respectively; P < 0.05). The average methylation index was similar in both intestinal metaplasia and adenoma but higher than in chronic gastritis (P < 0.05). Samples with a methylation index of ≥4 were seen in gastric carcinoma samples (13 of 64, 20.3%) and not in premalignant lesions.

We examined other normal tissues, including pediatric gastric mucosa, colon mucosa, breast tissue, and bile duct mucosa for the methylation of the three genes (DAP-kinase, THBS1, and TIMP-3) that were hypermethylated in a subset of chronic gastritis to see whether this alteration is a tissue-specific or age-related change. Pediatric gastric mucosa samples were obtained from the pediatric patients with dyspepsia and was histologically diagnosed as chronic gastritis without intestinal metaplasia. Table 4 summarizes the results. DAP-kinase was not hypermethylated in pediatric gastric mucosa, colon mucosa, and breast tissue except for bile duct mucosa (1/11, 9.1%). THBS1 was hypermethylated in pediatric gastric mucosa samples (4 of 40, 10%), normal colon mucosa samples (3 of 34, 8.8%), and bile duct mucosa samples (3 of 11, 27.3%) but not in breast tissue. TIMP-3 was hypermethylated in pediatric gastric mucosa only (6 of 48, 15%). The methylation frequencies of THBS1 and TIMP-3 were not different between chronic gastritis of adults (n = 69; mean age, 48 years) and children (n = 48, mean age, 11 years). DAP-kinase, which was hypermethylated in 25% of adult chronic gastritis samples, was not hypermethylated in pediatric gastric mucosa samples.

**Discussion**

In contrast to genetic alterations, epigenetic change has not been extensively studied because it has been considered to play a minor role in carcinogenesis. However, recent data has altered this view and led our attention to the epigenetic change that occurs in gastric carcinogenesis.

Our previous studies (5, 24) revealed that promoter hypermethylation of p16 and hMLH1 was strongly correlated with the lack of protein expression, indicating promoter hypermethylation as the main inactivation mechanism of these genes in gastric carcinoma. These strong associations have been reported by many other researchers (6, 8, 26). A study using gastric cancer cell lines also demonstrated methylation-associated gene inactivation of TIMP-3 (7). Although hypermethylation of DAP-kinase or THBS1 CpG islands was not studied previously in gastric carcinomas, gene inactivation by hypermethylation of the CpG islands have been reported in other tissues or...
The timing of hypermethylation during tumor development may vary among different genes and tumor types. On the basis of the observation that hypermethylation with the expression loss of hMLH1 was present in invasive gastric carcinoma but not in adjacent dysplastic tissues, an earlier study suggested that this was a late event in gastric carcinogenesis (6). However, the present study has shown that hMLH1 hypermethylation occurs in both intestinal metaplasia and gastric adenoma, although its frequency is low. When we performed immunohistochemical staining of hMLH1 protein on gastric adenomas with hMLH1 hypermethylation, one of six adenomas clearly demonstrated an absence of hMLH1 protein expression. This case showed allelic alterations of BAT-26 (data not shown). MSP is so sensitive that even one methylated allele in 100,000 unmethylated alleles can be detected (25). Therefore, it is quite probable that hMLH1 hypermethylation has a subclonal event in gastric adenoma and may not be detected by immunohistochemical staining in the five gastric adenomas.

Our results have demonstrated a 4-fold increase in the p16 hypermethylation frequency from gastric adenoma (11.5%) to gastric carcinoma (42.2%), suggesting that p16 hypermethylation plays a role in the malignant transformation. p16 hypermethylation in non-neoplastic gastric mucosae have been described in some studies (27, 28), although these studies did not provide the genetic alterations that have been found in intestinal metaplasia and gastric adenoma. Although the detailed histological features of the nonneoplastic gastric mucosae. The present study demonstrated p16 hypermethylation in intestinal metaplasia, but the frequency was very low (1 of 48 samples, 2.1%).

In the present study, intestinal metaplasia showed a higher hypermethylation index than that of chronic gastritis. Other studies have reported that genetic alterations have been found in intestinal metaplasia, including p53 or K-ras mutations and allelic losses of several loci (22, 23, 29). These findings suggest that a portion of intestinal metaplasia may have epigenetic change or genetic alterations and act as premalignant lesions. Hypermethylation of three genes was observed in 10.2% of intestinal metaplasia samples in the present study, and these cases might have a higher risk of developing gastric carcinomas. A prospective longitudinal study is required to confirm this possibility.

In contrast with the alleged phenotypic sequential change from intestinal metaplasia to gastric adenoma, our results have demonstrated similar methylation indices in both the intestinal metaplasia and gastric adenoma. This raises the possibility that intestinal metaplasia may not be a prestage for gastric adenoma in the CpG island methylation pathway. However, considering the limited number of assessed genes, methylation analysis of more candidate genes with CpG islands is necessary to clarify this issue. On the basis of the significant difference in the methylation index between gastric carcinoma and premalignant lesions, it can be speculated that accumulation of inactivated genes by aberrant methylation is important for malignant transformation from premalignant lesions.

The data in the present study showed less frequent methylation in early-step lesions and a statistically significant trend toward increasing methylation along the multistep carcinogenesis. This raises a possibility that more advanced malignancies may have acquired additional epigenetic changes during tumor progression. However, when we analyzed the correlation between the methylation index and Tumor-Node-Metastasis stage of gastric carcinomas, there was no statistical significance (data not shown). Furthermore, 16 (25%) of 64 gastric carcinomas had no methylation of the five tested genes. These findings argue against the acquisition of hypermethylation during tumor progression of gastric carcinomas. A previous study (28) reported that gastric carcinomas with hypermethylation of multiple loci had a relatively earlier stage than gastric carcinomas with no or less frequent hypermethylation.

The analysis of other normal tissue samples for the methylation status of DAP-kinase, THBS1, and TIMP-3 demonstrated hypermethylation of DAP-kinase or THBS1 or TIMP-3 in a small percentage of normal bile duct mucosa and colon mucosa but not in normal breast tissue. TIMP-3 was not methylated in these normal tissues. The results showed a tissue-type-specific difference of gene hypermethylation. This was reported in E-cadherin, which was frequently hypermethylated in normal gastric mucosa but rarely in normal esophageal mucosa (30). When the hypermethylation frequency of the three genes was compared between adult and pediatric chronic gastritis samples, DAP-kinase was not hypermethylated in pediatric samples, but there was no difference in the hypermethylation frequency of THBS1 or TIMP-3. This suggests that in gastric mucosa, DAP-kinase hypermethylation may be an age-related change.

In conclusion, we have studied promoter hypermethylation of several genes and determined the frequency and chronology of hypermethylation during multistep carcinogenesis from chronic gastritis to gastric carcinoma. We found that aberrant CpG island hypermethylation occurred in early stages and tended to increase along the multistep gastric carcinogenesis, although the timing and frequency of hypermethylation varied according to the gene.
CpG ISLAND METHYLATION IN PREMALIGNANT GASTRIC LESIONS

References

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