Impact of Catechol-\textit{O}-methyltransferase (\textit{COMT}) Gene Polymorphism on Promoter Methylation Status in Gastric Mucosa

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\textbf{Abstract.} DNA methylation is one of the major events in the early process of gastric carcinogenesis and also occurs in non-neoplastic gastric mucosa. Catechol-\textit{O}-methyltransferase (\textit{COMT}) catalyzes the methylation of various endobiotic and xenobiotic substances, and protects DNA from oxidative damage. The association between a common functional polymorphism of \textit{COMT} Val158Met and DNA methylation status in the stomach was investigated. Patients and Methods: One hundred and sixty-nine gastric mucosa samples from non-cancer patients were obtained by endoscopy. The promoter methylation status of \textit{p14} and \textit{p16} was determined by methylation-specific PCR (MSP). The \textit{COMT} Val158Met polymorphism was detected by PCR-restriction fragment length polymorphism (RFLP). Results: \textit{CpG} island methylation was observed in 32.5\% of the \textit{p14}, and 37.9\% of the \textit{p16}. The methylation status of both \textit{p14} and \textit{p16} was not associated with gender or age, while \textit{p16} methylation was strongly associated with \textit{Helicobacter pylori} infection (OR=4.71, 95\% CI=2.35-9.46, \textit{p}<0.0001). The \textit{Val/Val} genotype held a significantly higher risk of \textit{p16} methylation (OR=3.27, 95\% CI=1.05-10.25, \textit{p}=0.0418). Conclusion: The \textit{COMT} polymorphism may influence the susceptibility to gene methylation in the gastric mucosa. The promoter \textit{CpG} island of \textit{p16} gene, but not of \textit{p14} may be one of the specific regions whose methylation is closely influenced by the \textit{COMT} polymorphism.

Aberrant DNA methylation is an important mechanism in gene silencing. In many kinds of cancer, some genes seem to acquire aberrant methylation in their \textit{CpG} islands. Some genes are also methylated in non-neoplastic tissues with aging and this alteration is known as age-related methylation (1, 2). In addition, it has been shown that gene methylation may be present in non-neoplastic colorectal mucosa in patients with inflammatory bowel disease (3, 4), esophageal mucosa in patients with Barrett’s esophagitis (5, 6) and liver tissues in chronic hepatitis (7).

In the non-neoplastic human gastric mucosa, frequencies or levels of \textit{CpG} islands methylation of certain genes correlate with \textit{Helicobacter pylori} infection (8, 9), histological or serological severity of gastritis and gastric cancer occurrence (10-13), suggesting that aberrant DNA methylation is one of the major events which occurs early in the process of tumorgenesis in the stomach. These epigenetic events in the gastric mucosa may lead to transcriptional inactivation in specific genes and increase DNA damage or mutation and chromosomal instability.

\textit{p16(INK4a)} and \textit{p14(ARF)} are involved in the negative cell cycle regulation via the \textit{pRb} and \textit{p53} pathways, respectively. The genes for these two proteins have an independent first exon (exon 1a and 1B, respectively) but share exons 2 and 3 (14, 15). Methylation of \textit{p16} and \textit{p14} has been shown to be present in gastric cancer as well as premalignant lesions (16, 17). Thus, both genes may play crucial roles in cell cycle control, apoptosis and DNA repair in the stomach and their disorder may be closely associated with gastric carcinogenesis.

\textbf{Abbreviations:} \textit{COMT}, Catechol-\textit{O}-methyltransferase; MSP, methylation-specific polymerase chain reaction.

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\textbf{Key Words:} Methylation, \textit{Helicobacter pylori}, gastric mucosa, \textit{COMT}, polymorphism.
Catechol-O-methyltransferase (COMT) is expressed in various mammalian tissues including the stomach (18-20) and has been shown to catalyze the methylation of various endobiotic and xenobiotic substances preventing quinine formation and redox cycling, and therefore might protect DNA from oxidative damage (21, 22). A G to A transition, which results in an amino acid change from valine to methionine at codon 158, leads to COMT activity of the Met/Met genotype which is a quarter of that of the wild genotype, and heterozygous individuals exhibit intermediate enzyme activity (23).

Because of the important role that COMT plays with respect to preventing DNA damage, the polymorphism of COMT may influence the susceptibility to methylation in the human gastric mucosa.

In the present study, the methylation status of $p14$ and $p16$ in non-neoplastic gastric mucosa samples and its relation to the COMT Val158Met polymorphism was investigated.

**Patients and Methods**

*Tissue samples and DNA extraction.* The study population comprised 169 non-cancer patients, attending the Endoscopy Center of Fujita Health University Hospital from January 2005 to July 2007. All patients underwent upper gastroscopy as part of a health check, as a secondary procedure following barium X-ray examination for suspected stomach cancer, or for the investigation of abdominal discomfort. Patients who had severe systemic disease, or malignancy in the stomach or other organ were excluded from this study. Biopsy specimens were taken from the antrum along the greater curvature, from grossly non-pathological mucosa in all patients. The specimens were cut into two pieces. One of the pieces was fixed in 10% buffered formalin and embedded in paraffin for microscopic histological examination and the other part was immediately frozen and stored at −80°C until use. Histological analysis of all the selected biopsy samples also showed that these samples contained more than 70% of epithelial cells. Genomic DNA was extracted directly from the frozen specimens using a standard phenol/chloroform method. *H. pylori* infection status was assessed by serological, histological analysis or urea breath test. Patients were diagnosed as infected when at least one of the diagnostic tests was positive. The Ethics Committee of the Fujita Health University School of Medicine approved the protocol and prior, written informed consent was obtained from all participants.

**Bisulfite modification and methylation-specific PCR (MSP).** For the examination of DNA methylation, the genomic DNA was treated with sodium bisulfite using a BislFast DNA Modification Kit for Methylated DNA Detection (Toyobo, Co. Ltd., Osaka, Japan). MSP was carried out with the following primers: $p14$ methylated forward (MF), 5′-gtgtauggagggtggagc-3′ and $p14$ methylated reverse (MR), 5′-aaaaaccaactcggagca-3′ which amplify a 122-bp product; $p14$ unmethylated forward (UP), 5′-ttttgtgtaaaggtgttgagc-3′ and $p14$ unmethylated reverse (UR), 5′-cacaacacctctggagcagaa-3′ which amplify a 132-bp product (24); $p16$ methylated forward (MF), 5′-ttttaggggtggtggtggattg-3′ and $p16$ methylated reverse (MR), 5′-accaccagcgccaaccgagtaa-3′ which amplify a 149-bp product; $p16$ unmethylated forward (UF), 5′-tttttaggggtggtggtggattg-3′ and $p16$ unmethylated reverse (UR), 5′-ccaccccccatccctggagca-3′ which amplify a 151-bp product (25). The annealing temperature and times were determined using DNA from the peripheral blood of a young individual without *H. pylori* infection and DNA methylated with SsiI methylase (New England BioLabs Inc., Beverly, MA, USA). The MSP was carried out in a volume of 21 μL containing 0.1 μg of bisulfite-modified DNA. The DNA was denatured at 95°C for 5 minutes, followed by 33–35 cycles at 95°C for 30 s and according to the primers for 1 minute, and 72°C for 1 minute with a final extension at 72°C for 5 minutes. The MSP reactions were conducted using EX Taq HS (Takara Bio Inc., Shiga, Japan). The bands of MSP were detected by electrophoresis in 2.5% agarose gels stained with ethidium bromide (Figure 1).

**COMT Val158Met genotypes.** Using genomic DNA, the polymorphism at codon 158 in the COMT gene was determined by PCR-restriction fragment length polymorphism (RFLP) assays. The PCR primers 5′-tcgaggacccgactgattg-3′ and 5′-agttgctgaccaagttcagc-3′ were used to amplify a 217-bp fragment of COMT that contains the polymorphic NlaIII site as well as one other constant NlaIII site. PCR was performed in a reaction volume of 25 μL containing 200 ng of genomic DNA, 10 pmol of each primer, 200 ng of each dNTP and 0.6 units Taq DNA polymerase (Toyobo). The reaction mixture was first denatured at 95°C for 5 min and then amplified by PCR for 32 cycles at 95°C for 30 s, at 55°C for 30 s, and at 72°C for 1 min, followed by a 10 min extension at 72°C. Twelve μL of PCR product were incubated with 10 units of NlaIII (New England Biolabs) in a volume of 20 μL at 37°C overnight. NlaIII cuts only the low-activity variant (COMT158Val) in addition to a second constant cleavage site in the PCR product. Thus, homozygotes for COMT158Val generated fragments of 136 and 81bp, heterozygotes gave 136, 96, 81, and 40 bp fragments and homozygotes for COMT158Met generated 96, 81, and 40-bp fragments. The 40bp fragment ran off the gel during electrophoresis. The 3 genotypes were scored after running on a 3.5% agarose gel with ethidium bromide 10 μg/mL.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (mean±SD)</strong></td>
<td>60.0±13.1</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>94/75</td>
</tr>
<tr>
<td><em>H. pylori</em> infection (+/−)</td>
<td>77/92</td>
</tr>
<tr>
<td>Active ulcer disease</td>
<td>13</td>
</tr>
</tbody>
</table>

**Table I. Characteristics of the 169 patients.**
Although or both associated with methylation, the Met/Met genotype was significantly lower than 0.05 was considered statistical significant. The prevalence of patients in whom either p14 or p16 was methylated was also investigated.

### Table II. Association between p14 and p16 promoter methylation and age, gender, H. pylori infection and COMT Val158Met genotypes.

<table>
<thead>
<tr>
<th>Variable (n)</th>
<th>Age (Mean±SD) (years)</th>
<th>Gender (n)</th>
<th>H. pylori infection (n)a</th>
<th>COMT genotype (n)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>p14</td>
<td></td>
<td>Male (94)</td>
<td>Female (75)</td>
<td>Val/Val (80)</td>
</tr>
<tr>
<td>Unmethylated (114)</td>
<td>58.5±13.3</td>
<td>66</td>
<td>48</td>
<td>56</td>
</tr>
<tr>
<td>Methylated (55)</td>
<td>63.2±12.0</td>
<td>28</td>
<td>27</td>
<td>47</td>
</tr>
<tr>
<td>p16</td>
<td></td>
<td></td>
<td></td>
<td>Val/Met (75)</td>
</tr>
<tr>
<td>Unmethylated (105)</td>
<td>60.5±14.0</td>
<td>59</td>
<td>46</td>
<td>49</td>
</tr>
<tr>
<td>Methylated (64)</td>
<td>59.4±11.5</td>
<td>35</td>
<td>29</td>
<td>51</td>
</tr>
<tr>
<td>p14 or p16</td>
<td></td>
<td></td>
<td></td>
<td>Met/Met (14)</td>
</tr>
<tr>
<td>Unmethylated (69)</td>
<td>58.3±14.5</td>
<td>42</td>
<td>27</td>
<td>49</td>
</tr>
<tr>
<td>Either methylated (100)</td>
<td>61.2±11.9</td>
<td>52</td>
<td>48</td>
<td>45</td>
</tr>
<tr>
<td>Both p14 and p16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmethylated (150)</td>
<td>60.0±13.3</td>
<td>83</td>
<td>67</td>
<td>68</td>
</tr>
<tr>
<td>Both methylated (19)</td>
<td>60.6±11.6</td>
<td>11</td>
<td>8</td>
<td>12</td>
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</table>

p16, + vs. –: OR=4.71, 95% CI=2.35-9.46, p<0.0001; p14 or p16, + vs. –: OR=2.89, 95% CI=1.53-5.45, p=0.001; Both p14 and p16, + vs. –: OR=5.19, 95% CI=1.45-18.57, p=0.006; bp16, Met/met vs. Val/Val+Val/Met, OR=3.27, 95% CI=1.05-10.25, p=0.0418.

### Statistical analysis
Statistical analysis was conducted with two-sided chi-square for the comparison of promoter DNA methylation frequencies of p14 and p16 between two groups. The association between DNA methylation status of p14 and p16 and age was examined by the Mann-Whitney U-test. A probability value of less than 0.05 was considered statistical significant.

### Results

#### Study population
The characteristics of the participants is shown in Table I. After gastroscopy, 13 patients (7.7%) were diagnosed as having active peptic ulcer disease.

#### Association between methylation of p14 and p16 and gender, age, H. pylori infection and COMT Val158Met polymorphism
All 169 gastric mucosa samples were available for MSP analysis and COMT genotyping. CpG island methylation of p14 was observed in 55 (32.5%) and of p16 in 64 subjects (37.9%). The methylation status of both p14 and p16 was not associated with gender or age, while p16 methylation was strongly associated with H. pylori infection (OR=4.71, 95% CI=2.35-9.46, p<0.0001). The COMT genotype distribution in the 169 participants was 80 Val/Val (47.3%), 75 Val/Met (44.4%) and 14 Met/Met (8.3%).

### Discussion

Methylation of p16 was strongly associated with H. pylori infection. This observation is in line with previous studies (8-13). In regard to the CpG sites, the Met/Met genotype held a significantly higher risk of p16 rather than p14 promoter methylation.

The amino acid change at codon 158 (Val to Met) of COMT results in lower thermostability, with enzyme activity that is up to four times less than that from the wild-type allele (26). The presence of the Val/Val genotype has been considered to be favorable because it seems to lower the risk of developing non-Hodgkin lymphoma and estrogen-associated carcinomas in women (27-29), and because it is associated with a higher tendency to remain free from an increase in prostate-specific antigen in men with prostate cancer (30).

The mechanisms of gene methylation are unknown. Several factors may contribute to this methylation, such as exogenous carcinogens, generated reactive oxygen, and host genetic differences (31). One of the most important factors causing oxidative stress in the gastric mucosa is H. pylori infection, which induces chronic inflammation (32, 33). Indeed, the methylation of certain genes in non-neoplastic gastric mucosa correlates with H. pylori infection (8, 9), histological or serological severity of gastritis and
gastric cancer occurrence (11-13). However, not all patients with \textit{H. pylori} infection or gene methylation develop gastric cancer. This difference may be attributed to some genetic factors. The present results provided the first evidence that the genetic polymorphisms of \textit{COMT} may also be involved in DNA methylation in human gastric mucosa. Although the activity of \textit{COMT} in the serum or gastric mucosa was not investigated, it is possible that the \textit{COMT} polymorphism influences the activity and modifies the risk of DNA methylation in the gastric mucosa, and thus, influences the susceptibility to methylation-related carcinogenesis.

Meanwhile, no significant association between \textit{COMT} polymorphism and \textit{p14} methylation status was found. In addition, the \textit{COMT} genotype was also not associated with methylation of either \textit{p14} or \textit{p16}. The promoter CpG island of \textit{p16} gene, but not of \textit{p14} may be one of the specific regions whose methylation is closely influenced by \textit{COMT} polymorphism. However, why the interplay between \textit{COMT} polymorphism and aberrant hypermethylation is different in different genes is still unexplained. Only a more extensive understanding of the regulation of methylation in relation to gene expression and carcinogenesis will allow us to fully interpret our findings.

References


