The Glu346Lys polymorphism and frameshift mutations of the *Methyl-CpG Binding Domain 4* gene in gastrointestinal cancer


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MBD4 (Methyl-CpG Binding Domain 4) is a human DNA repair protein that may be involved in DNA mismatch repair. The polymorphisms and frameshift mutations in *MBD4* may influence cancer susceptibility and the development of cancer. The specific aim of this study was to investigate whether frameshift mutations of the *MBD4* gene and the codon 346 polymorphism were associated with microsatellite instability (MSI) and the risk for gastrointestinal cancer. We examined the MSI, frameshift mutations and polymorphisms of the *MBD4* gene in 84 patients with gastric cancers, 82 colorectal cancers and 299 healthy controls. MSI was found in 19 (22.6%) and 26 (31.7%) of the gastric and colorectal cancer samples, respectively. The mutation analysis revealed no frameshift mutations in the *MBD4* gene among the gastrointestinal cancers. The frequencies of genotypes: Glu/Glu, Glu/Lys and Lys/Lys were 41.7% (35/84), 41.7% (35/84) and 16.6% (14/84), respectively, in the gastric cancer cases, and 42.7% (35/82), 36.6% (30/82) and 20.7% (17/82), respectively, in the colorectal cancers. MSI was not associated with the *MBD4* codon 346 polymorphism and there was no significant difference in the frequency of the genotypes between healthy controls and gastric cancer patients (*P*=0.2748). However, the *MBD4* codon 346 polymorphism was significantly associated with the risk of colorectal cancer (*P*=0.0315). Our findings suggest that microsatellite instability may not be associated with frameshift mutations in the *MBD4* gene, and that the *MBD4* codon 346 polymorphism may play a role in colorectal cancer susceptibility in the Korean population.

Key words: MBD4, polymorphism, MSI, gastric cancer, colorectal cancer

DNA sequences referred to as microsatellites are one to six nucleotide motifs that are randomly repeated numerous times in the human genome. The instability of microsatellite repeated sequences has shown variation in the repeated sequence length of cancer specimens compared to normal DNA from the same patient [1]. Microsatellite instability (MSI) underlies the hereditary non-polyposis colorectal cancer syndrome that occurs due to a germline mutation in one of the mismatch repair genes [2–4]. In addition, MSI has been reported in breast cancer [5], non-small cell lung cancer [6], gastric cancer [7], and colorectal cancers [8], suggesting that MSI is a common genetic alteration in human cancers. The MSI phenotype is known to be caused either by mutations in the mismatch repair genes or by aberrant methylation of these genes [9, 10]. MSI affects not only intergenic non-coding repeated sequences, but also intronic and coding mononucleotide repeats, resulting in frameshift mutations in numerous genes [11–13].

The Methyl-CpG Binding Domain 4 (*MBD4*) protein is a methyl-CpG-binding endonuclease involved in the recognition of T/U-G mismatches at methyl-CpG nucleotides [14, 15]. The inactivation of *MBD4*, in mice, was shown to increase the mutation frequency by a factor of three and subsequently the formation of tumors [16]. Although inactivation of *MBD4* by itself is not responsible for cancer predisposition in knockout mice, the combination of an *MBD4* deficiency with a germline mutation in the *APC* gene has been shown to be associated with an increased number of tumors in the gastrointestinal tract and accelerated tumor progression [17]. *MBD4* plays an important role in the maintenance of genomic integrity by affecting three biological processes: base excision repair, DNA mismatch repair and the cell-cycle response to DNA damage [18]. Recently, frameshift mutations in the *MBD4* gene have been identified frequently in Japanese gastric MSI-H cancers [19]; however, these mutations were rare in Portuguese gastric cancers [20]. In addition, it has been suggested that polymorphisms of the *MBD4* gene may be associated with varying DNA repair capacity, as well as the risk for primary lung cancer and esophageal squamous cell carcinoma [21, 22]. Among the identified *MBD4* polymorphisms, the Glu346Lys (rs140693) polymorphism in exon 3 of the *MBD4* gene has
been associated with susceptibility to cancer [21, 22]. Thus, we hypothesized that frameshift mutations at the mononucleotide (A)10 repeat or a single nucleotide polymorphism (SNP) at codon 346, in exon 3 of the MBD4 gene, may be associated with the development of gastrointestinal cancers.

To determine whether frameshift mutations of the MBD4 gene are associated with the development of gastrointestinal cancers, we analyzed the presence of MSI and MBD4 mutations in 84 sporadic gastric carcinomas and 82 colorectal cancers. In addition, we evaluated the association between a SNP at codon 346 of the MBD4 gene and the risk of gastrointestinal cancer.

Materials and methods

Samples. A total of 84 methacarn-fixed gastric cancer specimens were examined in this study. Three tumors were early gastric cancers that were limited to the gastric mucosa and submucosa. Eighty-one tumors were advanced gastric cancers that invaded through the muscularis propria into the serosa. Histologically, 47 cases were the intestinal-type, and 37 were the diffuse-type of gastric cancer. For colorectal cancer, eighty-two methacarn-fixed and paraffin-embedded colorectal cancers were enrolled in this study. Twelve cases were from the right-side of the colon and 70 the left-side. No patient had a family history of gastric or colorectal cancer. The healthy control population consisted of 163 males and 136 females with a mean age of 44. To exclude ethnic differences, only Korean persons were included in this study. Informed consent was obtained from all of the patients whose tumors were used in the study, according to the Declaration of Helsinki. Approval was obtained from the institutional review board of The Catholic University of Korea, College of Medicine.

Microdissection and DNA extraction. The tumor cells were selectively procured from Hematoxylin & Eosin stained slides using a laser microdissection device (ION LMD, JungWoo University of Korea, College of Medicine). The surrounding normal gastric and colonic mucosal cells were also obtained to study the corresponding normal DNA from the same slides in all cases. DNA extraction was performed using a modified single step DNA extraction method, as described previously [23].

Single strand conformation polymorphism (SSCP) and DNA sequencing. Genomic DNAs from the tumor cells and corresponding normal cells were amplified with primers covering the mononucleotide (A)10 repeat and the E346K polymorphism in exon 3 of the MBD4 gene. The PCR primer pairs were as follows: 5’-AGACCCCTCAGTGTACAGTG-3’ and 5’-TTTGTTCGGATTTCTTCAGATTCT-3’. Numbering of the sequences from MBD4 was done with respect to the ATG start codon according to the genomic sequence from Genbank accession no. NM_003925. All cases were screened by SSCP analysis for SNPs, and the presence of aberrant bands from the tumor DNA were compared to the normal DNA. Each polymerase chain reaction (PCR) procedure was performed under standard conditions in a 10 µl reaction mixture containing 1 µl of the template DNA, 0.5 µM of each primer, 0.2 µM of each deoxynucleotide triphosphate, 1.5 mM MgCl₂, 0.4 unit of Ampli Taq gold polymerase (Perkin-Elmer, Foster City, CA, USA), 0.5 µCi of [³²P]dCTP (Amersham, Buckinghamshire, UK), and 1 µl of 10X buffer. The reaction mixture was denatured at 94°C for 12 min and then incubated for 35 cycles (denaturing at 94°C for 40 s, annealing at 56°C for 40 s and extension at 72°C for 40 s). A final extension step at 72°C was performed for 5 min. After amplification, the PCR products were denatured for 5 min at 95°C in a 1:1 dilution of sample buffer containing 98% formamide/5 mmol/L NaOH. These products were loaded onto a SSCP gel (FMc Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT, USA) containing 10% glycerol. After electrophoresis, the gels were transferred to 3 MM Whatman paper and dried. Autoradiography was then performed using Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). DNA showing mobility shifts were cut out of the dried gels and amplified for 35 cycles using the same primer sets. DNA sequencing, of the cases showing mobility shifts on the SSCP gel, was carried out using the fluorescent dideoxy chain termination method with an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions.

Microsatellite status. A standard panel of microsatellite markers, including mononucleotide (BAT-25 and BAT-26) and dinucleotide (D2S123, D5S346, and D17S250) repeats were used according to the Bethesda Guidelines for MSI testing [8]. Primers specific for each locus were used to amplify repeats and short flanking sequences in the template DNA by PCR. PCR amplification was performed with 300 nM of primers each with 1.5 mM MgCl₂, 100 µM deoxynucleotide triphosphate, 0.25 U of Taq polymerase, and 1 µl of DNA in a total volume of 20 µl. The PCR conditions were 95°C for 5 min followed by 35 cycles (95°C for 30 s, 47-62°C for 40 s and 72°C for 40 s), and a final elongation step at 72°C for 10 min. After the PCR, the samples were denatured in a buffer containing 95% formamide, 10 mM EDTA (pH 8.0), 0.05% xylene cyanol, and 0.05% bromophenol blue and heated to 95°C for 10 min followed by rapid cooling. The samples were then electrophoresed on 6% denaturing polyacrylamide gels containing 7 M urea for 3-4 hours at 60 W. After the electrophoresis, the gels were dried and exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA) for 12-24 hrs. All scoring was performed independently by two observers. A case was considered MSI-positive when at least 1 locus had a different mobility band (Fig. 1). A “high and low degree of MSI” (MSI-H and MSI-L) was defined as the presence of instability in ≥30% and <30% of the markers, respectively. The cases without an unstable marker were considered as “microsatellite stable” (MSS). All MSI-positive loci were confirmed by additional PCR and electrophoresis.

Statistical analysis. The Chi-square test was used to test the relationship between SNPs of the MBD4 gene and the MSI status of the gastrointestinal cancer, and the risk of gastrointestinal cancers. P values <0.05 were considered statistically significant.
The MBD4 polymorphism and mutations in gastrointestinal cancer

Results

Microsatellite status. We analyzed the allelic profiles of BAT-25, BAT-26, D2S123, D5S346, and D17S250 to establish the microsatellite status of the 84 gastric and 82 colorectal carcinomas. With these markers, we could assess the microsatellite status directly with the corresponding normal DNA. Because the BAT-25 and -26 allelic size variation of the DNA from normal individuals was in the range of 1 bp to 2 bp, tumors were considered to be MSI-positive, for these markers, when they showed allelic size variation of more than 2 bp.

The number of MSI-positive and MSS tumors for each cancer type is summarized in Table 1. Aberrantly migrating bands, on the sequencing gels, led to the identification of MSI in 19 (22.6%) and 26 (31.7%) of the gastric and colorectal cancer samples, respectively. MSI-H was found in 15 and 9 of the gastric and colorectal cancers, respectively.

Mutational analysis of the MBD4 gene. The presence of frameshift mutations, resulting in truncation of the corresponding protein, at the mononucleotide (A)10 tract, in exon 3, of the MBD4 gene was investigated using PCR-based SSCP and sequencing analysis. There was no aberrant SSCP pattern identified in this region. This finding suggests that there was no frameshift mutation of the MBD4 gene in the gastric and colorectal cancers. The experiments, including tissue microdissection, PCR, SSCP, and sequencing analysis, were repeated to ensure the reliability of the results (data not shown).

Single nucleotide polymorphism analysis of the MBD4 gene. The genotype frequencies of the MBD4 codon 346 polymorphism, from the Korean gastric and colorectal cancer patients and controls, are summarized in Table 2. The frequencies of genotypes Glu/Glu, Glu/Lys and Lys/Lys were 41.7% (35/84), 41.7% (35/84) and 16.6% (14/84), respectively, for the gastric

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Table 1. Genotype frequency of the MBD4 Glu346Lys polymorphism and microsatellite instability in gastrointestinal cancers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gastric cancer (n=84)</th>
<th>Colorectal cancer (n=82)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSS</td>
<td>MSI-L</td>
</tr>
<tr>
<td>GG</td>
<td>25 (29.8%)</td>
<td>4 (4.8%)</td>
</tr>
<tr>
<td>GA</td>
<td>29 (34.5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>AA</td>
<td>11 (13.1%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>65 (77.4%)</td>
<td>4 (4.8%)</td>
</tr>
</tbody>
</table>

*MSS, microsatellite stability
*MSI-L, microsatellite instability-low
*MSI-H, microsatellite instability-high
*Compared MSI with the frequencies of MBD4 genotype or allele

Table 2. Distribution of the MBD4 genotype in gastrointestinal cancer patients and controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy controls (n=299)</th>
<th>Gastric cancer patients (n=84)</th>
<th>Colorectal cancer patients (n=82)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Glu/Glu</td>
<td>Glu/Lys</td>
<td>Lys/Lys</td>
</tr>
<tr>
<td>Male</td>
<td>71 (23.7%)</td>
<td>70 (23.4%)</td>
<td>22 (7.4%)</td>
</tr>
<tr>
<td>Female</td>
<td>58 (19.4%)</td>
<td>69 (23.1%)</td>
<td>9 (3.0%)</td>
</tr>
<tr>
<td>Age</td>
<td>Glu/Glu</td>
<td>Glu/Lys</td>
<td>Lys/Lys</td>
</tr>
<tr>
<td>≤50</td>
<td>101 (33.8%)</td>
<td>117 (39.1%)</td>
<td>26 (8.7%)</td>
</tr>
<tr>
<td>&gt;50</td>
<td>28 (9.3%)</td>
<td>22 (7.4%)</td>
<td>5 (1.7%)</td>
</tr>
<tr>
<td>Histology</td>
<td>Glu/Glu</td>
<td>Glu/Lys</td>
<td>Lys/Lys</td>
</tr>
<tr>
<td>Diffuse</td>
<td>12 (14.3%)</td>
<td>15 (17.9%)</td>
<td>10 (11.9%)</td>
</tr>
<tr>
<td>Intestinal</td>
<td>23 (27.4%)</td>
<td>20 (23.8%)</td>
<td>4 (4.7%)</td>
</tr>
<tr>
<td>Site</td>
<td>Glu/Glu</td>
<td>Glu/Lys</td>
<td>Lys/Lys</td>
</tr>
<tr>
<td>Left</td>
<td>129 (43.1%)</td>
<td>139 (46.5%)</td>
<td>31 (10.4%)</td>
</tr>
<tr>
<td>Right</td>
<td>3 (3.7%)</td>
<td>3 (3.7%)</td>
<td>6 (7.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>132 (44.8%)</td>
<td>142 (47.9%)</td>
<td>37 (12.3%)</td>
</tr>
</tbody>
</table>
cancer cases and 42.7% (35/82), 36.6% (30/82) and 20.7% (17/82), respectively, for the colorectal cancers. In healthy individuals, the genotype frequencies were 43.1% (129/299), 46.5% (139/299) and 10.4% (31/299), respectively. For gastric cancer, there was no significant difference in the genotype frequencies between gastric cancer patients and the healthy controls (P=0.2748). When stratified by the histological type of gastric cancer, there were no statistically significant differences observed. There was a tendency for the Lys/Lys genotype to be more common in colorectal cancer patients than in the controls. A significant difference was observed in the genotype frequency of the MBD4 codon 346 polymorphism between the healthy controls and the colorectal cancer patients (P=0.0315).

When we compared the frequency of MSI with the genotype or allele frequency of the MBD4 codon 346 polymorphism (Table 1), there was no statistically significant difference for either the gastric cancer (P=0.5168 and P=0.3912) or the colorectal cancer cases (P=0.1453 and P=0.5569).

Discussion
Since tumors with MSI accumulate hundreds or thousands of mutations in mononucleotide repeats throughout the genome, the detection of insertion or deletion mutations in these mononucleotide repeats is indicative of widespread genomic damage with likely functional significance for the development of cancer [24, 25]. Frequent mutations in the mononucleotide (A)10 repeat of exon 3 of MBD4 have been previously described in MSI-H gastrointestinal cancers [19, 26]. However, rare mutations in the repeat region of the MBD4 gene, in MSI-positive gastric cancers, have also been reported [20]. This discrepancy led us to analyze the presence of MSI and frameshift mutations in MBD4 containing mononucleotide repeat mutations in gastrointestinal cancers. MSI-positive cancers were found in 19 out of 84 gastric cancers and 26 out of 82 colorectal cancers (Table 1). However, the MBD4 frameshift mutation was not detected in either the gastric or colorectal cancers. This finding suggests that a MBD4 frameshift mutation may not be associated with deficient DNA mismatch repair in Korean gastrointestinal cancers. Although there are several possible explanations for the above mentioned discrepancy in the frequency of MBD4 mutations, including racial differences in mutations, differing genetic backgrounds and different etiologies for the gastrointestinal cancers, our findings do not support a significant role for MSI of the MBD4 gene in Korean gastrointestinal tumorigenesis.

The association between this polymorphism and cancer risk has been examined in primary lung cancer and esophageal squamous cell carcinoma [21, 22]. However, the association of this polymorphism with the risk for gastrointestinal cancer has not been previously studied. In the present study, we found that there was a significant difference in the frequency of the MBD4 codon 346 genotype between healthy controls and colorectal cancer patients (P=0.0315), but not gastric cancer patients (P=0.2748). When stratified by histological type of gastric cancer, there was no significant difference in the MBD4 genotype frequency between the intestinal- and diffuse-type tumors (P=0.0598). The Lys/Lys homozygote carried a nearly two-fold increased risk, compared with the Glu/Glu homozygote. Therefore, our results suggested that the MBD4 Glu346Lys genotype was associated with an increased risk for colorectal cancer in Korean patients and may be an important factor in the predisposition for developing colorectal cancer. A previous study reported that the genotype distributions associated with the Glu carrier genotype corresponded to a significant increase in lung cancer risk [21]. We found that a Lys homozygous genotype was a risk factor for the development of colon cancer. Although the functional significance of this polymorphism requires further study, it is likely that different results are due to different cancer types, ethnic differences, environmental factors, and the relatively small number of cases included in studies, including this report.

In addition, we analyzed the association between MSI and the MBD4 Glu346Lys polymorphism in the gastric and colorectal cancers. There was no statistically significant difference between the MSI and MBD4 Glu346Lys polymorphism in the cases studied. This result suggests that the MBD4 Glu346Lys polymorphism may not be associated with the genomic stability of gastrointestinal cancer cells.

Even with the small number of cases included in this study, our findings suggest that frameshift mutations in the MBD4 gene may not play an important role in gastrointestinal cancers with MSI, and that the Glu346Lys polymorphism may be associated with the risk of colorectal cancer. Additional studies with a larger patient cohort are needed to confirm these initial observations. In addition, a functional analysis of the MBD4 Glu346Lys polymorphism is needed to broaden our understanding of the molecular pathogenesis of gastrointestinal cancer.

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References


