CpG Island Methylation of the MLH1, MGMT, DAPK, and CASP8 Genes in Cancerous and Adjacent Noncancerous Stomach Tissues

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Key Words: stomach cancer; methylation; MLH1; DAPK; MGMT; CASP8.

Summary. Background and Objective. Many factors are involved in the development of gastric adenocarcinoma. The CpG island methylation of apoptosis and mismatch repair genes by the loss of their function is important in gastric adenocarcinoma. The aim of this study was to determine the methylation frequency of MLH1, MGMT, CASP8, and DAPK in cancerous and adjacent noncancerous stomach tissues, to determine possible associations with the selected clinicopathological characteristics, and to identify possible correlation between the methylation of individual genes.

Material and Methods. The methylation status of MLH1, MGMT, DAPK, and CASP8 was investigated in 69 patients with gastric adenocarcinoma by using methylation-specific polymerase chain reaction. The associations between patients' clinical characteristics and methylation status were assessed.

Results. The methylation frequency of the MLH1, DAPK, MGMT, and CASP8 gene promoters in cancerous and adjacent noncancerous tissues was 31.9% and 27.5%; 47.8% and 46.4%; 36.2% and 44.9%; and 5.8% and 5.8%, respectively, but the differences were not significant. There was no significant association between the methylation status of the mentioned genes and clinicopathological characteristics, such as age, sex, tumor type by the Lauren classification, degree of differentiation G, and TNM staging. An inverse correlation between the methylation of the DAPK and MLH1 gene promoters in cancerous and surrounding noncancerous tissues was found.

Conclusions. The methylation of the MLH1, MGMT, DAPK, and CASP8 genes was found to occur both in cancerous and noncancerous stomach tissues. These findings provide additional insights into gene methylation patterns in gastric adenocarcinoma.

Introduction

Gastric cancer is the fourth most common malignancy and the third leading cause of cancer death in men and the fifth leading cause in women (1). Many factors are involved in the development of gastric adenocarcinoma. Genetic host (2) and environmental factors, including Helicobacter pylori (H. pylori) infection (3), Epstein-Barr virus (4), diet (5), synergize and promote carcinogenesis pathways. However, the regulatory mechanisms involved in the development of gastric cancer remain poorly understood.

An epigenetic event – CpG island methylation of apoptosis and mismatch repair genes by the loss of their function – plays an important role in the development and progression of gastric adenocarcinoma. Epigenetic alterations also affect the expression of cancer genes alone or in combination with genetic mechanisms. The cytosine methylation of CpG dinucleotides in gene promoters is a common cause of DNA silencing and transcriptional repression that can modulate the clinical features of gastric cancer. Recent studies in the Asian population have indicated an important role of gene methylation in the cancer development and clinical variables.

Death-associated protein kinase (DAPK) is a calcium/calmodulin-dependent serine/threonine kinase that participates in apoptosis pathways (6). DAPK methylation occurs more frequently in H. pylori-positive gastric cancer patients (7, 8). There is an inverse correlation between DAPK methylation and microsatellite instability (MSI) (9). Gene promoter methylation alone or combined with other methylated genes serves as a predictive marker (lower response rate to fluoropyrimidine-based chemotherapy, shorter progression-free survival [PFS])
in a metastatic setting), meanwhile DAPK methylation can be a prognostic marker related to shorter overall survival (10, 11).

**CASP8** is a member of the caspase family, which plays a central role in the execution phase of cell apoptosis. There is a lack of data about methylation frequency in the corresponding nontumor gastric tissue and associations with tumor histological characteristics and TNM grading.

The methylation of the DNA repair gene of O(6)-methylguanine-DNA methyltransferase (**MGMT**) is important for cancer development. Gene promoter methylation is associated with **cagA** and **vacA**, which are virulence factors of **H. pylori** infection (12). **MGMT** promoter methylation in patients with gastric carcinoma was found to be associated with the mutations of the KRas gene, tumor stage, and disease-free survival (DFS) (13). Recent data show that **MGMT** promoter methylation is also a prognostic marker, and it is related to poor prognosis (14).

The **MLH1** gene, similarly as **MGMT**, is responsible for the mismatch repair function. A significant association between MSI and **MLH1** methylation has been reported (9, 15–20).

The importance of methylation of the mentioned genes in the pathogenesis of gastric cancer is significant. More data are becoming available regarding its prognostic and predictive value. Methylation frequency varies greatly, and most data come from East Asia. Therefore, it is important to determine the methylation frequency of gastric cancer gene promoters in Europe, to compare it with the methylation frequency of surrounding tissues, to determine a possible association with clinicopathological characteristics, and to determine the association between the methylation of individual genes.

**Material and Methods**

**Study Population.** Patients with histologically confirmed gastric adenocarcinoma were recruited at the Hospital of Lithuanian University of Health Sciences during the period of 2009–2011. Tissue samples were obtained by endoscopy or surgical resection from the tumor and the tumor-free area, which was at least 2 cm distant from the tumor and which was confirmed to be without any tumor cell infiltration by a histological assessment. Gastric tissue specimens were frozen in liquid nitrogen after dissection and stored at –80°C until analysis. Tumors were staged according to the criteria of the 2002 UICC/AJCC staging system for gastric cancer (21), and histologically subtyped and graded according to the World Health Organization (22) and the Lauren classification (23). Written informed consent was obtained from all study participants. The study was approved by Kaunas Regional Research Bioethical Committee (protocol No. BE–2–16).

**Methylation-Specific Polymerase Chain Reaction.** DNA was extracted from 25–30 mg of frozen tissue using a ZS Genomic DNA™ Tissue Mini Prep Kit (Zymo Research, USA) according to the manufacturer's instructions. The methylation status of **MLH1**, **MGMT**, **DAPK**, and **CASP8** gene promoters was determined by bisulfite treatment of DNA. Bisulfite treatment was performed using an EZ DNA Methylation Gold Kit™ (Zymo Research, USA) according to the manufacturer's instructions/protocol. Human genomic DNA from peripheral blood lymphocytes treated with bisulfite served as a negative control. Human genomic DNA treated in vitro with Sss I methyltransferase (New England Biolabs, UK) was used as a positive control. The methylation status of the promoters was detected by methylation-specific polymerase chain reaction (MSP). The methylated and unmethylated DNA sequence primers are listed in Table 1. PCR was performed in a total volume of 20 μL, containing 10 μL Maxima® Hot Start PCR Master Mix (PCR buffer, dNTP, MgCl₂) with Hot Start Taq DNA polymerase (Thermo Fisher Scientific, USA), 10 μM of each primer (Metabion International AG, Germany), and 2 μL of converted DNA. MSP included 38–40 cycles starting at 94°C for 30 seconds, annealing at temperature appropriate for an individual gene (DAPK, 60°C; MLH1, 56°C; MGMT, 61°C; and CASP8, 58°C) for 1 minute, and extension at 72°C for 1 minute. The PCR products were separated by 3.5% gel electrophoresis. If both methylated and unmethylated signals appeared in a gel, the methylation of the gene was considered.

**Statistical Analysis.** Statistical analysis was carried out with the IBM SPSS Statistics 19 software (IBM SPSS Inc., Chicago, IL). Quantitative data are presented as mean and standard deviation (SD). For testing statistical hypothesis about the independence of two variables, the chi-square test or the Fisher exact test was used. A Spearman coefficient was calculated to determine correlation. The significance level of <0.05 was selected.

**Results**

The study population comprised 69 patients (39 men and 30 women) with a median age of 64.5 years (SD, 12.7; range, 23–87). The representative cases of methylation are shown in Fig. 1. The methylation frequencies of **MLH1**, **MGMT**, **DAPK**, and **CASP8** in the gastric cancer and paired nontumor tissues are presented in Table 2. The methylation of the **CASP8** gene promoter was found to be quite a rare event both in the gastric cancer and the surrounding noncancerous tissue. There were no significant differences in the methylation frequency of the gene promoters between the can-

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An inverse correlation between the methylation of the DAPK and MLH1 gene promoters was observed in the cancerous (Spearman coefficient, −0.28; \(P = 0.02\)) and surrounding noncancerous tissues (Spearman coefficient, −0.28; \(P = 0.04\)). No significant associations between the methylation status of the studied genes and clinicopathological characteristics were found (Table 3).

Discussion

Our study and the study by Ivanauskas et al. (26) provide more information about the importance of gene CpG island methylation in gastric cancer in our region. The methylation frequency of DAPK in gastric cancer tissues varies from 22% to 91% (6–7, 10, 27–31). A study by Ye et al. reported that the methylation frequency of the DAPK promoter was significantly higher in the gastric cancer tissue than the corresponding nontumor tissue (30). The results of our study show a similar methylation frequency of the DAPK gene promoter in cancerous and adjacent noncancerous tissue. There are data available showing that the methylation of apoptosis-related genes correlates with poorly differentiated tumors and the advanced TNM stage (31), but we did not find any significant association between the methylation of the DAPK gene promoter and any pathological characteristics (TNM stage, Lauren tumor type, degree of differentiation G, involvement of lymph nodes) as well as clinical characteristics (age, sex). These findings, however, could be biased by a small sample size in our study, making the comparison between different subgroups difficult.

Only few articles about the importance of meth-

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Table 1. Primers Used for Methylation-Specific Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Location of Primers*</th>
<th>Product Size (bp)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(MLH1) methylated</td>
<td>CGG ATA GCG ATT TTT AAC GC</td>
<td>CCT AAA ACG ACT ACT ACC CG</td>
<td>Chr 3: 37034769–37034833</td>
<td>64</td>
<td>24</td>
</tr>
<tr>
<td>(MLH1) unmethylated</td>
<td>AAT GAA TTA ATA GGA AGA GTG GAT AGT</td>
<td>TCT CTT CAT CCC TCC CTA AAA CA</td>
<td>Chr 3: 37034750–37034847</td>
<td>97</td>
<td>24</td>
</tr>
<tr>
<td>(MGMT) methylated</td>
<td>TTT CGA CGT TCG TAG GTT TTC GC</td>
<td>GCA CTC TTC CGA AAA CGA AAC G</td>
<td>Chr 10: 131265515–131265596</td>
<td>81</td>
<td>24</td>
</tr>
<tr>
<td>(MGMT) unmethylated</td>
<td>TTT GTG TTT TGA TGT TGT TAG GTT TTT GT</td>
<td>AAC TCC ACA CTC TTC CAA AAA CA AA A</td>
<td>Chr 10: 131265509–131265602</td>
<td>93</td>
<td>24</td>
</tr>
<tr>
<td>(DAPK) methylated</td>
<td>GGA TAG TCG GAT CGA GTT AAC ACG CCC TCC CAA ACG C</td>
<td>Chr 9: 90112798–90112896</td>
<td>98</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>(DAPK) unmethylated</td>
<td>GGA TAG TCG GAT CGA GTT AAC ACG CCC TCC CAA ACG C</td>
<td>Chr 9: 90112795–90112901</td>
<td>106</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>(CASP8) methylated</td>
<td>TAG GGG ATT CGG AGA TTT CGA</td>
<td>CGT ATA TCT ACA TTG GAA ACG A</td>
<td>Chr2: 202123060–202123380</td>
<td>320</td>
<td>25</td>
</tr>
<tr>
<td>(CASP8) unmethylated</td>
<td>TAG GGG ATT TGG AGA TTT CGA</td>
<td>CCA TAT ATA TCT ACA TTG CCA CAA A</td>
<td>Chr2: 202123060–202123383</td>
<td>323</td>
<td>25</td>
</tr>
</tbody>
</table>

*The location of primers and the length of PCR products were assessed with the help of primer design and search tool http://bisearch.enzim.hu.

![Fig. Representative methylation-specific polymerase chain reaction for the DAPK, CASP8, MLH1, and MGMT genes](image)

Table 2. Methylation Frequency of the \(MLH1\), MGMT, DAPK and CASP8 Gene Promoters in Gastric Cancer and Paired Nontumor Tissues

<table>
<thead>
<tr>
<th>Gene</th>
<th>GC (n = 69)</th>
<th>NT (n = 69)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(MLH1)</td>
<td>22 (31.9)</td>
<td>16 (27.5)</td>
<td>0.58</td>
</tr>
<tr>
<td>MGMT</td>
<td>25 (36.2)</td>
<td>31 (44.9)</td>
<td>0.29</td>
</tr>
<tr>
<td>DAPK</td>
<td>33 (47.8)</td>
<td>32 (46.4)</td>
<td>0.86</td>
</tr>
<tr>
<td>CASP8</td>
<td>4 (5.8)</td>
<td>4 (5.8)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Values are number (percentage). GC, gastric cancer; NT, nontumor tissue. Analyzed by two-sided Fisher exact or chi-square test.

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optosis-related genes, no significant association between the methylation of the CASP8 gene promoter and clinicopathological characteristics of patients with gastric cancer was found as well.

According to the published data, the methylation frequency of the MLH1 gene promoter in gastric cancer tissue varies greatly, i.e. from 14% to 65.3% (9, 34, 35). However, the methylation frequency of the MLH1 gene promoter determined in our study was higher compared to the previously published results in the European study by Balasiano et al. (31.9% and 14.2%, respectively) (34). Some data have indicated that the methylation of the MLH1 gene promoter occurs more frequently in the gastric cancer tissue compared with the adjacent noncancerous mucosa (35). In our study, the difference in the methylation frequency between cancerous and noncancerous tissues was 4%, but it was not significant. The methylation of MLH1 could be a diagnostic marker for gastric cancer, but further studies involving a larger number of patients are needed to confirm this, as the methylation frequency of MLH1 in cases of chronic gastritis is very low and does not reach 2% (24). A high methylation rate of surrounding noncancerous gastric tissues may indicate an association with local relapse frequency. This hypothesis should also be tested in future research. A Polish study reported that the MLH1 gene was hypermethylated more frequently in women than men (36), this was not confirmed in our study. Some authors detected the link between the methylation of the MLH1 gene promoter and the type of intestinal gastric cancer, lower clinical stage, absence of lymph node metastasis (9, 34, 35, 36), differences were compared by using the \( \chi^2 \) test. M, methylated; U, unmethylated.

### Table 3. Associations Between the Methylation of the MLH1, MGMT, DAPK, and CASP8 Gene Promoters in Gastric Cancer Tissues (N=69) and Clinicopathological Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MLH1 M</th>
<th>MLH1 U</th>
<th>P</th>
<th>MGMT M</th>
<th>MGMT U</th>
<th>P</th>
<th>DAPK M</th>
<th>DAPK U</th>
<th>P</th>
<th>CASP8 M</th>
<th>CASP8 U</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>14/22</td>
<td>11/28</td>
<td>0.41</td>
<td>11/25</td>
<td>10/25</td>
<td>0.19</td>
<td>13/21</td>
<td>14/21</td>
<td>0.07</td>
<td>4/3</td>
<td>14/20</td>
<td>0.09</td>
</tr>
<tr>
<td>Male</td>
<td>8/22</td>
<td>14/16</td>
<td>0.11</td>
<td>13/14</td>
<td>14/16</td>
<td>0.12</td>
<td>2/3</td>
<td>1/2</td>
<td>0.24</td>
<td>1/1</td>
<td>3/1</td>
<td>1.0</td>
</tr>
<tr>
<td>Female</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
<td>0.09</td>
<td>0.09</td>
<td></td>
<td>0.87</td>
<td>0.87</td>
<td></td>
<td>0.07</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>4/17</td>
<td>8/13</td>
<td>0.13</td>
<td>13/8</td>
<td>8/13</td>
<td>0.05</td>
<td>21/21</td>
<td>21/21</td>
<td>1.0</td>
<td>3/0</td>
<td>3/0</td>
<td>1.0</td>
</tr>
<tr>
<td>≤60</td>
<td>18/30</td>
<td>17/31</td>
<td>0.83</td>
<td>20/28</td>
<td>20/28</td>
<td>0.12</td>
<td>6/3</td>
<td>6/3</td>
<td></td>
<td>4/1</td>
<td>4/1</td>
<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>0.07</td>
<td>0.07</td>
<td></td>
<td>0.44</td>
<td>0.44</td>
<td></td>
<td>0.07</td>
<td>0.07</td>
<td></td>
<td>0.09</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis*</td>
<td>Positive</td>
<td>11/31</td>
<td>0.24</td>
<td>14/28</td>
<td>0.58</td>
<td></td>
<td>21/21</td>
<td>0.87</td>
<td></td>
<td>3/0</td>
<td>3/0</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>10/15</td>
<td>10/15</td>
<td>0.12</td>
<td>12/13</td>
<td>1/2</td>
<td></td>
<td>2/3</td>
<td>0.93</td>
<td></td>
<td>3/0</td>
<td>3/0</td>
<td></td>
</tr>
<tr>
<td>Lauren classification*</td>
<td>Intestinal</td>
<td>10/25</td>
<td>0.6</td>
<td>10/25</td>
<td>0.19</td>
<td></td>
<td>21/14</td>
<td>0.07</td>
<td></td>
<td>2/2</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>11/21</td>
<td>14/18</td>
<td>0.73</td>
<td>14/13</td>
<td>0.73</td>
<td></td>
<td>0.2</td>
<td>0.2</td>
<td></td>
<td>0.09</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Tumor differentiation*</td>
<td>Well-to-moderate</td>
<td>7/12</td>
<td>0.43</td>
<td>9/18</td>
<td>0.73</td>
<td></td>
<td>14/13</td>
<td>0.73</td>
<td></td>
<td>0.09</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>14/26</td>
<td>15/25</td>
<td>0.4</td>
<td>19/21</td>
<td>0.44</td>
<td></td>
<td>0.44</td>
<td>0.07</td>
<td></td>
<td>0.09</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Tumor (pT, cT)*</td>
<td>T1-2</td>
<td>4/9</td>
<td>0.93</td>
<td>6/7</td>
<td>0.4</td>
<td></td>
<td>9/4</td>
<td>0.09</td>
<td></td>
<td>0/1</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>T3-4</td>
<td>17/36</td>
<td>18/35</td>
<td>0.07</td>
<td>23/30</td>
<td>0.09</td>
<td></td>
<td>4/4</td>
<td>0.31</td>
<td></td>
<td>0.09</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

*Pathological tumor data from 3 patients was incomplete. Differences were compared by using the \( \chi^2 \) test.
37, 38). Contrary to these studies, our study failed to show the association between the methylation status of the mentioned gene and pathological characteristics of cancer such as tumor differentiation, tumor type by the Lauren classification, degree of differentiation G, and TNM staging. Data show that the methylation of the MLH1 gene promoter is associated with MSI (9, 15–19). Our results indicate that the methylation frequency of MLH1 inversely correlated with that of the DAPK gene promoter, which corresponds to the data presented in a study by Ferrasi et al. (9), reporting an inverse correlation between DAPK hypermethylation and MSI. This correlation was also confirmed in the surrounding noncancerous gastric tissue in our study.

Our study design has certain limitations. Further studies are needed to compare methylation patterns in gastric adenocarcinoma and adjacent tumor-free tissues with those of tissue specimens obtained from a healthy control group. The assessment of the gene methylation pattern in premalignant gastric lesions (atrophic gastritis and intestinal metaplasia) could also give additional insights in elucidating the role of methylation of the selected genes.

Conclusions
The methylation of the MLH1, MGMT, DAPK, and CASP8 gene promoters occurs in cancerous as well as noncancerous stomach tissues. An inverse correlation between the methylation of the MLH1 and DAPK promoter genes was found. Our findings provide additional insights in the puzzle of gene methylation patterns in gastric adenocarcinoma.

Statement of Conflict of Interest
The authors state no conflict of interest.

References

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