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

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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]

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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, $MgCl_2$) 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.

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-

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

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

*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.

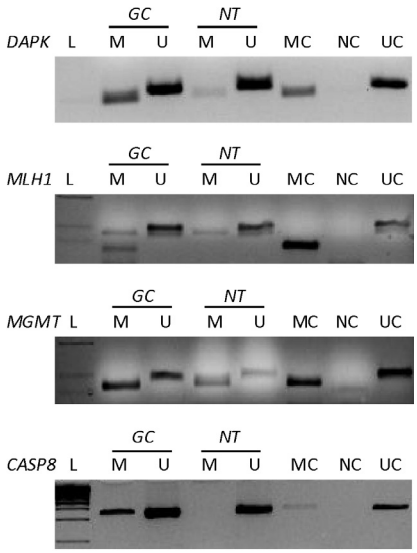

Fig. Representative methylation-specific polymerase chain reaction for the *DAPK, CASP8, MLH1,* and *MGMT* genes

L, DNA ladder marker, M, methylated allele, U, unmethylated allele, MC, methylated control, NC, negative control, UC, unmethylated control, GC, gastric cancer tissue sample, NT, nontumor adjacent gastric tissue sample.

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

Gene	GC n=69	NT n=69	*P*
MLH1	22 (31.9)	16 (27.5)	0.58
MGMT	25 (36.2)	31 (44.9)	0.29
DAPK	33 (47.8)	32 (46.4)	0.86
CASP8	4 (5.8)	4 (5.8)	1.0

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

cerous and adjacent noncancerous tissues.

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-

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

Characteristic	*MLH1*		*P*	*MGMT*		*P*	*DAPK*		*P*	*CASP8*		*P*
	M n=22	U n=47		M n=25	U n=44		M n=33	U n=36		M n=4	U n=65	
Sex												
Male	14	25	0.41	11	28	0.11	19	20	0.86	3	36	0.44
Female	8	22		14	16		14	16		1	29	
Age, years												
⩽60	4	17	0.13	8	13	0.83	13	8	0.12	3	18	0.07
>60	18	30		17	31		20	28		1	47	
Lymph node metastasis*												
Positive	11	31	0.24	14	28	0.58	21	21	0.87	3	39	0.6
Negative	10	15		10	15		12	13		1	24	
Lauren classification*												
Intestinal	10	25	0.6	10	25	0.19	21	14	0.07	2	33	0.93
Diffuse	11	21		14	18		12	20		2	30	
Tumor differentiation*												
Well-to-moderate	7	20	0.43	9	18	0.73	14	13	0.73	0	27	0.09
Poor	14	26		15	25		19	21		4	36	
Tumor (pT, cT)*												
T1-2	4	9	0.93	6	7	0.4	9	4	0.09	0	13	0.31
T3-4	17	36		18	35		23	30		4	49	

*Pathological tumor data from 3 patients was incomplete. Differences were compared by using the χ^2 test.
M, methylated; U, unmethylated.

ylation of the *CASP8* gene promoter in gastric cancer have been published. Our data showed that the methylation of the *CASP8* gene promoter was quite a rare event in gastric cancer and surrounding noncancerous tissues. In both paired tissues, the methylation frequency was around 6%, and no significant difference between the compared tissues was observed. The methylation frequency of the apoptosis-related *CASP8* gene promoter in this study was lower compared with that of the South Korean study that reported the methylation frequency of the *CASP8* gene promoter in the gastric cancer and healthy tissues to be 16.7% and 4.2%, respectively (32). No significant associations between the methylation frequency of the *CASP8* gene promoter and any pathological characteristics (TNM stage, Lauren tumor type, degree of differentiation G, involvement of lymph nodes) as well as demographic characteristics (age, sex) were found. The reported methylation frequency of the *MGMT* promoter in gastric cancer ranges from 6.9% to 61% (15, 27–28, 33–34), while it is reported to be 5.7% in the adjacent nontumor tissue (28). Our study showed a higher methylation frequency of *MGMT* in the nonmalignant (44.9%) than malignant (36.2%) tissues, but the difference was not significant. It could be related to technical aspects as our test was not quantitative but qualitative. Some data indicate that the methylation of the *MGMT* gene occurs more frequently in lymph node-positive gastric cancer (13, 35). Our results do not contradict the literature data, but are not statistically significant. In line with the above mentioned genes, no significant association between the methylation of the *MGMT* 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 tissues 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 Balassiano 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,

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.

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