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DNA Hypermethylation of Tumor-Related Genes in Gastric Carcinoma

The hypermethylation of the CpG islands is a common mechanism for the inactivation of tumor-related genes. In the present study, we analyzed the methylation status of genes for cell repair such as *hMLH1*, *MGMT*, and *GSTP1*, and a gastric cancer-specifically methylated DNA fragment, *MINT 25* in gastric cancer cases and control groups. The study population consisted of 100 gastric cancer patients (50 distal and 50 proximal carcinomas), and 238 healthy controls. All genes showed more frequent hypermethylation in the cases than in the control group (p<0.0001). We investigated the association between promoter hypermethylation and relevant parameters including age, gender, alcohol consumption, smoking, and family history. There was a common hypermethylation of *hMLH1* (p=0.008), *MGMT* (p=0.0001), and *GSTP1* (p=0.0003) in females. This study also demonstrates that hypermethylation was strongly associated with non-drinkers (*MGMT*, p=0.046 and *MINT 25*, p=0.049) and non-smokers (*hMLH1*, p=0.044; *MGMT*, p=0.0003; *MINT 25*, p=0.029). Moreover, the frequency of *MINT 25* hypermethylation increased with age (p=0.037), and *MGMT* methylation was frequently detected in distal gastric cancer than in proximal type (p=0.038). Our study suggested that promoter hypermethylation of the genes involved in cell repair system and *MINT 25* is associated strongly with some subgroups of primary gastric carcinoma.

Key Words : *Stomach Neoplasms; DNA Methylation; MLH1 Protein, mammalian; O (6)-Methylguanine-DNA Methyltransferase; Glutathione S-transferase pi; MINT 25*

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INTRODUCTION

Aberrant methylation patterns are one of the fundamental hallmarks of cancer cells. Tumor cells are generally hypomethylated relative to normal cells with regional hypermethylation. CpG islands are GC-rich areas of the genome corresponding to the promoter regions of genes and are associated with transcriptional activity (1). The methylation status of these CpG islands has been shown to be involved with oncogene activation and tumor suppressor gene inactivation. A recent study on the profile of promoter hypermethylation for 12 genes ($p16^{INK4A}$, $p15^{INK4B}$, $p14^{ARF}$, *p73, APC, BRCA1, hMLH1, GSTP1, MGMT, CDMI, TIMP3,* and *DAPK*) in 15 major tumor types revealed that one or more of the genes are hypermethylated in all tumor types (2). The profile of the promoter hypermethylation for the genes, however, differs in each cancer type, providing a tumor type- and gene-specific profile.

Aberrant methylation in tumor-related genes is frequently detected in gastric intestinal metaplasia with and without gastric cancer, suggesting their early involvement in the multistep progression of gastric carcinogenesis (3). The identification of genes targeted by hypermethylation may provide insights into the mechanisms for the inactivation of tumor-suppressive pathways in gastric cancer cases. In addition, hypermethylated genes may serve as targets for the development of new screening tests for cancer (4).

In the present study, we compared the hypermethylation of genes responsible for the cell repair system (*hMLH1, MGMT,* and *GSTP1*) in gastric cancer case and control study. We also analyzed *MINT 25* (methylated in tumors 25) which showed a high frequency of methylation in gastric carcinomas (5, 6). There are many reports that have shown frequent hypermethylation of these genes in gastric carcinoma, but its interaction with histological characteristics is still unclear. Thus, we evaluated the association between the hypermethylation of these genes and gastric cancer according to the risk factors such as *H. pylori* infection, alcohol consumption, smoking, family history, and their histological characteristics by the methylation-specific PCR (MSP) method.

MATERIALS AND METHODS

Subjects and genomic DNA purification

A case-control study of gastric cancer was performed in Daegu City. One hundred gastric cancer patients and two hundred thirty-eight healthy subjects participated in this study. Patients affected with gastric cancer were considered eligible if they had histologically diagnosed adenocarcinoma

of the stomach. The control group included subjects who had no current or previous diagnosis of cancer. Data included questionnaire data, and a review of medical records.

Whole blood samples of control group were used to isolate genomic DNA by the phenol-chloroform method (7). Tumor tissues were used to determine DNA methylation status of cancer patients. Frozen tumor tissues were ground and incubated at 50°C for 3 hr in a lysis buffer containing 0.5% of sodium dodecyl sulfate (SDS), followed by a phenol-chloroform method. The amount of DNA and their purity were determined by spectrophotometry.

Bisulfite modification

DNA methylation patterns in the CpG islands of the target genes were determined by chemical modification of unmethylated, but not methylated, cytosines to uracils, and subsequent PCR amplification using primers specific for either methylated or modified unmethylated DNA (8). The bisulfite-modification was performed according to Olek et al. (9). One microgram of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, WI, U.S.A.), treated with NaOH again, and precipitated with ethanol. DNA was resuspended in water and used immediately or stored at -20°C.

Methylation-sensitive PCR and bisulfite-PCR RFLP

Two μL of treated DNA were used for each PCR reaction. MSP showed the presence or absence of methylated genes of *hMLH1, MGMT,* and *GSTP1*. The methylation status of *MINT 25* was determined by bisulfite-PCR followed by restriction digestion. Two μL of DNA modified with bisulfite were amplified and 15 μL of the PCR products were then digested with *Rsa*I, which is specific to the methylated alleles by virtue of having CpG sites in their recognition sequence. After digestion, 10 μL of each product were directly loaded onto 2% agarose or 6% polyacrylamide gels and stained with ethidium bromide. Primer sequences and annealing temperatures are shown in Table 1.

Table 1. PCR primers used for MSP and bisulfite-PCR

Gene	M/U	S/AS	Sequences (5′→3′)	Size (bp)	Annealing Temp (°C)
MGMT	M	S	TTTCGACGTTCGTAGGTTTTCGCGC	81	59
		AS	ACTCTTCCGAAAACGAAACG		
	U	S	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	93	59
		AS	AACTCCACACTCTTCCAAAAACAAAACA		
hMLH1	M	S	ACGTAGACGTTTTATTAGGGTCGC	124	60
		AS	CCTCATCGTAACTACCCGCG		
	U	S	TTTTGATGTAGATGTTTTATTAGGGTTGT	112	60
		AS	ACCACCTCATCATAACTACCCACA		
GSTP1	M	S	TTCGGGGTGTAGCGGTCGTC	91	59
		AS	GCCCCAATACTAAATCACGAC		
	U	S	GATGTTTGGGGTGTAGTGGTTGTT	97	59
		AS	CCACCCCAATACTAAATCACAACA		
MINT25		S	TYGGTGTTTGTAAAGGGTTGGAAT	233	60
		AS	CCCRAACTAAAAACTAACTCRTAA		

M, methylated; U, unmethylated; S, sense; AS, antisense.

Statistical analysis

Cases and controls were described according to their basic sociodemographic, and clinicopathological factors. Patients who indicated that they had stopped smoking or drinking alcohol within the post 6 months were classified as current smokers or current alcohol drinkers. χ^2 tests and Fisher's exact tests were done using the software package SAS Release 8.01 for Windows to examine the differences in DNA methylation status. The corresponding tests on the cases and controls were carried out (p-values) to compare each factor.

RESULTS

DNA methylation in gastric carcinoma

We determined aberrant DNA methylation of *hMLH1,*

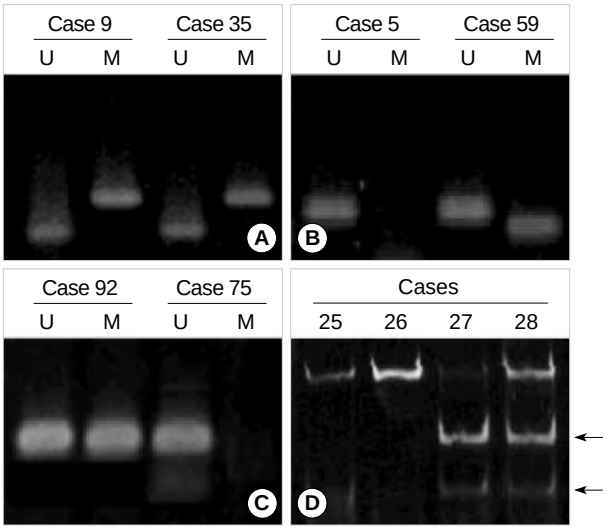

Fig. 1. Methylation analysis in gastric cancer. (A) *hMLH1*, (B) *MGMT*, and (C) *GSTP1* methylation were analyzed by methylation-specific PCR. The presence of visible PCR products in those lanes marked U indicate the presence of unmethylated genes; the presence of products in those lanes marked M indicate the presence of methylated genes. Cases 9, 35, 59, and 92 show methylated and unmethylated bands because of the heterogeneously methylated genes, and cases 5 and 75 do not have methylated genes. (D) *MINT 25* methylation analysis was performed by bisulfite-PCR and restriction digestion. Only methylated alleles will be digested by restriction enzymes, and they are indicated by arrows. Case 27 and 28 have homogeneously and, heterogeneously methylated *MINT 25* DNA fragments, respectively.

Table 2. The frequency of DNA hypermethylation in gastric cancer and control groups

	Frequency of hypermethylation (%)			
	hMLH1	*MGMT*	*MINT 25*	*GSTP1*
Cases (n=100)	26 (26.0)*	25 (25.0)*	19 (19.0)*	7 (7.0)*
Controls (n=238)	2 (0.8)	4 (1.7)	0 (0)	0 (0)

*All the *p* values are <0.0001.

Table 3. Concurrent hypermethylation of hMLH1 with other genes

hMLH1	Concurrent methylation (any genes)
Methylated (n=26)	16 (61.6%)*
Unmethylated (n=74)	23 (31.1%)

**p*=0.045.

MGMT, MINT 25, and *GSTP1* in 100 gastric cancer patients and 238 randomly chosen, healthy Koreans. The mean ages were 62 yr for cancer patients and 60.7 yr for controls. These genes were generally unmethylated in the control group, in particular, *MINT 25* and *GSTP1* showed no hypermethylation. All these genes, however, were aberrantly methylated in the cancer group at the following frequencies: 26 (26%) for *hMLH1*, 25 (25%) for *MGMT*, 19 (19%) for *MINT 25*, and 7 (7%) for *GSTP1*. All the *p* values are <0.0001, and the overall results are shown in Fig. 1 and Table 2. When we compared the relationship of the DNA methylation of *hMLH1* and other 3 genes, we found concurrent methylation of *hMLH1* and any of other 3 genes (p=0.045) (Table 3).

Association between the characteristics of patients and DNA methylation

We analyzed the methylation changes in the tumors and the questionnaire data obtained from the patients. The promoter hypermethylation of *hMLH1, MGMT,* and *GSTP1* were detected more frequently in women than in men and the frequencies are as follows: 44.4% vs. 15.6% for *hMLH1* (p=0.008), 50.0% vs. 10.9% for *MGMT* (0.0001), and 13.9% vs. 3.1% for *GSTP1* (p=0.0003). To determine the relationship between DNA hypermethylation and aging, we ranked the 100 gastric carcinomas into three groups according to age. The proportion of *MINT 25* methylation was increased with age (p=0.037). We compared the frequency of methylation after grouping the cases in two by tobacco smoking or never-smoking. It is interesting to note that the promoter hypermethylation of *MGMT* and *MINT 25* were detected less frequently in the alcohol consumption subgroup and the frequencies are as follows: 14.6% vs. 32.2% for *MGMT* (p=0.046), and 9.8% vs. 25.4% for *MINT 25* (p=0.049). When we compared the methylation after grouping in two by smoking or never smoking, we observed similar results in alcohol consumption, which showed a lower proportion *hMLH1* methylation (18.6% vs. 36.6%, p=0.044), *MGMT* (11.9% vs. 43.9%, p=0.0003), and *MINT 25* (11.9% vs. 29.3%, p=0.029). There was no significant difference in the methylation frequency between subgroups with and without gastric cancer family history. The proportion of *hMLH1* methylation in these two groups was 27.6% and 15.4%, respectively, but it is not statistically significant.

Another aim of this study was to investigate that the instances of promoter hypermethylation in gastric carcinoma are associated with histological parameters such as *H. pylori* infection, tumor location, and Lauren classification. In our study, *MGMT* showed hypermethylation more frequently in distal gastric carcinomas than in the proximal type (34% vs. 16%, p=0.038). These were summarized in Table 4.

Table 4. Association between the characteristics of the subjects and DNA hypermethylation

Variables	n	Frequency of hypermethylation (%)				
		hMLH1	*MGMT*	*MINT 25*	*GSTP1*	Any genes
Sex						
Female	36	16 (44.4)§	18 (50.0)§	8 (27.8)	5 (13.9)‖	28 (77.8)
Male	64	10 (15.6)	7 (10.9)	9 (14.1)	2 (3.1)	23 (35.9)
p values		*p*=0.008	*p*=0.0001		*p*=0.0003	*p*=0.037
Age (yr)						
<60	37	5 (13.5)	10 (25.6)	5 (12.8)§	3 (7.7)	18 (46.2)
60-69	38	13 (34.2)	9 (25.0)	5 (13.9)	3 (8.3)	17 (47.2)
>69	25	8 (32.0)	6 (24.0)	7 (28.0)	1 (4.0)	15 (60.0)
p values				*p*=0.037		
Alcohol consumption						
Yes*	41	8 (19.5)	6 (14.6)§	4 (9.8)§	2 (4.9)	16 (37.2)
No	59	18 (30.5)	19 (32.2)	15(25.4)	5 (8.5)	34 (59.6)
p values				*p*=0.046	*p*=0.049	
Smoking						
Yes†	59	11 (18.6)§	7 (11.9)§	7 (11.9)§	2 (3.4)	20 (32.3)
No	41	15 (36.6)	18 (43.9)	12 (29.3)	5 (12.2)	30 (78.9)
p values		*p*=0.044	*p*=0.0003	*p*=0.029		
Family history‡						
Yes	87	24 (27.6)	20 (23.0)	16 (18.3)	6 (6.9)	42 (49.4)
No	13	2 (15.4)	5 (38.5)	3 (23.1)	1 (7.7)	9 (60.0)
Histological characteristics						
H. pylori infection						
Yes	63	19 (30.2)	17 (27.0)	15 (23.8)	3 (4.8)	33 (51.6)
No	37	7 (18.9)	8 (21.6)	4 (10.8)	4 (10.8)	16 (44.4)
Location						
Distal	50	12 (24.0)	17 (34.0)§	12 (24.0)	2 (4.0)	29 (58.0)
Proximal	50	14 (28.0)	8 (16.0)	7 (14.0)	5 (10.0)	22 (44.0)
p values			*p*=0.038			
Lauren classification						
Intestinal	32	9 (28.1)	8/30 (25.0)	8 (25.0)	2 (6.3)	18 (60.0)
Mixed	22	5 (26.7)	4/22 (18.2)	1 (4.6)	4 (18.2)	9 (40.9)
Diffuse	46	12 (22.1)	13/45 (28.3)	10 (21.7)	1 (2.2)	23 (51.1)

*Current or ex-drinkers, †Current or ex- smokers, ‡One or more first-degree relatives with gastric cancer, §*p* values by chi square test, ‖*p* values by Fisher's exact test.

DISCUSSION

Methylation of the CpG islands of tumor suppressor genes

leading to their transcriptional inactivation is a highly consistent feature of tumorigenesis. In the present study, we demonstrated the distribution pattern of the aberrant methylation of *hMLH1, MGMT, MINT 25* and *GSTP1* in gastric cancer patients and controls. We also investigated the association between promoter hypermethylation and relevant parameters including age, gender, alcohol consumption, smoking, and family history. Other histological characteristics were also taken into consideration.

The transcriptional inactivation of *MGMT* by DNA methylation occurs in a wide spectrum of human tumors (10), whereas that of *hMLH1* is restricted to sporadic tumors with microsatellite instability such as colon (11), endometrial (12, 13), and gastric tumors (14). *MGMT* plays a major role in the repair of O^6-methylguanine DNA adducts. The loss of *MGMT* expression is rarely due to genetic mutation, but due to the methylation of discrete regions of the CpG island of the gene. Recently reported data indicated that *MGMT* protein expression levels were decreased by the promoter hypermethylation of *MGMT* in gastric carcinomas (15). Glutathione S-transferases (GSTs) are a family of enzymes involved in the detoxication of xenobiotics and oxygen radicals (16, 17). Recent studies have demonstrated that the expression of the *GSTP1* gene, one of the GST isoenzymes, is controlled by DNA methylation (18). *MINT 25* stands out as the specific methylation pattern in gastric tumors, and there was no methylation observed in either normal stomach or colon, or less than 10% of colorectal tumors (19). This suggests that it may play a special role in stomach neoplasia.

In the present study, the methylation of *hMLH1, MGMT, MINT 25,* and *GSTP1* in gastric cancer was detected more frequently than in the controls ($p<0.0001$). Previous report showed that concurrent hypermethylation of *hMLH1, CDH1, MGMT* and *COX2* gene promoters was more frequently observed in MSI-H gastric tumors, and the significant association between the concurrent hypermethylation and MSI-H was lost when *hMLH1* was excluded (20). We tried to compare the hypermethylation pattern between *hMLH1* and other genes analyzed in this study. Our result indicates that concurrent hypermethylation of *hMLH1* and the other three genes are a common event in gastric cancer ($p=0.045$). Further studies are necessary to determine the association between the inactivation of *hMLH1* gene promoters by hypermethylation and the microsatellite instability status of gastric carcinomas.

We investigated the association between promoter hypermethylation and relevant parameters including age, gender, alcohol consumption, smoking, and family history. We found that the promoter hypermethylation of *hMLH1, MGMT,* and *GSTP1* was detected more frequently in women than in men ($p<0.05$). A previous report showed that the methylation of *TIMP-3* was seen more frequently in women with lung cancer, whereas methylation of *DAPK* and $p16^{INK4a}$ was more common in men. These data suggest that some genes showed gender-specific methylation pattern, but the reason for this is unknown.

It has been described that aging is associated with the methylation of certain genes such as *hMLH1* (21) and estrogen receptor (22). In the present study the proportion of *MINT 25* methylation increased with age ($p=0.037$), and *hMLH1* also showed significant difference of methylation frequency between the two age groups of 69 yr apart. Previous studies showed that age-related methylation affects only a subset of genes, suggesting a gene-specific susceptibility in this process (23). Furthermore, there are significant tissue-specific differences in age-related methylation (23). *MINT 25*, which is strongly associated with gastric carcinoma, demonstrated for the first time in this report an age dependent methylation pattern.

In the present study, there was a significant association between methylation and smoking/alcohol consumption. The proportion of promoter methylation of genes increased significantly in the never-smoking or never-drinking subgroups ($p<0.05$). It was also shown that the incidence of *MGMT* promoter hypermethylation was significantly higher in never-smokers in lung adenocarcinomas (24). These result are, however, inconsistent with the previous study related with the promoter methylation pattern of tumor suppressor genes in head and neck squamous cell carcinoma (25). It could be suggested that hypermethylation is regulated differently by alcohol consumption and/or smoking in genes.

There was a difference in *hMLH1* methylation between subgroups with and without family history of gastric carcinoma, but this is not statistically significant. Since the size of these two subgroups is significantly different, it needs further investigation with more subgroups without gastric cancer family history.

A previous study showed that the p16 methylation in the distal stomach epithelium was higher than that in the proximal stomach (26). There have been reports showing that the microsatellite instability phenotype is linked with promoter hypermethylation of *hMLH1* and *MGMT* (27). Furthermore, many studies including with Korean patients showed that gastric cancer with microsatellite instability was associated with distal location (28-31). These reports suggest that hypermethylation is more susceptible in distal gastric carcinoma. The correlation mechanism between the microsatellite instability and DNA methylation needs to be uncovered. In our study, *MGMT* hypermethylation was detected more frequently in distal gastric carcinoma than in the proximal type ($p=0.038$).

A previous report suggested that there was a significant association between *hMLH1* promoter hypermethylation and intestinal type gastric carcinomas (32). Another study, however, showed that the frequency of *hMLH1* methylation was similar between intestinal and diffuse type gastric carcinomas (3). In our study, there was no association between *hMLH1* hypermethylation and the Lauren classification of gastric

carcinomas.

In conclusion, our study regarding promoter hypermethylation of genes involved in the cell repair system and *MINT 25* in primary gastric carcinomas shows the high frequency of methylation of *hMLH1, MGMT,* and *MINT 25*. This study also demonstrates that hypermethylation was strongly associated with females, and non-drinking/non-smoking subgroups. Moreover, the frequency of *MINT 25* hypermethylation increased with aging, and the methylation of *MGMT* was frequently detected in distal gastric cancer than in proximal cancer. The exact nature of the methylation defect in cancer cells should be defined by further studies.

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