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• *GASTRIC CANCER* •

Effects of dietary intake and genetic factors on hypermethylation of the *hMLH1* gene promoter in gastric cancer

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Abstract

AIM: Hypermethylation of the promoter of the *hMLH1* gene, which plays an important role in mismatch repair during DNA replication, occurs in more than 30% of human gastric cancer tissues. The purpose of this study was to investigate the effects of environmental factors, genetic polymorphisms of major metabolic enzymes, and microsatellite instability on hypermethylation of the promoter of the *hMLH1* gene in gastric cancer.

METHODS: Data were obtained from a hospital-based, case-control study of gastric cancer. One hundred and ten gastric cancer patients and 220 age- and sex-matched control patients completed a structured questionnaire regarding their exposure to environmental risk factors. Hypermethylation of the *hMLH1* gene promoter, polymorphisms of the *GSTM1*, *GSTT1*, *CYP1A1*, *CYP2E1*, *ALDH2* and *L-myc* genes, microsatellite instability and mutations of *p53* and *Ki-ras* genes were investigated.

RESULTS: Both smoking and alcohol consumption were associated with a higher risk of gastric cancer with hypermethylation of the *hMLH1* gene promoter. High intake of vegetables and low intake of potato were associated with increased likelihood of gastric cancer with hypermethylation of the *hMLH1* gene promoter. Genetic polymorphisms of the *GSTM1*, *GSTT1*, *CYP1A1*, *CYP2E1*, *ALDH2*, and *L-myc* genes were not significantly associated with the risk of gastric cancer either with or without hypermethylation in the promoter of the *hMLH1* gene. Hypermethylation of the *hMLH1* promoter was significantly associated with microsatellite instability (MSI): 10 of the 14 (71.4%) MSI-positive tumors showed hypermethylation, whereas 28 of 94 (29.8%) the MSI-negative tumors were hypermethylated at the *hMLH1* promoter region. Hypermethylation of the *hMLH1* gene promoter was significantly inversely correlated with mutation of the *p53* gene.

CONCLUSION: These results suggest that cigarette smoking and alcohol consumption may influence the development of *hMLH1*-positive gastric cancer. Most dietary factors and polymorphisms of *GSTM1*, *GSTT1*, *CYP1A1*, *CYP2E1*, *ALDH2*, and *L-myc* genes are not independent risk factors for gastric cancer with hypermethylation of the *hMLH1* promoter. These data also suggest that there could be two or more different molecular pathways in the development of gastric cancer, perhaps involving tumor suppression mechanisms or DNA mismatch repair.

Key words: Gastric cancer; Environmental carcinogens; Genetic polymorphisms; *hMTLH1*; Microsatellite instability; p53; Ki-ras

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INTRODUCTION

Gastric cancer is the most common cancer among Koreans. Environmental factors including cigarette smoking and dietary intake have been implicated in the etiology of gastric cancer[1-4]. Genetic polymorphisms of xenobiotic-metabolizing enzymes can also affect susceptibility to cancer. Several studies have reported that the genetic polymorphisms of metabolic enzymes, such as cytochrome p450 2E1 (*CYP2E1*)[5], glutathione S-transferase mu 1 (*GSTM1*)[6], glutathione *S*-transferase theta 1 (*GSTT1*)[7], aldehyde dehydrogenase 2 (*ALDH2*)[8], and L-myc proto-oncogene[9], and mutations of p53[10] and Ki-ras[11] genes are associated with the development of gastric cancer.

Promoter hypermethylation is an alternative mechanism of gene inactivation in carcinogenesis[12]. Several studies have suggested that aberrant methylation of the promoter causes transcriptional silencing of some important suppressor genes, such as *p16*[13], *E-cadherin*[14], and von Hippel Lindau (*VHL*) gene[15], and this has been implicated in the carcinogenic process in many cancers[12]. In addition, it was recently shown that hypermethylation of gene promoters increases along

the pathway of development from chronic gastritis, intestinal metaplasia, and adenomas to carcinomas of the stomach[16,17].

The *hMLH1* protein, a mismatch repair enzyme, maintains the fidelity of the genome during cellular proliferation. It has no known enzymatic activity and probably acts as a 'molecular matchmaker', recruiting other DNA-repair proteins to the mismatch repair complex[18]. Dysfunction of a mismatch repair system such as *hMLH1* and *hMSH2* could alter microsatellites, short tandem repetitive sequences[19]. Several reports have suggested that hypermethylation of the *hMLH1* promoter correlates with a loss of expression of the gene, resulting in microsatellite instability in gastric cancer[20,21].

There is evidence that diet may be associated with hypermethylation of the *hMLH1* gene promoter in gastric cancer. Aberrant hypermethylation of the *hMLH1* gene promoter is frequently observed in cancers of digestive organs such as the colon, rectum, and stomach[21,22], and decreased levels of folate, vitamin C, and niacin can induce hypermethylation of gene promoters[23]. These facts led us to hypothesize that genetic polymorphisms and environmental factors, such as cigarette smoking, alcohol consumption, and diet, may interact during the hypermethylation of the *hMLH1* gene promoter and in the carcinogenesis of gastric cancer. We studied the association between hypermethylation of the *hMLH1* gene promoter and environmental factors, genetic polymorphisms of major metabolic enzymes, genetic mutation of p53 and Ki-ras genes, and microsatellite instability in gastric cancer.

MATERIALS AND METHODS

Subjects

One hundred and ten individuals with gastric cancer and 220 age-matched (within 3 years) and sex-matched controls were enrolled in this study. Cases of cancer were all histologically confirmed from February 1997 to June 2002 at Chungbuk National University Hospital and Eulji University Hospital, Korea. Control subjects were selected from patients newly diagnosed with diseases other than cancer at the same hospitals or from individuals receiving routine medical examinations in Chungbuk National University Hospital. Table 1 shows the age and gender distribution of the subjects according to hypermethylation of the *hMLH1* gene promoter. The mean±SD ages were 59.81±11.23 years for cases and 59.60±11.03 years for controls. This study was conducted in accordance with the recommendations outlined in the Declaration of Helsinki and all subjects provided written informed consent.

Table 1 Gender and age distribution of cases and controls

	Cases	Controls
Gender		
Male	70	140
Female	40	80
Age (yr)		
–39	6	12
40–49	11	22
50–59	34	68
60–69	40	80
70–	19	38

Exposure to environmental factors

Trained interviewers interviewed subjects using a structured questionnaire within a month after the diagnosis of gastric cancer or benign diseases or at the time of the hospital visit for control subjects undergoing routine medical examination. The questionnaire included questions on demographic factors, smoking habits, alcohol consumption, and diet. Dietary data were collected using a semiquantitative food frequency table previously evaluated for validity and reliability[24]. All subjects were asked about their average frequency of consumption and portion size of 89 common food items during the year preceding the interview. These items were classified into 21 food groups having similar ingredients. The 21 food groups were as follows: cereals; potato; nuts; noodles; breads and cakes; vegetables; mushrooms; fruits; meats; eggs; fishes and shellfishes; stews; chicken; kimchi; soybean foods; soybean pastes; milk and dairy products; butter, cheeses, and margarine; jams, honey, candies, and chocolates; coffee and tea; seaweeds; and alliums.

The amount of calories, nutrients, vitamins, and minerals consumed for each food item was estimated by multiplying the intake amount of the food item and its nutrient value. The total intake of calories, nutrients, vitamins, and minerals was calculated for each subject by summing the respective calories, nutrients, vitamins, and minerals for each food item[25]. The intake amounts of these factors were adjusted for caloric intake using the method of Willett *et al*.[26].

Analysis of genetic polymorphisms

Genomic DNA was isolated from peripheral leukocytes by proteinase K digestion and phenol-chloroform extraction. A multiplex polymerase chain reaction (PCR) method[27] was used simultaneously to detect the presence or absence of the *GSTM1* and *GSTT1* genes with slight modification. The primers used were 5'-GAA GGT GGC CTC CTC CTT GG-3' and 5'-AAT TCT GGA TTG TAG CAG AT-3' for *GSTM1*, 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' and 5'-TCA CCG GAT CAT GGC CAG CA-3' for *GSTT1*, and 5'-CAA CTT CAT CCA CGT TCA CC-3' and 5'-GAA GAG CCA AGG ACA GTT AC-3' for *β-globin*, the internal reference gene. The PCR reactions were performed in 25 μL of a solution containing 50 ng of genomic DNA, 1× PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9.0), 1.5 mmol/L $MgCl_2$, and 0.1% Triton X-100], 5 pmoL of each primer, 80 μmol/L each dNTP, and 2.0 units *Taq* polymerase (Promega, Madison, WI). Amplifications were carried out in a thermocycler (MJ Research, Watertown, MA) as follows: 5 min of denaturation at 94 ℃, followed by 35 cycles consisting of denaturation at 94 ℃ for 1 min, annealing at 58 ℃ for 1 min, and extension at 74 ℃ for 1 min. PCR products were separated on 2% agarose gels with ethidium bromide. *GSTM1* and *GSTT1* genotypes were not scored unless the PCR product of the *β-globin* gene was evident.

The A4889G polymorphism in exon 7 of the *CYP1A1* gene was analyzed for each subject as described previously[28]. Briefly, PCR was performed using the primers 5'-GAA CTG CCA CTT CAG CTG TC-3' and 5'-GAA AGA CCT CCC AGC GGT CA-3'. The temperature program for the PCR

reaction was slightly modified. After initial denaturation for 5 min at 94 ℃, a thermal cycle consisting of denaturation for 90 s at 94 ℃, annealing for 90 s at 53 ℃, and extension for 30 s at 74 ℃, was repeated 35 times. The PCR products (187-bp fragments) were digested with *Hin*cII restriction enzyme at 37 ℃ overnight and subjected to electrophoresis on 12% polyacrylamide gels. PCR analysis resulted in the following genotype classification: a predominant homozygote (Ile/Ile), a heterozygote (Ile/Val), and a rare homozygote (Val/Val).

The 5'-flanking region polymorphism of the *CYP2E1* gene was analyzed using procedures described previously[29]. Briefly, PCR was performed using the primers 5'-CCA GTC GAG TCT ACA TTG TCA-3' and 5'-TTC ATT CTG TCT TCT AAC TGG-3'. Initial denaturation was performed at 94 ℃ for 5 min, followed by 35 thermal cycles consisting of denaturation for 1 min at 94 ℃, annealing for 1 min at 53 ℃, and extension for 30 s at 74 ℃. The 410-bp PCR product was digested with *Rsa*I at 37 ℃ overnight and subjected to electrophoresis on 2% agarose gels. The genotypes of *CYP2E1* were classified as follows: a predominant homozygote (c1/c1), a heterozygote (c1/c2), and a rare homozygote (c2/c2).

The *Mbo*II polymorphism of *ALDH2* was identified using a PCR-RFLP method[30] with slight modification. Briefly, PCR was performed using the primers 5'-CCA CAC TCA CAG TTT TCT CTT-3' and 5'-AAA TTA CAG GGT CAA CTG CT-3'. We used the same PCR conditions as in the *CYP1A1* gene analysis. The 134-bp amplicon was digested with *Mbo*II restriction enzyme at 37 ℃ overnight and subjected to electrophoresis on 15% polyacrylamide gels. The genotypes of *ALDH2* were identified as the predominant homozygote (NN), the heterozygote (ND), and the rare homozygote (DD).

The polymorphism of the *L-myc* gene was analyzed using procedures described previously[31]. Briefly, PCR was performed using the primers 5'-ACG GCT GGT GGA GTG GTA GA-3' and 5'-AAG CTT GAG CCC CTT TGT CA-3'. Initial denaturation was performed at 94 ℃ for 5 min, followed by 35 thermal cycles consisting of denaturation for 45 s at 95 ℃, annealing for 40 s at 60 ℃, and extension for 40 s at 74 ℃. The amplified 613-bp PCR product was directly digested with the restriction enzyme *Eco*RI at 37 ℃ overnight and separated by electrophoresis on 2% agarose gels. The genotypes of *L-myc* were classified as follows: a predominant homozygote (LL), a heterozygote (LS), and a rare homozygote (SS).

Methylation-specific PCR for hMLH1 promoter

Tissue samples from gastric cancer patients were immediately frozen and stored in liquid nitrogen until analysis. DNA was extracted using a DNA extraction kit (Promega) according to the manufacturer's instructions.

The promoter methylation status of the *hMLH1* gene was determined by methylation-specific PCR (MSP), as described previously[32]. MSP distinguishes unmethylated from hypermethylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracils. Briefly, 2 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were then purified using a Wizard DNA Purification Resin (Promega), treated again with NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was amplified using the primer pairs as follows: 5'-TTT TGA TGT AGA TGT TTT ATT AGG GTT GT-3' and 5'-ACC ACC TCA TCA TAA CTA CCC ACA-3' for the unmethylated reaction (124-bp), and 5'-ACG TAG ACG TTT TAT TAG GGT CGC-3' and 5'-CCT CAT CGT AAC TAC CCG CG-3' for the methylated reaction (115-bp)[32]. PCR was performed in a thermocycler (MJ Research) as follows: 5 min of denaturation at 95 ℃, then 35 cycles consisting of denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 30 s. PCR products were separated on 6% polyacrylamide gels with ethidium bromide. DNA from blood samples was used as a negative control for hypermethylated *hMLH1*.

Microsatellite instability

Microsatellite instability (MSI) was examined using BAT25 and BAT26 mononucleotide microsatellite markers. PCR was performed in a 25 μL reaction volume containing 50 ng of genomic DNA, 1× PCR buffer, 5 pmoL of each primer, 80 μmol/L each dNTP, 2.0 units *Taq* polymerase (Takara, Shiga, Japan), and 0.2 μCi of α-^{32}P-labeled dCTP. Amplifications were carried out as follows: 5 min of denaturation at 95 ℃, then 35 cycles consisting of denaturation at 95 ℃ for 50 s, annealing at 58 ℃ for 90 s, and extension at 72 ℃ for 90 s. Two microliters of PCR product was electrophoresed on 6% denaturing polyacrylamide gels containing 6 mol/L urea at room temperature. The gels were dried and autoradiographed on X-ray film. MSI-positive results were identified when the mobility of the microsatellite fragment amplified from tumor DNA differed from the corresponding blood DNA. Tumors were considered microsatellite instability-positive (MSI+) if they manifested instability at one or two loci or microsatellite instability-negative (MSI-) if no unstable microsatellite was found.

Sequencing of p53 *and* Ki-ras *genes*

Reverse transcription (RT)-PCR and direct sequencing methods were used to detect mutations in *p53* and *Ki-ras* genes. Briefly, tissues from gastric cancer patients were homogenized and RNA was isolated using TRIzol solution (Invitrogen Life Technologies, Grand Island, NY). RT-PCR to amplify *p53* and *Ki-ras* cDNA were performed using reagents purchased from Promega. Specific primers synthesized by Bioneer Company (Cheongju, South Korea), *Ex Taq* polymerase (Takara), dNTPs, $MgCl_2$, PCR buffer, and cDNA template were mixed and then amplified for 40 cycles at 95 ℃ for 30 s and at 72 ℃ for 1 min. The cDNA regions were amplified using primers 5'-TCT AGA GCC ACC GTC CAG GGA G-3' and 5'-AAC CTC AGG CGG CTC ATA GGG CA-3' for the +2-+810 region of *p53*, and 5'-ACC AGG GCA GCT ACG GTT TCC GT-3' and 5'-TCA GTC TGA ATC AGG CCC TTC TGT-3' for the +443-+1 317 region of *p53*. Exons 1 and 2 of the *Ki-ras* gene were amplified using primers 5'-GAC TGA ATA TAA ACG TGT GGT AG-3' and 5'-ACT GGT CCC TCA TTG

CAC TG-3'. Before the RT-PCR products were sequenced by cycle sequencing, a PCR purification kit (Boehringer Mannheim, Indianapolis, IN) was used to remove unwanted reagents from the PCR reaction. The purified PCR products were then directly cycle-sequenced using an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Data analysis

Calorie-adjusted intakes of foods, nutrients, vitamins, and minerals were categorized into low- and high-intake groups based on the median values of the control population. Alcohol consumption per week was calculated from questions about the types, frequency, and average amount of alcohol consumed. Alcohol consumption was categorized into three groups: none, ⩽280 g of alcohol/week, and >280 g of alcohol/week. Subjects who had smoked 20 cigarettes or more during their life were classified as smokers and those who had not were considered nonsmokers. Pack-year was used as an index of cumulative smoking.

The purpose of the study was to determine if dietary factors, genetic polymorphisms, MSI, and mutations of *p53* and *Ki-ras* genes were associated with hypermethylation of the *hMLH1* gene promoter. We used unconditional logistic analysis to compare the risk of exhibiting or not exhibiting hypermethylation of the *hMLH1* promoter in tumors and controls using the SAS System for Windows (Release 8.1). *P*-values less than 0.05 were considered significant.

RESULTS

There were significant differences according to the smoking history, pack-years, and higher weekly alcohol intake between patients with gastric cancers with hypermethylation of the *hMLH1* promoter and controls (Table 2). As the amount of cigarette smoking or alcohol drinking increased, the risk of gastric cancer with the *hMLH1* promoter hypermethylation (Table 3).

High consumption of potatoes and butter, cheese and margarine was associated with lower risk of gastric cancer with hypermethylation of the *hMLH1* promoter. In contrast, consumption of vegetables was associated with higher risk of gastric cancer with hypermethylation of the *hMLH1* promoter. High intake of mushrooms and fruits and low intake of cereals and butter, cheese and margarine were associated with higher risk of gastric cancer without hypermethylation of the *hMLH1* promoter (Table 4). Among the nutrients, vitamins, and minerals evaluated, high intake of protein, phosphorus, potassium, vitamin C, zinc, and calcium was associated with higher risk of gastric cancer without hypermethylation of the *hMLH1* gene promoter. However, the intake of nutrients, vitamins, and minerals

Table 2 Interaction between cigarette smoking and alcohol intake, and *hMLH1* gene promoter hypermethylation in gastric cancer

	Controls (*n*)	Cases without hMLH1 promoter hypermethylation (*n*)	Cases with hMLH1 promoter hypermethylation (*n*)	χ^2_{trend}
Smoking history				
Non-smoker	102	27	13	3.827
Smoker	117	42	24	
Odds ratio	Referent (1.00)	1.16 (0.62–2.17)	3.04[a] (1.29–7.19)	
Alcohol drinking				
Never	95	26	15	1.327
Ever	124	43	22	
Odds ratio	Referent (1.00)	1.07 (0.57–2.01)	2.11 (0.90–4.98)	

Odds ratio was adjusted for age and sex. [a]*P*<0.05 *vs* others.

Table 3 Interaction between amount of cigarette smoking and alcohol intake, and *hMLH1* gene promoter hypermethylation in gastric cancer

	OR[1] (95%CI[2])	
	Cases without hMLH1 promoter hypermethylation *vs* controls	Cases with hMLH1 promoter hypermethylation *vs* controls
Cumulative smoking		
0	1.00	1.00
1–15	0.96 (0.38–2.41)	0.86 (0.22–3.41)
16–34	0.92 (0.44–1.93)	0.75 (0.24–2.38)
35–	0.39[a] (0.16–0.93)	3.17[a] (1.20–8.42)
χ^2_{trend}	1.202	6.344[a]
Ethanol uptake per week (g / wk)		
0	1.00	1.00
⩽280	0.58 (0.29–1.14)	1.35 (0.51–3.55)
⩾281	0.68 (0.30–1.53)	3.94[a] (1.21–12.80)
χ^2_{trend}	0.830	5.419[a]

[1]Odds ratio estimated using a conditional logistic analysis. [2]Confidence interval. [a]*P*<0.05 *vs* others.

Table 4 Distribution of controls and cases with or without promoter hypermethylation of the hMLH1 gene according to their intake of food groups which were statistically significant

	Controls (*n*)	Cases without hMLH1 promoter hypermethylation (*n*)	Cases with hMLH1 promoter hypermethylation (*n*)
Cereal			
Low	110	45	17
High	109	24	20
Odds ratio	Referent (1.00)	0.56[a] (0.32–0.99)	0.94 (0.45–1.96)
Potato			
Low	109	36	27
High	110	33	10
Odds ratio	Referent (1.00)	1.00 (0.57–1.74)	0.30[b] (0.14–0.67)
Vegetable			
Low	110	29	12
High	109	40	25
Odds ratio	Referent (1.00)	1.42 (0.82–2.46)	2.17[a] (1.03–4.58)
Mushroom			
Low	110	26	18
High	109	43	19
Odds ratio	Referent (1.00)	1.85[a] (1.05–3.27)	0.89 (0.43–1.83)
Fruit			
Low	110	25	13
High	109	44	24
Odds ratio	Referent (1.00)	1.86[a] (1.06–3.27)	1.69 (0.81–3.54)
Butter, cheese, and margarine			
Low	110	49	24
High	109	20	13
Odds ratio	Referent (1.00)	0.45[b] (0.24–0.81)	0.44[a] (0.20–0.93)

Odds ratio was adjusted for age and sex. [a]*P*<0.05. [b]*P*<0.01 *vs* others.

did not differ significantly between patients with gastric cancers with the *hMLH1* promoter hypermethylation, those with gastric cancers without it, or controls (Table 5).

Genetic polymorphism of *GSTM1*, *GSTT1*, *CYP1A1*, *CYP2E1*, *ALDH2* and *L-myc* was not associated with development of gastric cancers with the *hMLH1* promoter hypermethylation or those without it (Table 6).

Hypermethylation of the *hMLH1* gene promoter was detected in 35.2% of patients with gastric cancer, in 13.6% of those with MSI, in 28.2% of those with mutations of *p53*, and in 4.9% of those with the *Ki-ras* gene (data not shown). Hypermethylation of the *hMLH1* promoter occurred in 10 of 14 MSI+ cases (71.4%) and in 28 of 94 MSI- cases (29.8%). We found a striking association between hypermethylation of the *hMLH1* promoter and MSI (Table 7). Hypermethylation of the *hMLH1* gene promoter was significantly inversely correlated with mutation of the *p53* gene (Table 7).

DISCUSSION

Cigarette smoking and alcohol consumption have been identified as risk factors for gastric cancer in some studies[33-36], although others have not found a causal relationship between these factors[37,38]. Data from our unconditional logistic models showed that both cigarette smoking and alcohol consumption play dominant roles in the development of gastric cancer with hypermethylation of the *hMLH1* promoter, but not in the development of cancer without hypermethylation of the promoter. This finding suggests that smoking- or alcohol-related biological pathways leading to the development of gastric cancer involve hypermethylation of the *hMLH1* promoter. Although it is unclear whether smoking induces hypermethylation of the *hMLH1* gene promoter in humans, recent reports indicate an association between DNA methylation and tobacco carcinogens in animal models[39,40]. Previous studies have also shown that smoking and alcohol consumption increase the risk of developing microsatellite-unstable tumors[41,42].

The exact mechanism of DNA hypermethylation by alcohol is unknown. However, it has been hypothesized that

Table 5 Distribution of controls and cases with or without promoter hypermethylation of the hMLH1 gene according to their intake of nutrients, vitamins, and minerals which were statistically significant

	Controls (*n*)	Cases without hMLH1 promoter hypermethylation (*n*)	Cases with hMLH1 promoter hypermethylation (*n*)
Protein			
Low	109	25	18
High	110	44	19
Odds ratio	Referent (1.00)	1.81[a] (1.03–3.17)	1.00 (0.49–2.02)
Phosphorus			
Low	109	25	20
High	110	44	17
Odds ratio	Referent (1.00)	1.82[a] (1.03–3.19)	0.77 (0.38–1.57)
Potassium			
Low	109	22	15
High	110	47	22
Odds ratio	Referent (1.00)	2.38[b] (1.32–4.26)	1.24 (0.60–2.56)
Vitamin C			
Low	110	19	13
High	109	50	24
Odds ratio	Referent (1.00)	2.78[b] (1.53–5.05)	1.74 (0.84–3.63)
Zinc			
Low	110	23	15
High	109	46	22
Odds ratio	Referent (1.00)	2.20[b] (1.23–3.91)	1.31 (0.64–2.70)
Calcium			
Low	110	22	15
High	109	47	22
Odds ratio	Referent (1.00)	2.32[b] (1.30–4.14)	1.34 (0.65–2.76)

Odds ratio was adjusted for age and sex. [a]$P<0.05$, [b]$P<0.01$ *vs* others.

Table 6 Distribution of controls and cases with or without promoter hypermethylation of the hMLH1 gene according to the genetic polymorphisms of *GSTM1*, *GSTT1*, *CYP1A1*, *CYP2E1*, *NAT2*, *ALDH2*, and *L-myc*

	Controls (*n*)	Cases without hMLH1 promoter hypermethylation (*n*)	Cases with hMLH1 promoter hypermethylation (*n*)
GSTM1			
Undeleted	90	21	13
Deleted	130	48	25
Odds ratio	Referent (1.00)	1.67 (0.93–3.00)	1.18 (0.56–2.47)
GSTT1			
Undeleted	117	32	17
Deleted	103	37	21
Odds ratio	Referent (1.00)	1.32 (0.76–2.29)	1.47 (0.72–2.98)
CYP1A1			
Ile / Ile	115	36	22
Ile / Val+Val / Val	104	33	15
Odds ratio	Referent (1.00)	1.02 (0.59–1.77)	0.74 (0.36–1.52)
CYP2E1			
c1 / c1	129	44	25
c1 / c2+c2 / c2	88	26	13
Odds ratio	Referent (1.00)	0.89 (0.51–1.56)	0.76 (0.37–1.59)
ALDH2			
NN	139	38	26
ND+DD	79	31	11
Odds ratio	Referent (1.00)	1.45 (0.83–2.52)	0.73 (0.34–1.56)
L-myc			
Low	52	20	9
High	164	48	29
Odds ratio	Referent (1.00)	1.59 (0.86–2.92)	1.56 (0.61–3.99)

Odds ratio was adjusted for age and sex.

Table 7 Frequencies of mutations of the *p53* and *Ki-ras* genes, and microsatellite instability according to *hMLH1* promoter hypermethylation

Gene	*hMLH1* promoter hypermethylation		OR[1] (95%CI[2])	χ^2	*P*
	Yes (%)	No (%)			
P53					
No	31 (81.58)	46 (65.71)	1.00	4.199	0.041
Yes	7 (18.42)	24 (34.29)	0.34 (0.12–0.95)		
Ki-ras					
No	37 (97.37)	58 (93.55)	1.00	0.407	0.524
Yes	1 (2.63)	4 (6.45)	0.47 (0.05–4.72)		
[3]MSI					
No	28 (73.68)	66 (92.86)	1.00	7.458	0.006
Yes	10 (26.32)	4 (7.14)	6.19 (1.67–22.88)		

[1]Odds ratio was adjusted for age and sex. [2]Confidence interval. [3]Microsatellite instability.

alcohol could influence carcinogenesis by influencing mucosal cell proliferation and related histological changes[43]. These changes have been associated with mucosal hyperregeneration, which may make the mucosa more susceptible to the action of other carcinogens such as cigarette smoke[43]. Therefore, alcohol consumption might increase the bioavailability of DNA-binding smoke components in the mucosa of the upper digestive tract, increasing the plasma levels of these compounds, or might modify the metabolism of pro-carcinogenic compounds by inducing specific metabolic pathways involving an aberrant mismatch repair system[44].

Folate deficiency is associated with hypermethylation of the H-cadherin promoter[45]. However, we found no significant association between folate intake and hypermethylation of the *hMLH1* promoter. Su and Arab reported that low folate intake is aggravated by high alcohol intake[46], probably because folate is degraded by acetaldehyde, an intermediate metabolite of alcohol[47]. van Engeland *et al*., suggested that intake of folate and alcohol is associated with changes in promoter hypermethylation in colorectal cancer[48]. Our data showing that alcohol intake increased the risk of gastric cancer with hypermethylation of the *hMLH1* promoter are consistent with these previous reports.

Most dietary factors, nutrients, vitamins, and minerals are not associated with gastric cancer with hypermethylation of the *hMLH1* promoter, although we found that a high intake of vegetables and low intake of potato and butter, cheese, and margarine were associated with increased likelihood of gastric cancer without hypermethylation of the *hMLH1* promoter, and high intake of mushrooms and fruits and low intake of cereals and butter, cheese and margarine were associated with higher risk of gastric cancer without hypermethylation of the *hMLH1* promoter. We cannot be certain that these results did not occur by chance, given the low number of comparisons. However, we observed that different dietary factors selectively affected the pathways to gastric cancer with or without hypermethylation of the *hMLH1* promoter. For example, a high intake of butter, cheese, and margarine was associated with a lower risk of gastric cancers either with or without hypermethylation of the *hMLH1* promoter. These findings agree with epidemiological data showing a relatively low incidence of gastric cancer in countries with consumption of high butter, cheese, and margarine[49]. Based on these facts, it could be suggested that butter, cheese and margarine decrease the risk of gastric cancer regardless of the hMLH1 promoter hypermethylation.

It has been reported that vitamin C can induce hypermethylation of gene promoters[23]. However, a higher intake of vitamin C is associated with an increased risk of gastric cancer in this present study. One of the main vitamin C sources for Koreans is kimchi, which has been reported as a potent risk factor for gastric cancer in some Korean epidemiologic studies[3]. Therefore, kimchi intake increases vitamin C intake amount, and, at the same time, the risk of gastric cancer.

Few epidemiological studies on gastric cancer have included genetic polymorphisms in the analysis or evaluated the association between genetic polymorphisms and hypermethylation of the *hMLH1* gene promoter. Several studies have reported an independent, increased risk of gastric cancer for the *GSTM1* null[7], *GSTT1* null[6], *CYP2E1* c1/c2 or c2/c2[50], *2-allele containing *ALDH2* genotypes[9], and shorter (s) allele-containing *L-myc*[10] genotypes. However, other studies have not found any association between gastric cancer and these genotypes[51-53]. We found no significant association between polymorphisms of *GSTM1*, *GSTT1*, *CYP1A1*, *CYP2E1*, *ALDH2*, and *L-myc* and the risk of gastric cancer with or without hypermethylation of the *hMLH1* promoter. These findings suggest that the genetic polymorphisms of the *GSTM1*, *GSTT1*, *CYP1A1*, *CYP2E1*, *ALDH2*, and *L-myc* genes might not be independent risk factors, but could act as effect modifiers of the risk of gastric cancer through environmental factors, such as dietary intake.

We examined the mononucleotide repeats BAT25 and BAT26 to detect genuine MSI because these repeats are considered as ideal diagnostic markers. Mononucleotide repeats are sufficient for the diagnosis of true MSI[54]. A consensus mononucleotide locus, BAT26 is altered in all tumors with genuine MSI[55,56]. We found that 10 of the 14 MSI+ gastric cancer cases (71%) in our patients were hypermethylated in the promoter region of *hMLH1*. We found a significant association between hypermethylation of the *hMLH1* promoter and MSI+ gastric carcinoma ($P = 0.006$), which is consistent with previous reports[21,57].

Point mutations in the *p53* tumor suppressor gene[58,59] and *ras* oncogenes[60,61] are frequently found in human and rodent tumors. Mutations of the *p53* and *Ki-ras* genes were detected in 28.2% and 4.9% of our patients with gastric cancer, respectively. We also found a significant inverse association between hypermethylation of the *hMLH1* gene promoter and *p53* mutations. Previous studies have reported a significantly lower incidence of *p53* gene alterations in MSI+ gastric cancer, in MSI+ colorectal cancers, and in colorectal cancer cell lines[62,63] than in MSI- gastric cancer[64,65]. Together, these data confirm the existence of alternative genetic pathways for gastric cancer, such as the classical 'tumor suppressor' pathway and the 'mismatch repair' pathway.

In conclusion, despite its limited size, this study suggests that cigarette smoking and alcohol consumption are significantly associated with higher risk of gastric cancer having hypermethylation of the *hMLH1* promoter. Polymorphisms of *GSTM1*, *GSTT1*, *CYP1A1*, *CYP2E1*, *ALDH2*, and *L-myc* genes were not associated with gastric cancers either with or without hypermethylation of the *hMLH1* promoter, suggesting that these polymorphisms operate along disease pathways other than those involving the mismatch repair system in gastric cancer. Our data also highlight the importance of aberrant methylation of the *hMLH1* promoter in causing MSI in gastric cancer. The negative association between hypermethylation of the *hMLH1* gene promoter and *p53* mutations suggests that there could be two or more different molecular pathways in the development of gastric cancer, such as tumor suppression mechanisms and DNA mismatch repair.

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