Aberrant CpG Island Hypermethylation of Chronic Gastritis, in Relation to Aging, Gender, Intestinal Metaplasia, and Chronic Inflammation

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Aberrant hypermethylation of promoter CpG islands is an important mechanism for the inactivation of tumor suppressor genes. CpG island hypermethylation occurs in relation to tumorigenesis or aging. Gastric cancer is one of the tumors with a high level of aberrant CpG island methylation. However, the data on the methylation status of normal gastric mucosa has been very limited. The present study attempted to compare the methylation status of nonneoplastic gastric mucosa, using clinicopathological parameters, including age, gender, *Helicobacter pylori* (*H. pylori*), acute and chronic inflammation, and intestinal metaplasia. Two hundred sixty-eight nonneoplastic gastric mucosa samples were studied for the methylation status of 11 genes (*COX-2*, *DAP-kinase*, *E-cadherin*, *GSTP1*, *MGMT*, *hMLH1*, *p14*, *p16*, *THBS1*, *TIMP3*, and *RASSF1A*), using methylation-specific PCR. CpG island hypermethylation was found in 53.7, 41, 37.7, 23.1, 18.7, 10.9, 10, 4.1, 3.4, 1.7, 0.4% for *DAP-kinase*, *E-cadherin*, *THBS1*, *TIMP3*, *p14*, *MGMT*, *p16*, *COX-2*, *GSTP1*, *hMLH1* and *RASSF1A*, respectively. Five genes (*DAP-kinase*, *E-cadherin*, *p14*, *THBS1*, and *TIMP-3*) showed a general progressive increase in the methylation frequency as a function of aging, whereas the other genes (*COX-2*, *GSTP1*, *MGMT*, *hMLH1*, *p16*, and *RASSF1A*) were rarely methylated. Male patients showed higher numbers of methylated genes than females (3.2 *vs*. 2.1, respectively, *P* = 0.002). Gastritis samples with marked intestinal metaplasia, showed higher numbers of genes methylated than those without (3.7 *vs*. 2.6, respectively, *P* = 0.021). Gastritis samples with marked infiltration of mononuclear cells displayed higher numbers of genes methylated than those with mild or moderate infiltration of mononuclear cells (3.4 *vs*. 2.5 or 2.5, respectively, *P* < 0.05). Our results demonstrated that many genes are methylated in the stomach as a function of age, and suggested that male gender, intestinal metaplasia, and chronic inflammation are closely associated with increased methylation in nonneoplastic gastric mucosa samples. (*Am J Pathol 2003, 163:1551–1556*)

Aberrant DNA methylation is a feature of human cancers, characterized by generalized hypomethylation and regional hypermethylation.[1–4] The regional hypermethylation involves CpG islands located in the promoter and upstream exons. Hypermethylation of CpG islands recruits methyl DNA binding proteins, and subsequently histone deacetylases.[5,6] Deacetylation of the histone backbones makes the DNA structure of the promoter into a closed chromatin structure, inaccessible to transcription factors, resulting in gene inactivation.[7,8] Aberrant hypermethylation of CpG islands located in the promoter, and/or upstream exons, acts as an alternative to genetic changes for the inactivation of tumor suppressor genes.[3,4]

CpG islands are normally protected from DNA methylation,[9] but in relation to cancer or aging, they are aberrantly methylated.[3,10] The stomach is one of the organs that shows frequent methylation of CpG islands of genes in nonneoplastic epithelial cells.[11–13] Many genes have been demonstrated to be methylated in nonneoplastic gastric mucosae, whether in association with gastric cancer or not.[11–13] Our previous study has demonstrated that these genes are not methylated in pediatric gastric mucosae, or at a frequency significantly lower than that in gastric mucosae from adults.[11] If these methylations are an age-related event, they would be expected to be progressively prominent with age. However, no study has ever displayed plots correlating the methylation frequency of genes in the normal stomach with age.

In our previous study, aberrant promoter CpG island methylation of some genes was a frequent event in nonneoplastic gastric mucosae.[11] The methylation status of these genes, in normal gastric mucosae, have not been
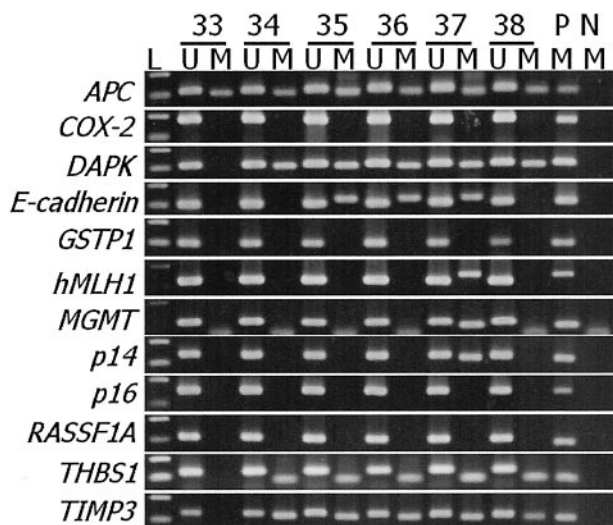
Figure 1. Electrophoresis of PCR products of representative samples. The PCR products in the **lanes** marked U show the presence of unmethylated templates of each gene, whereas the products in the **lanes** marked M indicate the presence of methylated templates. L, size marker (100-bp DNA ladder); P, positive control; N, negative control. Positive control is normal lymphocyte DNA treated with Sss1 methyl transferase before bisulfite modification. Negative control is distilled water without template DNA.

analyzed in relation to the histological parameters of chronic gastritis. The present study attempted to determine whether the histological parameters of chronic gastritis, as well as aging and gender, affect DNA methylation in nonneoplastic gastric mucosae. The aim of the present study was not to investigate the functional consequences of CpG island hypermethylation events, but to investigate the occurrence of CpG island hypermethylation in chronic gastritis in relation to aging or clinicopathologic variables. We examined 11 genes, whose expressions were frequently silenced by aberrant CpG island methylation in gastric, or other, cancers. The tested genes included those involved in cell-cycle regulation (*COX2*, *p14*, *p16*), signal transduction (*RASSF1A*), DNA repair or protection (*hMLH1*, *MGMT*, and *GSTP1*), apoptosis (*DAP-kinase*), and angiogenesis (*THBS1*), or those related to metastasis and invasion (*E-cadherin* and *TIMP-3*). MSP method was used for the analysis of the methylation frequency for the 11 genes, and the findings were correlated with the clinicopathological parameters.

Materials and Methods

A total of 268 archival samples from gastroscopic biopsies were collected from the Department of Pathology, Seoul National University Hospital, Seoul, Korea, and the Department of Diagnostic Pathology, Asan Medical Center, Seoul, Korea. The patients underwent endoscopic examination for the complaint of abdominal discomfort and diagnosed as having chronic gastritis. They did not have gastric cancers. The sex ratio was 160:108, and the ages ranged from 2 to 79 years. Through microscopic examination, hematoxylin and eosin-stained histological slides were scored for their histological parameters; *H. pylori*, chronic inflammation, acute inflammation, and in-

testinal metaplasia. The scoring was performed according to the modified Sydney system.[14] Giemsa-stained histological slides were microscopically examined for the densities of *H. pylori* in the biopsy sample.

DNA Extraction and Bisulfite Modification

Ten histological sections of 20-μm thickness were dewaxed in xylene, with subsequent ethanol treatment. After digestion with proteinase K (Invitrogen, Carlsbad, CA), the DNA was extracted with phenol/chloroform/isoamyl alcohol, and precipitated in ethanol. The purified DNA was subjected to bisulfite modification, as described by Herman et al[15] In brief, 2 μg of genomic DNA and 1 μg of salmon sperm DNA (as a carrier) (Invitrogen) were heated at 97°C for 6 minutes, and then cooled on ice. Fifteen μl of 1 mol/L NaOH was added to 35 μl of the denatured DNA solution, and this mixture was stored at room temperature for 15 minutes. Five hundred fifty μl of bisulfite (0.22 g) (Sigma-Aldrich, St. Louis, MO) and hydroquinone (0.08 mg) (pH 5.0) (Sigma-Aldrich) was added to the denatured DNA solution, and the mixture incubated at 55°C for 16 hours. After extraction with a JET-SORB gel extraction kit (Genomed, Bad Oeynhausen, Germany), the purified DNA (35 μl) was desulphonated with 15 μl of 1 mol/L NaOH. The modified DNA was precipitated in ethanol, and then resuspended in 50 μl TE buffer [10 mmol/L Tris and 1 mmol/L EDTA (pH 8.0)].

MSP (Methylation-Specific PCR)

The bisulfite-modified DNA (2 μl) was amplified with primers specific for either the methylated, or unmethylated, sequences. The methylated primers were selected because the primer sequences are located in upstream sequences of the genes (around transcription start sites or in the first exon) and the inverse relation between methylation and expression of the gene has been demonstrated in cancer cell or tissues by other studies using these primers. In a preliminary study, nine SNU gastric cancer cell lines were analyzed for the relationship between CpG island methylation and gene expression of the specific gene, using the same primer sets in the present study. The cell lines showed a loss or decrease of mRNA for the genes methylated, and re-expression of the genes after treatment of demethylating agent. The primer sequences of all genes for the methylated and unmethylated forms, and the annealing temperatures, cycle numbers and references, are summarized in Table 1. PCR was performed in 25-μl reaction volumes, containing 1X PCR buffer [16.6 mmol/L $(NH_4)_2SO_4$, 67 mmol/L Tris (pH 8.8), 6.7 mmol/L $MgCl_2$, 10 mmol/L β-mercaptoethanol], deoxynucleotide triphosphates (each at 0.2 mmol/L), primers (10 pmol each) and 1 unit of *Taq* polymerase. Seven μl of PCR products underwent electrophoresis on 2.5% agarose gel, then visualized under UV illumination using an ethidium bromide stain. Samples showing signals approximately equivalent to that of the size marker (7 ng/μl) were scored as methyl-

Table 1. Primer Sequences and PCR Conditions for MSP Analysis

Primer name*		Primer sequence (5′-3′) forward	Primer sequence (5′-3′) reverse	Product size (bp)	AT[†] (°C)	No. of cycles	References
COX-2	m	TTAGATACGGCGGCGGCGGC	TCTTTACCCGAACGCTTCCG	161	61	3516	
	u	ATAGATTAGATATGGTGGTGGTGGT	CACAATCTTTACCCAAACACTTCCA	171	61	35	
DAP-kinase	m	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98	60	35	17
	u	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	98	60	35	
E-cadherin	m	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	115	57	33	15
	u	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	97	53	33	
GSTP1	m	TTCGGGGTGTAGCGGTCGTC	GCCCCAATACTAAATCACGACG	91	59	35	18
	u	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAACA	97	59	35	
MGMT	m	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	81	59	35	19
	u	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	93	59	35	
hMLH1	m	TATATCGTTCGTAGTATTCGTGT	TCCGACCCGAATAAACCCAA	153	60	33	20
	u	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124	60	33	
p14	m	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	122	60	33	21
	u	TTTTTGGTGTTAAAGGGTGGTGTAGT	CACAAAAACCCTCACTCACAACAA	132	60	33	
p16	m	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65	33	15
	u	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60	33	
RASSF1A	m	GTGTTAACGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA	93	60	35	22
	u	TTTGGTTGGAGTGTGTTAATGTG	CAAACCCCACAAACTAAAAACAA	105	60	35	
THBS1	m	TGCGAGCGTTTTTTTAAATGC	TAAACTCGCAAACCAACTCG	74	62	35	23
	u	GTTTGGTTGTTGTTTATTGGTTG	CCTAAACTCACAAACCAACTCA	115	62	35	
TIMP3	m	CGTTTCGTTATTTTTTGTTTTCGGTTTTC	CCGAAAACCCCGCCTCG	116	59	35	24
	u	TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	CCCCCCAAAAACCCCACCTCA	122	59	35	

*m, methylated sequence; u, unmethylated sequence.
[†]AT, annealing temperature.

ated. Samples giving faint positive signals were repeated three times and only those samples with consistent positive signals were scored as methylated. Normal peripheral blood lymphocytes, obtained from patients with no evidence of cancer ($n = 20$), were used as a control group. Twelve men and 8 women, with a median age of 46 years (range, 23 to 68), were included in this group. The 11 genes were not methylated in the normal peripheral blood lymphocytes.

Statistical Analysis

All statistical calculations were made using the SPSS software (version 10.0 SPSS, Chicago, IL). Student's *t*-test or analysis of variance test and χ^2 test were used to compare the numbers of methylated genes, and the frequency of CpG island hypermethylation for the tested genes, respectively, in chronic gastritis by anatomical site, gender, age, and scores of histological parameters of the modified Sydney system (*H. pylori*, acute inflammation, chronic inflammation, and intestinal metaplasia). Of the collected samples, those from the patients younger than 40 years showed a skewed distribution of intestinal metaplasia, *H. pylori*, and gender. Pediatric patients showed no intestinal metaplasia at all, and very low frequencies of *H. pylori*-positivity, whereas young patients (third and fourth decades) showed high frequencies of *Helicobacter*-positivity and low frequencies of intestinal metaplasia. When the distribution of the scores for each histological parameter was analyzed by decades, for the patients over 40 years of age, the distribution was not skewed for any of the histological parameters and thus, the statistical analysis of the methylation frequency, with respect to histological parameters, was confined to the patients over 40 years of age ($n = 169$).

Results

Two hundred sixty eight nonneoplastic gastric mucosa samples were studied for the methylation status of 11 genes (*COX-2*, *DAP-kinase*, *E-cadherin*, *GSTP1*, *MGMT*, *hMLH1*, *p14*, *p16*, *RASSF1A*, *THBS1*, and *TIMP3*) using methylation-specific PCR (Figure 1). CpG island methylation was found in 53.7% for *DAP-kinase*, 41% for *E-cadherin*, 37.7% for *THBS1*, 23.1% for *TIMP3*, 18.7% for *p14*, 10.9% for *MGMT*, 10% for *p16*, 4.1% for *COX-2*, 3.4% for *GSTP1*, 1.7% for *hMLH1*, and 0.4% for *RASSF1A*. The frequencies of CpG island hypermethylation for the genes tested in each age group are displayed in Figure 2. The numbers of genes methylated for the 11 genes tested ranged from 0.4 to 3.3, exhibiting a general progressive increase with aging (Figure 2A). Five genes (*DAP-kinase*, *E-cadherin*, *p14*, *THBS1*, and *TIMP-3*) showed a general increase in the methylation frequency as a function of aging (Figure 2B), whereas the other genes (*COX-2*, *GSTP1*, *MGMT*, *hMLH1*, *p16*, and *RASSF1A*) were rarely methylated (Figure 2C).

When the number of genes methylated, or the frequency of CpG island methylation, of each gene tested was compared in chronic gastritis patients (aged over 40 years, $n = 169$), by gender and histological parameters (Table 2 and Figure 3), the male patients (mean age, 58 years; $n = 108$) showed higher numbers of methylated genes than the females (mean age, 57 years; $n = 61$) (3.2 *vs*. 2.1, respectively, Student's *t*-test, $P = 0.002$). *DAP-*
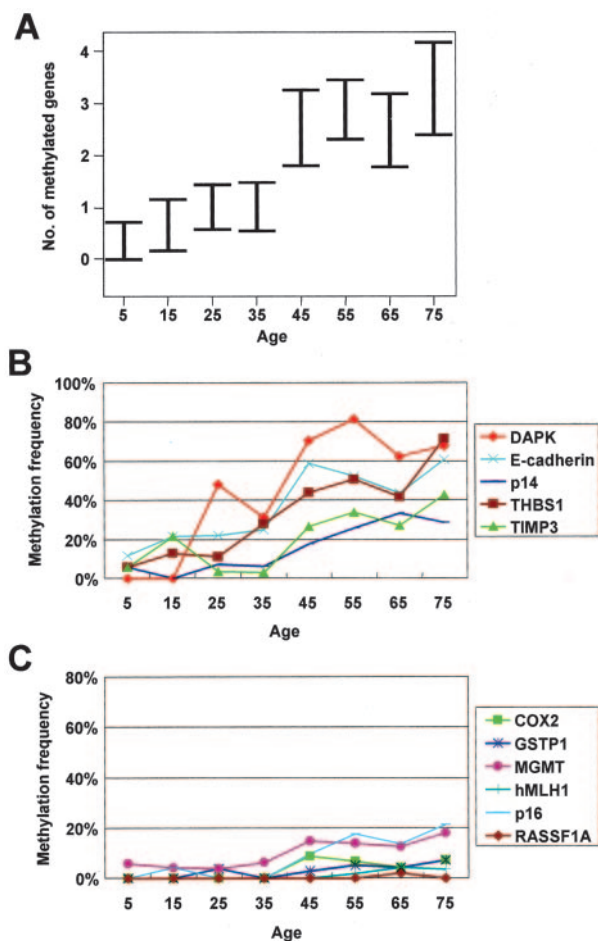
Figure 2. The number of methylated genes in 10-year age groups. Results are given as mean ± SE (**A**). The methylation frequency of *DAP-kinase, E-cadherin, p14, THBS1,* and *TIMP-3* increased with aging (**B**) but that of *COX2, GSTP1, MGMT, hMLH1, p16,* and *RASSF1A* did not (**C**).

kinase, E-cadherin, THBS1, and *TIMP-3* were more frequently methylated in the male patients than in females, with the difference in the methylation frequency reaching statistical significance ($P < 0.05$). No significant difference was found in the numbers of the genes methylated between subjects either with, or without, *H. pylori.* The subjects with marked intestinal metaplasia showed higher numbers of genes methylated than the subjects

Table 2. The Number of Genes Methylated in Chronic Gastritis According to Grades of Histologic Parameters

	Absent	Mild	Moderate	Marked
Helicobacter	2.9	2.7	2.7	2.7
pylori	($n = 84$)	($n = 5$)	($n = 26$)	($n = 54$)
Intestinal	2.5*	2.9†	2.8‡	3.7*,†,‡
metaplasia	($n = 91$)	($n = 31$)	($n = 24$)	($n = 23$)
Acute	2.4	3.3	2.7	2.9
inflammation	($n = 41$)	($n = 37$)	($n = 71$)	($n = 20$)
Chronic		2.5	2.5§	3.4§
inflammation		($n = 35$)	($n = 78$)	($n = 57$)

*, absent vs. marked, $P = 0.015$ by Student's t-test.
†, mild vs. marked, $P = 0.161$ by Student's t-test.
‡, moderate vs. marked, $P = 0.165$ by Student's t-test.
§, moderate vs. marked, $P = 0.01$ by Student's t-test.

without (3.7 *vs.* 2.6, $P = 0.021$, Student's *t*-test). Samples with a marked degree of mononuclear cell infiltration displayed higher numbers of genes methylated than samples with mild or moderate degrees of chronic inflammation (3.4 *vs.* 2.5 or 2.5, $P = 0.022$, analysis of variance test). *DAP-kinase, E-cadherin,* and *THBS1* were more frequently methylated in samples with a marked degree of mononuclear cell infiltration than in samples with mild or moderate degrees of chronic inflammation, with the difference in the methylation frequency of the respective genes reaching statistical significance. However, no statistical difference in the numbers of genes methylated was noted in the samples in relation to the degree of neutrophilic infiltration. The numbers of genes methylated were 2.6, 3.2, and 2.9 for samples from the antrum, body, and cardia, respectively, but they showed no significant difference.

Discussion

Aberrant CpG island methylation is a frequent finding in human cancers, and an important molecular mechanism of inactivation of tumor suppressor genes in the development of human cancers, regardless of the tissue type.[3,4,25] Against the original findings of the lack of CpG island methylation in normal tissues, CpG island methylation is often found in histologically normal tissues, in relation to aging.[26] Aging-related methylation has been demonstrated for the *ER* gene by Issa et al[27] and for multiple genes by Ahuja et al.[28] The susceptibility to aging-related CpG island methylation differs among genes and tissues.[28] The present study has demonstrated that *DAP-kinase, E-cadherin,* and *THBS1* were methylated at frequencies higher than 35%, whereas *COX-2, GSTP1, hMLH1,* and *RASSF1A* were methylated at frequencies less than 5% in nonneoplastic gastric mucosae. When the genes frequently methylated in nonneoplastic gastric mucosae were analyzed for their methylation status in normal tissues of the colon, liver, or prostate, they were found to be rarely methylated (data not shown). This finding exhibits a tissue-, or gene-difference, for aging-related methylation. The finding that the stomach is one of the normal tissues with a high frequency of aging-related methylation, is consistent with the finding that gastric cancer is one of the tumors with a high frequency of CpG island methylation.[29] The causes for such a difference among tissue types remain unclear, but it may be related to the accessibility of the tissue to exogenous agents; the stomach is directly exposed to exogenous agents. Dietary factors or heavy metals may be possible candidates. Issa[30] has suggested that diets high in fiber might be associated with reduced levels of *ER* methylation in colonic mucosa. *In vitro* studies have demonstrated that exposure to nickel causes CpG island methylation.[31,32]

In addition to exogenous agents, endogenously-produced materials, including reactive oxygen species, are suspected as possible causes for aging-related methylation in the stomach.[10,33] Epithelial cell injury, caused by *H. pylori,* implicates several pathways, with oxygen-radical injury being one.[34,35] However, in this study, no difference was noted in the frequencies of CpG island methylation for the genes tested between chronic gastritis with, and without,
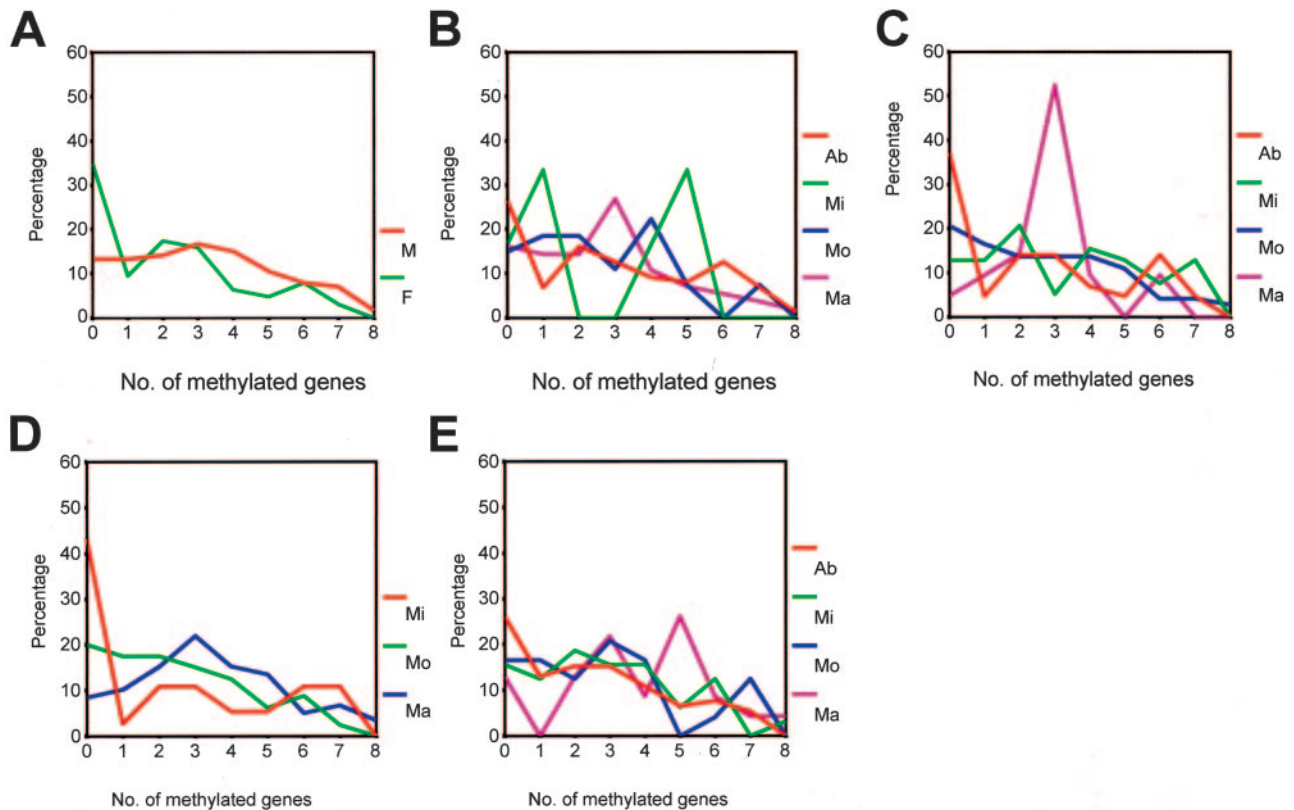
Figure 3. The number of genes methylated was analyzed in relation to clinicopathologic parameters: gender (**A**), *H. pylori* density (**B**), grade of acute inflammatory cell infiltration (**C**), grade of chronic inflammatory cell infiltration (**D**), and grade of intestinal metaplasia (**E**). Results are given as percentage of methylation-positive samples. Ab, absent; Mi, mild; Mo, moderate; Ma, marked.

H. pylori. The present study has a limitation in the evaluation of *H. pylori*, as the absence of *H. pylori* in the biopsy materials did not exclude the possibility of the presence of *H. pylori* in the adjacent sites that were not biopsied, or the previous residence of *H. pylori* in the same biopsied sites. However, the urea breath test, which was performed on a small proportion of the patients, showed correlation with the results of histological assessment of *H. pylori*.

The present study has demonstrated a significant difference in the number of genes methylated between male and female patients. The average number of genes methylated was 3.2 and 2.1 in male and female patients, respectively. However, when the numbers of genes methylated was compared in gastric cancers by gender, no difference was noted (data not shown). It is plausible that the gender-difference of the extent of methylation in chronic gastritis may be related to the gender-difference in the incidences of gastric cancers. It is currently unclear why such a gender difference for the extent of methylation exists in chronic gastritis. Larger studies are needed to validate the gender difference in aging-related methylation in nonneoplastic gastric mucosae.

In the present study, a general progressive increase in the numbers of the genes methylated, in relation to aging, was displayed. The fact that gastric cancer occurs in older people and has a high frequency of CpG island methylation, suggests the link of aging and cancer via increase in CpG island methylation. In the present study, a patient-to-patient variation in the extent of DNA methylation existed in

the same age group. Among the patients aged over 40 years, 15% showed CpG island methylation at more than 50% of the genes tested. Considering that nonneoplastic gastric mucosae, from patients with gastric cancers, harbor higher levels of CpG island methylation than nonneoplastic gastric mucosae from patients without gastric cancer,[12,13] these individuals with higher levels of CpG island methylation are expected to be at increased risk of tumor formation. A prospective study is required to confirm the risk.

The present study has demonstrated that many genes are frequently methylated in chronic gastritis. The methylation detected by MSP might not affect gene expression although MSP analysis using the same primer sets displayed an inverse relationship between methylation and expression of mRNA of the corresponding gene in gastric cancer cell lines and bisulfite-sequencing of MSP products of each of the genes from chronic gastritis samples showed that all of the CpG sites, in between both primers, were also methylated (data not shown). This is because the methylation of the CpG sites along CpG islands might be patchy and incomplete or the methylation detected by MSP might affect only a few cells over 1000 cells in each sample[15] and the vast majority of the cells might not harbor the CpG island methylation of the specific gene. The present study just demonstrated the presence of the methylated allele but did not say about the quantitative prevalence of the methylated allele. It is apparent that the occurrence of the methylated alleles of some genes increased with aging or was asso-

ciated with some clinicopathologic variables (gender, intestinal metaplasia, and chronic inflammation), but it is unknown whether the proportion of the methylated alleles of the genes does so. For the quantitative determination of the methylated allele, microdissection of the epithelial cells and quantitative assay, including Ms-SNuPE[36] or MethyLight,[37] should be performed.

In conclusion, we have studied chronic gastritis samples, from pediatric to geriatric patients, for the methylation status of the 11 genes using MSP assay, and determined the methylation frequency of the genes as a function of age, and correlated them with the clinicopathologic parameters. We found that aberrant CpG island methylation occurred frequently in chronic gastritis, which showed aging relationship, and that aging-related methylation was gene type-specific; *DAP-kinase*, *E-cadherin*, *p14*, *THBS1*, and *TIMP3* all showed a progressive increase of methylation frequency with aging, whereas *COX-2*, *GSTP1*, *MGMT*, *hMLH1*, *p16*, and *RASSF1A* were rarely methylated, with no aging relationship. Increased numbers of genes methylated were closely associated with male gender, marked chronic inflammation, marked intestinal metaplasia, as well as aging.

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