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Increased Frequency of CpG Island Methylator Phenotype and CDH1 Methylation in a Gastric Cancer High-Risk Region of China[1]

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Abstract

This study aimed to profile the methylation statuses of *CDH1/E-cadherin* and five CpG island methylator phenotype (CIMP)-associated genes (*p16*, *hMLH1*, *MINT1*, *MINT2*, and *MINT31*) in gastric specimens of 47 Dalian long-term residents with and 31 without gastric cancers (GCs). CIMP patterns were classified as CIMP-H with over three methylated genes, CIMP-L with one to two methylated genes, and CIMP-N without methylation. Of 47 GC cases, 24 (51.1%) were CIMP-H, 18 (38.3%) were CIMP-L, and 5 (10.6%) were CIMP-N, whereas 5 of 21 (23.8%) premalignant lesions were CIMP-H and 15 (71.4%) were CIMP-L. CIMP-L was found in 75% (12/16) of GC-adjacent mucosa and in 38.7% (12/31) of mucosa from GC-free patients. CDH1 methylation occurred in 48.9% (23/47) of cancer, in 23.8% (5/21) of premalignant, and in 25% (4/16) of noncancerous tissues and was correlated with patients' age ($P = .01$), lymph node metastasis, and CIMP severity ($P = .000$–$.028$). Our results demonstrated that the frequencies of CIMP-H in Dalian GCs, CIMP-L, and *p16* methylation in GC-adjacent tissues and in GC-free mucosa were much higher than those reported previously, indicating the elevated methylation pressure in this GC high-risk region. The close correlation between *CDH1* methylation and CIMP severity suggests the necessity of their combination in GC prevention and earlier diagnosis.

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Introduction

Gastric cancer (GC) is one of the commonest malignancies in the world and the major cause of cancer-related deaths in China [1]. There are several GC at-risk regions in China including Dalian, a coastal and mountainous area located at the far south of Northeast China. Although great effort has been paid in early prevention, diagnosis, and treatments, GC remains the first killer malignancy among Dalian residents due to later diagnosis and lack of timely surveillance measures. Multiple regional oncogenic factors have been proposed during the past decades and diet habits are one of them [2]. Epidemiologic studies demonstrated that people in this area were accustomed to eating toasted or salted seafood and meat and preparing preserved vegetables for the long winter season. These foods are rich in gastrocarcinogens such as heterocyclic amines, polycyclic aromatic hydrocarbons, and the well-known DNA methylators such as nitrite and benzo(*a*)pyrene [3,4]. Apparently, a long-term consumption of those foods may enhance the risk of genetic and epigenetic alterations [5]. The high GC incidence and the traditional diet habits in this region provide unique resource for investigating molecular aspect of gastrocarcinogenesis.

DNA hypermethylation is the main machinery of epigenetic modulation of gene expression [6], especially for those induced by chemical reagents [7,8]. DNA methylation happens in definite sequences because most of the methylation occurs on the cytosine of CpG site, where the 5′–CG–3′ dinucleotide sequences appear infrequently in the genome [9]. Because the 5′-promoter region of most genes are rich

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in CpG sequences [10], their methylation statuses determine the activity of gene transcription [6,11]. A body of evidence demonstrated that alterations of DNA methylation are closely related with cancer formation, particularly with those raised from the organs exposed directly to environmental carcinogens [7]. Because methylated genes rarely come alone but in group [12], Toyota et al. [13,14] introduced the concept of CpG island methylator phenotype (CIMP) through the study of aberrant methylation in colorectal and GCs, in which five to seven methylator-sensitive genes were included for evaluating the methylation statuses in cancers and for correlating the CIMP pattern(s) with tumor risk and prevention [15,16].

So far, no comprehensive study has been performed on Dalian GCs concerning CIMP profiling and its potential relationship with the high GC incidence. Cyclooxygenase-2 (COX-2) has been shown to play oncogenic roles during stepwise gastrocarcinogenesis, and its expression is correlated with *Helicobacter pylori* infection, nuclear factor–kappa B activation, and Wnt signaling [17–19]. However, despite the existence of those *COX-2* stimulators, *COX-2* expression remains infrequent in Dalian GCs due to hypermethylation of the *COX-2* promoter [20]. These data exclude the feasibility of COX-2–targeting therapy for the prevention of Dalian GCs and, meanwhile, highlight a hypermethylation-related gastrocarcinogenic option in this GC at-risk region. We therefore hypothesized that, in addition to *COX-2* hypermethylation, global methylation status in gastric epithelial cells might also be altered, which would be favorable for GC initiation and progression. This hypothesis was tested in this study by profiling the patterns of CIMP and *CDH1* methylation in GC mass, GC surrounding tissue, and grossly normal-looking epithelium of the surgical specimens as well as the endoscopic gastric mucosa of GC-free patients.

Materials and Methods

Sample Collection and Treatment

Forty-seven surgical specimens from 34 male (mean age 61.6 ± 9.2 years) and 13 female (mean age 61.7 ± 8.8 years) GC patients were selected from the Frozen Gastric Tissue Bank of the Cancer Institute, Dalian Medical University (DMU), Dalian, PR China. Gastric mucosa were obtained from 31 cancer-free patients and were provided after the test of *H. pylori* infection and/or frozen section–based pathologic examination. All of the patients were long-term Dalian residents who never received preoperative chemotherapy. The tissue samples were selected from the GC mass, GC surrounding tissue, and grossly normal-looking epithelium of the surgical specimens. They were trimmed to suitable sizes on ice, snap-frozen immediately in liquid nitrogen, and stored at −85°C until use. All tissue preparations were completed within 20 minutes of tissue removal. After obtaining patients' consent, fresh gastric biopsies were collected from either the Operation Rooms at DMU First Affiliated Hospital or the Gastroendoscopic Department of DMU Second Affiliated Hospital. The frozen tissue blocks were sectioned in 5-μm thickness, fixed in cold acetone for 20 minutes, and subjected to hematoxylin and eosin (H&E) staining for pathologic reexamination and tissue composition evaluation. According to pathologic findings, the gastric tissues were classified into well-differentiated intestinal-type gastric cancer (i-GC), poorly differentiated diffuse-type gastric cancer (d-GC), premalignant lesions including intestinal metaplasia and atrophic gastritis, and normal-looking noncancerous mucosa. Meanwhile, a piece of placenta tissue was obtained after getting the permission from a labored donor.

Genomic DNA Preparation and Sodium Bisulfite Treatment

Sample preparations were conducted according to the findings of pathologic examination. When a tissue block showed uniform composition, it was sectioned directly for DNA isolation. When multiple tissue components were found in the same tissue block, the border of the target histologic region was marked precisely for cell type–defined sample preparations with the manual dissection method established in our laboratory [21,22]. In total, 16 to 24 pieces of 3-μm target frozen fragments were collected after sectioning and were placed immediately in a 1.5-ml Eppendorf tube containing 300 μl of TE buffer (pH 8.0, 1% sodium dodecyl sulfate), digested with 5 μl of proteinase K (10 mg/ml), and subjected to conventional DNA extraction using phenol/chloroform extraction and ethanol precipitation.

For chemical DNA modification, 5 μl of sample DNA (0.4 μg/μl) was mixed with 40 μl of pure water, heated at 97°C for 6 minutes, and interacted with 5 μl of 2 M NaOH at 37°C for 10 minutes, mixed with 30 μl of freshly prepared 10 mM hydroquinone (H9003; Sigma, St. Louis, MO) and 520 μl of 3 M sodium bisulfate (S9000; Sigma), and then incubated at 50°C in darkness for 16 to 20 hours. The chemical-modulated sample DNA was subjected to desalt purification using the Wizard DNA Clean-Up System (A7280; Promega, Madison, WI). The purified DNA was dissolved with 50 μl of pure water, mixed with 5.5 μl of 3 M NaOH at 37°C for 5 minutes, precipitated in ethanol, and dissolved in 30 μl of pure water for use. A placenta DNA sample was incubated with Sss1 methyltransferase (New England Biolabs Inc., Beverly, MA) at 37°C for 4 hours followed by bisulfite treatment.

Methylation-Specific Polymerase Chain Reaction (PCR)

The sequences of methylation-specific PCR (MSP) primers and the conditions of PCR reaction for individual genes are listed in Table 1 [23]. PCR amplifications were conducted using TaKaRa PCR amplification Kit (TaKaRa Biotechnology Inc. Dalian, China) by the following procedures: 30 μl of reaction solution was prepared, which contains 3 μl of 10× buffer (Mg^{2+} Plus), 4 μl of 2.5 mM deoxyribonucleotide triphosphate, 0.5 μl of respective 20 μM upstream and downstream primers, 1 U *Taq* polymerase (5 U/μl), and 5 μl of DNA template. The reaction condition was as follows: 94°C for 5 minutes; 94°C for 1 minute; 53 to 65°C for 1 minute and 72°C for 1 minute for 35 to 40 cycles; and 72°C for 5 minutes. The PCR products were separated in 6% polyacrylamide gel and were then observed and photographed under UV illumination (BioSpectrumAC BioImaging Systems; Ultra-Violet Products Inc., Upland, CA). Distilled pure water was used as negative control [23] and the placenta DNA was treated with Sss1 methyltransferase was used as positive control for methylated allele in each of MSP reactions [24].

Criteria of CIMP Classification

In this study, *p16*, *hMLH1*, *MINT1*, *MINT2*, and *MINT31* were chosen and the hot loci of CpG island methylation in their promoter regions were examined for CIMP identification. According to the suggestion of Jean-Pierre Issa's Laboratory [25], the grades of methylation were classified into three types: CIMP-high (CIMP-H) when three to five genes were found with methylation; CIMP-low (CIMP-L) when one or two genes were methylated; and CIMP-none (CIMP-N) if none of the five genes was methylated. As indicated in Figure 1, DNA samples isolated from the GC tissues of G-89-T and G-64-T showed CIMP-H pattern, whereas their premalignant counterparts revealed CIMP-L, and the noncancerous mucosa showed CIMP-N.

Table 1. Primer Sequences and MSP Reaction Conditions for Individual Genes.

Gene	Primer Sequence	Annealing Temperature (°C)
p16	Methylated	65
	Sense 5′–TTATTAGAGGGTGGGGCGGATCGC–3′	
	Antisense 5′–GACCCCGAACCGCGACCGTAA–3′	
	Unmethylated	60
	Sense 5′–TTATTAGAGGGTGGGGTGGATTGT–3′	
	Antisense 5′–CAACCCCAAACCACAACCATAA–3′	
MINT1	Methylated	55
	Sense 5′–AATTTTTTTATATATATTTTCGAAGC–3′	
	Antisense 5′–AAAAACCTCAACCCCGCG–3′	
	Unmethylated	55
	Sense 5′–AATTTTTTTATATATATTTTTGAAGTGT–3′	
	Antisense 5′–AACAAAAAACCTCAACCCCACA–3′	
MINT2	Methylated	60
	Sense 5′–TTGTTAAAGTGTTGAGTTCGTC–3′	
	Antisense 5′–AATAACGACGATTCCGTACG–3′	
	Unmethylated	60
	Sense 5′–GATTTTGTTAAAGTGTTGAGTTTGTT–3′	
	Antisense 5′–CAAAATAATAACAACAATTCCATACA–3′	
MINT31	Methylated	60
	Sense 5′–TGTTGGGGAAGTGTTTTTCGGC–3′	
	Antisense 5′–CGAAAACGAAACGCCGCG–3′	
	Unmethylated	64
	Sense 5′–TAGATGTTGGGGAAGTGTTTTTTGGT–3′	
	Antisense 5′–TAAATACCCAAAAACAAAACACCACA–3′	
hMLH1	Methylated	60
	Sense 5′–GATAGCGATTTTTAACGC–3′	
	Antisense 5′–TCTATAAATTACTAAATCTCTTCG–3′	
	Unmethylated	60
	Sense 5′–AGAGTGGATAGTGATTTTTAATGT–3′	
	Antisense 5′–ACTCTATAAATTACTAAATCTCTTCA–3′	
CDH1	Methylated	57
	Sense 5′–TTAGGTTAGAGGGTTATCGCGT–3′	
	Antisense 5′–TAACTAAAAATTCACCTACCGAC–3′	
	Unmethylated	53
	Sense 5′–TAATTTTAGGTTAGAGGGTTATTGT–3′	
	Antisense 5′–CACAACCAATCAACAACACA–3′	

The primer sequences and reaction conditions were cited from Park et al. [23] and Herman et al. [26].

Meanwhile, the CpG island methylation in the *CDH1* promoter region was checked using the PCR primers and reaction condition described elsewhere [26] and the results were compared with CIMP patterns.

Collection and Analyses of CIMP-Related Data

The related clinical data were obtained from the clinical records achieved in the Case Muniment Room of DMU Affiliated Hospitals. Patients' gender, age, Lauren's classification, the degree of tumor differentiation, clinical stage/tumor, node, and metastasis staging, and *H. pylori* infection were recorded. Mann-Whitney test was used to analyze the distribution of three CIMP patterns as well as *CDH1* methylation in GC, premalignant, and noncancerous groups, and their relation with patients' gender, age, GC subtypes, tumor, node, and metastasis staging, and *H. pylori* infection. Spearman relative analysis was employed to evaluate the relation of the extent of CIMP and *CDH1* methylation.

Results

Differential Methylation Patterns of Five CIMP-Related Genes during Stepwise Gastrocarcinogenesis

The same parameters (*p16*, *hMLH1*, *MINT1*, *MINT2*, and *MINT31*) proposed by other investigators were employed here as CIMP-related genes [23]. As shown in Table 2, MSP analysis performed on the tissues obtained from gastrectomy specimens revealed that the incidences of *p16* methylation were 18.8% (3/16) in noncancerous, 57.1% (12/21) in premalignant, and 63.8% (30/47) in GC tissues, respectively. The detection rates of GCs and premalignant lesions were significantly different with their noncancerous counterpart ($P = .002$ and $P = .020$). The rates of *hMLH1* methylation were 25% (4/16), 47.6% (10/21), and 53.2% (25/47) in noncancerous, premalignant, and

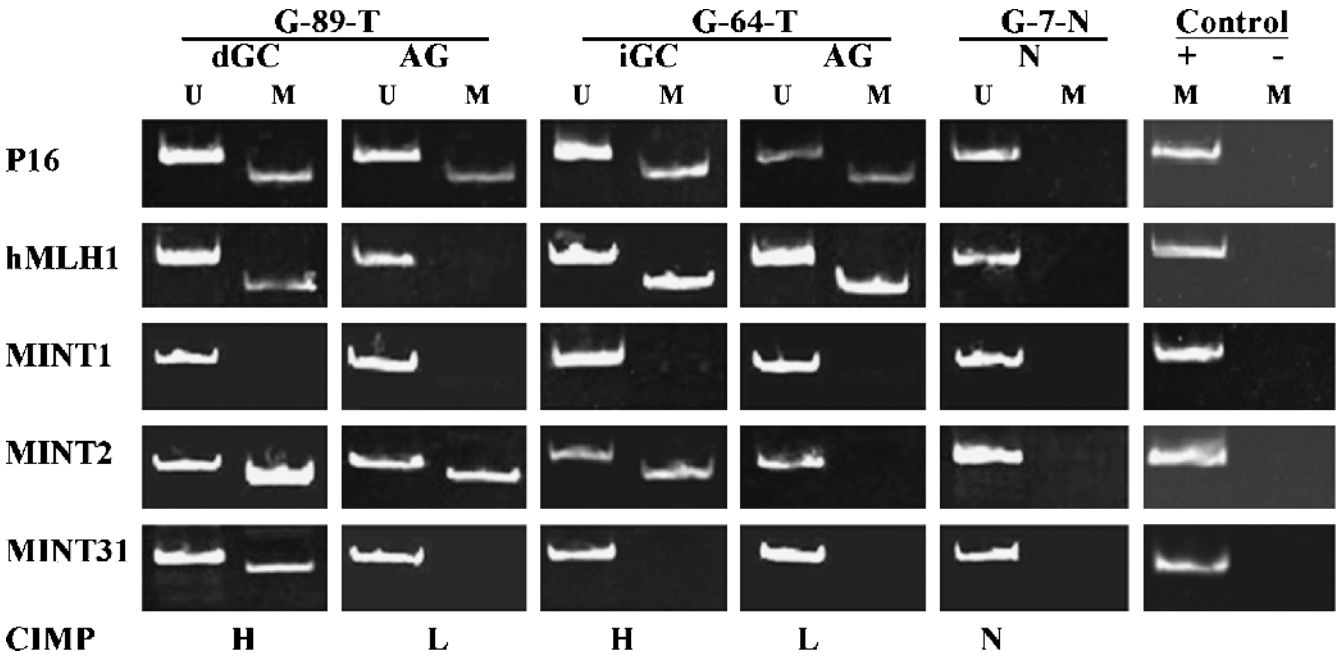

Figure 1. Illustration of CIMP-H, CIMP-L, and CIMP-N and their distribution in different gastric tissues. U and M indicate the PCR products amplified with the primers for unmethylated and methylated sequences, respectively. *d-GC*, diffuse gastric cancer; *i-GC*, intestinal gastric cancer; *AG*, chronic atrophic gastritis. Normal placenta DNA treated with Sss1 methyltransferase was used as positive control (+) and distilled water without template DNA as negative control (−) for methylated loci.

Table 2. Methylation Statuses of *p16*, *hMLH1*, *MINT1*, *MINT2*, *MINT31*, and *CDH1* in Different Gastric Tissues.

Locus	Methylation Frequencies				*P*
	Endoscopic (*n* = 31)	Noncancerous (*n* = 16)	Premalignant (*n* = 21)	GC (*n* = 47)	
p16	29% (9/31)	18.8% (3/16)	57.1% (12/21)	63.8% (30/47)	.008
hMLH1	9.7% (3/31)*	25% (4/16)	47.6% (10/21)	53.2% (25/47)	.151
MINT1	6.5% (2/31)*	25% (4/16)	42.9% (9/21)	44.7% (21/47)	.375
MINT2	0% (0/31)*	12.5% (2/16)	33.3% (7/21)	40.4% (19/47)	.126
MINT31	9.7% (3/31)*	18.8% (3/16)	14.3% (3/21)	25.5% (12/47)	.56
CDH1	9.7% (3/31)*	25% (4/16)	23.8% (5/21)	48.9% (23/47)	.031

*Significant difference of methylation incidences between the noncancerous mucosa of the patients with and without gastric cancers (Mann-Whitney test, *P* = .000–.008).

GC groups, respectively. In the case of MINTs, the methylation rates of *MINT1* were 25% (4/16) in noncancerous, 42.9% (9/21) in premalignant, and 44.7 (21/47) in GC groups; *MINT2*, 12.5% (2/16) in noncancerous, 33.3% (7/21) in premalignant, and 40.4 (19/47) in GC groups; and *MINT31*, 18.8% (3/16) in noncancerous, 14.3% (3/21) premalignant, and 25.5 (12/47) GC groups. Unlike the results of *p16* analysis, Mann-Whitney test revealed that no significant difference between the *hMLH1*, *MINT1*, *MINT2*, or *MINT31* methylation rates could be found among the three histologic groups when they were compared in pair ($P > .05$).

Distinct CIMP Patterns of GC, Premalignant, and Noncancerous Groups

As summarized in Figure 2, and Table 3, the frequencies of CIMP-H were 51.1% (24/47) in GC group, 23.8% (5/21) in premalignant lesions, and 0% (0/16) in noncancerous mucosa. CIMP-L was 38.3% (18/47) in GCs, 71.4% (15/21) in premalignant, and 75% (12/16) in noncancerous tissues, respectively. CIMP-N was 10.6% (5/47) in GCs, 4.8% (1/21) in premalignant, and 25% (4/16) in noncancerous tissues. The statistical analyses revealed that the incidences of CIMP-H were significantly different ($P = .001$) between premalignant (23.8%, 5/21) and GC groups (51.1%, 24/47). However, no statistical difference could be established between diffuse GCs (59.3%, 16/27) and intestinal GCs (40%, 8/20) ($P = .282$) and between the primary GCs with (50%, 15/30) and without lymph node metastases (50%, 5/10) ($P = .435$).

Frequent CIMP-L in the Mucosa of GC-Free Patients

Among the 31 gastric biopsies obtained from GC-free patients, 9 were found with mild and 22 were with moderate to severe chronic gastritis (Figure 3). Furthermore, 20 were positive in *H. pylori* infection, 10 were negative, and 1 was without relevant record. There were nine cases (29%) with p16 methylation, three (9.7%) with *hMLH1*, two (6.5%) with *MINT1*, none with *MINT2*, and three (9.7%) with *MINT31*. None of those samples exhibited CIMP-H, whereas 12/31 (38.7%) were found with CIMP-L. Distribution of CIMP-L in mild (2/9, 22.2%) and severe gastritis (10/22, 45.5%) was different but without statistical significance ($P = .367$). In comparison with the data obtained from the noncancerous mucosa of GC-bearing patients, the frequencies of *hMLH1*, *MINT1*, *MINT2*, and *MINT31* methylation were significantly lower ($P = .000–.008$) except that of p16 methylation ($P = .323$).

No Relevance of CIMP Patterns with Patients' Gender, Age, and **H. pylori** *Infection*

Analysis of CIMP patterns in male and female GC patients revealed that CIMP-H was 47.1% (16/34), CIMP-L was 38.2% (13/34), and CIMP-N was 14.7% (5/34) among male patients, and 61.5% (8/13), 38.5% (5/13), and none among females, respectively (Table 3). No significant difference of these parameters could be established between the two groups ($P = .317$). The 47 GC cases checked here were divided into two age groups: the younger (less

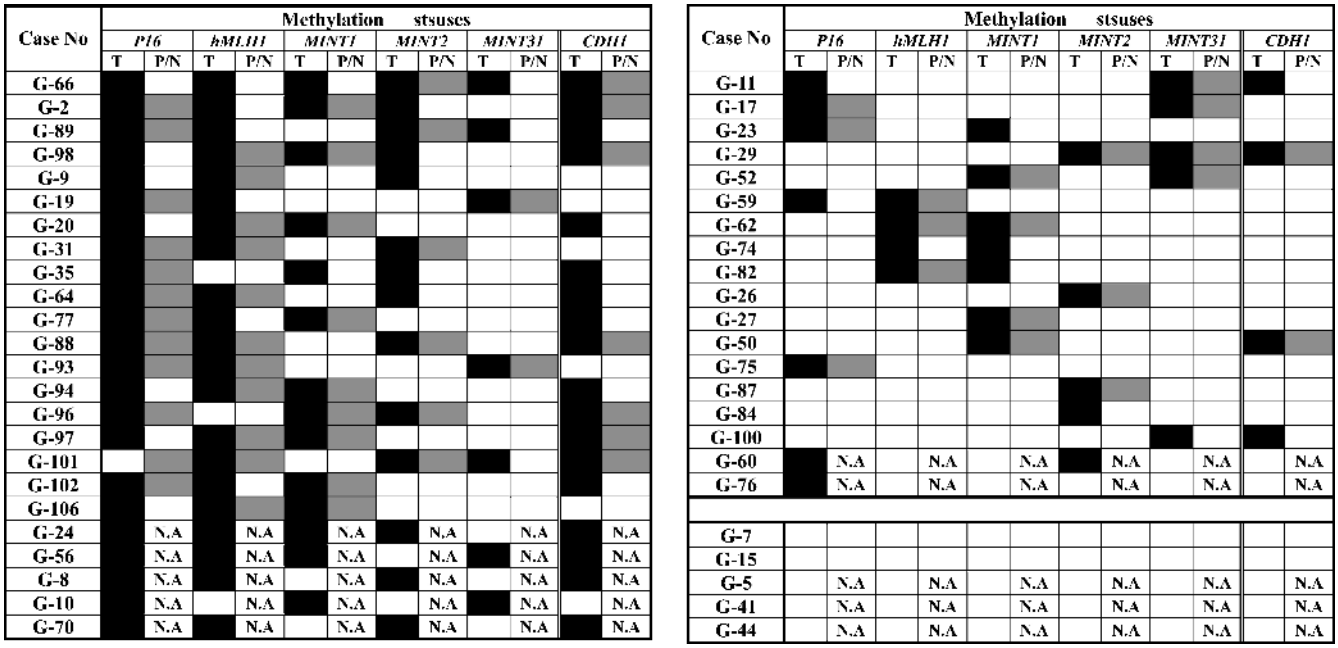

Figure 2. Profiling of CIMPs in gastric cancers raised from Dalian, China. ■, the locus with methylation among GC tissues; ▨, the locus with methylation among premalignant/noncancerous gastric mucosa; and □, the locus without methylation. *N.A.*, tissue sample was not available.

Table 3. Differential CIMP and *CDH1* Methylation Patterns during Stepwise Gastrocarcinogenesis and Their Correlation with Patients' Personal Parameters.

Parameters	No.	CIMP			*P*	*CDH1** Methylation (%)	*P*
		H (%)	L (%)	N (%)			
Gender							
Male	34	16 (47.1)	13 (38.2)	5 (14.7)	.317	16 (47.1)	.108
Female	13	8 (61.5)	5 (38.5)	0 (0)		7 (53.8)	
Age (years)							
< 50	12	4 (33.4)	7 (58.3)	1 (8.3)	.154	2 (16.7)	.010
≥ 50	35	20 (57.2)	11 (31.4)	4 (11.4)		21 (60)	
Histology							
Noncancerous	16	0 (0)	12 (75)	4 (25)	.001	4 (25)	.031
Premalignant	21	5 (23.8)	15 (71.4)	1 (4.8)		5 (23.8)	
Cancer	47	24 (51.1)	18 (38.3)	5 (10.6)		23 (48.9)	
d-GC	27	16 (59.3)	8 (29.6)	3 (11.1)	.282	16 (59.3)	.100
i-GC	20	8 (40)	10 (50)	2 (10)		7 (35)	
Differentiation							
Poor	32	16 (50)	13 (40.6)	3 (9.4)	.236	*19 (59.4)*	.335
Well	7	4 (57.1)	2 (28.6)	1 (14.3)		*2 (28.6)*	
Lymph node metastasis							
+	30	15 (50)	11 (36.7)	4 (13.3)	.435	19 (63.3)	.028
–	10	5 (50)	5 (50)	0 (0)		2 (20)	
H. pylori infection							
+	8	3 (37.5)	3 (37.5)	2 (25)	.079	3 (37.5)	.112
–	5	5 (100)	0 (0)	0 (0)		4 (80)	

*Significant difference of *CDH1* methylation incidences between CIMP-H and CIMP-L/N groups (Spearman relative analysis, $P = .000$).

than 50 years old) and the elder (over 50 years old) groups [12]. The incidences of i-GC and d-GC were 50% (6/12) and 50% (6/12) in the younger and 40% (14/35) and 60% (21/35) in the elder group, respectively, showing similar i-GC and d-GC incidences of the two groups ($P = .157$). Of 12 younger cases, 4 (33.4%) were found with CIMP-H, 7 (58.3%) with CIMP-L, and 1 (8.3%) with CIMP-N, whereas 20 (57.2%) of 35 elder GC patients were with CIMP-H, 11 (31.4%) with CIMP-L, and 4 (11.4%) with CIMP-N. Although CIMP-H frequency of the elder patients was higher than that of the younger patients, this difference had no statistical significance ($P = .154$).

Among the 47 GC cases, 8 were diagnosed as *H. pylori*–positive (HP^+) and 5 as *H. pylori*–negative (HP^-). Notably, all of the HP^- cases showed CIMP-H in comparison with 37.5% (3/8) of CIMP-H in HP^+ tissues. However, a statistical difference of CIMP-H incidences could not be established ($P = .079$) between the two groups due to the limited case number. To further address this issue, the test of *H. pylori* infection was performed on the noncancerous mucosa of 30 GC-free patients, which revealed that 20 were found with and 10 without *H. pylori* infection. The same frequencies of CIMP-L (40%) and CIMP-N (60%) were found in both HP^+ and HP^- groups.

Close Correlation of CIMP-H with CDH1 Hypermethylation

CDH1 promoter methylation was found in 48.9% (23/47) of GC samples, and 75% (18/24) of this methylation occurred in CIMP-H and 21.7% (5/23) in CIMP-L/N GC group, revealing a significant difference of *CDH1* methylation rates between the two groups ($P = .000$; Table 3). The incidences of *CDH1* methylation were apparently lower in the premalignant (23.8%; 5/21) and noncancerous tissues (25%; 4/16) of GC patients ($P = .031$). About 35% (7/20) of i-GC were found with methylated *CDH1* in comparison with 59.3% (16/27) in d-GC ($P = .100$), indicating that the loss of E-cadherin due to the increased *CDH1* methylation in d-GCs may lead to the diffuse phenotype. Additionally, *CDH1* methylation was found in 63.3% (19/30) of primary GCs with lymph node metastases, whereas in 20% (2/10) of those without lymph node metastases ($P = .028$). *CDH1* methylation was also correlated with the patients' age ($P = .010$), because its frequencies were apparently different in the younger (16.7%, 2/12) and the elder GC groups (60%, 21/35). In contrast, no statistical significance of *CDH1* methylation incidences was found between male (47.1%, 16/34) and female (53.8%, 7/13) patients ($P = .108$). The results from the noncancerous mucosa of 31 GC-free patients revealed that 3 cases (9.7%) were found with *CDH1* methylation, and all of them fell into the elder and CIMP-L groups.

Discussion

Altered gene silencing due to DNA hypermethylation is a universal epigenetic event in great majority of human malignancies, which, unlike the situation of hereditary diseases, happens to multiple genes in cancer cells and the genes affected usually vary in chemical- and disease-dependent fashions [3,4,27]. It is therefore necessary to elucidate the methylation statuses of a panel of representative genes in an individual disease. To achieve this goal, CIMP was introduced by Toyota et al. [13]. Data from gastroenterological cancers revealed that a combination of *MINT1*, *MINT2*, and *MINT31* with tumor suppressor *p16* and mismatch repair gene *hMLH1* is suitable in evaluating the general methylation status in these sorts of tumors [14,25,28]. To make our results more comparable with the published data, we employed this evaluation system to check the unknown CIMP pattern in Dalian GCs and to see whether the CIMP pattern here was different from those identified in other places.

To have a clearer view in the correlation of DNA methylation with GC formation in this GC high-risk region, different histologic tissues of GC surgical specimens and the endoscopic gastric mucosa of GC-free patients were collected and examined. It has been believed that GC-adjacent noncancerous mucosa and chronic gastritis rarely had *p16* (0–10%) and *hMLH1* (0–1.7%) methylation, even in the case of GCs of high-risk regions [29,30]. In contrast, the methylation incidences of *p16* and *hMLH1* in the same tissue types of Dalian GC patients were 18.8% and 25%, respectively. Accordingly, the methylation rates of *MINT1* (25%) and *MINT2* (12.5%) in those samples were also higher than the ones (5% and 7%) reported by other investigators [28]. These results implicate the existence of certain epigenetic variations in different GC high-risk regions and the elevated methylation pressure in Dalian region presumably due to its epidemiologic features as mentioned in the Introduction section. This speculation was further supported by the data obtained from the inflammatory mucosa of 31 GC-free patients, because 29% of them were found with *p16* methylation, 9.7% with *hMLH1*, and even GC-associated *MINT1* and *MINT31* methylation could be detected in the rates of 6.5% and 9.7%, respectively. Moreover, *p16* and *MINT1* or *MINT31* methylation appeared even in the mucosa of 18-year-old male and 27-year-old female patients, and *hMLH1* in a 26-year-old male. If we suppose the relatively high *p16* and *hMLH1* methylation incidences in GC-adjacent mucosa is somewhat influenced by the neoplastic changes and/or tumorigenic microenvironment, the findings from GC-free patients reflect the frequent preexistence of abnormally methylated genes in this GC high-risk population. Because *p16* plays important roles in controlling cell outgrowth [31] and *hMLH1* in repairing the nucleotide sequences

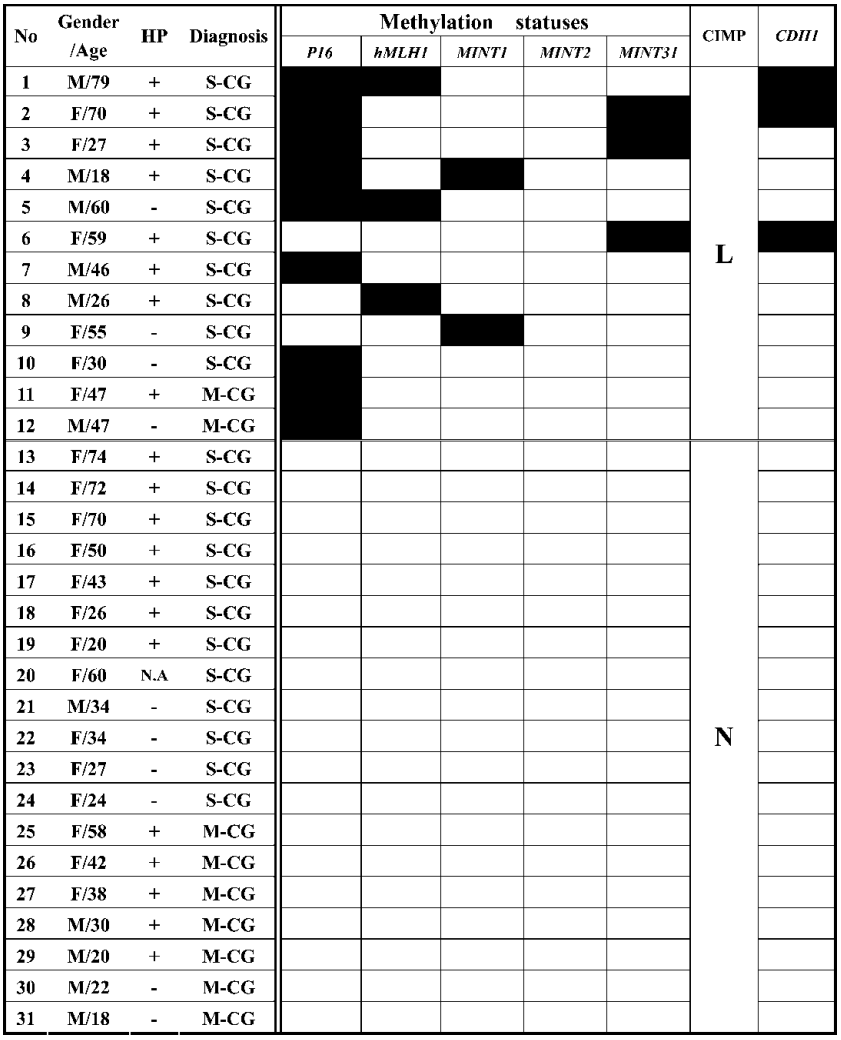

Figure 3. Personal backgrounds and CIMP statuses of gastric biopsies of GC-free patients. The dark and light blocks indicate the genes with and without methylation, respectively. *M-CG*, mild chronic gastritis; *S-CG*, moderate to severe chronic gastritis; *N.A.*, the record of *H. pylori* test was not available.

damaged/altered by external and intrinsic factors [32], it is reasonable to suppose that the cells without those proteins may gain longer life span and/or more chance of additional genetic aberrations.

The CIMP data from previous GC studies revealed that the frequencies of CIMP-H were from 31% to 51% in GC tissues, whereas only 4% to 16% in GC-adjacent premalignant or normal mucosa [28,33]. It was therefore proposed that CIMP-H happened in the later stage of gastrocarcinogenesis. In this study, CIMP-H was found preferentially in Dalian GCs (51.1%) and at a higher rate among premalignant lesions (23.8%, 5/21), suggesting the accumulation of multiple loci methylations during malignant transformation. Although none of the noncancerous mucosa exhibited CIMP-H, the incidence of CIMP-L (75%) in those tissues was remarkably higher than the ones (17–38%) reported previously [28,33,34] because of the early appearance of methylation(s) of the five checked genes. Based on the low frequency of *COX-2* expression due to promoter hypermethylation in Dalian GCs, we had assumed the possible high methylation background in this GC high-risk population [20]. The results of the current study provided further supporting data for this speculation and motivated us to check whether the gastric mucosa of GC-free patients showed the similar methylation tendency.

The data about CIMP patterns in different gastric lesions have been accumulated in recent years, including the ones from other GC popular regions [33,34]. However, a simultaneous analysis on gastric samples from the patients with and without GCs in a high-risk population has been rarely reported. This work was conducted using endoscopic biopsies of 31 GC-free outpatients. Similar with the situation of GC-adjacent noncancerous mucosa, a high rate (38.7%) of CIMP-L and absence of CIMP-H was found in those samples. It was noted that 2 of 12 CIMP-L cases appeared in the patients with mild gastritis and 7 in the patients are less than 50 years old. These findings thus provide additional evidence for the high methylation status of Dalian long-term residents, and further hold the notion that DNA methylation may occur at the early stage of gastrocarcinogenesis. Although the local factor(s) leading to the high methylation status remains to be figured out, early detection of methylation-sensitive genes such as *p16* and exploration of reliable method to erase the methylation from those genes would have potential value in GC prevention of this region.

Generally, the genes affected by hypermethylation can be classified into two categories: cancer-associated genes as Type-C and age-related genes as Type-A [13]. The five genes used for CIMP profiling

here are Type-C genes [35] that are sensitive to environmental methylators [25]. However, their responses to other GC-related factors like *H. pylori* infection are still in dispute. Maekita et al. [36] found high levels of aberrant DNA methylation in *H. pylori*–infected gastric samples, whereas Kang et al. [30] reported that this epigenetic event was unrelated with *H. pylori* infection. The results obtained from the gastric samples of both GC-bearing and GC-free patients revealed that the CIMP status is related neither with *H. pylori* infection nor with patients' age, gender, as well as GC subtypes. These data thus suggest that Type-C genes are more suitable for CIMP profiling because of their preferential methylations in cancer-associated lesions regardless of the differences of other personal factors. Additionally, our results indicate two possibilities: 1) the gastrocarcinogenic effect(s) of *H. pylori* may not depend on DNA methylation pathway or 2) certain powerful methylation element(s) might exist universally and cause hypermethylation of Type-C genes in Dalian gastric samples irrespective of the presence or absence of *H. pylori* infection. In this context, more careful evaluation will be required to address the association of HP infection and increased severity of DNA methylation in GC high-risk regions.

Downregulation of *CDH1* is a typical malignant biomarker observed in a wide variety of cancers including GCs [37]. Multiple genetic factors can inhibit *CDH1* expression, and hypermethylation in *CDH1* promoter region is one of the main reasons [38]. Because *CDH1* methylation usually appeared in elder patients, it was regarded as a kind of age-related methylation (Type-A methylation) [35]. In parallel with CIMP analysis, the status of *CDH1* methylation was checked here to elucidate the potential link of *CDH1* methylation to CIMP patterns and to see whether this sort of methylation occurred earlier or more frequently in a GC high-risk population. In agreement with previous reports [35,39,40], we found increased frequency of *CDH1* methylation in GC samples and the gastric mucosa of GC-free patients in an age-related pattern. However, it is difficult to conclude the causality of *CDH1* methylation with gastrocarcinogenesis because: 1) the methylation event of *CDH1* unnecessarily occurs concurrently in GC and noncancerous tissues of the same patients and 2) *CDH1* methylation is frequently found in CIMP-H GC cases. Moreover, *CDH1* methylation is closely related with GC dissemination because of its presence in 63.3% (19/30) of primary GCs with lymph node metastases, but only 20% (2/10) in those without lymph node metastases. All these data suggest the cancer-promoting feature of *CDH1* downregulation/silencing and the existence of other methylation element(s) in the transformed cells beyond cell aging. Therefore, we would rather regard *CDH1* methylation as Mixed type (Type-M) because of its compatibility with age- and cancer-associated features. To make the results more informative and substantial, a combination of *CDH1* with Type-C genes would be necessary in CIMP profiling.

In summary, our comprehensive CIMP profiling revealed a high CIMP-H incidence in GC samples and more frequent CIMP-L in premalignant and noncancerous mucosa of the patients with and without GCs, suggesting the persistent methylation pressure and increased CIMP grade in this GC at-risk region. Among the five CIMP-associated genes so far checked, *p16* seems more sensitive to methylator(s) because of its overall methylation rate and the earlier onset of its methylation in stepwise gastrocarcinogenesis. *CDH1* methylation was closely related with either aging or malignant phenotypes of gastric epithelial cells. Therefore, an integration of *CDH1* with conventional CIMP-related genes is recommended in methylation assay. Because several options for profiling methylation patterns of GCs have been available [14,28,30,34], it would be worthwhile to compare their applicability in GC risk assessment of the Dalian region based on the current study.

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