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High CpG island methylator phenotype is associated with lymph node metastasis and prognosis in gastric cancer

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Several studies have found that the promoter CpG island is frequently methylated in gastric cancer. The CpG island methylator phenotype (CIMP) defines concordant methylation of multiple promoter CpG island loci in a subset of gastric cancer. However, the relationship between CIMP and lymph node metastasis in gastric cancer is unknown. Our study aimed to characterize the role of CIMP in lymph node metastasis. Clinical specimens from 120 patients were analyzed and PCR was used to detect the methylation status of five genes (*ALX4*, *TMEFF2*, *CHCHD10*, *IGFBP3*, and *NPR1*). We measured the level of mRNA for the five genes by real-time RT-PCR. Microsatellite instability and *Helicobacter pylori* infection status were assayed by capillary electrophoresis and real-time PCR, respectively. DNA methylation in the five genes was correlated with low expression of the respective mRNA. With CIMP as the dependent variable, CIMP-high gastric cancer tended to show more distant lymph node metastasis, higher pathologic tumor classification, more pathologic metastasis, and higher pathologic TNM status. Microsatellite instability and *H. pylori* status were not significant predictors of prognosis. CIMP-high gastric cancer showed significantly worse survival compared with that of CIMP-low/CIMP-negative gastric cancer ($P < 0.001$). Our results show that there is an association between CIMP status and lymph node metastasis in gastric cancer and CIMP-high was an independent prognostic factor. (*Cancer Sci* 2012; 103: 73–79)

Gastric cancer is the second most common cause of global cancer mortality, accounting for >700 000 deaths annually.[1] Despite a steady decline in global incidence, gastric cancer still causes prominent morbidity and mortality in China. The clinical outcome of surgery in combination with chemotherapies largely depends on the stage of the gastric cancer. Although the molecular mechanisms of gastric cancer carcinogenesis remain unclear, epigenetic alteration through promoter methylation is known to play an important role in the development of this cancer that inhibit the expression of tumor suppressor genes. Currently, DNA methylation markers have been used in early detection, prognosis, and prediction of response to cancer therapy.[2,3]

DNA methylation has been studied extensively in gastric cancer.[4–7] However, most studies have focused on aberrant methylation in a single gene. Because methylated genes rarely occur singly, and more often in groups, the concept of a CpG island methylator phenotype (CIMP) in gastric and colorectal cancer was introduced,[2] in which five to seven methylation-sensitive genes were included for evaluating the methylation status in cancer and for correlating the CIMP with tumor risk and prevention. The CIMP was defined as a subset of malignancies that show widespread hypermethylation of multiple promoter CpG island loci. Several scientists have used their own CIMP marker panels for the determination of CIMP status, however, producing some inconsistent results.[8–12]

In this study we tried to evaluate the role of hypermethylation of multiple tumor-related genes such as *ALX4*, *TMEFF2*, *CHCHD10*, *IGFBP3*, and *NPR1* in gastric cancer. *ALX4* methylation frequently occurs in colorectal cancer tissue as well as in patients' serum.[13] Aberrant methylation of the *TMEFF2* gene inhibits the transforming growth factor β signaling pathway, and the gene plays an important role in human cancers.[14–19] Using gene knockdown under *in vitro* conditions, it is thought that *CHCHD10* is involved in oxidative phosphorylation and plays an important role in complex IV activity.[20] *NPR1* is regarded as a major natriuretic peptide receptor and its activation produces the second messenger cGMP, which plays a key role in maintaining blood pressure and cardiovascular homeostasis. It has been shown that *NPR1* methylation is associated with survival in colorectal cancer[21] and hepatocellular carcinoma.[22] *IGFBP3* is the main carrier of insulin-like growth factors (IGFs) in the circulation, where this complex regulates the biologic function of IGFs.[23] Hypermethylation of the *IGFBP3* promoter is a frequent phenomenon and strongly associated with prognosis of non-small-cell lung cancer and ovarian cancer.[24,25]

In this study, we investigated the prevalence of the methylation of *ALX4*, *TMEFF2*, *CHCHD10*, *IGFBP3*, and *NPR1* among gastric cancer tissues and its relation to various clinicopathological characteristics. In addition, to clarify the characteristics and their underlying mechanisms of gastric tumors with hypermethylation of the five genes, we also measured levels of expression of mRNAs for these genes by real-time RT-PCR and evaluated microsatellite instability (MSI) and *Helicobacter pylori* status. Finally, we evaluated the prognostic significance of CIMP status in gastric carcinomas.

Materials and Methods

Tissue samples. One hundred and twenty samples were obtained from patients with newly diagnosed primary tumor at the Gastric Cancer Center, First Affiliated Hospital, Sun Yet-sen University (Guangzhou, China) between December 2003 and August 2009. These samples were from primary surgery, and the patients did not receive previous chemotherapy. Tumor sampling was carried out specifically for *in vitro* testing and was approved by the Ethical Research Committee, Sun Yet-sen University. Tumor samples were transferred from the operating room to the laboratory within 30 min and stored in liquid

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nitrogen for later use. As a control, endoscopic gastric biopsies from 10 patients with chronic gastritis also were included.

DNA extraction and sodium bisulfite modification. Genomic DNA was isolated from gastric cancer specimens in liquid nitrogen using using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Bisulfite treatment of DNA was carried out with an EpiTect bisulfite kit (Qiagen) according to the manufacturer's protocol. Bisulfite-treated DNA was used as a template in subsequent MethyLight PCR analyses.

Methylation analysis of multiple genes. After genomic DNA was treated with sodium bisulfite, the methylation levels of five genes (*ALX4*, *TMEFF2*, *CHCHD10*, *IGFBP3*, and *NPR1*, as well as β-actin as an internal marker) was analyzed using MethyLight PCR. Real-time PCR-based DNA methylation assay (MethyLight assay) was validated and carried out as described earlier.[26–28] We used a percentage of methylated reference (PMR) cut-off value of 4 to define positive versus negative methylation and to determine DNA methylation frequencies (PMR > 4) for each CpG island locus.

An aliquot of 2 μL was amplified with a primer set along with the TaqMan probe specific to methylated sequences. All PCR experiments were carried out in a volume of 25 μL using the EpiTect MethyLight PCR kit (Qiagen) with 96-well plates and an ABI 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The primer and probe sequences are shown in Table 1. Each reaction contained 12.5 μL Master Mix for methylation-specific real-time PCR analysis, 0.5 μM each primer, and 2.0 μL bisulfite-treated DNA in a total volume of 25 μL at 95°C for 10 min, followed by 45 cycles of 94°C for 30 s, and 60°C for 45 s in *ALX4*, *TMEFF2*, *CHCHD10*, *IGFBP3*, *NPR1*, and β-actin. The latter was used to normalize for input DNA. These experiments were carried out in triplicate and the mean value was then calculated. Every PCR experiment included serial dilutions of a positive control for construction of the calibration curve, a positive and a negative DNA sample, and water blanks. CpGenome Universally Methylated DNA (Chemicon, Temecula, CA, USA) was used as a positive control for methylation, and CpGenome Universal Unmethylated DNA (Chemicon) was used as a negative control. The methylation value of target genes in the specimens was determined as the relative methylation ratio (methylation level of target gene/β-actin in sample)/(methylation level of target gene/β-actin in positive control DNA).

RNA isolation and real-time RT-PCR for determining mRNA expression. Total RNA was extracted from gastric cancer specimens using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Nucleic acid concentrations were determined using RiboGreen (Molecular Probes, Eugene, OR, USA). The RNA was stored at −80°C until further use.

To analyze expression of the five genes, we carried out real-time RT-PCR as described previously.[29] The primer and probe sequences are shown in Table 1. The mRNA expression level was detected by real-time one-step RT-PCR using TaqMan probe specific for the five genes. Real-time one-step RT-PCR was carried out on an ABI 7500 (Applied Biosystems) using standard 25 μL Universal PCR Master Mix on 1–2 μg total RNA. Reaction conditions were 50°C for 15 min, 95°C for 8 min, 40 cycles at 94°C for 30 s, with an annealing temperature of 55°C for 45 s. No-template control was included in each assay. β-actin was used as an endogenous control and vehicle control was used as a calibrator. Each sample was run in triplicate. The comparative threshold cycle method was used to calculate the relative changes in the expression of five genes.

Table 1. Primers and probes for DNA methylation and mRNA detection

Gene	Status	Primer and probe sequence	Length (bp)
ALX4	Methylation	5′-TTAGGTATGAATGTTGAGATTTGCG-3′	83
		5′-CTACGACACCGAACTATAATAAACG-3′	
		5′-FAM-TTATTGCGAGTCGTCGGTCGTTGTTATGG-BHQ1-3′	
TMEFF2	Methylation	5′-GTTATCGTCGTCGTTTTTGTTGTC-3′	87
		5′-GACTTCCGAAAAACACAAAATCG-3′	
		5′-FAM-CGCGGGATGTTTAGTAGTTCGTTGTTCGG-BHQ1-3′	
CHCHD10	Methylation	5′-AGGTTTCGTTCGGGGTTTCG-3′	88
		5′-AAACGACGACAACGATACTATCG-3′	
		5′-FAM-ACAAATACCGCAACGCTTATCACAACCGA-BHQ1-3′	
IGFBP3	Methylation	5′-GTTTCGGGCGTGAGTACGA-3′	119
		5′-GAATCGACGCAAACACGACTAC-3′	
		5′-FAM-TCGGTTGTTTAGGGCGAAGTACGGG-BHQ1-3′	
NPR1	Methylation	5′-GCGGGTAATTTGACGGTAGTCG-3′	126
		5′-CAACAAATCGAAACGCGCCTTC-3′	
		5′-FAM-AAACCAACTCCACGACGAATCCCACGC-BHQ1-3′	
ALX4	mRNA	5′-GGGAACAGCTGGCCATGA-3′	95
		5′-AAAACGCTCCCGCTTCCT-3′	
		5′-FAM-CCCGCGTGCAGGTCTGGTTCC-BHQ1-3′	
TMEFF2	mRNA	5′-CTGCTTTCCCTACCTCCTTAAG-3′	110
		5′-TTTACAGGTGTTGGTGTCACAG-3′	
		5′-FAM-ACTGCCAAACGCCCACCGG-BHQ1-3′	
CHCHD10	mRNA	5′-TCCTGCACCCACCTCTACC-3′	81
		5′-CCTCACTTCCAATCCCAGCTA-3′	
		5′-FAM-CGCCGACAGCCAGACCACAAC-BHQ1-3′	
IGFBP3	mRNA	5′-TGATACAACTGTGGCCATGACT-3′	105
		5′-TCCCTGAGCCTGACTTTGC-3′	
		5′-FAM-CTCTCCCGGAGGCCAAACCCA-BHQ1-3′	
NPR1	mRNA	5′-ATACCTGAAAATTGATAGCA-3′	105
		5′-GTCCCATTGTAGTTCAGTA-3′	
		5′-FAM-AACCCTGAAGGCACCATTCT-BHQ1-3′	

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The relative change of gene expression was calculated using the following formula: fold change in gene expression, $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ (\text{tumor samples}) - \Delta Ct\ (\text{vehicle control})]}$, where ΔCt = Ct (detected gene) – Ct (β-actin) and Ct represents the threshold cycle number.

Microsatellite instability and *H. pylori* status. The MSI status was determined using a consensus panel of five reference microsatellite markers (BAT25, BAT26, D2S123, D3S546, and D17S250) by a previously described method.[12,30] When no marker was altered the tumors were defined as microsatellite stable. When only one marker was altered, the tumors were defined as low MSI. When two or more markers were altered those were defined as high MSI.

Helicobacter pylori infection was analyzed by detecting urease A gene of *H. pylori* genomic DNA in the gastric cancer mucosa using real-time PCR. The *H. pylori* specific primers and probe were: forward primer, 5′-ATGAAGTGGGTATTGAAGC GAT-3′, reverse primer, 5′-TTAAGAACAACTCACCAGGA ACTA-3′, and probe, 5′-FAM-CCTCAATAGGGGTATGCAC GG-BHQ1-3′.

Statistical analysis. All clinicopathologic variables were used as categorical variables. Differences in continuous variables between two groups were evaluated by Student's *t*-test, and differences in categorical variables were evaluated by the chi square -test. Associations between clinicopathological parameters and CIMP status were analyzed using Pearson's chi square-test and Fisher's exact test. Disease-free survival was measured from the date of resection of gastric cancer to the date of event or the last follow-up date before 11 May 2011. Event was defined as recurrence, death due to any cause, or development of a second primary gastric cancer. The Kaplan–Meier method was used to calculate and display disease-free survival curves, and the log–rank test was used to determine differences among all groups. The Cox proportional hazards regression method was used to determine independent prognostic factors. All statistical tests were done using the SPSS software package, version 17 (SPSS, Chicago, IL, USA). All *P*-values were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Gene methylation and CIMP status in gastric cancer. We studied 120 patients based on sample availability. Mean age was 58 years (range, 25–86 years), 80 patients were male (66.7%). The clinicopathologic features of the patients analyzed by CIMP are summarized in Table 2.

The methylation status of 120 gastric cancer samples in five DNA methylation markers was detected by MethyLight technology and PMR values were calculated for each sample and MethyLight reaction. Bisulfite genomic DNA sequencing of representative methylated PCR products of each of the five genes showed that all cytosines at non-CpG sites were converted to thymine (representative result shown in Fig. 1A).

The methylation frequencies of the five genes analyzed were 62.5% for *ALX4*, 70% for *TMEFF2*, 39.2% for *CHCHD10*, 58.3% for *IGFBP3*, and 42.5% for *NPR1*. The average number of methylated genes per tumor was 2.73. The results indicated that hypermethylation of these loci is a common event in gastric cancer. Representative results of MethyLight of *ALX4*, *TMEFF2*, *CHCHD10*, *IGFBP3*, and *NPR1* are shown in Figure 1B. We confirmed that DNA methylation of each gene was correlated with low expression of the respective mRNA (Table 3).

For descriptive purposes, CIMP status was classified as: CIMP-negative (CIMP-N) if none of the evaluated genes were methylated; CIMP-low (CIMP-L) if fewer than four genes were methylated; and CIMP-high (CIMP-H) if four or more genes were methylated. Based on this classification, concordant methylation of multiple genes (CIMP-H) was present in 15% (18 of 120) of tumors, CIMP-L in 78.3% (94 of 120), and CIMP-N in 6.7% (8 of 120) (Table 2). There were no differences in age, sex, tumor gross, histologic grade, pathologic lymph node status, MSI, or *H. pylori* infection status among the three groups. However, when three subtypes were correlated with lymph node metastasis, pathologic tumor classification, pathologic metastasis status, and stage (pTNM), CIMP-H gastric cancers tended to show more distant metastasis and higher tumor stage (Table 2).

Table 2. Clinicopathologic features of gastric carcinomas with CpG island methylator phenotype (CIMP) status

Characteristic	CIMP-H	CIMP-L	CIMP-N	*P*†
No. of patients	18	94	8	
Age group				
<60 years	9	45	3	0.890
≥60 years	9	49	5	
Sex				
Male	10	65	5	0.516
Female	8	29	3	
Gross				
Borrmann I	1	7	0	0.186
Borrmann II	4	30	3	
Borrmann III	8	56	4	
Borrmann IV	5	5	1	
Borrmann V	0	6	0	
Histologic grade				
G1 Well differentiated	1	4	1	0.658
G2 Moderately differentiated	7	32	2	
G3 Poorly differentiated	10	58	5	
G4 Undifferentiated	0			
Lymph node metastasis				
Negative	0	24	4	<0.001
<3 cm from tumor	5	41	4	
≥3 cm from tumor	13	29	0	
Pathologic tumor classification				
pT1	0	8	5	<0.001
pT2	1	10	2	
pT3	7	60	1	
pT4	10	16	0	
Pathologic lymph node status				
pNo	3	33	6	0.229
pN1	10	36	2	
pN2	3	15	0	
pN3	2	10	0	
Pathologic metastasis status				
pM0	9	83	8	0.001
pM1	9	11	0	
Stage (pTNM)				
Stage IA	0	5	5	<0.001
Stage IB	1	11	2	
Stage II	1	15	0	
Stage IIIA	1	24	1	
Stage IIIB	1	12	0	
Stage IV	14	27	0	
MSI				
MSI-H	1	16	2	0.244
MSI-L	2	21	0	
MSI-N	15	57	6	
Helicobacter pylori				
Positive	4	16	1	0.902
Negative	14	78	7	

H, high; L, low; MSI, microsatellite instability; N, negative.
†Statistical significance determined using Pearson's χ^2-test and Fisher's exact test.

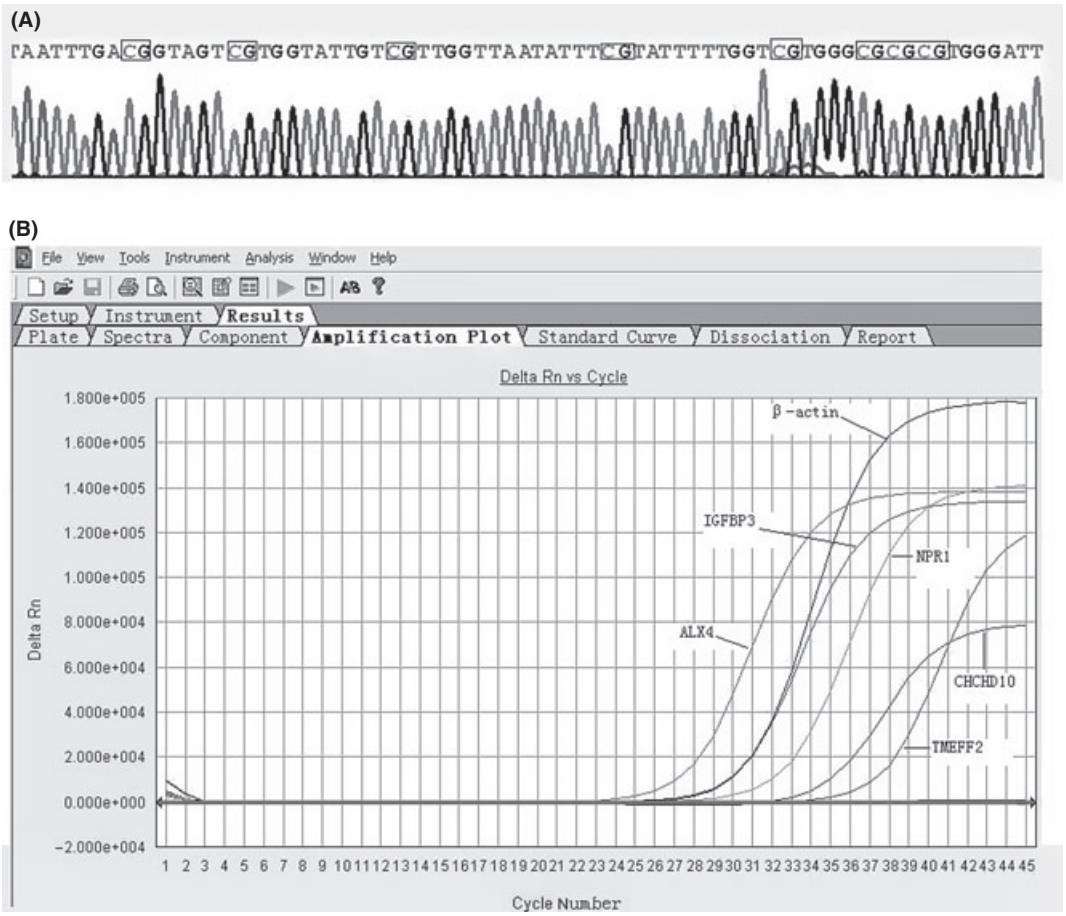

Fig. 1. (A) Sequencing analysis of methylated PCR products of the *NPR1* gene. All CpG sites were methylated and C to T transition was observed by bisulfite modification. To indicate methylation status, the wild-type CpG sites were squared. Methylated CpG sites appear as CG. (B) Methylation analysis of five genes in gastric cancer by MethyLight PCR. The gene studied is indicated.

Table 3. Relationship between DNA methylation and mRNA expression in gastric cancer

Gene	Methylation status	No. cases	mRNA level†	*P*‡
ALX4	M	75	0.20 ± 0.03§	0.017
	U	45	2.48 ± 0.12	
TMEFF2	M	84	1.14 ± 0.11	0.001
	U	36	8.26 ± 2.04	
CHCHD10	M	47	0.04 ± 0.01	0.005
	U	73	0.89 ± 0.05	
IGFBP3	M	70	0.13 ± 0.02	0.005
	U	50	0.74 ± 0.14	
NPR1	M	51	0.05 ± 0.03	0.005
	U	69	0.48 ± 0.08	

†Mean values and standard errors for all gastric cancer samples including those that are methylated (M) and unmethylated (U).
‡Statistical significance determined using the Mann–Whitney *U*-test.
§Units are arbitrary, and we calculated the respective mRNA expression level by standardization with 1 μg total RNA of NCI-N87 gastric cancer cells, taken as 1.0.

Microsatellite instability and *H. pylori* infection. Representative examples of MSI in gastric cancer are shown in Figure 2. The prevalence of MSI-high (MSI-H) was 15.8% (19 of 120), MSI-low (MSI-L) was 19.2% (23 of 120), and MSI-stable was 65% (78 of 120) in gastric carcinomas. There was no statistical difference between MSI status in tumors with evaluated clinicopathologic features, including age, sex, tumor histology, and pathologic stage (data not shown).

Helicobacter pylori was detected in 21 of 120 tumors (17.5%) using real-time quantitative PCR. No differences were found in the number of methylated genes between *H. pylori*-negative patients and *H. pylori*-positive patients (data not shown).

Survival analysis. In univariate analysis, Borrmann stage ($P < 0.001$), lymph node metastasis ($P < 0.001$), tumor stage (pTNM) ($P < 0.001$), and CIMP-H ($P < 0.001$) were statistically significant predictors for overall survival (Table 4). Histologic grade, MSI, and *H. pylori* infection status were not significant prognostic factors. By Kaplan–Meier survival analysis and the log–rank test, patients who had CIMP-H gastric tumors showed significantly worse survival than patients with CIMP-L/CIMP-N tumors (Fig. 3).

Borrmann stage, lymph node metastasis, stage (pTNM), and CIMP were included in multivariate Cox regression analysis (Table 4). Patients with CIMP-H gastric tumors tended to have worse survival than patients with CIMP-L/CIMP-N gastric tumors and the difference was significant ($P < 0.001$).

Discussion

The relationship between CIMP and tumor pathology is unclear. It seems reasonable that the biologic functions of each associated gene are also simultaneously silenced because DNA methylation is associated with low expression of the respective mRNA. Among the five marker genes used in the present study,

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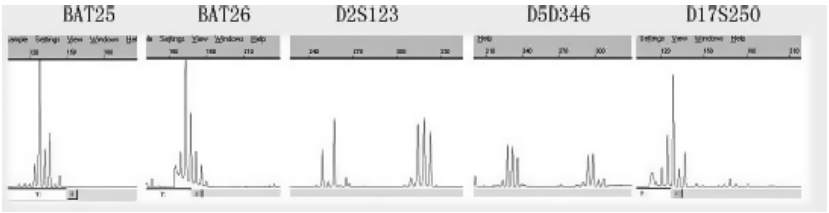

Fig. 2. Representative examples of microsatellite instability in gastric carcinoma. A microsatellite instability-high tumor had allelic shifts in two microsatellite markers (D2S123 and D5S346).

Table 4. Univariate and multivariate survival analysis in gastric cancer (*n* = 120)

Variables	No. patients	No. deaths	Univariate analysis†	Multivariate analysis‡	
			χ^2 (*P*)	Hazard ratio (95% CI)	*P*
Age group					
<60 years	57	23	0.136 (0.712)		
≥60 years	63	25			
Gross					
Borrmann I + II	35	7	20.158 (<0.001)	Reference	
Borrmann III	68	28		1.612 (0.688–3.779)	0.272
Borrmann IV + V	17	13		3.587 (1.339–9.612)	0.011
Histologic grade					
G1 + G2	47	18	0.049 (0.825)		
G3	73	30			
Lymph node metastasis					
Negative	28	5	22.345 (<0.001)	Reference	
<3 cm from tumor	50	17		0.949 (0.322–2.793)	0.924
≥3 cm from tumor	42	26		1.438 (0.489–4.225)	0.509
Stage (pTNM)					
Stage IA + IB	24	1	44.676 (<0.001)	Reference	
Stage II	16	3		2.583 (0.257–25.978)	0.420
Stage IIIA	26	9		10.476 (1.266–86.700)	0.029
Stage IIIB	13	5		11.715 (1.337–102.668)	0.026
Stage IV	41	30		22.182 (2.75–178.940)	0.004
CIMP					
N + L	102	32	91.108 (<0.001)	Reference	
H	18	16		12.688 (5.249–30.671)	<0.001
MSI					
MSI-N	78	34	3.608 (0.058)	NA	NA
MSI-L	23	12		NA	NA
MSI-H	19	2		NA	NA
Helicobacter pylori					
Negative	99	38	1.287 (0.257)	NA	NA
Positive	21	10		NA	NA

CI, confidence interval; CIMP, CpG island methylator phenotype; H, high; L, low; MSI, microsatellite instability; N, negative; NA, not applicable; Reference, represented in the contrast matrix as a row of zeros in multivariate analysis.
†Log–rank test. ‡Cox's proportional hazards model.

ALX4 and *TMEFF2* were reported as methylated in our previous colorectal cancer research by multiplex MethyLight assay.[28] The methylation of *ALX4* and *TMEFF2* is regarded as an early event during tumor carcinogenesis.[13,16] *IGFBP-3* expression may be protective against the development of gastric adenocarcinoma by preventing the formation of intestinal metaplasia and improve the prognosis of gastric cancer.[31] The relationship between the methylation of *CHCHD10* and *NPR1* and gastric cancer clinical outcome is not clear so far. In this study, CIMP-H including *CHCHD10* and *NPR1* methylation with lymph node metastasis, pathologic tumor classification, pathologic metastasis status, and pathologic TNM status show significant difference. In particular, patients with CIMP-H show more distant lymph node metastasis, which can be explained by the fact that the methylation of *ALX4* and *TMEFF2* is an early event, the methylation of *IGFBP-3*, *CHCHD10*, and *NPR1* may be a later event associated with the lymph node metastasis. A project designed to clarify the concrete role of *CHCHD10* and *NPR1* methylation in lymph node metastasis of gastric cancer is underway.

Furthermore, most previous studies of methylation in gastric cancer focus on the prognostic significance of methylation of a single gene.[4–7] The current research about the clinical outcome of CIMP-positive gastric cancer is controversial.[2,9,11,24] Most previous reports show that CIMP was related with better prognosis, but was not an independent prognostic factor on multivariable analysis, except in the report published by Seog-Yun Park *et al.*[11] The present study shows that CIMP was closely associated with poor prognosis of gastric cancer patients and CIMP-H was an independent prognostic marker. The differences might

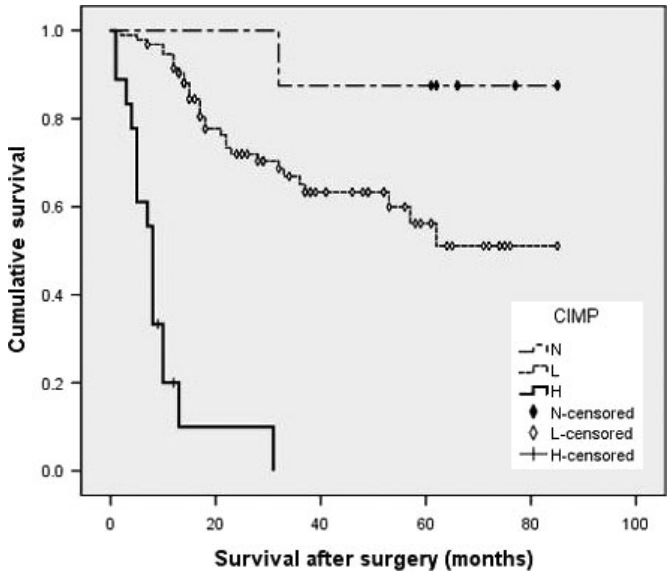

Fig. 3. Kaplan–Meier survival curves in gastric cancers (n = 120) according to CpG island methylator phenotype status. H, high; L, low; N, negative.

be attributed to two reasons. One is the different CIMP marker panels. Most CIMP reports used *MINT1*, *MINT2*, *MINT12*, *MINT25*, and *MINT31* as the CIMP marker panel, but the CIMP marker panel in this study is *ALX4*, *TMEFF2*, *CHCHD10*, *IGFBP3*, and *NPR1*. The other is the different methods used to analyse the status and level of target gene methylation. Compared with combined bisulfite restriction analysis, methylation-specific PCR, and quantitative methylation-specific PCR, MethyLight assay is more accurate and more sensitive. However, the number of markers used in the CIMP panel in this study (five) was small, and the number of cases (120) was too few, therefore, it is necessary to launch a large-scale study including more CIMP markers to attest the detailed clinicopathological features of tumors with CIMP.

Several studies have found that multiple gene methylation correlates with *H. pylori* infection in non-neoplastic gastrointestinal tissues.[32,33] However, several studies have shown that *H. pylori* infection induced the altered DNA methylation in gastric cancer.[34,35] However, in this study, *H. pylori* infection was not a differential influence on CIMP including *ALX4*, *TMEFF2*, *CHCHD10*, *IGFBP3*, and *NPR1* in gastric cancer.

Controversial results were reported by a different research group regarding the relationship between MSI, DNA replication errors, and clinical prognosis.[12,36,37] In this study, MSI-H was present in 15.8% of gastric cancers. There was no relationship between MSI status and clinicopathologic characters including CIMP. Compared with MSI-L/MSI-stable tumors, overall survival was slightly worse but without statistically significant difference (P = 0.058) in patients with MSI-H tumors. The MSI status of gastric cancer in this study was not a significant predictor of prognosis. It is possible that CIMP in this study did not include DNA repair genes such as *hMLH1*, as has been reported previously.[12]

In conclusion, using the methylation profile of five unique genes (*ALX4*, *TMEFF2*, *CHCHD10*, *IGFBP3*, and *NPR1*) as marker genes, we found that CIMP-H was associated with lymph node metastasis, pathologic tumor classification, pathologic metastasis status, and pathologic TNM status. CIMP-H was an independent prognostic factor in gastric cancer. However, more studies are needed to validate the role of CIMP and elucidate its mechanism in gastric cancer.

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Disclosure Statement

The authors have no conflict of interest.

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