CpG island methylator phenotype and *Helicobacter pylori* infection associated with gastric cancer

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Abstract

**AIM:** To investigate the association between the CpG island methylator phenotype (CIMP) and serum *Helicobacter pylori* (*H. pylori*) levels for clinical prediction of gastric cancer (GC) progression.

**METHODS:** We analyzed the serum CIMP status of 75 patients with GC using a methylation marker panel and a methylation-specific polymerase chain reaction. Serum samples from 40 healthy persons were examined at the same time. The genes examined were *APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK*, and *E-cad*. *H. pylori* infection in serum was assayed with an anti-*H. pylori* immunoglobulin G antibody test and a rapid urease test.

**RESULTS:** The frequencies of high-level methylation in GC tissues for the seven genes were: 48% for *APC*, 57.33% for *WIF-1*, 56% for *RUNX-3*, 50.67% for *DLC-1*, 52% for *SFRP-1*, 54.67% for *DKK*, and 48% for *E-cad.*

The frequencies in GC serum were 30.67% for *APC*, 34.67% for *WIF-1*, 37.33% for *RUNX-3*, 29.33% for *DLC-1*, 33.33% for *SFRP-1*, 32% for *DKK*, and 26.67% for *E-cad.* CIMP+ (defined as ≥3 methylated genes) was associated with 47 (62.67%) GC tissue samples and 44 (58.67%) GC serum samples. CIMP+ was not associated with non-neoplastic mucosal tissues or the serum of healthy persons. Of the 75 GC cases, 51 (68%) were *H. pylori*+, and 24 (32%) were *H. pylori*-. Of the 51 *H. pylori*+ cases, 36 were CIMP+ and 15 were CIMP-. In contrast, for the 24 *H. pylori*- cases, 11 were CIMP+, and 13 were CIMP-. The difference was significant between the *H. pylori*+ and *H. pylori*- groups ($\chi^2 = 4.27, P < 0.05$). Of the 51 *H. pylori*+ GC patients, 34 were CIMP+ and 17 were CIMP-, while among the 24 *H. pylori*- GC cases, 10 were CIMP+ and 14 were CIMP-. The difference was significant between the *H. pylori*+ and *H. pylori*- groups ($\chi^2 = 4.21, P < 0.05$). A 2-year follow-up showed significant difference in the rates of metastasis and recurrence between *H. pylori*+/*CIMP*+ cases and the *H. pylori*+/*CIMP*- cases or *CIMP* cases associated with *H. pylori* assayed in serum ($P < 0.05$). However, there were no significant differences in survival rates between the two groups.

**CONCLUSION:** *H. pylori*+/*CIMP*+ cases are associated with higher rates of metastasis and recurrence than *H. pylori*+/*CIMP*- cases. Serum may be useful for examining CIMP status.

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**Key words:** CpG island methylator phenotype; *Helicobacter pylori*; Serum; Prognosis; Gastric cancer

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INTRODUCTION

Gastric cancer (GC) is one of the most common human tumors and is the second-leading cause of cancer-related deaths worldwide[1]. GC remains a major clinical challenge because it has a poor prognosis and limited treatment options due to its relative resistance to radiotherapy and chemotherapy[2].

DNA methylation is an enzyme-mediated chemical modification that occurs in cytosine-guanine dinucleotide-rich areas (CpG islands) in gene promoter regions. Aberrant promoter hypermethylation leads to loss of gene function in tumors[3]. In several types of cancers, including GC, global hypomethylation and promoter hypermethylation in specific genes are associated with genomic instability and inactivation of tumor-suppressor genes[4]. The methylation pattern(s) of multiple genes can provide useful information regarding global epigenetic alterations[5]. The hypermethylated subtype associated with tumors, denoted that the CpG island methylator phenotype (CIMP) for which multiple genes are concurrently methylated, is a novel marker of tumor progression[6-8].

Helicobacter pylori (H. pylori) infection of the stomach is associated with an increased risk for gastric carcinoma[9]. Several studies have demonstrated that H. pylori infection is associated with gene promoter hypermethylation and gene-type-specific methylation profiles involved in the multistep process of carcinogenesis[10,11]. H. pylori infection may induce methylation of the Trefoil factor family 2 and E-cadherin promoters in GC[12]. Certain studies have shown that methylation levels in GC patients are one to two orders of magnitude greater in H. pylori+ individuals than in H. pylori- individuals[13]. In infected mucosae, aberrant methylation and subsequent silencing of tumor suppressor genes, including p16, E-cadherin, and hMLH1, are strongly correlated with subsequent cancer risk[14].

In the present study, we focused on the relationship between H. pylori infection and CIMP status in GC risk. We did not specifically study the relationship between CIMP and H. pylori infection or the clinical value of evaluating CIMP associated with H. pylori infection.

We analyzed the relationship between serum CIMP of 75 GC patients and H. pylori infection in GC. The examined genes were APC, WIF-1, RUNX-3, DLC-1, SFRP-1, DKK, and E-cad, which were selected because they had been found to be frequently methylated in GC and other malignancies The aim of this study was to evaluate the clinical significance of serum CIMP and H. pylori-infection levels for the prediction of GC progression.

MATERIALS AND METHODS

Patients and specimens

Between 2008 and 2009, tumor samples, adjacent non-neoplastic tissues, and sera from 75 Chinese GC patients were collected during surgical resection at the Nantong Tumor Hospital. Samples from 40 healthy people were used as matched controls. The controls had no self-reported history of cancer and could frequently be matched to the cases in age (± 5 years), gender, and residential area. After resection, tissue specimens were immediately frozen in liquid nitrogen and stored at -70 °C. Patients consisted of 53 men and 22 women, ranging in age from 31 to 76 (52 ± 7) years. Questionnaire data and blood samples were also collected from all patients and controls. After centrifugation, isolated serum samples were stored at -70 °C. The diagnosis of GC was confirmed by pathological examination and magnetic resonance imaging and/or computerized tomography. Tumor-node-metastasis (TNM) stage was classified according to the 6th edition TNM classification of the American Joint Committee on Cancer. Written informed consent was obtained from each patient and healthy control, and the local ethics committee approved the study protocol.

DNA extraction and sodium bisulfite

DNA from serum (200 µL per sample) and tissue samples was treated with proteinase K and then extracted with phenol-chloroform according to the manufacturer’s instructions (Shanghai ShineGene Molecular Biotech, Inc., Shanghai, China) and stored at -20 °C. A final elution volume of 50 µL was collected. The extracted DNA was treated with sodium bisulfite using EZ DNA Methylation kit reagents (Zymo Research, Orange, CA, United States) to convert all unmethylated cytosines to uracils. Bisulfite-modified DNA was suspended in 10 µL of elution buffer and stored at -20 °C until methylation-specific polymerase chain reaction (PCR) (MSP) was performed.

MSP

MSP was used to determine the methylation status of CpG islands in genes after bisulfite treatment[15,16]. The methylation status of the promoters of APC, WIF-1, RUNX-3, DLC-1, SFRP-1, DKK, and E-cad was determined with a two-step amplification/detection MSP protocol. PCR products were electrophoresed through 2% agarose gels, stained with ethidium bromide, and visualized with ultraviolet illumination. All experiments were performed in duplicate. Table 1 lists the sequences of the PCR primers used.

H. pylori assay

H. pylori infection was assayed with the reagents of a serum anti-H. pylori immunoglobulin G (IgG) antibody test (PBM, PRINCETON, United States) and a rapid urease test (Fujian Sanqiang Biological and Chemical Co., Ltd,
Table 1 Primer sets for nested methylation-specific polymerase chain reaction

| Gene  | Forward sequence (5’→3’) | Reverse sequence (5’→3’) |
|-------|--------------------------|--------------------------|
| APC   | F: GTCATTATGATCAGGTTGTTGTTGTC | R: CCAATCCAAATACCCCCAACAAC |
| M     | F: TGATGCGGCAGATGGTTGTTGTTGTC | R: TCCATCTCTTACCTGTCGTC |
| WIF-1 | F: GGGTGTTTTATATGGTGTTGTTGTTGTC | R: AAAAACTAACAACAAAAAATACCAAAAC |
| M     | F: CGGTTGTTTTATATGGTGTTGTTGTTGTC | R: ACTAATCCGGAAAATACAGCA |
| DLC-1 | F: TTAATGACGACGAGTTGTTGTTGTTGTC | R: AACCCCAACAACACAAAACCCACCAC |
| M     | F: TTACGGAGGGGCGGTCGTACGG | R: AAAAAAGCCCGACGCGAGCCGAGCC |
| DKK   | F: GACGTGTTTTATATGGTGTTGTTGTTGTC | R: CCAACATCTACAAAACTACCAAAAC |
| M     | F: GTGTCGAGGGGCGGTCGTACGCG | R: AAGCGTTACCGACTCCGGAGCC |
| RUNX-3| F: TTAATGACGACGAGTTGTTGTTGTTGTC | R: ACAATCCCGGTCCCGAGCC |
| M     | F: TTACGGAGGGGCGGTCGTACGCG | R: TAAATCCCGACCTACCCGAC |

M: Methylated sequence; U: Unmethylated sequence; F: Forward sequence.

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Table 2 Number of samples with methylated genes for gastric cancer, adjacent non-neoplastic tissue, and serum from gastric cancer patients and healthy controls

| Gene | Tumor tissues (n = 75) | Serum from GC patients (n = 75) | Non-neoplastic tissues (n = 72) | Serum from healthy controls (n = 40) | \( \chi^2 \) |
|------|-----------------------|-------------------------------|--------------------------------|----------------------------------|---------|
| APC  | 36%                   | 23                            | 3                              | 0                                | 36.21   |
| WIF-1| 43%                   | 26                            | 4                              | 0                                | 45.28   |
| DLC-1| 38%                   | 22                            | 3                              | 0                                | 39.49   |
| DKK  | 41%                   | 24                            | 2                              | 0                                | 47.79   |
| SFRP-1| 39%                 | 25                            | 4                              | 0                                | 38.29   |
| E-cad| 36%                   | 20                            | 3                              | 0                                | 36.21   |
| RUNX-3| 42%                 | 28                            | 5                              | 0                                | 40.64   |

*P < 0.05 vs the number of methylated gene promoters for tumor and non-neoplastic tissue samples with the \( \chi^2 \) test. GC: Gastric cancer.

Follow-up examination

Patients were followed up to 2 years after surgery. The last follow-up was on September 12, 2010. Patients had not received chemotherapy prior to surgery. The median follow-up period was 17.5 mo (range, 8-28 mo). Patients were given a physical examination, abdominal ultrasonography, and chest X-ray, and serum was collected and tested for associated tumor markers. During the first year, local recurrence and the development of distant metastasis was monitored every 3 mo with computerized tomography and/or magnetic resonance imaging. Of the 75 patients, 13 (18.67%) died from cancer-related causes. We tracked all but three of the remaining patients during the entire study period. Seventeen patients (22.67%) had GC recurrence found on computerized tomography Fourteen patients (18.67%) had GC metastases. Sixty-two patients were still alive at the time of the last follow-up report.

Statistical analysis

The SPSS 13.0 software package (SPSS, Inc., Chicago, IL, United States) was used for statistical analysis. Values for the clinical and biological characteristics of the patients are expressed as mean ± SD. Comparison was done with the Student’s t test. The \( \chi^2 \) test was used to compare the incidence of methylation. All \( P \) values are two-sided, and a \( P \) value < 0.05 was considered statistically significant.

RESULTS

Clinical data

The median tumor size was 3.8 cm (range 2.5-9.6 cm). According to the Edmondson-Steiner classification system, six cases were classified as grade I, 22 as grade II, 40 as grade III, and seven as grade IV. According to the 6th edition TNM classification of the American Joint Committee on Cancer, 24 cases were classified as grade I, 31 as grade II, and 20 as grade III.

Methylation of tumor-associated genes in GC

We examined the methylation status of seven tumor-associated genes (APC, WIF-1, RUNX-3, DLC-1, SFRP-1, DKK and E-cad) from tissue and serum samples of GC patients and from serum samples of controls. The overall results are summarized in Table 2. No methylated gene promoters from control serum were detected. Methylation occurred more frequently in the GC DNA than in adjacent non-neoplastic mucosal tissues, and the same relationship was observed for serum samples and non-neoplastic tissues. Methylation was detected in one or more of the genes in 69 (92%) of the 75 cases. The frequencies of high-level methylation in GC tissue and serum were ≥ 15% for the seven genes: 48% for APC, 57.33% for WIF-1, 56% for RUNX-3, 50.67% for DLC-1, 52% for SFRP-1, 54.67% for DKK, and 48% for E-cad in tissue. In serum, the frequencies were 30.67% for APC, 34.67% for WIF-1, 37.33% for RUNX-3, 29.33% for DLC-1, 33.33% for SFRP-1, 32% for DKK, and 26.67% for E-cad.

According to the criteria used in a related study, the CIMP status of our 75 GC samples was classified as CIMP+ (≥ 3 methylated genes) or CIMP- (two or fewer methylated genes). In this study, because the cutoff value was 3, the average number of methylated genes found
Table 3  CpG island methylator phenotype associated with Helicobacter pylori infection in 75 gastric cancer tissue and serum samples

| CIMP | H. pylori infection in tissue samples | H. pylori infection in serum samples |
|------|----------------------------------|----------------------------------|
|      | Negative (−) | Positive (+) | Negative (−) | Positive (+) |
| Negative (−) | 13 | 15 | 14 | 17 |
| Positive (+) | 11 | 36 | 10 | 34 |

*P < 0.05 vs the frequencies of CIMP between H. pylori+ and H. pylori- samples in serum with the χ² test. H. pylori: Helicobacter pylori; CIMP: CpG island methylator phenotype.

was 3.6 in tumor tissue and 2.0 in serum. For tumor tissue samples, 47 (62.67%) were classified as CIMP+, and 28 (37.33%) were classified as CIMP-. For serum samples, 44 (58.67%) were classified as CIMP+, and 31 (41.33%) were classified as CIMP-. No non-neoplastic tissues or serum from healthy controls were classified as CIMP+.

H. pylori in GC

We determined how many of the 75 GC patients were infected with H. pylori and found 51 (68%) to be H. pylori+ and 24 (32%) to H. pylori-.

Of the 51 H. pylori+ GC cases examined in tissues, 36 were CIMP+ and 15 were CIMP-. In contrast, of the 24 H. pylori- GC cases, 11 were CIMP+ and 13 were CIMP-. There was a significant difference between the H. pylori+ and H. pylori- groups (χ² = 4.27, P < 0.05). When the sera from the 51 H. pylori+ GC cases were examined, 34 were CIMP+ and 17 were CIMP-. In contrast, of the 24 H. pylori- GC cases, 10 were CIMP+ and 14 were CIMP-. There was a significant difference between the two groups (χ² = 4.21, P < 0.05). The overall results are summarized in Table 3.

Prognostic significance of CIMP associated with H. pylori infection in GC

After the two-year follow-up, the metastasis rates were found significantly different between the H. pylori+/CIMP+ and the H. pylori+/CIMP- cases when analyzed in serum (P < 0.05). The tumors of H. pylori+/CIMP+ patients frequently metastasized. The recurrence rates were significantly higher in the H. pylori+/CIMP+ group than in the H. pylori+/CIMP- group when serum was examined (P < 0.05). There were no differences in survival rates between H. pylori+/CIMP+ cases and H. pylori+/CIMP- cases examined in serum (P > 0.05). The overall results are summarized in Table 4.

DISCUSSION

Epigenetic alterations have been suggested to be significant initiating events in tumorigenesis. Aberrant methylation of promoter DNA regions rich in CpG islands is the key step in epigenetic gene silencing. Abnormal gene expression may be an early event in tumorigenesis and a potential biomarker for early detection. In several studies, aberrant DNA methylation in gastric biopsies from H. pylori+ patients was found to be correlated with a greater gastric cancer risk. Methylhyation level of promoters in severeral genes in the gastric mucosa is 5-300 times higher in infected than in uninfected individuals. In infected mucosa, the aberrant methylation and subsequent silencing of tumor suppressor genes has been strongly correlated with subsequent cancer risk.

Notably, the term “CIMP” has been used in a variety of ways in the context of gastric carcinoma. The hypermethylator phenotype may be related to patient-specific factors, such as exposure to carcinogens or genetic predisposition. The relationship between H. pylori and methylation of multiple genes has been demonstrated, but little attention has been paid to the association of CIMP and H. pylori with respect to gastric cancer or the relationship between CIMP in serum and the presence of H. pylori in gastric cancer.

We collected tissue and serum samples from GC patients and healthy controls to examine the methylation status of seven tumor-associated genes (APC, WT1, RUNX-3, DLC-1, SFRP-1, DKK, and E-cad). We found that the promoters of these genes from healthy control serum were not methylated. The frequencies of high-level methylation in GC tissue and serum were at least 15% for these seven genes. Thus, examining the methylation status of multiple genes may help diagnose early GC. We also found that promoter methylation in serum DNA was highly specific for the cancer-related genes and that the results in serum samples were similar to tissue samples. We also determined the CIMP status of 75 GC samples and found a good concordance between serum and tumor CIMP status.

Of the 75 GC cases, 51 (68%) were H. pylori+ and 24 (32%) were H. pylori- Thus, H. pylori infection was substantial in GCs, suggesting that eradicating H. pylori infection may be essential to prevent or treat GC.

The prognostic roles of CIMP status have been evaluated in several cancer types. In esophageal adenocarcinoma, CIMP is associated with a poor prognosis. In GC, concordant methylation of multiple genes/loci (CIMP-H)
H. pylori infection is also a factor in the prognosis of GC. We found that CIMP was associated with H. pylori infection. At the end of the 2-year follow-up, we found that the rates of metastasis differed significantly between H. pylori+/CIMP+ and H. pylori+/CIMP- cases when examining the serum (P < 0.05). The tumors of H. pylori+/CIMP+ cases frequently metastasized. The recurrence rates were also significantly higher in the H. pylori+/CIMP+ cases than in the H. pylori+/CIMP- cases when serum was examined (P < 0.05). There were no significant differences in survival rates between H. pylori+/CIMP+ and H. pylori+/CIMP- cases examined in serum (P > 0.05). Therefore, aberrant DNA hypermethylation may be frequently associated with chronic inflammation, but not with H. pylori infection. Modulation levels of pleiotropic regulators suggested that DNA methylation plays an important role in host response to H. pylori infection. H. pylori promotes genetic instability via decreasing expression of DNA repairing genes. We observed that CIMP+ in conjunction with H. pylori infection was associated with metastasis and recurrence of GC, but not the survival rate after the 2-year follow-up period.

In summary, our study shows that promoter methylation in serum DNA was highly specific and that serum and tissue samples yielded similar results. Therefore, CIMP detection in serum, rather than in tumor tissues, may be used as a reliable index for predicting the clinical course of patients with GC.

This study is limited by a relatively small number of patients, cancer-related genes, as well as follow-up period of less than 2 years. Thus, larger-scale multi-gene studies with an extended follow-up period are needed to validate our results.

**Innovations and breakthroughs**

In the present study, the authors focused on the relationship between H. pylori infection and CIMP status in GC risk. The authors analyzed the relationship between serum CIMP of 75 patients with GC and H. pylori infection in GC. The examined genes were APC, WIF-1, RUNX-3, DCL-1, SFRP-1, DKK, and E-cad, which were selected because they had been found to be frequently methylated in GC and other malignancies. This study evaluated the clinical significance of serum CIMP and H. pylori-infection levels for the prediction of GC progression.

**Applications**

It was found in this study that H. pylori+/CIMP+ cases were associated with higher metastasis and recurrence rates than H. pylori+/CIMP- cases. CIMP detection in serum, rather than in tumor tissues, may be used as a reliable index for predicting the clinical course of patients with GC.

**Terminology**

Epigenetic changes: Hentable changes in gene structure without changing the gene sequence; CpG islands: CpG rich areas located in the promoter regions of many genes; CpG island methylation: The addition of a methyl group to a cytosine residue that lies next to guanine within CpG dinucleotides; CIMP: The hypermethylated subtype in tumors, where multiple genes are concurrently methylated.

**Peer review**

This manuscript was well designed and executed, and data were summarized well.

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