Genetic, Epigenetic, and Clinicopathologic Features of Gastric Carcinomas with the CpG Island Methylator Phenotype and an Association with Epstein–Barr Virus

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BACKGROUND. The CpG island methylator phenotype (CIMP), which is characterized by simultaneous methylation of the CpG islands of multiple genes, has been recognized as one of the important mechanisms in gastrointestinal carcinogenesis.

METHODS. Methylation of the 5 methylated-in-tumors (MINT) loci and 12 tumor-related genes in 78 primary gastric carcinomas was examined using combined bisulfite-restriction analysis. Epstein–Barr virus (EBV)-associated gastric tumors were detected using real-time polymerase chain reaction analysis followed by an evaluation of the correlations between CIMP status, EBV-association, and genetic alteration of p53 and K-ras.

RESULTS. The methylation profiles of 12 genes showed nonrandom methylation, supporting the presence of CIMP in gastric carcinoma. No p53 mutations were detected among CIMP-H tumors, and no EBV association was detected in tumors that showed mutation of p53 and K-ras. In a multiple logistic regression model with CIMP-H as the dependent variable, proximal location (p = .011), diffuse type (p = .019), and less advanced pathologic TNM status (p = .043) contributed significantly to CIMP-H. Patients who had CIMP-N gastric tumors had a significantly worse survival than patients who had CIMP-H tumors (p = .004) or CIMP-L tumors (p = .012). EBV-associated tumors were associated strongly with CIMP-H, hypermethylation of tumor-related genes, and no p53 or K-ras mutation.

CONCLUSIONS. CIMP status appeared to be associated with distinct genetic, epigenetic, and clinicopathologic features in gastric carcinomas. The finding that gastric carcinomas arose through different molecular pathways may affect not only tumor characteristics but also patient prognosis.

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KEYWORDS: gastric carcinoma, DNA methylation, molecular profiling, clinicopathologic features, Epstein–Barr virus.

Gastric carcinoma is one of the most common human neoplasms and is the second leading cause of cancer-related death in the world.1 Promoter hypermethylation that leads to epigenetic silencing of multiple genes has been recognized as an important mechanism in gastrointestinal carcinogenesis. In that regard, promoter methylation

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CpG island methylator phenotype status appeared to be associated with distinct genetic, epigenetic, and clinicopathologic features in patients with gastric carcinoma. The finding that gastric carcinomas arise through different molecular pathways may affect not only tumor characteristics but also patient prognosis.
of the so-called CpG islands, which are CpG dinucleotide-rich areas located within the promoters of approximately 60% of human genes, usually is associated with long-term, irreversible epigenetic silencing of X-linked and imprinted genes, but it also is known that CpG promoter methylation silences tumor-related genes. Furthermore, concordant promoter hypermethylation of multiple genes, which is termed descriptively as the "CpG island methylator phenotype" (CIMP), was described recently in both gastric and colorectal carcinomas.

Helicobacter pylori (HP) infection of the stomach is associated with an increased risk of gastric carcinoma. Although HP infection is extraordinarily common, gastric adenocarcinoma occurs in only a minority of infected individuals. In addition, Epstein–Barr virus (EBV) is a ubiquitous herpes virus that infects most children during early childhood and causes few if any symptoms. However, EBV also is involved in a subset of gastric carcinomas, although its specific role in carcinogenesis remains unclear. It has been shown that p16 cyclin dependent kinase 4A inhibitor (p16INK4A) expression is absent significantly more often in EBV-negative gastric carcinoma than with EBV-associated gastric carcinoma and that this loss is associated with not only p16INK4A methylation but also with methylation of other tumor-suppressor genes. In addition, evidence suggests a close association between aberrant methylation and the entry of foreign viral DNA into host cells.

The most frequently observed genetic alteration in gastric carcinoma is mutation of the p53 gene, which is believed to play a central role in carcinogenesis of the stomach. By contrast, there is wide variation in the frequency of K-ras mutations in upper and lower gastrointestinal tract tumors, with several studies showing the frequency in gastric carcinomas to be quite low. The frequency of the occurrence of p53 and/or K-ras mutations in gastric tumors with multiple concordant methylation events and in EBV-associated gastric tumors remains unknown.

In the current study, we evaluated the methylation status of 12 tumor-related genes along with the 5 methylated-in-tumors (MINT) loci. In addition, to clarify the characteristics of gastric tumors with hypermethylation of MINT loci and to shed light on their underlying mechanisms, we initially assessed their clinicopathologic features, including EBV and HP status and then analyzed the genetic alterations of p53 and K-ras. Finally, we evaluated the prognostic significance of CIMP status in gastric carcinomas.

### MATERIALS AND METHODS

#### Cell Lines and Specimens

The gastric carcinoma cell lines that were used in this study were obtained from the Japanese Collection of Research Biorepositories (Tokyo, Japan) or the American Tissue Type Collection (Manassas, VA) and then were cultured in the appropriate medium. The 78 gastric tumor specimens and their paired normal tissue specimens were from 78 randomly selected Japanese patients; these specimens were removed surgically, immediately frozen, and stored at −80°C until they were used. Informed consent was obtained from all patients before the samples were collected. DNA was extracted using the standard phenol/chloroform method. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA).

#### Reverse Transcription-Polymerase Chain Reaction

Five micrograms of total RNA were reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen); then, polymerase chain reaction (PCR) analysis was carried out as described previously. The integrity of the cDNA was confirmed by amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described previously. To analyze restoration of chondroitin sulfate proteoglycan 2 (CSPG2), MKN74, and KATOIII, cells were incubated for 72 hours with 0.2 μM 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma, St. Louis, MO), which is a methyltransferase inhibitor.

#### Combined Bisulfite Restriction Analysis

Combined bisulfite restriction analysis (COBRA) consists of a standard sodium bisulfite treatment and PCR amplification followed by restriction enzyme digestion and a quantification step. We applied COBRA to assess gene methylation using primers that were designed to amplify the regions around the transcription start sites of the target genes. Bisulfite modification was carried out as described previously. Briefly, 2 μg of genomic DNA were incubated with 3 M sodium bisulfite (Sigma) for 16 hours; then, the DNA was purified using a DNA Purification System (Promega, Madison, WI), and it was stored at −20°C until it was used. Selected for study were the 5 MINT loci (MINT1, MINT2, MINT12, MINT25, and MINT31), which were examined as described previously, and 12 tumor-related genes. The primer sequences, annealing temperatures, and restriction enzymes that were used are listed in Table 1. Initially, PCR was carried out in a reaction medium that contained 1× methylation-specific PCR (MSP) buffer (67 mM Tris-HCl, pH 8.8; 16.6 mM (NH₄)₂SO₄; 6.7 mM MgCl₂; and 10 mM...
### TABLE 1

**Primer Sequences Used in the Current Study**

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Annealing Temperature in °C (No. of Cycles)</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>MINT1</td>
<td>55 (35)</td>
<td>Taq I</td>
</tr>
<tr>
<td>MINT2</td>
<td>60 (3), 58 (4), 56 (5), 54 (26)</td>
<td>BstU I</td>
</tr>
<tr>
<td>MINT12</td>
<td>64 (3), 61 (4), 58 (5), 55 (26)</td>
<td>Tai I</td>
</tr>
<tr>
<td>MINT25</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>BstU I</td>
</tr>
<tr>
<td>MINT31</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>Afa I</td>
</tr>
<tr>
<td>CHFR</td>
<td>55 (3), 53 (4), 51 (5), 49 (26)</td>
<td>Nru I</td>
</tr>
<tr>
<td>HRK</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>BspT104 I</td>
</tr>
<tr>
<td>SLC5A8</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>Taq I</td>
</tr>
<tr>
<td>SOST</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>EcoR I</td>
</tr>
<tr>
<td>TIG1</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>Hinf I</td>
</tr>
<tr>
<td>P53</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>EcoR I</td>
</tr>
<tr>
<td>MINT indicates methylated in tumor loci; F, forward primer; R, reverse primer.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Y/H11032*  

Gastric CA with CIMP and Association to EBV/Kusano et al.
Detection of the EBV Genome and HP

To detect the EBV genome in gastric tumors, we performed real-time PCR using 2 sets of primers that detect BamHl-W and the EBV-encoded protein EBNA, as described previously.21 Both fluorescent probes contained a 3’-blocking phosphate group to prevent their extension during PCR. A calibration curve was run in parallel using DNA extracted from the EBV-positive Raji cell line (American Type Culture Collection) as a standard. Consistent results were obtained with both systems. HP infection was identified by conducting histologic review of hematoxylin and eosin-stained tissue specimens and PCR assays. A patient was classified as histopathologically HP-positive on the basis of the presence of curved, rod-shaped bacteria on the tumor neighboring and/or antral gastric mucosa. Genomic DNA from normal mucosa was analyzed using PCR with primers that were derived from the internal 411-base-pair fragment of the urease A gene, as described by Clayton et al.22

Mutational Analysis

Mutations in codon 12 or 13 of K-ras were detected by direct sequencing of PCR products after the amplification of K-ras exon 2. Mutations of p53 were detected by single-strand conformation polymorphism followed by direct sequencing. Genomic DNA was amplified using exon-specific primers for p53 exons 2 through 11; the primer sequences were obtained from the literature23 and were used with only minor modification. The mutations that were identified were examined using a GenePhor Electrophoresis System (Amersham Biosciences, Uppsala, Sweden) and a DNA Silver Staining Kit (Amersham Biosciences). Shifted bands were excised from gels and reamplified using the same sets of primers. The resultant PCR products were purified using a DNA Purification System (Promega); then, the mutated sequences were determined by direct sequencing using a Big Dye Terminator v3.1 Cycle Sequencing Kit with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Statistical Analysis

The κ statistic was used to describe the correspondence between tumors that had high CIMP methylation (CIMP-H) or EBV-association and methylation of each of the 12 tumor-related genes studied. It is accepted generally that κ values from 0.0 to 0.4 indicate poor correspondence, values from 0.41 to 0.6 indicate moderate correspondence, values from 0.61 to 0.8 indicate substantial correspondence, and values >0.8 indicate near perfect correspondence. The hypothesis that κ = 0.0 was tested using the exact test. Each tumor was classified using tumor location, macroscopic type; lymphatic invasion; venous invasion (Japanese Gastric Cancer Association, 1998)24; the pathologic tumor, lymph node, metastasis (pTNM) classification (5th edition, 1997)25; and the Lauren classification.26 CIMP status was compared using the Student t test for age; the Mann–Whitney U test for tumor size, pT status, pN status, and disease stage; and the Fisher exact test for gender, tumor location, macroscopic type, histology, lymphatic invasion, venous invasion, pM status, EBV association, lymphoepithelioma-like carcinoma, HP status, and p53 and K-ras mutation. Survival was assessed using the Kaplan–Meier method; survival curves were compared using the log-rank test. The Fisher exact test was carried out using SAS (SAS Institute Inc., Cary, NC), and all other statistical analyses (indicated in the text) were carried out using SPSS software (version 11.0; SPSS Inc., Chicago, IL). All tests were 2-tailed, and values of P <.05 were considered significant.

RESULTS

Gene Methylation and CIMP Status in Gastric Carcinoma

Of the 12 tumor-related genes that were studied, 8 genes were selected for the current analysis based on the finding that their methylation occurs exclusively in cancerous tissues and not in adjacent normal gastric mucosa. These included p16INK4A, the human MutL homologue 1 gene (hMLH1), the p57 cyclin-dependent kinase 2 inhibitor gene (p57KIF2), the helicase-like transcription factor gene (HLTF), the mitotic checkpoint gene CHFR, the human harakiri gene (HRK), the solute carrier family 5 (iodine transporter) member 8 gene (SLC5A8), and the BCL2/adenovirus E1B 19-kDa interacting protein 3 gene (BNIP3), all of which are inactivated by methylation in gastric carcinoma. The other 4 genes that were studied, CSPG2, paired box gene 5β (PAX5β), suppressor of cytokine signaling 3 gene (SOCS-3), and tazarotene-induced gene 1 (TIG1), all have been
shown to be methylated in various human malignancies other than gastric carcinoma. We deemed that a gene was methylated if it showed a methylation density ≥10%. No methylation was detected in normal mucosa adjacent to tumors. Confirmation of methylation-induced silencing of affected genes in gastric carcinoma cell lines was followed by methylation analysis of the primary tumors. Representative results that show the methylation and expression of CSPG2 and TIG1 in various gastric carcinoma cell lines and primary gastric carcinomas are depicted in Figure 1.

To evaluate CIMP status, we classified gastric tumors, first, based on the presence or absence of methylation within each of the five MINT loci and, second, based on the numbers of methylated loci—i.e., tumors were classified as CIMP-H (4 or 5 MINT loci showed methylation), CIMP-low (CIMP-L) (from 1 to 3 MINT loci showed methylation), or CIMP-negative (CIMP-N) (no MINT loci showed methylation). Of the 78 primary gastric tumors studied, 19 tumors were classified as CIMP-H, 39 tumors were classified as CIMP-L, 20 tumors were classified as CIMP-N, and the frequency of MINT locus methylation ranged from 23.1% (MINT12) to 64.1% (MINT25). In addition, we determined the methylation status of 12 tumor-related genes and found that the frequency of their methylation ranged from 6.4% (hMLH1) to 48.7% (CSPG2). The methylation profiles and the EBV and HP status of the 78 gastric tumors studied are summarized in Figure 2.

CIMP Status and the Frequency of Methylation of Tumor-Related Genes

Figure 3 shows that the frequency of methylation in 11 of 12 tumor-related genes studied was much greater in CIMP-H tumors compared with the frequency of methylation in CIMP-L or CIMP-N tumors. The exception was SOCS-3, which was methylated slightly more frequently in CIMP-L tumors than in CIMP-H tumors. To calculate the expected number of tumors that would arise for a given number of methylated loci (from 0 to 12 loci), we created a web-based program, “Tool for Gene Methyl Possibility” (TGMP) (http://info.bio.sunysb.edu/methyl.html), which worked on the assumption that methylation of individual genes occurs independent of the methylation of other genes. We then compared the observed distribution of tumors that had each number of methylated loci with the expected distribution among our 78 tumor specimens; the expected distribution was calculated using the TGMP program based on the methylation frequency of the aforementioned 12 genes (Fig. 4). In this comparison, tumors with five or more methylated loci were pooled into one group, because the number of these tumors in the expected distribution was less than five. There were no tumors with 7, 11, or 12 methylated loci in the observed distribution. The expected distribution was unimodal, with a peak at 2 loci per tumor occurring in 22.5 tumors. By contrast, the observed distribution was not unimodal and differed significantly from the expected distribution (goodness-of-fit test: chi-square statistic, 92.576; 5 degrees of freedom; P < .0005).

To evaluate the correlation between the number of methylated MINT loci (CIMP status) and the number of methylated loci among the 12 tumor-related genes in more detail, the 78 tumor specimens first were divided into 6 groups based on the number of methylated MINT loci (from 0 to 5 loci) in each tumor. Then, which the average numbers of methylated tumor-related genes were determined (Fig. 5). One-way analysis of variance (ANOVA) showed that the numbers of methylated tumor-related genes were affected
significantly by the numbers of methylated MINT loci (F[5, 72] = 16.376; P < .0005), and a post-hoc Tukey test showed that, in tumors that had 4 or 5 methylated MINT loci, there also were significantly greater numbers of methylated loci among the 12 tumor-related genes than among tumors that had fewer methylated MINT loci. The statistic, which indicated the degree of correspondence between CIMP-H and methylation of the 12 genes, varied from 0.006 to 0.544 (Table 2). Correspondence was moderate for the HRK, HLTF, BNIP3, p16INK4A, TIG1, and PAX5β genes but was poor for the other 6 genes. In addition, we evaluated the correspondence between EBV association and gene methylation (Table 2) and found that it was substantial for p16INK4A and TIG1 and moderate for p57KIP2, HLTF, PAX5β, and HRK.

**CIMP Status and the Frequency of EBV Association and Genetic Alteration of p53 and K-ras**

EBV was detected in 10 of 78 tumors (12.8%) using real-time quantitative PCR. Mutations in p53 were found in 19 of 78 tumors (24.4%), including 14 tumors that contained single point mutations between exons 4 and 10 and 4 tumors that contained from 1 to 21 base pair deletions between exons 4 and 7. K-ras mutations were detected at codon 12 in 4 of 78 gastric tumors, a GGT → GAT substitution was found in 3 tumors, and a GGT → GTT substitution was found in 1
tumor. No K-ras mutations were detected at codon 13. The frequencies of EBV association, p53 mutation, and K-ras mutation in the 78 gastric tumors are summarized in Figure 2. The median number of methylated loci of the 12 tumor-related genes was significantly greater in EBV-associated gastric tumors than in EBV-negative gastric tumors (7 loci vs. 1 locus; \(P < .0005\); Mann–Whitney U test). All EBV-associated tumors belonged to the CIMP-H group, with 10 of 19 CIMP-H tumors (52.6%) showing an EBV-association. Tumors with p53 and/or K-ras mutations showed neither hMLH1 methylation nor EBV association, with the exception of 1 tumor (KG12): In other words, these genetic alterations almost never occurred together with hMLH1 methylation or EBV association. The correlation between CIMP status and genetic alteration of the p53 and K-ras genes is summarized in Table 3. No p53 mutations were detected among CIMP-H tumors, whereas 19 of 59 CIMP-L/CIMP-N tumors (32.2%) showed p53 mutation (\(P = .004\)). Conversely, no significant difference was noted in the frequency of K-ras mutation between CIMP-H tumors and CIMP-L/CIMP-N tumors (\(P = .248\)).

CIMP Status and Clinicopathologic Characteristics

Bearing in mind the results described above, our objective was to gain a better understanding of the clinicopathologic characteristics of CIMP by comparing
DISCUSSION

It is noteworthy that the term CIMP has had a variety of usages in the context of gastric carcinoma. Its original definition was based on quantitative techniques that were relatively insensitive to methylation levels in normal mucosa. However, careful quantitative evaluation showed that many genes that are highly methylated in carcinoma also show a low but measurable degree of methylation in normal mucosa. Conversely, in studies using MSP, which is a nonquantitative and highly sensitive method, the incidence of methylation was substantially higher. For instance, the frequency of p16\textsuperscript{INKA}\textsuperscript{A}\textsuperscript{A} methylation in gastric carcinoma detected using MSP was reportedly 27%, 33%, 37, or 42%, rates much higher than what we obtained in the current study by using COBRA.

We compared the observed distribution of tumors that carried each number of methylated tumor-related genes with the expected distribution, which was calculated using our TGMP program. If methylation of these genes occurred independently of one another, the observed distribution should have resembled the expected distribution. Our finding of a significant difference between the observed and expected distributions suggests that promoter methylation of tumor-related genes in gastric tumors is not a random occurrence. In that regard, we defined CIMP status on the basis of the degree of methylation in the five MINT loci. One-way ANOVAs showed that tumors with 4 or 5 methylated MINT loci (CIMP-H) had significantly greater numbers of methylated loci among the 12 tumor-related genes that among tumors that had fewer methylated MINT loci (CIMP-L or CIMP-N), a finding that validated our operational definition of the threshold between CIMP-H tumors and CIMP-L tumors (between 3 and 4 methylated MINT loci).
TABLE 3
Clinicopathologic Features of Gastric Carcinomas with High CpG Island Methylator Phenotype Expression and Low or Negative CpG Island Methylator Phenotype Expression: Univariate Analysis*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (No. of Patients)</th>
<th>CIMP-H (No. of Patients)</th>
<th>CIMP-L/CIMP-N (No. of Patients)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>No. of patients</td>
<td>78</td>
<td>19</td>
<td>59</td>
<td>.580</td>
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<tr>
<td>Mean age ± SD (yrs)</td>
<td>64.5 ± 12.0</td>
<td>65.9 ± 13.3</td>
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<td>.580</td>
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<td>Gender</td>
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<td>Male</td>
<td>52 (66.7)</td>
<td>12 (63.2)</td>
<td>40 (67.8)</td>
<td>.782</td>
</tr>
<tr>
<td>Female</td>
<td>26 (33.3)</td>
<td>7 (36.8)</td>
<td>19 (32.2)</td>
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<td>Tumor size (cm)</td>
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<td></td>
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<tr>
<td>Range</td>
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<td>2.3-20.5</td>
<td>2.3-21.0</td>
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<td>7.0</td>
<td>7.5</td>
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<td>Upper one-third</td>
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<td>8 (42.1)</td>
<td>14 (23.7)</td>
<td>.005</td>
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<td>Middle one-third</td>
<td>23 (29.5)</td>
<td>7 (36.8)</td>
<td>16 (27.1)</td>
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<td>Lower one-third</td>
<td>33 (42.3)</td>
<td>4 (21.1)</td>
<td>29 (49.2)</td>
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<td>Macroscopic type</td>
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<td>21 (35.6)</td>
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<td>Type 3</td>
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<td>Diffuse</td>
<td>42 (53.8)</td>
<td>14 (73.7)</td>
<td>28 (47.5)</td>
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<tr>
<td>Lymphatic invasion</td>
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<td>.233</td>
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<td>7 (36.8)</td>
<td>13 (22.0)</td>
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<tr>
<td>Positive</td>
<td>58 (74.4)</td>
<td>12 (63.2)</td>
<td>46 (78.0)</td>
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<td>Venous invasion</td>
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<td>.009</td>
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<tr>
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<td>23 (39.0)</td>
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<td>5 (26.3)</td>
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<td>3 (15.8)</td>
<td>2 (3.4)</td>
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<td>28 (35.9)</td>
<td>6 (31.6)</td>
<td>22 (37.3)</td>
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<tr>
<td>pT4</td>
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<td>pN3</td>
<td>12 (15.4)</td>
<td>1 (5.3)</td>
<td>11 (18.7)</td>
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<td>Pathologic metastasis status</td>
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<td>pM0</td>
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<td>18 (94.7)</td>
<td>48 (81.4)</td>
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</tr>
<tr>
<td>pM1</td>
<td>12 (15.4)</td>
<td>1 (5.3)</td>
<td>11 (18.8)</td>
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<tr>
<td>Stage (pTNM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IA</td>
<td>3 (3.8)</td>
<td>3 (15.8)</td>
<td>0 (0.0)</td>
<td>.034</td>
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<tr>
<td>Stage IB</td>
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<td>3 (15.8)</td>
<td>12 (20.3)</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>16 (20.5)</td>
<td>5 (26.3)</td>
<td>11 (18.6)</td>
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<tr>
<td>Stage IIIA</td>
<td>13 (16.7)</td>
<td>4 (21.1)</td>
<td>9 (15.3)</td>
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<tr>
<td>Stage IIIB</td>
<td>8 (10.3)</td>
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<td>6 (10.2)</td>
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<tr>
<td>Stage IV</td>
<td>23 (29.5)</td>
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<td>21 (35.6)</td>
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<tr>
<td>Epstein-Barr virus</td>
<td></td>
<td></td>
<td></td>
<td>&lt;.0005</td>
</tr>
<tr>
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<td>10 (12.8)</td>
<td>10 (52.6)</td>
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</tr>
<tr>
<td>Negative</td>
<td>68 (87.2)</td>
<td>9 (47.4)</td>
<td>59 (100.0)</td>
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</tr>
<tr>
<td>p53 mutation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>19 (24.4)</td>
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<td>19 (32.2)</td>
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<tr>
<td>Negative</td>
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<td>40 (67.8)</td>
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<td>K-ras mutation</td>
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<td>2 (10.5)</td>
<td>2 (3.4)</td>
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<tr>
<td>Negative</td>
<td>74 (94.9)</td>
<td>17 (89.5)</td>
<td>57 (96.6)</td>
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<tr>
<td>Helicobacter pylori</td>
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<td>65 (83.3)</td>
<td>17 (89.5)</td>
<td>48 (81.4)</td>
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</tr>
<tr>
<td>Negative</td>
<td>13 (16.7)</td>
<td>2 (10.5)</td>
<td>11 (18.6)</td>
<td></td>
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</tbody>
</table>

* CIMP-H indicates high CpG island methylator phenotype methylation; CIMP-L/CIMP-N, low/negative CpG island methylator phenotype methylation; SD, standard deviation; pTNM, pathologic tumor, lymph node, metastasis status according to the International Union Against Cancer classification system.
We observed no significant difference in the age or gender of patients with CIMP-H tumors and patients with CIMP-L/CIMP-N tumors. The strong association between CIMP-H and EBV-associated gastric carcinoma appears to have influenced the result of the multiple logistic regression model, because diffuse type tumors and a proximal location were selected as significant variables for CIMP-H tumors. Evaluation of the correspondence between EBV association and methylation of the 12 genes studied showed the highest correspondence with the p16INK4A gene (0.706): The product of that gene is an inhibitor of G1/S phase transition, the loss of which promotes uncontrolled cell growth. The p16INK4A gene is a common target of inactivation by epigenetic mechanisms in gastric carcinoma.4,39-41 In our current results, p16 INK4A was methylated frequently in CIMP-H tumors (52.6%) but rarely was methylated in CIMP-L/CIMP-N tumors (6.8%). It is worth noting that the frequency of p16INK4A methylation in EBV-associated tumors was remarkably high (90.0%). Thus, it appears that epigenetic silencing of this gene is associated strongly with the development of EBV-associated gastric carcinoma.

Approximately 50% of CIMP-H gastric carcinomas are EBV-negative; presumably, CIMP-H tumors without EBV association are mediated by different, as yet unknown mechanisms. Compared with EBV-positive CIMP-H tumors, it appeared that EBV-negative CIMP-H tumors were less likely to be diffuse carcinomas or to be located in the upper one-third of the stomach, although there was no meaningful prognostic difference between the two groups. No lymphoepithelioma-like carcinomas were detected among the EBV-negative tumors. A subset of CIMP gastric tumors are microsatellite instability-positive tumors because of hMLH1 methylation.4,5 Kang et al. reported that EBV-positive gastric tumors are subset of CIMP-positive tumors, although only 2 of 21 EBV-positive tumors (9.5%) showed hMLH1 methylation.11 This is consistent with our finding that the carcinogenesis of EBV-associated gastric tumors rarely involves hypermethylation of multiple genes without involvement of hMLH1 methylation.

It is plausible that EBV may activate a methylation pathway that affects multiple genes during gastric carcinogenesis; however, the molecular mechanism underlying EBV-related aberrant methylation currently is

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**TABLE 4**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>β</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric tumor location</td>
<td>Upper one-third/middle one-third/lower one-third</td>
<td>−1.035</td>
<td>.011</td>
</tr>
<tr>
<td>Histology (Lauren)</td>
<td>Intestinal/diffuse</td>
<td>1.635</td>
<td>.018</td>
</tr>
<tr>
<td>Venous invasion</td>
<td>Negative/positive</td>
<td>−1.242</td>
<td>.098</td>
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<tr>
<td>Stage (pTNM)</td>
<td>Stage I/Stage IB/Stage II/Stage IIIA/Stage IIIIB/Stage IV</td>
<td>−0.529</td>
<td>.037</td>
</tr>
</tbody>
</table>

*β* indicates logistic regression coefficient; pTNM, pathologic Tumor, Lymph Node, Metastasis status according to the International Union Against Cancer classification system.

**TABLE 5**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CIMP-H</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of patients</td>
<td>EBV-Positive</td>
<td>EBV-Negative</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Mean age ± SD, y</td>
<td>65.9 ± 13.3</td>
<td>64.0 ± 11.6</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Tumor location</td>
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<td></td>
</tr>
<tr>
<td>Upper GI tract</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Middle GI tract</td>
<td>4</td>
<td>3</td>
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<tr>
<td>Lower GI tract</td>
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<td>4</td>
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<tr>
<td>Histology (Lauren)</td>
<td></td>
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</tr>
<tr>
<td>Intestinal</td>
<td>1</td>
<td>5</td>
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<tr>
<td>Diffuse</td>
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<td>4</td>
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<tr>
<td>Stage (pTNM)</td>
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<td>2</td>
</tr>
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<td>Stage IB</td>
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</tr>
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<td>Stage II</td>
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</tr>
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<tr>
<td>Stage IIIB</td>
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</tr>
<tr>
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<td>LELC</td>
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<td>Negative</td>
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<td>Helicobacter pylori</td>
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<td>Positive</td>
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<td>8</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
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</tbody>
</table>

* CIMP-H indicates high CpG island methylator phenotype methylation; EBV, Epstein-Barr virus; SD, standard deviation; GI, gastrointestinal; pTNM, pathologic Tumor, Lymph Node, Metastasis status according to the International Union Against Cancer classification system; LELC, lymphoepithelioma-like carcinoma.
unknown. Consistent with the idea that oncogenic viruses induce aberrant methylation of tumor suppressor genes, Shivapurkar et al. reported that methylation was completely absent in Simian virus 40 (SV40)-uninfected and EBV-infected peripheral blood mononuclear lymphocytes but that the presence of SV40 in hematologic malignancies was associated with promoter methylation of tumor suppressor genes. Moreover, Soejima et al. reported that malignant transformation of normal human bronchial epithelial cells that expressed telomerase, SV40 large-T antigen, and activated Ras led to DNA methyltransferase 3b expression, which was correlated with the methylation and down-regulation of tumor suppressor genes.
Determining precisely how EBV infection leads to methylation in gastric carcinogenesis seems to be a key question to be addressed in future studies.

Previous studies identified differences in the frequency of p53 mutation in EBV-associated malignancies, including Burkitt lymphoma, posttransplantation lymphoma, and nasopharyngeal carcinoma. Consistent with the finding that p53 is overexpressed infrequently in EBV-associated gastric carcinomas, no p53 mutations were detected among the 10 EBV-associated gastric tumors we studied, all of which were classified as CIMP-H, suggesting that an EBV viral gene product may interfere with some functions of p53 by eliminating a section for mutational inactivation. Szekely et al. reported that, on binding in vitro, the EBV-encoded protein EBNA-5 (alternatively designated EBNA-LP) forms a molecular complex with both p53 and retinoblastoma (Rb) proteins, thereby inactivating the tumor suppressor pathway. This means that, in EBV-associated gastric carcinoma, the p53/Rb pathway can be inactivated without mutation of the p53 gene. Still, 42 of 78 tumor specimens (53.8%) in the current study showed no EBV association, no methylation of hMLH1, and no genetic alterations of the p53 or K-ras genes. Thus, these traits do not explain the mechanism of carcinogenesis in approximately 50% of our 78 tumors.

Finally, our clinicopathologic study using multivariate analysis revealed that less advanced pTNM stages contributed significantly to CIMP-H. Although venous invasion differed significantly between CIMP-H and CIMP-L/CIMP-N in univariate analysis, it was not selected as a significant variable in the multiple logistic regression model of CIMP-H, because positive venous invasion was linked strongly to advanced pTNM stages. Furthermore, among the 20 tumors that were classified as CIMP-N, 6 tumors (30.0%) showed distant metastasis to either the liver or peritoneum at the time of resection, whereas 5 of 39 CIMP-L tumors (12.8%) and only 1 of 19 CIMP-H tumors (5.3%) showed such distant metastasis. Thus, the clinical characteristics of CIMP-H, CIMP-L, and CIMP-N gastric carcinomas appear to differ. An interesting question in this regard is whether patients who have hypomethylated tumors tend to present at more advanced stages and, thus, have poorer prognoses. Because the answer to that question is affirmative, the five MINT loci may be useful not only as hypomethylation markers but also as hypomethylation markers and may serve as valuable prognostic markers that facilitate decisions about whether chemotherapy is indicated for an individual patient.

REFERENCES


