Prognostic Significance of CpG Island Methylator Phenotype and Microsatellite Instability in Gastric Carcinoma

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ABSTRACT

Purpose: The influence of molecular characteristics in prognosis of gastric cancer remains unclear. The aim of this study was to evaluate the prognostic value of the CpG island methylator phenotype (CIMP) and microsatellite instability (MSI) in gastric cancer.

Experimental Design: We studied the methylation profiles of tumor suppressor gene p16, DNA mismatch repair gene hMLH1, and four CpG islands (MINT1, MINT2, MINT25, and MINT31) using bisulfite/methylation–specific PCR, and MSI using five microsatellite markers in 83 resected gastric carcinomas. The CIMP and MSI status were compared with clinicopathologic features and overall survival.

Results: Concordant methylation of multiple genes/loci (CIMP-high) was present in 31% of tumors and in 4% of non-neoplastic mucosa, CIMP-low in 55% and 17%, and CIMP-negative in 13% and 79%, respectively (P < 0.001). The prevalence of MSI-high, MSI-low, and MS-stable in tumor was 19%, 17%, and 64%, respectively. MSI status was closely associated with hMLH1 hypermethylation and CIMP status (P = 0.001). In univariate analysis, overall survival was predicted by pathologic stage (P < 0.001), R0 resection (P = 0.0002), MINT31 methylation (P = 0.04), and CIMP-high status (P = 0.04). MSI status of tumor was not a significant predictor of prognosis. Although CIMP status seemed to be a prognostic predictor of gastric cancer, only pathologic stage remained a significant predictor of prognosis on multivariate analysis (P < 0.001).

Conclusions: Our results indicate that there is an association between CIMP status and MSI status in gastric cancer. Concordant methylation of multiple genes/loci (CIMP-H) is associated with better survival but is not an independent predictor of prognosis in resected gastric cancer.

INTRODUCTION

The incidence of gastric cancer has declined; however, it continues to be the second most common malignant neoplasm across the world and the second leading cause of cancer death (1). Gastric cancer also remains a major clinical challenge because it has a poor prognosis and limited treatment options due to its relative resistance to radiotherapy and chemotherapy. Currently, tumor histology and pathologic stage based on the depth of tumor invasion, lymph node involvement, and metastasis (tumor-node-metastasis classification) are the major prognostic variables used in clinical management of gastric cancer patients. However, tumor with similar morphology may differ in biological aggressiveness, prognosis, and response to therapy. Therefore, understanding of the molecular profiles may help to identify early tumor markers, lead to novel treatment modalities, and improve the prognosis.

Epigenetic silencing of tumor-related genes due to CpG island hypermethylation has emerged as one of the most important epigenetic alterations in cancer development (2). CpG islands are 0.5- to 2-kb regions rich in cytosine-guanine dinucleotides and are present in the 5′ promoter region of approximately half of all human genes. Methylation of cytosines within CpG islands is associated with loss of gene expression by transcriptional repression and is observed in various tumors as well as physiologic conditions such as X chromosome inactivation (3) and the aging process (4). Aberrant CpG island methylation in tumor-related genes such as cyclin-dependent kinase inhibitors (CDKI), the p16 gene on chromosome 9p21(5), and in the DNA mismatch repair gene hMLH1 on chromosome 3p21 has been found in gastric tumors (6). Tumors with concurrent hypermethylation in multiple loci have been defined as CpG island methylation phenotype-high (CIMP-H) and have been reported in acute myeloid leukemia, colorectal carcinoma, pancreatic carcinoma, and gastric carcinoma (7–10).

In the stomach, previous studies have shown that hypermethylation of gene promoters progressively increases with histopathologic progression from chronic gastritis, intestinal metaplasia, and adenoma to carcinoma (11, 12). These findings suggest that the presence of concurrent hypermethylation of gene promoters may be a distinct pathway in gastric carcinogenesis and play an important role in gastric cancer progression (13). Recently, it has been reported that concurrent hypermethylation of gene promoters is associated with a microsatellite instability-high (MSI-H) phenotype in gastric cancers (14, 15). Although mutations in the DNA mismatch repair genes hMSH2 and hMLH1, are rare in gastric carcinoma (16, 17), methylation of hMLH1 promoter region CpG island is associated with loss of hMLH1 expression in the majority of gastric cancers exhibiting MSI (5, 12, 17) and occasionally in non-neoplastic surrounding gastric mucosa (18). The clinical significance of MSI status in gastric cancer, however, remains controversial. Some reports have shown associations between MSI-H in gastric cancers and intestinal type histology, prominent lymphoid
infiltration, older age, antral tumor location, lower prevalence of lymph node metastasis, and better prognosis (19, 20). However, other studies have argued there was no significant correlation between the presence of MSI and any of the clinicopathologic characteristics in gastric cancer (21–23). To date, most studies have focused mainly on methylation status on the isolated genes, and the prognostic significance of concurrent gene methylation in gastric cancer remains unclear. The aim of present study was to evaluate the prognostic significance of the CIMP status and MSI in gastric cancer.

MATERIALS AND METHODS

Patient Population

This study included surgical resection specimens of 83 gastric cancer patients identified from the surgical pathology files in the Department of Pathology at the M.D. Anderson Cancer Center between 1986 to 1998. This study is approved by Institutional Review Board. All of the patients had primary gastric carcinoma and underwent gastrectomy with lymph node dissection. No preoperative neoadjuvant therapy was given to any of these patients. The mean follow-up period was 52.7 ± 5.7 months. The histology of the tumors was assessed using Lauren’s classification (24).

DNA Extraction

Microdissection using 27.5-gauge needle was done on H&E-stained slides prepared from formalin-fixed and paraffin-embedded tissue for both gastric carcinoma and corresponding non-neoplastic mucosa. Genomic DNA was extracted from microdissected tissue as described previously (25). In brief, microdissected tissue was treated with 100 μL of lysis buffer containing 0.5% Tween 20 (Boehringer Mannheim, Mannheim, Germany), 40 μg of proteinase K (Boehringer Mannheim), 50 mmol/L of Trizma base (pH 8.9), and 2 mmol/L of EDTA, and the samples were incubated at 56°C overnight. Proteinase K was inactivated by incubating the samples at 100°C for 10 minutes. The extracted DNA was stored at −80°C.

Bisulfite Treatment of DNA and Methylation-Specific PCR

The methylation status of tumor suppressor gene p16, DNA mismatch repair gene hMLH1, and four CpG islands (MINT1, MINT2, MINT25, and MINT31) was determined by bisulfite treatment of DNA followed by methylation-specific PCR, as described with modification (26). The selection of these genes and loci was based on a previous study that showed these loci had high sensitivity and specificity for the detection of hypermethylation in cancer and offered excellent discrimination for CIMP status (10). The primer sequences of each gene and locus tested and the conditions of the PCRs have been previously described (12). In brief, 1 μg of microdissected genomic DNA was denatured with 2 mol/L of NaOH at 37°C for 10 minutes, followed by incubation with 3 mol/L of sodium bisulfite (pH 5.0) at 50°C for 16 hours in the dark. After that treatment, DNA was purified using a DNA cleanup kit (Promega, Madison, WI) as recommended by the manufacturer, incubated with 3 mol/L of NaOH at room temperature for 5 minutes, precipitated with 10 mol/L of ammonium acetate and 100% ethanol, washed with 70% ethanol, and finally resuspended in 20 μL of distilled water. Methylation status of each gene/locus was determined using 2 μL of bisulfite-treated DNA as template for each PCR reaction and primers specific for methylated and unmethylated alleles. Amplification was carried out in a GeneAmp PCR System 9700 thermocycler (Perkin-Elmer, Norwalk, CT) with PCR cycling conditions optimized for each primer set. A 10-μL aliquot of amplified PCR product was electrophoresed on 2.5% agarose gels and visualized by ethidium bromide staining. DNA from RKO and Colo320 colon cancer cell lines (American Type Culture Collection, Manassas, VA) was used as a positive control for methylation and distilled water was used as a negative control.

Criteria for CIMP Status

CIMP status was classified as CIMP-negative (CIMP-N) if none of the evaluated genes/loci were methylated; CIMP-low (CIMP-L) if <50% of genes/loci were methylated; and CIMP-high (CIMP-H) if ≥50% of genes/loci were methylated. These criteria were based on the previously published study (7) in which CIMP-positive cases were methylated at the majority of MINT loci and p16, but CIMP-negative cases were rarely methylated at any MINT loci and never methylated at p16.

Microsatellite Instability Analysis

MSI analysis was done by evaluating two mononucleotide repeat markers (BAT25 and BAT26) and three dinucleotide repeat markers (D2S123, D5S346, and D17S250) as recommended by the National Cancer Institute workshop for MSI (27). Each marker was PCR-amplified in a separate 15 μL reaction containing 9 μL True Allele PCR Premix (Applied Biosystems, Foster City, CA), 10 pmol each of 6-FAM–labeled forward and unlabeled reverse primers, and 10 ng template DNA. PCR was done in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). The reaction conditions were as follows: initial denaturation at 95°C for 8 minutes, 45 cycles of (94°C for 45 seconds, 55°C for 45 seconds, 55°C for 45 seconds, 72°C for 1 minute), followed by a final extension at 72°C for 30 minutes. Approximately 1 to 2 ng of each PCR product were mixed with a 1:40 dilution of formamide-LIZ500 size standard and visualized on an ABI 3730 DNA Analyzer (Applied Biosystems). Allelic size alterations were detected using GeneMapper Software Version 3.0 (Applied Biosystems). Samples were considered positive for MSI when alternate-sized bands were present in the tumor DNA but absent in the respective control mucosal DNA. Tumors were classified as high level MSI (MSI-H) if ≥2 markers had allelic shifts, low level MSI (MSI-L) if only one of the five markers had allelic shift, and microsatellite-stable (MS-stable) when no marker showed allelic shift (27).

Statistical Analysis

Comparisons of categorical variables, including sex, tumor histology, methylation and MSI status, race/ethnicity, tumor location, lymph node involvement, and pathologic stage
were made using the $\chi^2$ test; a Fisher’s exact test was used when testing smaller samples. Survival was assessed by the Kaplan-Meier method and compared using the log-rank test. Multivariate survival analysis, which included the variables pathologic stage, CIMP status, and resection status, was carried out using the Cox Proportional Hazards Model. For all of the tests, $P < 0.05$ was regarded as statistically significant in a two-tailed test.

RESULTS

Methylation Profile and CIMP Status

Representative examples of methylation at $hMLH1$, $p16$, MINT1, MINT2, MINT25, and MINT31, and CIMP status are shown in Fig. 1. The methylation frequency was significantly higher in tumor than in paired non-neoplastic mucosa for $p16$, $hMLH1$, MINT1, MINT2, MINT25, and MINT31 (Table 1). Concordant methylation of multiple genes/loci (CIMP-H) was present in 31% (26 of 83) of tumor, CIMP-L in 55% (46 of 83), and CIMP-N in 13% (11 of 83, Table 2). In contrast, CIMP-H was present in 4% (3 of 82) of non-neoplastic mucosa, CIMP-L in 17% (14 of 82), and CIMP-N in 79% (65 of 82), $P < 0.001$. Methylation of $hMLH1$ was associated with methylation of MINT1, MINT2, MINT25, and MINT31 (Table 2). In addition, MINT31 methylation was associated with methylation in MINT1 and MINT2 (Table 2).

There were no statistical differences between CIMP status in tumors with evaluated clinicopathologic features, including age, race, sex, tumor histology, tumor location, and pathologic stage (Table 3).

Microsatellite Instability

The representative examples of MSI-H, MSI-L, and MS-stable carcinomas are shown in Fig. 2. The prevalence of MSI-H was 19% (15 of 81), MSI-L17% (14 of 81), and MS-stable 64% (52 of 81) in gastric carcinomas. There was an association between MSI-H status and $hMLH1$ methylation: methylation of $hMLH1$ was present in 73% (11 of 15) of MSI-H tumors, but in 0% (0 of 14) of MSI-L tumors, and 4% (2 of 52) of MS-stable tumors ($P < 0.001$), Table 4). A similar significant association between MSI status and CIMP status was also noted: CIMP-H was present in 73% (11 of 15) of MSI-H tumors, but in 14% (2 of 14) of MSI-L tumors and 23% (12 of 52) MS-stable tumors ($P < 0.001$, Table 4). There was no statistical difference between MSI status in tumors with evaluated clinicopathologic features, including age, race, sex, tumor histology, tumor location, and pathologic stage (Table 3).

Survival Analysis

In univariate analysis, pathologic stage ($P < 0.0001$), R0 resection ($P = 0.0002$), MINT31 methylation ($P = 0.04$), and CIMP-H ($P = 0.04$) were statistically significant predictors for overall survival (Table 5; Fig. 3). Patients with MSI-H showed a trend toward better survival than MSI-L and MS-stable, but the differences were not statistically significant ($P = 0.15$, Table 5). Histologic type based on Lauren’s classification and single gene/locus methylation of $hMLH1$, $p16$, MINT1, MINT2, and MINT25 were not significant prognostic factors.

Pathologic stage, resection status, and CIMP status were included in multivariate Cox regression analysis (Table 6). Pathologic stage was the only independent ($P < 0.001$) predictor for overall survival.

DISCUSSION

Epigenetic silencing of tumor-related genes due to methylation of gene promoter regions plays an important role in carcinogenesis in the stomach (1, 10). Concordant methylation of multiple genes (termed CIMP) has been reported in carcinomas from diverse sites, including uterine cervix, colorectum, biliary tree, pancreas, bladder, nasopharynx, esophagus, and stomach (7, 9, 11, 13, 28–33). The prognostic roles of CIMP status have been evaluated in several cancer types. In colorectal cancer, Cpg island methylation of multiple tumor-related genes was associated with distinct clinicopathologic features like right-sided tumor location, female sex, older age, high tumor grade, mucinous histology, and MSI (34), but CIMP status had no prognostic significance in stage II or III colorectal cancer treated by surgery alone (35). In contrast, CIMP-positive phenotype can independently predict better survival after 5-fluorouracil–based chemotherapy in stage III colorectal cancer (36). In esophageal adenocarcinoma, concurrent multiple gene methylation, including
methylation of p16, MGMT, DAP-K, TIMP-3, E-cadherin, ER, and APC, was associated with poor prognosis (37).

The prognostic role of CIMP status in gastric carcinomas is unclear. Most previous studies of methylation in gastric cancer have focused on the carcinogenesis pathway or prognostic significance of methylation of single gene (38, 39), and there is little information about the prognostic significance of concordant gene methylation. It has been reported in gastric cancer that CIMP-H was more frequent in early-stage tumors than CIMP-L or CIMP-N; 6 of 23 CIMP-H tumors were early stage, whereas none of 32 CIMP-L or CIMP-N tumors were early stage (10). In the present study, CIMP-H was present in 31% of gastric carcinosmas, similar to the frequency of 41% reported by Toyota et al. (10). However, in the present study, CIMP status was not associated with pathologic stage or any of the clinicopathologic features (Table 3).

Table 2 Association of methylation among genes and loci in gastric carcinomas

<table>
<thead>
<tr>
<th>P</th>
<th>p16</th>
<th>MINT1</th>
<th>MINT2</th>
<th>MINT25</th>
<th>MINT31</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMLH1</td>
<td>0.98</td>
<td>&lt;0.0001</td>
<td>0.001</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>p16</td>
<td>–</td>
<td>0.56</td>
<td>0.69</td>
<td>0.62</td>
<td>0.62</td>
</tr>
<tr>
<td>MINT1</td>
<td>–</td>
<td>–</td>
<td>0.07</td>
<td>0.48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MINT2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.49</td>
<td>0.008</td>
</tr>
<tr>
<td>MINT25</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Table 3 Association among CIMP, MSI, and clinicopathologic variables in gastric carcinomas

<table>
<thead>
<tr>
<th>Race</th>
<th>CIMP</th>
<th>MSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asian</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>African American</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Hispanic</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>White</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>Sex</td>
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<td></td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Mid</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Distal</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>Limits</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tumor Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>Intestinal</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>Pathologic Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>II</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>IV</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>23</td>
<td>34</td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>12</td>
</tr>
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</table>
In present study, we found that tumors with methylation of MINT31 and CIMP-H predict a better overall survival in univariate analysis (Table 5). However, in multivariate analysis, pathologic stage was the only independent predictor for overall survival \( (P < 0.001) \), and CIMP-H was not significant \( (P = 0.08, \text{Table 6}) \). It is not clear why methylation of multiple genes/loci (CIMP-H) predicts poor prognosis in esophageal adenocarcinomas (37), but shows a trend toward better prognosis in gastric carcinomas in the present study. One possible explanation for the difference is that methylation profiles are site dependent. For example, methylation of \( hMLH1 \) is more frequent in gastric carcinomas than in esophageal adenocarcinomas and may explain the rarity of MSI-H in esophageal adenocarcinomas (40). In addition, comparison among different studies is difficult due to evaluation of different genes or loci. In this study, we have used 6 CpG islands, including tumor suppressor gene \( p16 \), DNA mismatch repair gene \( hMLH1 \), and four CpG islands (MINT1, MINT2, MINT25, and MINT31) specific for gastric cancer as described by Toyota et al. (10). Methylation of CpG island has been reported in gastric cancer for other genes such as \( O^6 \)-methylguanine-DNA methyltransferase (\( MGMT \)), death-associated protein (\( DAP \))-kinase, thrombospondin-1 (\( THBS1 \)), tissue inhibitor of metalloproteinase 3 (\( TIMP-3 \)), \( HPP1 \), and \( p14 \) genes (41–43). A more comprehensive evaluation of methylation profile in these genes is needed to clarify their roles. Moreover, the CIMP status may vary due to histologic heterogeneity in the

**Fig. 3**  In univariate analysis, Kaplan-Meier estimates of overall survival among patients with gastric cancer according to the methylation status of MINT31, the CIMP status, and the MSI status. A, patients whose tumor had methylation of MINT31 had significantly better overall survival than patients whose tumor lacked methylation of MINT 31 \( (P = 0.04) \). B, patients with a tumor with CIMP-H had significantly better overall survival than patients with a tumor that was CIMP-L and CIMP-N \( (P = 0.04) \). C, patients with a tumor that had MSI-H had a trend toward better overall survival than those with MSI-L and MSS, but the differences were not statistically significant \( (P = 0.15) \). D, patients with low pathologic stage showed significantly better overall survival \( (P < 0.001) \).
same tumor, as shown in gastric cancer that different frame-shift mutations can occur in histologically heterogeneous MSI-positive tumor (44).

The observation that tumors with methylation of MINT31 have tendency toward better survival is interesting due to the nature of the marker, MINT31 is present at 2 kb upstream of the CACNA1G, a T-type calcium channel gene, and methylation of MINT31 was correlated with methylation of hMLH1, MINT1, and MINT2 (Table 3). The prognostic significance of methylation of a single CpG island is unclear, but it has been reported that methylation of the MGMT gene was associated with advanced stage and poor prognosis in gastric cancer (39), but methylation of p16 was not associated with any clinicopathologic features or worse prognosis (38), similar to our results.

MSI due to DNA replication errors has been widely observed in a variety of sporadic tumors, in addition to tumors associated with hereditary nonpolyposis colorectal cancer syndrome due to germ line mutation in a mismatch repair gene (45–48). MSI has been identified in 7% to 50% of gastric carcinomas with geographic variation in prevalence (49) and may occur early in gastric carcinogenesis (50). In gastric cancers, MSI-H tumors have been associated with intestinal type histology, prominent lymphoid infiltration, older age, antral tumor location, lower prevalence of lymph node metastasis, and better prognosis in some reports (51, 52), but not in others (21–23). In the present study, MSI-H was present in 19% of the gastric carcinomas, and there was no association between MSI status and any of the clinicopathologic features including tumor histology, location, and pathologic stage. However, there was a significant correlation between MSI-H and methylation of hMLH1 and CIMP-H, as has been shown previously (10). Overall survival was slightly better but was not statistically significantly different ($P = 0.15$) in patients with MSI-H tumors as compared with patients with MSI-L and MS-stable tumor.

In conclusion, concordant methylation in multiple genes/loci (CIMP-H) was present in 31% of gastric carcinomas, and MSI-H was present in 19% of gastric carcinomas and was associated with $hMLH1$ hypermethylation and CIMP-H. Our results suggested that methylation of CIMP-H was associated with better overall survival, but was not an independent prognostic factor in resected gastric carcinomas.

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