Prognostic Analysis of E-Cadherin Gene Promoter Hypermethylation in Patients with Surgically Resected, Node-Positive, Diffuse Gastric Cancer

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ABSTRACT

Purpose: Recent investigations have demonstrated that hypermethylation is a frequent mechanism for silencing tumor suppressor genes. This is a potentially reversible epigenetic change, and it is the target of a novel class of antineoplastic compounds with demethylating activity. Better understanding of the clinical implications of hypermethylation will allow the optimal planning of future trials with demethylating drugs. In this perspective, we investigated whether hypermethylation in the CDH1 promoter region is correlated with poor prognosis of patients with surgically resected, node-positive, diffuse gastric cancer.

Experimental Design: Consecutive cases of diffuse gastric cancer were considered eligible for study entry. Additional inclusion criteria were radical surgery with a minimum of D1 lymphadenectomy, complete follow-up information, and availability of tumor specimens for methylation-specific PCR and immunohistochemistry analyses.

Results: CDH1 promoter hypermethylation was found in 40 of 73 cases (54%), and it was significantly associated with worse prognosis. In patients with and without hypermethylation, the 5-year event-free survival rate was 30% and 62%, respectively, and the 5-year overall survival rate was 35% and 67%, respectively. CDH1 promoter hypermethylation retained its prognostic role for disease-free survival ($P < 0.001$) and overall survival ($P < 0.001$) in multivariate analysis. Immunohistochemistry showed a significant association between CDH1 methylation and E-cadherin expression ($P < 0.001$).

Conclusions: This study shows adverse prognostic effect of CDH1 promoter hypermethylation in patients with diffuse gastric cancer. This form of cancer, and other types with frequent hypermethylation and silencing of critical tumor suppressor genes, would make appropriate targets for the testing of novel compounds with demethylating activity.

INTRODUCTION

CDH1 is a tumor suppressor gene located on chromosome 16q22.1. The mature E-cadherin protein is a transmembrane homodimer that is localized mainly to the adherens junctions of epithelial cells. E-cadherin plays a fundamental role in maintaining cell differentiation, polarity, and normal tissue architecture (1, 2).

Specific germ-line truncating mutations in CDH1 characterize the hereditary diffuse gastric cancer syndrome (3), and somatic CDH1 mutations occur in sporadic gastric carcinomas at a high frequency (4, 5). In both hereditary diffuse gastric cancer syndrome cases and sporadic gastric carcinomas, E-cadherin immunoreactivity is frequently abolished (2). According to the “two-hit” hypothesis, this is the result of an inactivating mutation in a CDH1 allele, and a second hit, which down-regulates the remaining wild-type allele (6).

In mammalian DNA, through the action of methyltransferase enzymes, methyl groups can be added to the cytosines in CpG dinucleotides. CpG dinucleotides occur at high density in stretches of DNA termed “CpG islands” (7). About half of all human genes have CpG islands in their 5′-promoter regions, and in normal tissues, methylation is limited to exceptional situations (embryogenesis, development, and differentiation to adult cells; Ref. 7). Aberrant CpG island methylation and transcriptional silencing of tumor suppressor genes frequently occur in human neoplasms (7, 8). However, concomitant up-regulation of DNA methyltransferase activity is not always detected, illustrating that the mechanisms that induce methylation in the human genome are only partially understood (8).

Hypermethylation in CDH1 occurs in 40–80% of primary human gastric carcinomas, especially in the poorly differentiated, diffuse histotype (2, 9). It is considered as a common inactivating second hit for CDH1 (10, 11), and the presence of hypermethylation is associated frequently with E-cadherin down-regulation (9, 11–13). In recent years, several retrospec-
ative studies have found a more aggressive phenotype and poor prognosis in gastric cancer patients with E-cadherin-negative tumors (14–20).

Interestingly, hypermethylation is a potentially reversible epigenetic change, and, therefore, has become the target for novel anticancer drugs (21). Several demethylating compounds have been investigated in Phase II trials in hematological and solid neoplasms over the last decades (22), and second-generation drugs of this family will be evaluated in the near future (23).

Early clinical trials with such agents have shown disappointing results (22), but notably, none of these studies stratified patients for the methylation status of genes involved in the critical stages of tumorigenesis and tumor progression. Therefore, better knowledge on the frequency of hypermethylation, its involvement in the silencing of tumor suppressor genes, and the final influence on the natural history of the disease could lead to the optimal use of demethylating compounds.

According to this background and the lack of information on the prognostic role of CDH1 promoter hypermethylation, we sought to investigate the relationship between the epigenetic change of CDH1 and the outcome of patients with surgically resected, diffuse gastric cancer.

MATERIALS AND METHODS

Human Samples and Clinicopathologic Data. In this retrospective analysis, the study population consisted of consecutive patients with node-positive, diffuse gastric cancer who underwent surgery between 1994 and 1998. None of the patients had received preoperative or adjuvant chemotherapy. Additional inclusion criteria were radical gastrectomy with a minimum of D1 lymphadenectomy, availability of paraffin-embedded specimens of the primary tumor, and complete follow-up information.

According to the policy of participating institutions, the follow-up consisted of interim history, physical examination, hematological studies, carcinoembryonic antigen levels, and diagnostic imaging (chest X-ray and abdominal ultrasonography) every 4 months in the first year, and every 6 months in the second through fifth years. Patients underwent upper endoscopy 6 months after surgery and every 12 months thereafter. Abdominal and pelvic computed tomographic scan was performed for corroborative evidence of relapse. The recurrences of gastric carcinoma had to be proven by cytology biopsy or surgery.

Before study inclusion, all of the cases were reviewed by two pathologists (I. B. and P. M.) for confirmation of diagnosis, staging, and grading. The 1997 revision of the American Joint Committee on Cancer manual was used for the classification of each case. The study was performed in a blind fashion, so that patient outcome was unknown to investigators performing immunohistochemistry and methylation analyses. The study was carried out in accordance with the Institutional Review Board requirements for retrospective investigations.

Analysis of CDH1 Promoter Hypermethylation. Fifteen 5-mm thick paraffin sections of gastric tissue samples were used for DNA extraction. Careful microdissection of gastric carcinoma cells from representative tumor areas was performed from the slides. Genomic DNA from microdissected tissue was isolated by the high pure PCR template preparation kit (Boehringer Mannheim Corp., Indianapolis, IN; Ref. 24). Treatment with sodium bisulfite was used to induce chemical modification of genomic DNA and allowing identification of aberrant DNA methylation in CpG islands. In this procedure, the unmethylated cytosine nucleotides are converted to uracil, whereas methylated cytosine nucleotides remains unchanged. The Intergen CpGenome DNA modification kit (Intergen, Purchase, NY) was used for the bisulfite modification procedure (24). Subsequently, the

![Fig. 1](image-url)  
**Fig. 1** Gel electrophoresis of methylation-specific-PCR using CDH1 methylated-specific primers (A): lack of visible product in the normal mucosa (M) and presence of a product in the tumor (T); – and + correspond to water and the positive control, respectively. Analysis of the same case using unmethylated-specific primers (B): a visible product is present in the normal mucosa (M) and the tumor (T); – and pl correspond to water and the DNA from peripheral lymphocytes, respectively. Experimental estimation of methylation-specific-PCR sensitivity for CDH1 promoter methylation (C). After bisulfite conversion, lymphocyte DNA with unmethylated CDH1 promoter was mixed with increasing amounts of DNA extensively methylated with SssI CpG methylase (New England Biolabs, Beverly MA). Numbers above lanes indicate the percentage of methylated DNA that is present in the mixture. U and M indicate unmethylated and methylated products, respectively. As low as 1% of methylated CDH1 promoter can be detected.
methylation-specific PCR (MS-PCR) was performed by adding the bisulfite-modified DNA to PCR reactions and using specific primers for either the methylated or the unmethylated CDH1 promoter. Primers and conditions for the MS-PCR have been described previously (9, 25). In vitro methylated DNA (Intergen) was used as a positive control for methylation, and water and DNA from peripheral lymphocytes of healthy individuals were used as negative controls. The results of the MS-PCR were scored when there was a clearly visible band on the gel with the methylated primers (Fig. 1A) and the unmethylated primers (Fig. 1B). Electrophoresis results were interpreted by two independent investigators, and in case of discrepancy, the opinion of a third investigator was sought. All of the PCRs were repeated twice, and a random sample of cases underwent external repetition of methylation analysis to ensure the reproducibility of the results (B. H., H. M., and P. G.).

**Immunohistochemistry.** The avidin-biotin complex peroxidase method was used for immunostaining (26), and the anti-E-cadherin monoclonal antibody (clone HECD-1; Zymed Laboratories, San Francisco, CA) was used for detecting the E-cadherin protein on sections from paraffin-embedded tissue (27). For the negative control, the primary antibody was replaced with mouse IgG. Slides with normal gastric mucosa were used as positive controls. Furthermore, positive E-cadherin staining in the adjacent noninvolved gastric mucosa served as an internal positive control. Necrotic areas and areas where the tissue had a deteriorated morphology were excluded. For the purpose of the analysis and according to the criteria used in previous studies (16, 19), the results of immunohistochemistry were classified according to the proportion of positive tumor cells. When >90% of cancer cells showed homogenous membranous E-cadherin staining, the tumor was considered positive; when 10–90% of cancer cells were positively stained, the tumor was deemed with partially reduced E-cadherin expression; tumors with <10% of positive cancer cells were considered as E-cadherin negative (Fig. 2, A and B).

**Statistical Methods.** Statistical analyses were performed to correlate the methylation analysis with the disease-free survival and the overall survival of patients. Overall survival was measured from the date of surgery until death from any cause. Disease-free survival was measured from the date of surgery to the time of confirmed relapse or death from any cause. The Kaplan-Meier method was adopted to estimate survival curves. Differences between survival curves were studied by the log-rank test. The Cox proportional hazards model was used to assess the prognostic importance of hypermethylation adjusting for the following clinicopathological features, age, gender, tumor size and location, degree of lymph nodal involvement, tumor stage, and hypermethylation. Contingency tables were analyzed by the $\chi^2$ test. All of the values were two-sided, and statistical significance was defined as $P < 0.05$.

**RESULTS**

The study population consisted of 73 patients with surgically resected, node-positive, diffuse gastric cancer who underwent radical surgery between June 1994 and January 1998 (Table 1). During the follow-up (range, 63–108 months), 39

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of the 73 patients included in the study and distribution of data in 40 methylated cases and 33 unmethylated cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Total</td>
</tr>
<tr>
<td>&lt;45 years</td>
<td>24 (33%)</td>
</tr>
<tr>
<td>≥45 years</td>
<td>49 (67%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>40 (54%)</td>
</tr>
<tr>
<td>Female</td>
<td>33 (46%)</td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
</tr>
<tr>
<td>Cardia</td>
<td>10 (14%)</td>
</tr>
<tr>
<td>Noncardia</td>
<td>63 (86%)</td>
</tr>
<tr>
<td>Stage grouping</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>30 (41%)</td>
</tr>
<tr>
<td>Stage IIIA-B</td>
<td>43 (59%)</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
</tr>
<tr>
<td>Disease free</td>
<td>34 (47%)</td>
</tr>
<tr>
<td>Relapsed</td>
<td>39 (53%)</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td></td>
</tr>
<tr>
<td>Cadherin positive</td>
<td>30 (41%)</td>
</tr>
<tr>
<td>Cadherin reduced/ negative</td>
<td>43 (59%)</td>
</tr>
</tbody>
</table>

NS, not significant.
patients had tumor recurrence, and 35 patients had died at the time of analysis. All of the patients had received palliative chemotherapy with fluorouracil and folinic acid (16 patients); fluorouracil, folinic acid, and cisplatin (25 patients); fluorouracil, folinic acid, cisplatin, and epirubicin (21 patients); and fluorouracil, folinic acid, cisplatin, and mitomycin (11 patients).

CDH1 promoter hypermethylation was found in 40 cases (54%), and it was not detected in the remaining 33 cases (46%). In patients aged >45 years there was a higher frequency of hypermethylated tumors than in younger patients (Table 1). A significant association was found between the results of immunohistochemistry and the CDH1 promoter methylation status (Table 1). Also, patients with reduced or lost E-cadherin expression showed worse event-free survival and overall survival than patients with preserved protein expression (data not shown).

**DISCUSSION**

The results of the present investigation should be considered in a clinical perspective. Over the last 3 decades, demethylating agents for the treatment of solid tumors have been tested in Phase II studies with disappointing results. These trials showed promising response rates and durable responses in hematological malignancies, whereas low response rates and stabilization of disease, at most, were observed in solid neoplasms (9, 22). Studies with first-generation demethylating drugs such as azacytidine were started in the 1980s (22), when the knowledge on hypermethylation of tumor-related genes and methods for its assessment were largely unknown. Today, we know that

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**Table 2** Cox multivariate analysis for disease-free and overall survival in the 73 patients

<table>
<thead>
<tr>
<th></th>
<th>RR*</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease-free survival</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size (&lt;50 mm vs. ≥50 mm)</td>
<td>—</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>Number of positive nodes (≤6 vs. &gt;6)</td>
<td>2.96</td>
<td>2.0–4.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tumor location (cardia vs. noncardia)</td>
<td>—</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor stage (II vs. IIIA–B)</td>
<td>3.24</td>
<td>2.35–4.46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypermethylated vs. unmethylated</td>
<td>2.28</td>
<td>1.41–3.71</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size (&lt;50 mm vs. ≥50 mm)</td>
<td>—</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>Number of positive nodes (≤6 vs. &gt;6)</td>
<td>2.11</td>
<td>1.14–3.93</td>
<td>0.009</td>
</tr>
<tr>
<td>Tumor location (cardia vs. noncardia)</td>
<td>—</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor stage (II vs. IIIA–B)</td>
<td>2.55</td>
<td>1.53–4.25</td>
<td>0.003</td>
</tr>
<tr>
<td>Hypermethylated vs. unmethylated</td>
<td>1.94</td>
<td>1.23–3.06</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*RR, relative risk; CI, confidence interval; NS, not significant.
hypermethylation is a relevant mechanism for silencing tumor suppressor genes, but it does not occur at the same frequency in all of the human neoplasms (7). Hematological malignancies may have particularly high levels of CpG island methylation (28), whereas solid tumors show variable methylation profiles (7, 29). These data may partially explain the erratic results from early clinical trials with demethylating drugs. In addition, they suggest that demethylating drugs may be more effective in patients in whom hypermethylation has silenced one or more critical tumor suppressor genes.

Thus far, investigations on CDH1 promoter hypermethylation in gastric carcinomas have aimed at elucidating the effect of this event on tumorigenesis. In the present study, we report the first evidence that CDH1 promoter hypermethylation has a strong prognostic role in patients with diffuse gastric cancer. This finding is consistent with immunohistochemistry data from retrospective studies, which indicated the unfavorable outcome of gastric cancer patients whose tumors showed down-regulation of E-cadherin expression (14–20). In the largest of these studies involving 413 cases, Gabbert et al. (16) found that patients with E-cadherin-negative tumors had significantly worse 3- and 5-year survival rates than patients with E-cadherin-positive tumors. Our observation, together with the remarkable frequency (40%–80%) of this epigenetic change in sporadic diffuse gastric carcinomas (2), suggest an attractive setting for testing novel demethylating agents.

An alternative explanation for our observation of a correlation between CDH1 methylation and survival is that CDH1 promoter hypermethylation is a surrogate marker for CpG island methylation in multiple tumor suppressor genes (methylator phenotype), which cooperatively influence tumor behavior. However, regardless of the number and identity of any genes that may be methylated in parallel with CDH1, it is well established that E-cadherin down-regulation alone will contribute significantly to the process of tumor progression (30).

Assessing hypermethylation in multiple genes for prognostic purposes is costly and time consuming, whereas a single determination in CDH1 in diffuse gastric carcinomas may identify patients with poor prognosis and be relevant to the decision making in clinical practice. Immunohistochemistry for E-cadherin expression may also represent a convenient prognostic marker, and possibly, a surrogate indicator for the presence of hypermethylation in CDH1. However, the interpretation of E-cadherin staining may be too ambiguous and show insufficient correlation with the CDH1 methylation status (9–11) to be used for this purpose.

Our combined analysis of CDH1 hypermethylation and E-cadherin immunohistochemistry showed good correspondence between the two methods in 40 cases, but 5 methylated tumors maintained E-cadherin expression and 8 unmethylated cases showed reduced or negative E-cadherin expression. This discordance has been observed in previous studies (9, 11–13). The few methylated cases that maintained E-cadherin immunoreactivity may be explained by incomplete methylation of the CDH1 promoter or the occurrence of methylation on a single allele. The observation of reduced immunoreactivity in the absence of CDH1 hypermethylation is consistent with E-cadherin down-regulation through other mechanisms including mutations (4, 5), transcriptional repression of CDH1 (e.g., via snail; Refs. 31, 32), post-translational modification by direct or indirect phosphorylation of adherens junction components (e.g., B-catenin), or receptor tyrosine kinase-associated endocytosis and degradation of E-cadherin (33, 34). In the clinical setting, unsuccessful activity of demethylating therapies targeted to CDH1 might be observed in cases with partial CDH1 promoter hypermethylation and/or silencing of E-cadherin due to other molecular mechanisms.

Additional concerns for interpreting results and widening applications of hypermethylation in solid tumors are related to the fact that the best method for its assessment has not been defined yet, and today, it remains a high-cost, investigational assay. Under optimal experimental conditions (Fig. 1C), a sensitive MS-PCR should be able to detect low numbers of methylated tumor cells (i.e., 1–5%), which could be sufficient, after their selection and expansion, to influence the natural history of the disease. When used in retrospective studies, the analysis of tumor methylation necessitates DNA isolation from archival, paraffin-embedded material, and even under steady MS-PCR conditions a variable sensitivity of the test may occur.

In conclusion, the present data support the adverse prognostic effect of CDH1 promoter hypermethylation in diffuse gastric cancer and emphasize the potential clinical relevance of additional investigations in this field (35). This information can be relevant to future studies with novel demethylating drugs and for identifying high-risk gastric cancer patients who could benefit from adjuvant chemotherapy. The role of CDH1 hypermethylation in normal gastric mucosa (36), in precancerous gastric lesions (37, 38), in patients aged ≥45 years (36), and with concomitant Helicobacter pylori infection (39) may represent additional fields of investigation for chemopreventive strategies aiming at the preservation of the E-cadherin function.

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