Combined analysis of E-cadherin gene (CDH1) promoter hypermethylation and E-cadherin protein expression in patients with gastric cancer: implications for treatment with demethylating drugs


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Background: Hypermethylation is studied as a new, relevant mechanism for silencing tumor suppressor genes. It is a potentially reversible epigenetic change and it is the target of novel anticancer compounds with demethylating activity. In this perspective, we investigated E-cadherin gene (CDH1) promoter hypermethylation in gastric carcinomas and its correlation with E-cadherin protein expression.

Methods: Consecutive cases of gastric carcinoma with assessable paraffin-embedded tumor blocks and paired normal mucosa were considered eligible for study entry. CDH1 promoter hypermethylation and E-cadherin protein expression were determined by methylation-specific polymerase chain reaction and immunohistochemistry, respectively.

Results: CDH1 promoter hypermethylation was found in 20 out of 70 gastric carcinomas and the epigenetic change occurred in the early, as well as in the locally advanced disease. In five cases, hypermethylation was also detected in the normal mucosa. Eighteen out of 20 hypermethylated tumors were of the diffuse histotype (P = 0.0001). Of 24 tumors with reduced or negative E-cadherin expression, 19 were hypermethylated and 5 were unmethylated (P = 0.0001).

Conclusions: CDH1 promoter hypermethylation frequently occurs in gastric carcinomas of the diffuse histotype and it is significantly associated with downregulated E-cadherin expression. The knowledge on the hypermethylation status of tumor suppressor genes may be relevant to the development of demethylating drugs and novel chemopreventive strategies in solid tumors.

Key words: gastric neoplasms, gastric carcinoma, E-cadherin, methylation, tumor suppressor gene

Introduction

The E-cadherin gene (CDH1) is a tumor suppressor gene located on chromosome 16q22.1. The mature E-cadherin protein is a transmembrane homodimer mainly located in the adherens junctions of the cell surface; it plays a fundamental role in maintaining cell differentiation and the normal architecture of epithelial tissues [1, 2]. Recent research has shown specific germline truncating mutations in CDH1 to be the cause of hereditary diffuse gastric cancer syndrome (HDGC) [3]. Somatic inactivating mutations in CDH1 also occur in ≥50% of sporadic gastric carcinomas [4, 5]. In both HDGC cases and sporadic gastric carcinomas, E-cadherin expression is frequently reduced or lost [2]. According to the ‘two-hit’ hypothesis, this is the result of a truncating mutation in one CDH1 allele, and a second hit, which inactivates the remaining wild-type allele. To date, hypermethylation in the CDH1 promoter region seems to be the commonest second event that knocks out CDH1 [6–8].

Methyltransferases are enzymes that add methyl groups to cytosine followed by guanosine in small DNA regions called ‘CpG islands’. CpG islands are detectable in the 5’ promoter region of about half of all human genes. In normal tissues, CpG island methylation is limited to exceptional situations (embryogenesis, development and differentiation to adult cells) [9, 10], but recent studies have found increased DNA methyltransferase activity, aberrant CpG island methylation and transcriptional silencing of tumor suppressor genes in human neoplasms [11]. Interestingly, hypermethylation is a potentially reversible epigenetic change, and it is the target of novel anticancer therapies with demethylating compounds [9, 10]. Early clinical trials with such agents showed disappointing results, but notably, these studies included patients with a broad range of tumor histologies, and their enrollment...
started in the 1980s, when knowledge about hypermethylation in disease-related genes was lacking [9, 10].

Silencing of CDH1 due to hypermethylation may represent an attractive setting for the testing of novel demethylating agents. Epigenetic change seems to occur quite frequently in gastric carcinomas and the loss of E-cadherin expression was found to correlate with different stages of the disease and its correlation with E-cadherin modification of genomic DNA with sodium bisulfite followed by methylation normalization of genomic DNA was mandatory for study entry.

In this field before a tailored therapy for reversing CDH1 hypermethylation and restoring E-cadherin function in gastric carcinomas can be employed.

In addition to exploring CDH1 promoter hypermethylation in the intestinal and diffuse histotype of gastric carcinomas, we wished to document the frequency of this epigenetic change at different stages of the disease and its correlation with E-cadherin protein expression.

Materials and methods

Human samples and clinicopathological data

In this retrospective analysis, the study population consisted of consecutive patients with gastric cancer who underwent surgery from 1998 to 2002. Availability of paraffin-embedded specimens of the primary tumor and paired normal mucosa was mandatory for study entry.

Before study inclusion, all 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 for Cancer Staging (AJCC) manual was used for the classification of each case. The study was performed blind, so that patients’ characteristics were unknown to investigators performing immunohistochemistry and methylation analyses.

DNA extraction from tissue

Procedures for DNA extraction from tissue have been described previously [13]. In brief, 15 paraffin sections, each 5-mm thick, of gastric tissue samples were used for DNA extraction. Gastric tissues were retrieved using xylene and alcohol. The areas rich in gastric tumor cells were selected and carefully microdissected from the slides. Genomic DNA from microdissected tissue was isolated using the High Pure PCR Template Preparation kit (Boehringer Mannheim, Indianapolis, IN).

Bisulfite treatment and methylation-specific PCR

Aberrant DNA methylation in CpG islands was determined by chemical modification of genomic DNA with sodium bisulfite followed by methylation-specific PCR (MS-PCR). The bisulfite modification procedure was carried out using a Intergen CpGenome DNA Modification kit (Intergen, Purchase, NY). In brief, 1 μg genomic DNA was denatured with sodium hydroxide and then chemically modified with sodium bisulfite for 20 h. Unmethylated cytosine is converted to uracil, whereas methylated cytosine remains unchanged. Modified DNA was recovered by ethanol precipitation and resuspended in PCR-grade water. In vitro methylated DNA (Intergen) was used as a positive control for methylation, and water was used as a negative control. Specific primers for either the methylated and unmethylated CDH1 promoter were as follows: methylated DNA, 5′-GGTAGATTTTTTGGTTAATTAGCTTAC-3′ and 5′-CATAACTAACCAGAAACGCCG-3′; unmethylated DNA, 5′-GGTAGGGGTAATTTTTGGTTAATTAGCTTAC-3′ and 5′-ACCCTAACCAGAAACGCCG-3′ [8]. Briefly, 2 μl bisulfite-modified DNA was amplified in a total volume of 25 μl containing 1 × PCR buffer II (Perkin-Elmer, Boston, MA), 2 mM MgCl2, 0.25 mM deoxynucleotide triphosphate, 1 μM each primer and 1 U AmpliTaq Gold polymerase (Perkin-Elmer) at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 30 s, the specific annealing temperature for 45 s, and 72°C for 45 s. PCR product (10 μl) was loaded on 10% nondenaturing polyacrylamide gel stained with ethidium bromide and visualized under ultraviolet (UV) illumination. Samples were scored as methylated when there was a clearly visible band on the gel with the methylated primers. Electrophoresis results were interpreted by two independent investigators, and in case of discrepancy, the opinion of a third investigator was sought.

Immunohistochemistry

Immunohistochemical detection of E-cadherin on sections from paraffin-embedded tissue was performed as previously described [13]. In brief, two deparaffinized sections were incubated overnight at 4°C with anti-E-cadherin monoclonal antibody (clone HEDC-1; Zymed Laboratories, San Francisco, CA), diluted 1:100 in phosphate-buffered saline (PBS). Next, the slides were incubated with biotinylated goat anti-mouse IgG for 20 min (LSAB kit; DAKO, Copenhagen, Denmark) and biotinylated goat antirabbit IgG. Immunostaining was done with dianaminobenzidine (Dako) solution with hydrogen peroxide for 1 min. For the negative control, the primary antibody was replaced with mouse immunoglobulin G. Slides with normal gastric mucosa were used as positive controls. Furthermore, positive E-cadherin staining in the adjacent non-involved gastric mucosa served as an internal positive control. Necrotic areas and areas where the tissue had deteriorated morphology were excluded. For the purpose of the study and according to previous analyses [13], the results of immunohistochemistry were classified according to the staining pattern (membranous or cytoplasmic) and the percentage of positive cells. E-cadherin positive cases were those with preserved, homogenous membranous staining in all tumor cells. E-cadherin was defined as partially reduced in cases with faint membranous staining and/or membranous staining, but with a decreased percentage of positive tumor cells. Negative E-cadherin cases were those which lacked E-cadherin expression or cytoplasmic staining.

Statistical methods

Statistical analysis was performed to correlate the results of methylation analysis to the clinico-pathological characteristics of patients and to the results of E-cadherin protein expression by immunohistochemistry. Contingency tables were analyzed using the Chi-square test. All the values were two-sided and statistical significance was defined as values of P <0.05.

Results

The study population consisted of 70 patients with gastric cancer of which 39 were men and 31 women. Median age was 52 years (range 30–77). According to Lauren’s classification, 32 and 38 tumors were of the intestinal and diffuse histotypes, respectively. All patients underwent surgical resection of the primary tumor which was early gastric cancer (EGC) in 10 patients, pT1b–TN0M0 in 25 patients and pT1–3N0–3M0 in 35 patients.

Results from MS-PCR assays found CDH1 promoter hypermethylation in 20 of 70 gastric carcinomas, and in five cases the epigenetic change also occurred in the non-neoplastic mucosa. Immunohistochemistry showed reduction or loss of E-cadherin expression in 24 of 70 cases. Table 1 shows the data of methylation and immunohistochemistry analyses and the distribution of results across different stages and histology subtypes. CDH1 promoter hypermethylation was found in EGC cases as well as in locally-advanced disease, including node-positive tumors. Notably, 18 of 20 cases of CDH1 hypermethylation occurred in tumors of the diffuse histotype. Indeed, the distribution of hypermethylated
and unmethylated tumors among intestinal and diffuse gastric carcinomas (Table 2) was statistically significant ($P = 0.0001$).

Immunohistochemistry demonstrated 46 cases with positive E-cadherin expression, eight cases with partially reduced E-cadherin expression and 16 cases with lack of E-cadherin expression. A significant association was found between the results of immunohistochemistry and the $CDH1$ promoter methylation status (Table 3). In particular, in 20 cases with hypermethylation, E-cadherin expression was negative or partially reduced in 19 cases and positive in one case. In the 50 cases without hypermethylation, E-cadherin expression was negative or partially reduced in five cases and positive in 45 cases ($P = 0.0001$).

**Discussion**

Hypermethylation has emerged as a possible mechanism for the silencing of tumor suppressor genes and it is currently under investigation in basic and clinical oncology research [11]. In particular, reversibility of hypermethylation in pre-clinical models [14, 15] led to the development of demethylating agents for anticancer therapy [9, 10]. In early clinical trials, demethylating compounds showed unsatisfactory results in solid tumors and the lack of meaningful activity in this setting was initially attributed to unfavourable pharmacokinetics and/or intrinsic inefficacy of these drugs [9, 10]. Actually, hypermethylation in disease-related genes does not occur at the same frequency in all human neoplasms [9, 10]: hematological malignancies may have particularly high levels of CpG islands methylation [16], while unsteady methylation profiles characterize solid tumors [17]. Given this, one explanation for the failure of such agents in early phase II trials could be the lack of a target (hypermethylation) in the tumor. In fact, clinical trials with first generation demethylating drugs, such as azacytidine, were started in the 1980s when the extent of hypermethylation in tumor-related genes and methods for its assessment were largely unknown.

To the best of our knowledge, this is the largest combined analysis of $CDH1$ promoter hypermethylation and E-cadherin expression in gastric cancer and these data should be considered before testing novel demethylating agents in patients with the diffuse histotype. Differences in the frequency of $CDH1$ promoter hypermethylation between intestinal and diffuse gastric carcinomas has been shown in previous studies [7, 8, 18]. In addition, Machado et al. [7] found loss of plasma membrane E-cadherin expression in nine diffuse gastric carcinomas with $CDH1$ mutations; six of these cases showed $CDH1$ promoter hypermethylation. Tamura et al. [8] studied E-cadherin expression by Western blot analysis in 12 gastric carcinomas with variable histologies: loss or marked reduction of E-cadherin expression was found in four of the six hypermethylated tumors.

In the present study, one tumor with $CDH1$ methylation maintained E-cadherin expression and five tumors lacking $CDH1$ methylation showed reduced or negative E-cadherin expression. The genotype–phenotype discordance in 8.5% of cases might be explained at the molecular level. Hypermethylation can occur in a

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<th>Table 1. Results of methylation and immunohistochemistry analyses in different stage groups and histology</th>
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<tr>
<td>EGC</td>
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<td>Diffuse ($n = 4$)</td>
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<td>pT2–N0M0</td>
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<td>Diffuse ($n = 18$)</td>
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<td>Intestinal ($n = 17$)</td>
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Cad–, cadherin negative; Cad+, cadherin positive; EGC, early gastric cancer.

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<th>Table 2. Results of $CDH1$ methylation analysis in intestinal and diffuse gastric carcinomas</th>
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$P = 0.0001$

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<th>Table 3. Results of $CDH1$ methylation analysis in E-cadherin positive and negative cases</th>
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<td>Hypermethylated</td>
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$P = 0.0001$
CDH1 allele carrying an inactivating somatic mutation, while the function of the remaining CDH1 allele is preserved; in this case, E-cadherin protein expression is maintained despite a positive methylation analysis. On the other hand, if somatic mutations inactivate both CDH1 alleles, and/or alternative molecular mechanisms knock out CDH1, loss of E-cadherin expression can be found even in unmethylated tumors. Indeed, recent experimental data have indicated new molecular pathways which can lead to downregulation/disassembly of E-cadherin complexes: transcriptional repression of CDH1 (e.g. via snail) [19, 20], post-translationally by direct or indirect phosphorylation of adherens junction components (e.g. β-catenin), or RTK-associated endocytosis and E-cadherin degradation [21, 22].

The presence of CDH1 promoter hypermethylation in apparently non-neoplastic gastric mucosa and in EGC cases suggests a possibility of innovative chemopreventive strategies aiming at the preservation of E-cadherin function, such as Helicobacter pylori eradication [23]. In previous studies, Waki et al. [24], Leung et al. [25] and To et al. [26] found CDH1 promoter hypermethylation in gastric carcinomas, intestinal metaplasia and normal gastric mucosa; interestingly, in one of these studies, the epigenetic change was age-dependent and its frequency increased in persons who were ≥42 years of age [24]. From this perspective, additional investigations should be addressed to the analysis of CDH1 promoter hypermethylation in concomitant conditions that may result in the reduction of E-cadherin expression [27].

In conclusion, patients with diffuse gastric cancer may represent an ideal setting for testing novel demethylating drugs. However, it must be considered that CDH1 promoter hypermethylation cannot be the exclusive mechanism for E-cadherin silencing, and therefore, the failure in the clinical activity of demethylating drugs may be observed in some cases even in the presence of the methylator phenotype. Another attractive setting for exploiting the activity of demethylating compounds is chemoprevention and further investigations are needed to clarify the significance of hypermethylation in the non-neoplastic gastric mucosa and precancerous conditions.

References