E-Cadherin Gene Promoter Hypermethylation in Primary Human Gastric Carcinomas

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Background: E (epithelial)-cadherin, the cell adhesion molecule also considered a potential invasion/metastasis suppressor, is mutationally inactivated in nearly half of all undifferentiated-scattered (diffuse-type) gastric carcinomas. In addition, silencing of E-cadherin by CpG methylation within its promoter region has been reported in several gastric carcinoma cell lines. We investigated the methylation status of the E-cadherin promoter region in 53 primary human gastric carcinomas. *Methods:* Hypermethylation of the E-cadherin promoter was determined by utilizing methylation-specific polymerase chain reaction (PCR)–single-strand conformation polymorphism (MSP–SSCP) analysis followed by direct sequencing of PCR products. Expression of E-cadherin was studied by western blot analysis. All statistical tests were two-sided. *Results:* Hypermethylation of the E-cadherin promoter was evident in 27 (51%) of 53 primary gastric carcinomas examined by MSP–SSCP. It occurred more frequently in carcinomas of the undifferentiated-scattered type (in 15 [83%] of 18) than in other histologic subtypes (in 12 [34%] of 35) (P = .0011, Fisher's exact test), and it was present at similar rates in early (in six [60%] of 10) versus advanced (in 21 [49%] of 43) carcinomas (P = .73, Fisher's exact test). Methylation occurring at all cytosine–guanosine sequences (CpGs) near the transcription start site was confirmed in six of six tumors examined by bisulfite–DNA sequencing, including two early gastric carcinomas. In addition, loss or diminished expression of E-cadherin was confirmed by western blotting in four of the six tumor tissues demonstrating hypermethylation. *Conclusions:* The E-cadherin promoter frequently undergoes hypermethylation in human gastric cancers, particularly those of the undifferentiated-scattered histologic subtype. E-cadherin promoter hypermethylation is associated with decreased expression and may occur early in gastric carcinogenesis. [J Natl Cancer Inst 2000;92:569–73]

E (epithelial)-cadherin is a member of a family of transmembrane glycoproteins responsible for calcium-dependent cell-to-cell adhesion that also appear to play roles in organogenesis and morphogenesis *(1)*. E-cadherin complexes and connects actin filaments with α-, β-, and γ-catenins, which are themselves involved in tumorigenesis, making the E-cadherin-mediated cell adhesion system a likely target of inactivation in human tumors [for review, *see (2)*]. Somatic alterations of the E-cadherin gene are more frequent in gastric carcinomas of the undifferentiated-scattered (diffuse) type *(3–5)* and in lobular breast carcinomas *(6,7),* both of which show histologic features consistent with loss of cell-to-cell adhesiveness, than in any other tumor type. In addition, point mutations of E-cadherin are frequently accompanied by loss of the wild-type allele, leading to

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complete gene inactivation [for review, *see (8)*]. Finally, germline mutations of this gene have been reported in diffuse-type gastric cancer families from both New Zealand and Europe *(9,10)*. Thus, E-cadherin satisfies criteria for classification as a tumor suppressor gene in the human stomach *(2)*.

In addition to the classic two-hit inactivation mechanism alluded to above, E-cadherin can be silenced by cytosine–guanosine (CpG) sequence methylation in human tumor cell lines, including gastric carcinoma cell lines, as well as in primary carcinomas of the breast and thyroid *(11–15)*. Aberrant DNA methylation of promoter region CpG islands serves as an alternative mechanism to coding region mutation for the inactivation of tumor suppressor or other tumor-related genes, including retinoblastoma (Rb), von Hippel–Lindau (VHL), p16 (also known as INK4a), p15 (also known as INK4b), hMLH1 (also known as human mut-L homologue), and E-cadherin *(12,14,16,17)*. Furthermore, *in vitro, de novo* methylation of the E-cadherin promoter region may involve the entire CpG island of the promoter, including the central area of highest CpG density near the transcriptional start site *(14)*.

In this study, we investigated the methylation status of the E-cadherin promoter in primary human gastric carcinomas. We found that this promoter is frequently hypermethylated (particularly in gastric tumors of the undifferentiated-scattered type), that hypermethylation occurs early in gastric carcinogenesis, and that hypermethylation uniformly affects a large CpG-rich region within the E-cadherin gene promoter.

MATERIALS AND METHODS

Tumor tissues and control samples. Fifty-three gastric carcinomas and their corresponding normal tissues were obtained surgically from 53 Japanese patients at the Iwate Medical University, Morioka, Japan. This study was approved by the Human Subjects Committee of the Iwate Medical University, and each subject signed an informed consent form before tissue was obtained. The tumors included 24 differentiated and 29 undifferentiated carcinomas (18 scattered-type tumors and 11 adherent-type tumors) and constituted 10 early (depth of invasion limited to the mucosa or submucosa) and 43 advanced (invasive) carcinomas. DNA was extracted by standard methods *(18)*. DNAs from MCF-7 and from MDA231 breast cancer cell lines were used as unmethylated and methylated controls, respectively *(12)*.

Methylation-specific polymerase chain reaction (PCR)–single-strand conformation polymorphism (MSP–SSCP) analysis. DNA methylation patterns in the E-cadherin promoter region were determined by MSP–SSCP *(12)*. MSP distinguishes unmethylated from methylated alleles of a given gene based on sequence alterations produced by bisulfite treatment of DNA, which converts unmethylated but not methylated cytosines to uracil, and subsequent PCR using primers specific to either methylated or unmethylated DNA *(12)*. We used SSCP after bisulfite modification to assess the clonal expansion of differentially or partially methylated sequences, reasoning that they should possess unique mobilities. Primer sequences were 5′-GGTGAATTTTTAGTTAATTAGCGGTAC-3′ and 5′-CATAACTAACCGAAAACGCCG-3′ for methylated DNA and 5′-GGTAGGTGAATTTTTAGTTAATTAGTGGTA-3′ and 5′-ACCCATAACTAACCAAAAACACCA-3′ for unmethylated DNA. The PCR-amplified region for methylated (204 base pairs [bp]) and unmethylated (211 bp) alleles contained 19 CpG dinucleotides, including five CpGs at the primer annealing sites. Primer sets spanned the transcriptional start site and were designed to include methylation sites that corresponded best with transcriptional silencing of the E-cadherin gene in published literature *(19)*. Briefly, 1 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were then purified with the use of Wizard DNA purification resin (Promega Corp., Madison, WI), again treated with NaOH, precipitated with ethanol, and resuspended in water. Extracted DNA samples (100 ng each) were amplified in 25 μL of buffer (16.6 mM ammonium sulfate, 67 mM Tris [pH 8.8], 6.7 mM MgCl$_2$, and 10 mM 2-mercaptoethanol) containing 10 pM of each primer, 0.2 mM of each deoxynucleoside triphosphate, and 0.5 μL of [α-^{32}P]deoxycytidine triphosphate. Reactions were heated to 95 °C for 5 minutes before the addition of 1 U of *Taq* polymerase (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). PCR was performed for 35 cycles in a thermal cycler (RoboCycler; Stratagene Cloning Systems, La Jolla, CA) consisting of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds, and extension at 72 °C for 30 seconds, followed by a final 4-minute extension at 72 °C. Then, 2 μL of the PCR product was diluted 10-fold with gel-loading buffer, heated to 94 °C for 2 minutes, and stored on ice until analysis. Electrophoresis was performed on a 6% neutral polyacrylamide gel at 60 W for 4 hours. The gel was dried and exposed to X-ray film at –80 °C overnight.

DNA-sequencing analysis. Major and minor SSCP bands from six different tumors were excised from gels and subjected to a second round of PCR amplification with the use of the same primers as those used in the primary PCR, as previously described *(20)*. These PCR products were purified and sequenced with the use of an automated DNA sequencer (type 373A; Applied Biosystems, Foster City, CA). The matching GenBank accession number was L34545.

Western blot analysis. Sections of frozen tissue were prepared in ice-cold RIPA buffer (i.e., 1× Tris-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) at a concentration of 200 μg/mL, with 10 mg/mL phenylmethylsulfonyl fluoride in isopropanol added at 10 μL/mL, with aprotinin (Sigma Chemical Co., St. Louis, MO) added at 30 μL, and with 100 mM sodium orthovanadate added at 10 μL/mL. The protein concentration of soluble supernatants from each sample was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Eighty micrograms of each sample protein was loaded per lane on 4%–15% Tris–HCl polyacrylamide gels (Bio-Rad Laboratories) and subjected to electrophoresis at 100 volts for 3 hours at room temperature. A second identical gel was simultaneously subjected to electrophoresis for Coomassie brilliant blue staining to confirm equal loading of proteins *(18)*. Protein was transferred to Trans-Blot transfer medium nitrocellulose membranes (Bio-Rad Laboratories) by electroblotting. After the blocking of nitrocellulose membranes in 5% nonfat dry milk in phosphate-buffered saline at room temperature for 1 hour, E-cadherin protein was detected by incubating it at room temperature for 1 hour with a mouse monoclonal antibody against human E-cadherin (Clone 36; Transduction Laboratories, Lexington, KY) at a 1 : 2000 dilution. This was followed by incubating this mixture with an anti-mouse secondary antibody conjugated to horseradish peroxidase at room temperature for 1 hour. Bands were visualized by ECL (enhanced chemiluminescence; Amersham Life Science Inc., Arlington Heights, IL) and exposed to Kodak BioMax MR film for 10–30 seconds.

Statistical analyses. Statistical analyses were performed with the use of Fisher's exact test by Statview 4.5 and superANOVA software for the Macintosh (SAS Institute Inc., Cary, NC). Two-sided tests were used to calculate P values.

RESULTS

MSP–SSCP and sequencing analyses. Hypermethylated alleles were present in 27 (51%) of 53 primary gastric carcinomas. This hypermethylation was more frequent in carcinomas of the undifferentiated-scattered type (83%; 15 of 18) than in those of other histologic subtypes (34%; 12 of 35) ($P = .0011$, Fisher's exact test) and was present at a similar rate in early (60%; six of 10) versus advanced (49%; 21 of 43) carcinomas ($P = .73$; Table 1). Only two patterns of mobility were detected by MSP–SSCP (Fig. 1). These two species of SSCP bands from six different tumors were directly sequenced; one species was always entirely methylated, even in early gastric carcinomas, while the other species was always entirely unmethylated (*see* Fig. 2 for sequencing histograms). These results suggested that partially methylated alleles were not present in these tumors.

Western blot analysis. Western blotting was performed in 12 tumor tissues of variable histologies: six methylated and six unmethylated. Loss or marked reduction of E-cadherin expression was observed in four of the six hypermethylated tumors: three undifferentiated-

Table 1. Association of hypermethylation of the CpG island in the E-cadherin promoter with histologic type or stage

	Methylation status	
	Methylated	Unmethylated
Histologic subtype		
Undifferentiated-scattered	15	3
Others (24 differentiated and 11 undifferentiated-adherent)	12	23
Stage		
Early	6	4
Advanced	21	22

*Two-sided $P = .0011$ by Fisher's exact test.
†Two-sided $P = .73$, not significant by Fisher's exact test.

Fig. 1. Methylation-specific polymerase chain reaction (PCR)–single-strand conformation polymorphism analysis of the E-cadherin promoter region in six undifferentiated-scattered gastric carcinomas (M162, M224, M226, M158, M275, and M169), three undifferentiated-adherent gastric carcinomas (M270, M157, and M267), and three differentiated gastric carcinomas (M206, M208, and M216). Methylated alleles were present predominantly in undifferentiated tumors. Only two patterns of mobility (methylated or unmethylated) occurred. MDA231 (MDA) and MCF-7 cell lines served as controls for the methylated and unmethylated alleles, respectively. M = methylated PCR products; U = unmethylated PCR products.
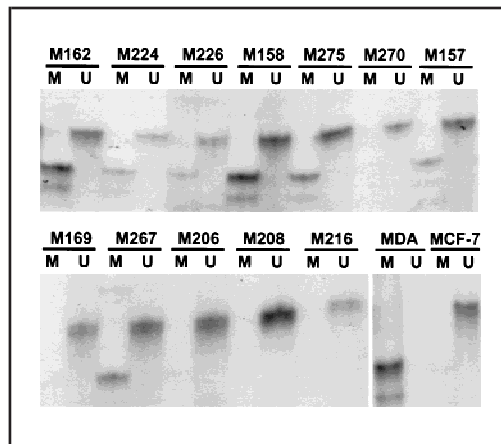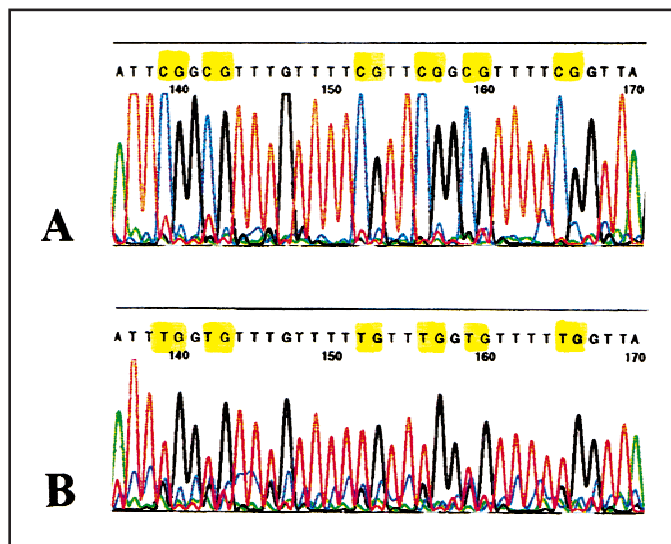

Fig. 2. Sequencing histograms (matching GenBank accession No. L34545) of entirely methylated (**A**) and unmethylated (**B**) E-cadherin alleles from a primary human gastric carcinoma (M162). All CpG sites were methylated in panel A; all CpG sites were unmethylated in panel B, resulting in the sequence "TpG" after bisulfite treatment.

Fig. 3. Western blot analysis of expression of the 120-kilodalton (kDa) E-cadherin protein in three tumor samples, including two undifferentiated-scattered tumors (M162 and M275) and one differentiated tumor (M276) exhibiting loss or reduction of E-cadherin expression associated with hypermethylation, and matching normal tissues. Additional bands (approximately 150 kDa) are nonspecific and have been reported on other western blots *(15)*. T = tumor; N = normal tissue.

E-cadherin expression in 10 (83%) of 12 tumor tissues.

DISCUSSION

Our observations demonstrate that E-cadherin promoter hypermethylation occurs frequently in primary gastric carcinomas, especially in tumors of the undifferentiated-scattered histologic type, and is associated with diminished expression of E-cadherin. Our findings corroborate previous immunohistochemical studies of E-cadherin in primary gastric carcinomas *(21–23)*, in which reduction of E-cadherin expression was observed in 54%–100% of undifferentiated carcinomas. However, these previous reports did not evaluate E-cadherin promoter hypermethylation. Among these reports, Oka et al. *(21)* studied Japanese patients with gastric carcinoma and found reduced E-cadherin expression in 46 (85%) of 54 undifferentiated tumors and in 18 (30%) of 61 differentiated tumors, rates actually similar to the rates of hypermethylation reported in our study.

Inactivation of the E-cadherin gene via the classic two-hit mechanism is most prevalent in gastric carcinomas of the undifferentiated-scattered type, occurring in nearly half of tumors of this type *(3,5)*. The high prevalence of hypermethylation and the reduced expression of E-cadherin, coupled with this high historic mutation rate, strongly suggest that inactivation of E-cadherin plays a dominant role in the development and/or progression of diffuse/undifferentiated gastric carcinomas. However, because disruption of the E-cadherin-mediated cell adhesion system may occur by other mechanisms, it is possible that genetic and epigenetic changes in other molecules, such as α- and β-catenins, are also involved *(2)*.

scattered and one differentiated tumor (Fig. 3). E-cadherin was expressed in one tumor of the undifferentiated-adherent type and in one signet-ring cell carcinoma of the undifferentiated-scattered type, even though a hypermethylated allele was present. In contrast, all six unmethylated tumors expressed similar amounts of E-cadherin compared with their matching normal tissues, regardless of histology. Specifically, two unmethylated carcinomas of the undifferentiated-scattered type expressed amounts of E-cadherin similar to those expressed by their corresponding normal tissues. Therefore, methylation status was predictive of
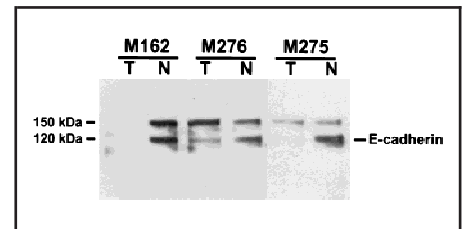
Moreover, precise E-cadherin promoter regions affected by DNA hypermethylation have not been extensively studied. Graff et al. *(14)* studied time-dependent *de novo* methylation of E-cadherin CpG islands *in vitro* and observed that methylation originating in either flanking region spread with continued cell passage to eventually include the central region of highest CpG density, probably because it was protected by Sp1 elements. It has been suggested that DNA methylation may differ among genes, tumor types, patients with the same tumor type, or even individual tumor cells within the same patient *(24)*. For example, intraindividual and interindividual heterogeneity of p15 gene promoter hypermethylation was reported in patients with acute myelogenous leukemia (AML) *(24,25);* in contrast, p16 gene CpG islands were either entirely methylated or unmethylated in malignant melanomas *(26)*. Results of MSP–SSCP and direct sequencing in our study proved the presence of entirely methylated but not partially methylated alleles in gastric carcinomas of the undifferentiated-scattered type, suggesting that partially methylated alleles were not present or did not clonally expand in these tumor samples. Our results are consistent with previous results in AML patients, showing a paucity of clonal expansion of partially methylated p15 alleles *(24),* and with other results, demonstrating uniform p16 methylation patterns in melanomas *(26)*. Nevertheless, it is possible that we failed to detect partially methylated alleles because our methylation-specific PCR primers did not amplify DNA samples in which the primer annealing site *per se* was partially methylated.

Recently, hypermethylation of the hMLH1 gene promoter associated with loss of gene expression has been reported in gastric carcinomas *(27,28)*. These findings were observed in invasive carcinomas but not in adjacent carcinoma *in situ* or dysplastic tissues, suggesting that hypermethylation occurred late in tumor development *(27)*. Furthermore, it has also been suggested that early hypermethylation of this gene may occur in a partial or in a monoallelic form, only later progressing to become complete or biallelic *(28)*. Finally, although the unmethylated status of hMLH1 generally is associated with expressed protein, some hypermethylated tumors expressed hMLH1 in previous studies *(27,28)*. Similarly, we found two E-cadherin-expressing tumors exhibiting complete promoter hypermethylation. We speculate that, in these specimens, a small population of hypermethylated tumor cells coexisted with unmethylated tumor cells. In fact, simultaneous coexistence of methylated and unmethylated E-cadherin alleles has been reported in tumor-derived cell lines *(15)*.

The timing of hypermethylation during tumor development may vary among different genes and tumor types. As described above, hypermethylation of hMLH1 was a late event, occurring in invasive gastric carcinomas but not in adjacent carcinomas *in situ (27)*. In contrast, hypermethylation of p16 has been reported in 75% of carcinomas *in situ* adjacent to squamous cell lung carcinomas *(29)*. Our study showed that the E-cadherin promoter is hypermethylated even in intramucosal carcinomas of the stomach and, therefore, may constitute an early event in gastric carcinogenesis.

In conclusion, the E-cadherin gene is inactivated by genetic and epigenetic mechanisms in the great majority of primary human gastric carcinomas of the undifferentiated-scattered type *in vivo*. In conjunction with historical data showing point mutations of E-cadherin in both germ-line DNA and somatic DNA of families and individual patients with gastric carcinoma, our results support the hypothesis that, overall, E-cadherin may represent one of the most definitive tumor suppressor genes thus far identified in the human stomach.

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