Establishment and Validation of Real-Time Polymerase Chain Reaction Method for *CDH1* Promoter Methylation

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Aberrant methylation of the promoter region has emerged as the major mechanism for silencing tumor suppressor genes. However, for some genes, such as E-cadherin (*CDH1*), methylation and protein expression demonstrate considerable heterogeneity, making correlations difficult. We compared methylation and protein expression status of *CDH1* in 56 primary breast carcinomas using semiquantitative assays. Aberrant *CDH1* methylation was studied by methylation-specific polymerase chain reaction (MSP) and semiquantitative real-time MSP assays. The *Cdh1* expression was investigated by immunostaining on archival formalin-fixed sections from 34 primary carcinomas and their accompanying normal epithelium and preinvasive and metastatic lesions. Membrane-specific *Cdh1* expression in the neoplastic cells was quantified by image analysis using an automated cellular imaging system and a continuous score. Aberrant promoter methylation of the *CDH1* was present in 24 of 56 (43%) breast carcinomas by MSP assay. There was excellent concordance between the standard MSP assay and the real-time assay (91%, $P < 0.0001$). The concordance between loss of *Cdh1* expression and *CDH1* methylation by standard MSP was 71% ($P = 0.02$). Furthermore, there was a strong correlation between the semiquantitative assays for methylation and protein expression ($r = 0.47$, $P = 0.005$). We conclude that promoter methylation of *CDH1* significantly correlated with the *Cdh1* expression level, demonstrating that epigenetic silencing is a valid pathway for silencing of tumor suppressor genes in primary breast carcinomas. (*Am J Pathol* 2002, 161:629–634)

mediates homophilic, Ca^{2+} -dependent intercellular adhesion that is essential for the maintenance of normal tissue architecture.¹ Among the cadherin family membranes, reduced expression of *Cdh1* and *Cdh13* is common in a variety of human tumors^{2,3} including breast carcinoma^{4–9} and they are regarded as tumor suppressor genes. Inactivation of these genes are hypothesized to be an important step in the progression from tumor formation to invasion and metastasis.¹⁰ These genes and some other cadherin family members are located on the long arm of chromosome 16 (16q),¹¹ a chromosomal location that is frequently deleted in breast and other types of carcinomas.^{12–16}

On the basis of Knudson's¹⁷ "two-hit" hypothesis, both alleles of tumor suppressor genes have to be inactivated for loss of expression. Allelic loss of one copy is often associated with methylation, mutation, or some other form of inactivation of the second allele. Aberrant methylation of the promoter regions of tumor suppressor genes seems to be the major mechanism of gene silencing in human tumors.¹⁸ Aberrant methylation of the *CDH1* promoter region has been observed in cancers of the breast, liver, prostate, lung, and stomach.^{18–23} Some genes such as *CDH1* demonstrate considerable heterogeneity of methylation and protein expression.²⁴ *Cdh1* expression in primary human breast cancers reflects a heterogeneous and unstable pattern of promoter region methylation, which begins early before invasion.²⁴ Such plasticity renders correlations between methylation and expression difficult, especially when the standard nonquantitative methylation-specific polymerase chain reaction (MSP) assay is used.

In an effort to overcome some of these problems, we developed a semiquantitative real-time MSP assay and correlated our findings with the standard MSP assay. We also correlated it with a semiquantitative assay for protein expression using an image analyzer and immunostained sections.

Supported by an Early Detection Research Network grant (5U01CA8497102) from the National Cancer Institute, Bethesda, MD.

Accepted for publication May 15, 2002.

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The cadherins are a family of transmembrane glycoproteins expressed on the surface of epithelial cells and

Materials and Methods

Clinical Samples

Surgically resected specimens from 42 primary breast carcinomas, 38 cases of infiltrating ductal carcinoma, and 4 cases of infiltrating lobular carcinoma and 17 corresponding nonmalignant breast tissues from these patients were obtained from the Tumor and Tissue Repository at the Hamon Center. DNAs of 14 biopsy samples (13 cases of infiltrating ductal carcinoma and 1 case of infiltrating lobular carcinoma) of breast carcinoma were obtained from Senegal, Africa. The pathologist selected areas of viable appearing tissues consisting primarily of tumor cells. In general, the tumor tissues contained ~50% tumor cells, although the percentage varied considerably. Epithelial cells from buccal swabs were obtained from 10 healthy nonsmoking volunteers. Paraffin blocks and slides were available from 34 of the resection samples and these were used for immunostaining. Collection of all specimens was obtained in accordance with procedures approved by the Human Subjects Committees of the participating institutions.

DNA Extraction and Bisulfite Treatment

Genomic DNA was obtained from primary carcinomas and nonmalignant cells by digestion with proteinase K (Life Technologies, Inc., Grand Island, NY) for 24 hours at 37°C, followed by extraction with phenol:chloroform (1:1).²⁵ For bisulfite treatment, 1 µg of genomic DNA was denatured by NaOH and modified by sodium bisulfite, which converts all unmethylated cytosines to uracil while methylated cytosines remain unchanged.²⁶ The modified DNA was purified using a Wizard DNA purification kit (Promega, Madison, WI), treated with NaOH to desulfonate, precipitated with ethanol, and resuspended in water.

Standard MSP Assay

Aberrant promoter methylation of *CDH1* was determined by the method of MSP assay as reported by Herman and colleagues²⁷ using primers specific for *CDH1*-methylated and -unmethylated sequences.²⁸ The polymerase chain reaction (PCR) amplicon of the methylated form encompassed nucleotides 945 and 1122 in the sequence of GenBank accession no. L34545. DNA from the lung cancer cell line NCI-H187 was used as a positive control for methylated alleles. Water blanks were included with each assay. PCR products were visualized on 2% agarose gels stained with ethidium bromide. Results were confirmed by repeating bisulfite treatment and MSP assays for all samples.

Semiquantitative Real-Time MSP Assay

Sodium bisulfite-treated genomic DNA was amplified by fluorescence-based real-time MSP by using TaqMan technology (Perkin Elmer Corp., Foster City, CA) as described previously.^{29,30} We performed the MSP with the Gene Amp

5700 Sequence Detection System (Perkin Elmer Corp.). In brief, oligonucleotide primers were designed to specifically amplify bisulfite-converted DNA within the promoter of the *CDH1* gene, and a probe was designed to anneal specifically within the amplicon during extension. For the internal reference gene, *MYOD1*, the primers and probe were designed to avoid CpG nucleotides. Thus, amplification of *MYOD1* occurs independent of its methylation status, whereas the amplification of *CDH1* is proportional to the degree of cytosine methylation within the amplicon. The methylation ratio was defined as the ratio of the fluorescence emission intensity values for the *CDH1* PCR products to those of the *MYOD1* PCR products, multiplied by 1000. This ratio was used as a measure for the relative level of methylated *CDH1* alleles in the particular sample. The sequences of the primers and probe used to amplify and detect methylated *CDH1* were 5'-AATTTTAGGTTA-GAGGGTTATCGCGT-3' (forward primer), 6FAM-5'-CGC-CCACCCGACCTCGCAT-3'-TAMRA (probe), and 5'-tc-cccaaacgaaactaacgac-3' (reverse primer). The amplicon encompassed nucleotides 842 and 911 in the sequence of GenBank accession no. L34545. The sequences of the primers and probe used to amplify and detect *MYOD1* were 5'-CCAACTCCAAATCCCCTCTCTAT-3' (forward primer), 6FAM-5'-TCCCTTCCTATTCTAAATCCAACCTA-AATACCTCC-3'-TAMRA (probe), and 5'-TGATTAATTGATTGGGTTTAGAGAAGGA-[primers]3 (reverse primer). Semi-quantitative real-time MSP assays were performed in a reaction volume of 25 µl by using components supplied in a TaqMan PCR Core Reagent Kit (Perkin-Elmer Corp.). Separate amplification assays were performed for *CDH1* and *MYOD1*; each assay was performed in duplicate. The final reaction mixtures contained the forward and reverse primers at 600 nmol/L each; the probe at 200 nmol/L; 200 µmol/L each of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate; 400 µmol/L deoxyuridinetriphosphate; 5.5 mmol/L MgCl₂; 1× TaqMan Buffer A; 1 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Corp.); and 3 µl bisulfite-converted genomic DNA. PCR was performed under the following conditions: 95°C for 12 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. We used DNA from NCI-H187 cells in which *CDH1* is methylated (positive control), DNA from NCI-H1395 cells in which *CDH1* is not methylated (negative control), and two wells that contained water instead of DNA (control for PCR specificity). We used serial dilutions of the positive control DNA to create a standard curve (1 to 1000 ng).

Immunohistochemistry and Automated Cellular Imaging

Immunostaining was performed at room temperature and performed on the DAKO Autostainer (DAKO, Carpinteria, CA). Reagents were used as supplied in the Envision Plus Detection Kit (DAKO). DAKO Target Retrieval Solution, pH 6.0, was used. Optimum primary antibody dilutions were predetermined using known positive control tissues. A known positive control section was included in

Figure 1. Representative examples of MSP assay in primary breast carcinoma. Results of testing for the unmethylated (*CDH1* U) and methylated forms (*CDH1* M) of the *CDH1* gene are illustrated. The unmethylated form of *CDH1* was run as a control for DNA integrity, and all samples yielded a strongly positive (++) band. The methylated bands demonstrated heterogeneity, with some being absent (-), weakly positive (+), or strongly positive (++) for the methylated form, the corresponding results of the semiquantitative ratio of the real-time MSP assay are provided. P, positive control; N, negative control (water blank).

each run to assure proper staining. Staining was performed using a DAKO Autostainer.

Evaluation of immunostaining was performed using a recently developed system of image analysis, the Automated Cellular Imaging System (ACIS) (ChromaVision Medical Systems, Inc., San Juan, CA)³¹ according to the manufacturer's instructions. The ACIS system consists of an automated robotic bright-field microscope module, a computer, and a Windows NT-based software interface. The robotic microscope module scans the immunohistochemically stained slides, and a computer monitor displays the digitized tissue images. The ACIS system, as described on the manufacturer's website (www.chromavision.com), is able to distinguish cell membrane staining from cytoplasmic staining, using so-called color-space transformation proprietary technology. Only tumor cells were selected for analysis by image analysis. An average score for 10 selected areas most positive for immunostaining was calculated for each sample, and a continuous score generated for the entire sample set.

Data Analysis

Statistical differences between groups were examined using Fisher's exact tests. The quantitative ratios of different groups were compared using the Mann-Whitney *U* nonparametric test. Correlation value was analyzed by the Pearson correlation test. All statistical tests were two-sided. For all tests, probability values of <0.05 were regarded as statistically significant.

Results

Standard MSP Assay

MSP assay for *CDH1* was performed in 56 infiltrating breast carcinomas (51 ductal and 5 lobular) and 17 corresponding nonmalignant breast tissues and 10 samples of buccal mucosa. The representative MSP assay examples were illustrated in Figure 1. In breast carcinoma samples, which consisted of mixtures of tumor cells and nonmalignant cells, either only the unmethylated band

Figure 2. The relationship between quantitative ratio by semiquantitative real-time MSP assay and standard MSP assay. MSP results were classified into three groups based on the intensity of MSP product: negative (-, white circle), relatively weak positive (+, gray circle); and relatively strong positive (++, dark gray circle). Dotted lines indicate the mean of each group. *n* = sample number. Values of 0.01 really represent completely negative samples.

was present or both methylated and unmethylated bands were present. The presence of unmethylated *CDH1* promoter sequences in all of the tissues analyzed reflected the presence of contaminating nonmalignant cells and confirmed the integrity of the DNA in these samples.

Fifty-six breast cancer specimens were analyzed by standard nonquantitative MSP assay. Of these 24 (43%) were positive. The various intensity of the bands demonstrated heterogeneity, and 12 (21%) of the tumors gave a relatively weak (+) band and 12 (21%) gave a strong (++) band. Of interest, all 5 infiltrating lobular carcinomas (100%) and 19 of 51 (37%) of infiltrating ductal carcinomas were methylated ($P = 0.02$). Seventeen corresponding adjacent nonmalignant tissues were available and of these two, 12% gave weak bands. There was no methylation detected in DNAs from the buccal mucosa ($n = 10$) of healthy nonsmoking volunteers. Examples of the bands are presented in Figure 1.

Real-Time MSP Assay

All of the 56 available breast cancer specimens were analyzed by semiquantitative real-time PCR. No fluorescent signal could be detected in 14 (25%) samples. Of the 42 samples that gave signals, the values ranged from 0.1 to 24.1 (median, 2.7; mean, 5.5).

Correlation between Standard and Real-Time MSP Assays

We correlated the results of the two MSP assays (Figure 2). The mean value (by real-time MSP assay) of the 32 samples scored negative by the standard MSP assay was 0.4, the mean value of the 12 samples scored as weak positive was 5.3, and the mean value of the 12 samples scored as strong positive was 12.8. The mean

Figure 3. Immunostaining of Cdh1 protein in breast carcinomas and adjacent tissues. **A:** Normal ducts showing uniform strong positive Cdh1 immunostaining. **B:** Normal duct showing strong immunostaining, whereas duct with apocrine metaplasia (arrow) shows decreased staining. **C:** Normal ducts showing loss of expression except for one focus of cells (arrow). **D:** Homogeneously strong positive staining in ductal carcinoma *in situ*. **E:** Clusters of metastatic ductal carcinoma cells in afferent lymphatics of an axillary lymph node showing heterogeneous expression (arrow). **F:** Completely Cdh1-negative lobular carcinoma. Normal ducts (arrows) surrounded by malignant cells also show loss of expression. *CDH1* methylation status by standard MSP assay was negative in the corresponding tumors of the samples illustrated in **A**, **B**, and **D** and positive in samples illustrated in **C**, **E**, and **F**. Numbers below figures indicate sample identification number. Original magnifications: $\times 200$ (**A**, **F**); $\times 400$ (**B**, **C**); $\times 600$ (**E**); $\times 800$ (**D**).

values of the three groups were significantly different (Figure 2).

Of the 32 samples scored negative by standard MSP assay, 14 samples lacked a fluorescent signal and were scored negative by real-time MSP assay. Of the remaining 18 samples, the mean value was 0.7, and the mean value for the whole group was 0.4. Based on our results we selected a value of 1.0 as the cutoff between methylation-negative and -positive samples. Using this value, the concordance between the two assays was 91%.

Loss of Cdh1 Expression by Immunostaining

Immunostaining for Cdh1 was performed on a subset of 34 primary breast carcinoma samples and their adjacent nonmalignant tissues and lymph node metastases, when available. Although all of these samples were examined microscopically, only the primary tumors were scored by image analysis.

Immunostaining was limited to epithelial cells (normal, premalignant, malignant). Stromal cells, including lymphocytes and blood vessels, were completely negative. Of the 34 samples, the majority of the samples (20, 59%) demonstrated heterogeneity of staining in the primary

tumor, and 12 (35%) were homogeneously negative, including all 4 (100%) of infiltrating lobular carcinomas. Only two samples (6%) demonstrated strong staining throughout the tumor. The corresponding *in situ* components and lymph node metastases also showed heterogeneity. The heterogeneity of staining indicated the need for a quantitative scoring system based on both the staining intensity and the percentage of positive cells. In general, the normal ducts and lobules demonstrated intense uniform staining when the tumor stained homogeneously, but showed focal loss of staining when the tumor demonstrated heterogeneity. Ducts in the vicinity of negatively stained tumors usually also demonstrated a negative pattern. Of interest, foci of hyperplasia and apocrine metaplasia stained less intensely and less uniformly than the corresponding normal ducts. Examples of these staining patterns are demonstrated in Figure 3.

Using the ACIS image analysis system, Cdh1 immunostaining was scored as a continuous variable with values within the range 0 to 5 Cdh1 staining index units. The range of the 34 test samples was 0 (12 samples) to 2.7, and the mean value of all samples was 0.86. We correlated the protein expression values with degree of methylation as determined by real-time PCR (Figure 4). There

Figure 4. The correlation of Cdh1 staining index (CSI) and by semiquantitative real-time MSP assay (quantitative ratio).

was a inverse correlation between these two sets of values, with a r value of 0.47 and a P value of 0.005. However, four breast cancers were completely negative for both methylation and protein expression.

Discussion

CDH1 is a potent invasion/tumor suppressor of breast cancer, and partial or complete loss of expression has been found to correlate with poor prognosis in breast cancer patients.³² Cdh1 is inactivated in a high percentage of breast cancers, especially lobular carcinomas. The *CDH1* gene is located on human chromosome 16q22.1, a region frequently demonstrating allelic loss in sporadic breast cancer,³³ thus providing a mechanism of inactivation of the second allele. Because methylation of *CDH1* is a dynamic, plastic process affected by a variety of local conditions,²⁰ down-regulation of its protein product is frequently heterogeneous, complicating interpretation of expression and its correlation with clinicopathological parameters. Variations in the overall level of methylation may be the result of 1) a variable degree of methylation in the promoter sequence of the gene; 2) mixtures of methylated and unmethylated tumor cells; and 3) varying percentages of nontumor and tumor cell mixtures. In addition, protein expression may be down-regulated by mechanisms other than methylation.

There was an excellent correlation between the non-quantitative standard MSP assay and the semiquantitative real-time PCR assay. The wide range of positive values by the real-time PCR assay reflected the known heterogeneity of expression, a finding that was confirmed by immunostaining. There was a significant inverse relationship between real-time PCR and image analysis results. Although methylation seemed to be the major mechanism of gene silencing, other mechanisms including gene mutations (especially in lobular carcino-

mas)^{33–38} or via the transcription repressor factor Snail³⁹ may play a role.

Our semiquantitative real-time MSP and image analysis assays may help interpret the dynamic and heterogeneous process of methylation and its relationship to protein expression.

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