E-Cadherin Expression Is Silenced by DNA Hypermethylation in Human Breast and Prostate Carcinomas


Abstract

Expression of the Ca⁺⁺-dependent, homotypic cell:cell adhesion molecule, E-cadherin (E-cad), suppresses tumor cell invasion and metastasis in experimental tumor models. Decreased E-cad expression is common in poorly differentiated, advanced-stage carcinomas. These data implicate E-cad as an "invasion suppressor" gene. The mechanism by which E-cad is silenced in advanced stage carcinomas is unclear. In this report, we show that: (a) the 5' CpG island of E-cad is densely methylated in E-cad-negative breast and prostate carcinoma cell lines and primary breast carcinoma tissue but is unmethylated in normal breast tissue; (b) treatment with the demethylating agent, 5-aza-2'-deoxycytidine, partially restores E-cad RNA and protein levels in E-cad-negative breast and prostate carcinoma cell lines; and (c) an E-cad promoter/CAT construct is expressed in both E-cad-positive and -negative breast and prostate carcinoma cell lines, indicating that these cells have the active transcriptional machinery necessary for E-cad gene expression. Our data demonstrate that frequent loss of E-cad expression in human breast and prostate carcinomas results from hypermethylation of the E-cad promoter region.

Introduction

E-cad is a Mr 120,000 glycoprotein that, when complexed with α-, β-, and γ-catenins, mediates Ca⁺⁺-dependent, homotypic cell:cell adhesion (1). Loss of E-cad expression and the subsequent loss of homotypic cellular adhesiveness may be a critical step in the ability of epithelial tumor cells to invade and metastasize. E-cad expression is decreased or absent in poorly differentiated carcinomas of the breast, prostate, colon/rectum, stomach, esophagus, kidney, pancreas, liver, thyroid, and ovary and in squamous cell carcinomas of the head and neck (2–11). In breast and prostate carcinoma, loss of E-cad is related to tumor aggressiveness (2, 3). In experimental tumor models, restored expression of E-cad promotes epithelial differentiation characteristics and inhibits invasion in vitro and in vivo, implicating E-cad as an "invasion suppressor gene" (12–14).

The mechanism by which E-cad expression is lost is presently unclear. Allelic loss of the E-cad gene locus (16q22) has been reported in 30–50% of breast, prostate, and hepatocellular carcinomas (11, 15, 16). Extensive analyses reveal that mutations within the E-cad coding sequence in breast, gastric, and gynecological cancers are rare (17–19). Additionally, in human mammary epithelial cells, expression of the E-cad gene can be down-regulated by overexpression of the c-erb-B₂ proto-oncogene cDNA (20). Alternatively, aberrant hypermethylation of 5' CpG islands within proximal promoter regions has been implicated as a mechanism by which tumor suppressor genes can be inactivated. Examples of this mechanism have been best demonstrated for the VHL and p16 tumor suppressor genes (21, 22). Indeed, a CpG island has been identified within the 5' proximal promoter region of the E-cad gene (23), and one recent study of colon, liver, bladder, and gastric carcinoma cell lines has correlated aberrant hypermethylation of HpaII sites in this CpG island with decreased E-cad expression (24). In this report, we have investigated, in detail, whether E-cad expression in breast and prostate carcinomas might be silenced by aberrant hypermethylation of the 5' CpG island within the proximal promoter region. We now show that hypermethylation of the E-cad 5' CpG island accounts for loss of this "invasion suppressor" molecule in cells from breast and prostate carcinomas.

Materials and Methods

Cell Culture and Treatment. All cell lines were cultured in 80-cm² tissue culture flasks (Nunc) or 10-cm² tissue culture plates (Sarstedt). Breast cancer cell lines (T47D, MDA-MB-468, MDA-MB-231, MCF-7, MCF-7/ADR, ZR-75-1, Hs578t, and HBL100) were routinely cultured in DMEM (GIBCO-BRL, Bethesda, MD) supplemented with 5% FCS. Prostate cancer cell lines (DuPro, Du145, PC-3, LNCaP, TSUPrl, and FNC) were cultured in RPMI (GIBCO-BRL) supplemented with 10% FCS. Selected cell lines were treated with a final concentration of 0.5 µM AzaC for 3 days (Sigma Chemical Co., St. Louis, MO).

Restriction Digests and Southern Blot Analysis. Genomic DNA (5–8 µg) was isolated, electrophoresed, transferred to Zetaprobe nylon membrane (Bio-Rad, Richmond, CA), and hybridized as described previously (21, 22). Fifteen units of each enzyme were used/µg genomic DNA. All restriction enzymes were purchased from New England Biolabs (Cambridge, MA).

To generate a DNA probe for Southern blotting, a 769-bp PCR fragment corresponding to nucleotides 403–1172 of the E-cad proximal promoter region (25, 26) was amplified from normal fibroblast DNA using the following primers and conditions: 5'-GGAGGCCAAGGCAGGAGGATCGC-3' (upstream); 5'-CGAGAGGCGAGCGGAGGATCGC3' (upstream); 5'-CGAGAGGCTGGCGGCTCAAGGG-3' (downstream); 95°C for 35 s, 68°C for 45 s, and 72°C for 1 min for 40 cycles. The 769-bp amplified product was then subcloned into the TA PCR cloning vector as per the manufacturer's instructions (Stratagene, La Jolla, CA). A 377-bp PstI fragment prepared from the amplified product, which encompasses the active 5' proximal promoter (Fig. 1; Ref. 25), was used as the probe for all Southern blots (Fig. 1).

Western Blot Analysis. Western blots were performed as described previously (27) using 50 µg protein/lane. The mouse monoclonal anti-E-cad antibody, HECD, was purchased from Zymed (San Francisco, CA). A 377-bp PstI fragment prepared from the amplified product, which encompasses the active 5' proximal promoter (Fig. 1; Ref. 25), was used as the probe for all Southern blots (Fig. 1).
samples were analyzed, including two samples from breast carcinoma.

Results

Fig. 1 provides a methylation-sensitive restriction map of the 1500-bp CpG island extending from the promoter region through intron 1 of the E-cad gene. To assess whether methylation of the E-cad CpG island may be related to expression, we mapped the methylation-sensitive restriction sites in breast and prostate carcinoma cell lines, normal breast tissue, and primary breast carcinoma (Fig. 2 and Table 1). We first matched methylation patterns of the E-cad promoter with well-documented expression profiles of E-cad in established breast and prostate cancer cell lines (29, 30). The E-cad-positive breast and prostate cancer cell lines are unmethylated at each site tested within the 5' CpG island (breast: MCF-7, T47D, and ZR-75-1; prostate: PC-3, LnCaP, and Du145). In contrast, all but one of the E-cad-negative breast and prostate cancer cell lines are methylated at each site examined (breast: MCF-7/ADR, MDA-MB-231, MDA-MB-435, Hs578T, and HBL 100; prostate: DuPro, TSUPr1, and FNC; Fig. 2, A and B; summarized in Table 1). MDA-MB-468, a breast cancer cell line with reduced E-cad expression despite having an unmethylated promoter, is discussed below.

To ensure that the E-cad CpG island is not methylated in DNA from normal tissue and that the above findings are not limited to cultured cells, the methylation status of the E-cad CpG island was determined for DNA isolated from normal breast tissue and primary breast cancer tissue. DNAs isolated from five normal breast tissue samples were unmethylated at all sites examined within the CpG island (summarized in Table 1). Changes in methylation, however, could be detected by Southern analysis of DNA isolated from primary tumor tissue samples, although these samples were not enriched for tumor versus normal cells. A total of 12 breast carcinoma tissue samples were analyzed, including two samples from breast carcinoma.

Metastases. The majority of these 12 samples were analyzed at the HhaI, SacII, and EagI sites depicted in Fig. 1 (summarized in Table 1). Each sample had prominent bands, resulting from completely unmethylated sites which may be attributed to associated normal tissue within the samples. However, many samples revealed distinct methylation at the HhaI site (55%; Fig. 2C), at the SacII site (45%; SacII site; Fig. 2D), and the EagI site (67%). Methylation at the HhaI site was also evident in a primary prostate tumor sample (PT-i; Fig. 2C; Table 1) previously shown not to express E-cad (3). These data demonstrate that hypermethylation of the E-cad CpG island is tumor specific and is not limited to cultured cells.

Transient Transfection Analysis. The promoterless CAT reporter construct and designated E-cad/CAT. One μg pR5V-BGal and 2 μg pRSV-CAT (positive control), pBLCAT3 (promoterless CAT as a negative control) or E-cad/CAT was transfected into cells with lipofectamine reagent as per the manufacturer’s recommendations (GIBCO-BRL). Cells were harvested 48 h after removal of lipofectamine. βGal activity was measured using a βGal activity kit (Promega, Madison, WI). CAT activity was measured as described previously (28). All CAT activity values were normalized to βGal activity for each plate.

If methylation of the E-cad CpG island is responsible for suppressing E-cad expression, treatment of the breast and prostate cancer cell lines with the demethylating agent, AzaC, might reexpress active expression. Treatment of cell lines with AzaC reactivates the expression of the VHL and p16 tumor suppressor genes for cell lines in which the 5' CpG islands are methylated (21, 22). Slight reactivation of E-cad expression was evident in the methylated breast cancer cell lines, Hs578T and MDA-MB-231, as evidenced by low levels of E-cad protein detected by Western blots only after AzaC treatment of cells (Fig. 3). Greater activation was detected for the methylated prostate cancer cell line, TSUPr1 (Fig. 3). Prostate cancer cell line DuPro, which is also methylated, expressed low basal levels of E-cad protein but much higher levels after AzaC treatment of cells (Fig. 3). Partial reactivation in the same cell lines was also confirmed by reverse transcription-PCR and by FACS analysis for one prostate and one breast cancer cell line (for DuPro, less than 1% of cells were positive prior to AzaC treatment, whereas 14% of cells were positive after AzaC treatment; for Hs578T, 1.5% of cells were positive prior to AzaC treatment, whereas 15% were positive after AzaC treatment). E-cad gene reactivation could also be clearly detected by indirect immunofluorescence. Surface localization of E-cad was evident in numerous clusters of cells from MDA-MB-231, Hs578T, TSUPr1 (data not shown), and DuPro (Fig. 3) after a 3-day treatment with AzaC. The level of demethylation at the HhaI site, as quantitated by phosphoimaging scanning of blots, ranged from 11% in Hs578T, where only minimal protein was detected (Fig. 3), to 45% in DuPro, in which E-cad expression was sharply increased after AzaC treatment (Fig. 3). AzaC treatment of the E-cad-positive cell lines
Methylation Silences E-Cadherin Expression

MCF-7 and PC-3, which are unmethylated, did not affect E-cad expression as evidenced by FACS analysis, Western blot analysis, reverse transcription-PCR, or indirect immunofluorescence (data not shown).

Aberrant hypermethylation of CpG islands is thought to silence transcription by altering local chromatin structure and preventing the binding of transcription factors to their respective promoter elements. In this manner, aberrant hypermethylation may block transcription without affecting the expression or activity of transcription factors (31, 32). If aberrant hypermethylation is responsible for silencing E-cad expression in this manner, then the cell lines with methylated E-cad CpG islands should retain the active transcriptional machinery necessary for E-cad gene expression. To test this hypothesis, a CAT reporter gene construct driven by the well-characterized murine E-cad proximal promoter region, known to contain maximal activity in both murine and human cells (26), was transfected into two prostate cell lines, DuPro and PC-3, and three breast cancer cell lines, Hs578t, MCF-7/Adr, and MDA-MB-468/Adr. The MCF-7/Adr, Hs578t, and MDA-MB-468/Adr cell lines express high levels of E-cad/NEO oncoprotein (R. X., and P. P.; data not shown), which has been shown to down-regulate transcription of the E-cad gene via decreased transcription factor activity (20). Hence, the mechanism associated with reduced expression of E-cad in this line should be different from that in the others.

As shown in Fig. 4, MCF-7, PC-3, DuPro, and Hs578t express substantial CAT activity, whereas MDA-MB-468 does not. Interestingly, the methylated cell lines, DuPro and Hs578t, express slightly less (2-fold) CAT activity than the unmethylated lines, MCF-7 and PC3. However, this reduction cannot solely account for the vast differences in expression of the endogenous E-cad gene between these cell lines. That the methylated cell lines do not express E-cad endogenously, but are able to express CAT activity from the exogenous E-cad/CAT construct, implies that methylation acts in cis to silence
Table 1 Mapping of the methylation-sensitive restriction sites in the E-cad 5' CpG island

<table>
<thead>
<tr>
<th>E-cad 5' CpG island</th>
<th>Tha1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HpaI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HpaII&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SacI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>EagI&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>LNCaP&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> ND, not determined; M, methylated sites; P, partially methylated sites; U, the presence of unmethylated sites only.

<sup>b</sup> Cell lines that express E-cad.

The flanking restriction for ThaI1, HhaI, and HpaII was PstI. The flanking restriction for SacI and EagI was EcoRI. All blots were probed with the 377-bp PstI fragment representing the active promoter region of E-cadherin (26). N1 through N5 are normal breast tissue samples. BT1 through 10 are independent primary breast carcinoma tumor samples. MBT1 and 2 are independent metastatic breast tumor samples. MBT1 and T2 are unrelated to BT1 and 2. PT-1, E-cad-negative prostate tumor tissue sample (3).

Discussion

Data in this report demonstrate that loss of expression of the E-cad gene, which encodes an invasion suppressor protein for which loss is integral to the progression of carcinomas, is accompanied by aberrant methylation of a 5' promoter region CpG island in human breast and prostate carcinoma cells. Methylation of the E-cad CpG island correlates not only with a lack of expression in breast and prostate carcinoma cell lines (Table 1) but also is evident in more than 50% of primary breast carcinomas throughout the entire E-cad 5' CpG island (Table 1). The direct involvement of hypermethylation in the suppression of E-cad gene expression is supported by the observation that expression of the E-cad gene can be reactivated by treatment with the demethylating agent, Azac, in four carcinoma cell lines. Finally, these cell lines, which do not express an endogenously methylated E-cad gene, can substantially utilize an E-cad promoter/CAT construct, indicating that they retain much of the active transcriptional machinery required for E-cad expression. This latter finding further implicates methylation of the endogenous promoter as a functional, cis-acting block to expression. However, our data cannot rule out the additional possibility that trans-acting mechanisms work in concert with methylation to suppress E-cad gene expression. Indeed, the
prostate cancer cell line TSUP1, which has a fully methylated E-cad CpG island, is unable to express a similar E-cad promoter CAT construct (25) but can express E-cad following AzaC treatment (Fig. 3).

As we were completing our current study, Yoshira et al. (24) reported similar data, indicating that hypermethylation of HpaII sites in the E-cad promoter is associated with loss of E-cad expression in colon, bladder, liver, and gastric carcinoma cell lines. Taken together, these two reports demonstrate that hypermethylation of the CpG island within the 5′ proximal promoter is a prevalent mechanism by which E-cad expression is inactivated in multiple types of carcinoma. This report provides further evidence that aberrant DNA hypermethylation critically alters gene expression patterns in cancers, thereby promoting tumor progression. Finally, these data indicate that loss of E-cad expression from tumor cells, a critical step in the acquisition of invasiveness and the subsequent metastatic spread of carcinomas, results from the potentially reversible process of aberrant DNA methylation. These findings suggest a potential therapeutic strategy for preventing or reducing the metastatic dissemination of epithelial tumors.

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References

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