Transcriptional Defects Underlie Loss of E-Cadherin Expression in Breast Cancer

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Abstract

Decreased expression of E-cadherin (E-cad), a calcium-dependent cell adhesion molecule, has been seen in many different epithelial cancers. Although somatic mutations in the E-cad gene have been identified in a small subset of tumors, in the majority of cancers, the mechanisms underlying loss of E-cad expression are poorly understood. We have cloned the human E-cad promoter and defined its critical components in functional assays. In eight human breast cancer cell lines, there was a striking correlation between endogenous E-cad gene expression and the E-cad promoter activity observed following the introduction of reporter gene constructs into the lines. These and other observations suggest that defects in trans-acting pathways regulating E-cad expression are the primary basis for the loss of its expression in most breast cancers. The results have significant implications for understanding the gene expression differences that underlie tumor heterogeneity and progression events in breast and other epithelial cancers.

Introduction

E-cad, a calcium-dependent transmembrane protein of roughly M, 120,000, regulates epithelial cell-cell interactions at specialized regions of the plasma membrane called adherens junctions (1, 2). The function of E-cad depends critically upon its ability to link to the submembrane cytoskeletal matrix through its interactions with other proteins, such as α-, β-, and γ-catenin/plakoglobin (1–5). Alterations in the structure or expression of E-cad or the catenins have been found to promote aberrant cell-cell interactions in vitro (1, 2, 5, 6). Decreased or undetectable levels of E-cad expression have frequently been seen in immunohistochemical studies of many different epithelial cancers (reviewed in Ref. 2). In some cancers, loss of E-cad expression has been associated with the loss of differentiated features in tumor cells and/or increased propensity of the cells to invade and metastasize to distant sites. In addition, the restoration of E-cad expression following E-cad gene transfer has been shown to inhibit the invasive and metastatic properties of the cells in in vitro and animal model systems (7–9).

The E-cad gene is located on chromosome 16q in a region that is frequently affected by allelic loss in several cancer types (10–13). Somatic mutations in the E-cad gene have been identified in more than 30% of gastric cancers of diffuse subtype, about 5–10% of endometrial and ovarian cancers, and about 5–10% of breast cancers, particularly those of lobular type (2, 14–17). The mutations identified include missense, nonsense, and splice mutations, as well as deletions. Nevertheless, in the majority of cancers in which E-cad expression is altered, the mechanisms accounting for its reduced or absent expression are poorly understood.

Two recent studies have presented apparently discordant conclusions on the mechanisms underlying loss of E-cad expression in cancer. A study by Graff et al. (18) concluded that E-cad expression was silenced in breast and prostate cancers by hypermethylation of the E-cad promoter sequences, whereas the findings of Hennig et al. (19) implied that the silencing of E-cad promoter activity in several different cancer types was due to loss of factor binding and/or chromatin rearrangement in the regulatory region. We report here the results of our studies to address the mechanisms underlying the loss of E-cad expression in breast cancer. Our findings of the human E-cad promoter suggest that defects in trans-acting pathways regulating E-cad gene expression are the primary mechanisms underlying loss of E-cad expression in breast cancer.

Results

Analysis of Human E-cad Promoter Activity. Previous studies have identified the transcription start sites for the murine and human E-cad genes (20, 21). In addition, several elements in the murine E-cad gene that regulate its expression in epithelial cells have been defined, including a 5′ promoter region, located within the 100-bp region immediately upstream of the transcription start site, and an enhancer region in the first intron (19, 20, 22, 23). Within the murine 5′ promoter region, a CCAAT-box and two candidate AP-2 binding sites in a GC-rich region have been characterized. In addition, a 12-bp palindromic element, located in the 5′ promoter region and called E-Pal, appears to be critical in directing epithelial-specific expression of E-cad (22, 23). Although the GC-rich region and CCAAT box are well con-

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3 The abbreviations used are: E-cad, E-cadherin; AzaC, 5′-aza-2′deoxy- cytidine; Luc, luciferase; CMV, cytomegalovirus.
served in the human E-cad promoter region, the E-pal element is less well conserved (Ref. 21 and data not shown).

In an effort to further define the elements in the human E-cad promoter that are responsible for its transcriptional activity, we generated a panel of reporter gene constructs in which human E-cad 5' flanking sequences of various extents were cloned upstream of the firefly Luc gene (Fig. 1A). We then characterized the Luc activities generated by these constructs following their transfection into MCF-7 and MDA-MB-361 cells, two breast cancer cell lines with high levels of endogenous E-cad expression. Similar activity profiles were obtained with the panel of constructs following transfection into each of the cell lines, although only the results for the MCF-7 cell line are shown in Fig. 1B. The constructs Ecad3/Luc, Ecad4/Luc, Ecad5/Luc, and Ecad6/Luc all generated Luc activities much greater than the reporter construct lacking any insert (i.e., pGLBasic/Luc; Fig. 1B). The activities of most E-cad reporter constructs were even greater than the activity of a positive control vector containing the major late promoter of adenovirus (pMLP/Luc). The Ecad2/Luc vector lacks the E-cad transcription start site, and as expected, it failed to yield detectable levels of Luc following transfection (Fig. 1B). We also found that the Ecad7/Luc construct had weak activity. This observation implied that although the CCAAT and GC-rich elements present in the 70-bp region upstream of the E-cad transcription start site were sufficient for promoter activity, the elements conferred relatively weak activity compared to those present in constructs containing more 5' E-cad sequences. We also noted that an E-cad reporter construct containing roughly 2.5 kb of E-cad 5' flanking sequences had very reduced promoter activity (data not shown), suggesting that inhibitory elements may be present upstream of the more proximal E-cad promoter elements.

Activity of E-cad Promoter Constructs Parallels Endogenous Gene Activity. Studies were undertaken with a subset of the Ecad/Luc constructs in a panel of eight breast cancer cell lines to further assess the relationship between reporter gene activity and endogenous E-cad expression in the lines. As noted above, the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs had considerably more activity than the Ecad7/Luc construct in MCF-7 cells and MDA-MB-361 cells. As shown in Fig. 2, these findings were confirmed and extended to an additional breast cancer cell line with moderate to high levels of endogenous E-cad expression (BT474; Ref. 16). However, in four breast cancer lines lacking endog-
enous E-cad expression and without evidence for mutational inactivation of the E-cad gene (BT549, HS578t, MDA-MB-231, and MDA-MB-435S; Ref. 16), the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc vectors had considerably less activity (Fig. 2).

SKBR3 cells lack E-cad expression because a substantial portion of the E-cad gene is affected by homozygous deletion (16). Given that the homozygous deletion in SKBR3 is clearly sufficient for complete inactivation of E-cad, the pathways and transacting factors regulating E-cad promoter activity might be expected to be intact in SKBR3. Consistent with this prediction, we found that transfection into the SKBR3 cell line, we found that the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs all had considerably greater activity than the Ecad7/Luc construct (Fig. 2). Additional studies comparing the activities of the Ecad3/Luc and Ecad5/Luc constructs with that of a control CMV-Luc vector further established that the E-cad promoter activities closely paralleled endogenous E-cad activity in the eight cell lines (Fig. 3). In toto, our studies indicate that trans-acting factors that interact with sequences between 70 and 277 bp upstream of the E-cad transcriptional start site are likely to be critical in regulating E-cad expression in breast epithelial cells. Defects in the expression or regulation of one or more of these transacting factors appear to be a major contributor to the loss of E-cad expression in a number of breast cancer cell lines.

**AzaC Treatment Fails to Reactivate E-cad Expression.**

As discussed above, the previous studies of Graff et al. (18) had shown that hypermethylation of the E-cad proximal promoter region was correlated with decreased E-cad expression in a panel of breast and prostate cancer cell lines. In addition, these authors reported that treatment of selected breast and prostate cancer cell lines with the demethylating agent AzaC reactivated E-cad expression. In particular, using Western blot and immunofluorescence studies, the authors found minimal reactivation of E-cad expression in the MDA-MB-231 and HS578t breast cancer cell lines following exposure of the cells to 0.5 μM AzaC for 3 days (18).

We sought to assess E-cad expression by Western blot analysis in five E-cad-negative breast cancer cell lines following exposure of the cells to various levels of AzaC. All five lines lacked detectable E-cad mutations (16). As shown in Fig. 4, we failed to detect E-cad expression by Western blot analysis in any of the E-cad-negative breast cancer cell lines treated with 1 or 3 μM AzaC for 5 days, including the two breast lines (i.e., MDA-MB231 and HS578t) studied by Graff et al. Two factors complicate definitive interpretation of the effects of AzaC treatment on endogenous E-cad expression. First, our Western blot analysis may have been somewhat less sensitive than the Western blot and immunofluorescence studies of Graff et al. (18). Second, similar to the findings of Graff et al. (18), we found that AzaC treatment had essentially no detectable effects on the methylation status of the proximal E-cad promoter in the breast cancer cell lines (data not shown). Although these caveats should be borne in mind, our results clearly demonstrate that E-cad expression cannot be reactivated to the levels seen in E-cad-positive breast cancer cell lines by brief AzaC treatment (Fig. 4).

**Factors Regulating E-cad Promoter Activity.**

Although our transfection studies with unmethylated report gene constructs indicate that trans-acting defects are likely to be the predominant mechanism underlying the loss of E-cad promoter activity in breast cancers, we sought to explore the possibility that methylation of CpG dinucleotide sites in the E-cad promoter region might cooperate with transcriptional defects to further extinguish E-cad expression. We compared the Luc activities of an unmethylated E-cad reporter gene vector to the Luc activities generated by the vector following its in vitro methylation with purified bacterial HhaI methylase or HpaII methylase. High levels of Luc activity were generated by the unmethylated Ecad5/Luc vector in the E-cad-positive MDA-MB-361 cell line, whereas in vitro methylation of the vector with HhaI or HpaII methylase markedly decreased Luc activity (Fig. 5). In the E-cad-negative cell line MDA-MB-435S, no significant effects on promoter activity were seen when the unmethylated Ecad5/Luc reporter construct was compared to the same vector methylated in vitro with HhaI or HpaII methylase.

Hennig et al. (19, 23) have provided evidence that AP-2 or an AP-2-related factor binds to two tandem sites in the...
Fig. 4. E-cad expression in breast cancer cell lines is not reactivated by treatment with AzaC. Western blot analysis was carried out to assess E-cad expression in cell lines following a 5-day treatment with AzaC at 0, 1, or 3 μM. Top, identity of the cell lines; arrow, relative mobility of E-cad; left, molecular weight markers (in thousands). The cell lines BT474, MDA-MB-361, and MCF-7 are E-cad-positive, and the other five cell lines are E-cad-negative.

<table>
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<tr>
<th>Cell Line</th>
<th>BT474</th>
<th>MDA-MB-361</th>
<th>BT549</th>
<th>DU447S</th>
<th>HS578t</th>
<th>MDA-MB-231</th>
<th>MDA-MB-435S</th>
<th>MCF-7</th>
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<tr>
<td>AzaC Conc (μM)</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
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<tr>
<td>E-cad</td>
<td>220</td>
<td>97</td>
<td>60</td>
<td></td>
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<td>MDA-Luc</td>
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<td>E-cad-Luc</td>
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Fig. 5. Methylation of the E-cad promoter inhibits its activity. The relative Luc activity of the Ecads5/Luc reporter gene construct was assessed in the MDA-MB-435S and MDA-MB-361 cell lines following in vitro methylation with either HhaI or Hpall methylase. Because transfection efficiencies differed among the lines, the Luc activity generated by the Ecads5/Luc construct was compared to the Luc activity of a control CMV-Luc reporter construct (pUHC-13-3). Luc activities were determined by three independent experiments, and all Luc activities were normalized for β-galactosidase activity. Columns, mean ratio of the luciferase activities; bars, SD.

GC-rich region of the murine E-cad promoter and that this region is critical in the regulation of E-cad expression, although their findings also suggest that the GC-rich region functions together with the CCAAT-box and E-pal elements to confer tissue-specific expression of E-cad. On the basis of the studies of AP-2 expression in breast cancer cell lines carried out thus far (24), there is little evidence for a correlation between E-cad expression and AP-2 expression, although only a limited number of cell lines have been studied. Nevertheless, to more directly assess the role of AP-2 in regulating E-cad expression in breast cancer, we transfected an AP-2 cDNA together with the Ecads5/Luc vector into two E-cad-negative cell lines. The AP-2 cDNA failed to activate the E-cad promoter in either the MDA-MB-231 or HS578t cell lines (Fig. 6). A small, but reproducible, decrease in E-cad promoter activity was seen in the BT474 E-cad-positive cell line following transfection of the AP-2 cDNA. The findings imply that loss of AP-2 activity is not likely to underlie the frequent loss of E-cad expression in breast cancer.

Discussion
A sizable fraction of breast cancers have been found to have reduced or absent E-cad expression, although mutations in the E-cad gene have only been identified in a very small...
subset of the E-cad-negative cancers (2, 16, 17). In an effort to further understand the mechanisms underlying loss of E-cad expression, we have cloned the human E-cad promoter and have undertaken sequence and functional analysis of its elements in breast cancer cells. We found that the Ecad7/Luc reporter construct, although it contains the proximal 70 bp of E-cad sequence upstream of the transcription start site and the previously defined E-cad regulatory elements (I.e., CCAAT box, two candidate AP-2 binding sites, and E-pal element), had only weak promoter activity. In contrast to the weak activity seen with the Ecad7/Luc construct, the Ecad6/Luc construct, containing a 275-bp fragment of E-cad proximal promoter sequences, had nearly the promoter activity of constructs containing considerably larger E-cad 5’ fragments of 500-1350 bp (e.g., Ecad3/Luc and Ecad5/Luc). Thus, the findings imply that critical regulatory elements in the human E-cad promoter reside in the approximately 200-bp region immediately upstream of the previously defined minimal promoter elements. Furthermore, we noted a striking relationship between the endogenous E-cad gene activity in breast cancer cell lines and the promoter activities of the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs when they were introduced into the cells. Our studies strongly suggest that defects in signaling pathways or trans-acting transcription factors that regulate E-cad expression are likely to be the primary mechanisms underlying the loss of E-cad expression in breast cancers.

Our results are consistent with those of two previous studies in which murine E-cad promoter activity was correlated with endogenous E-cad expression in several human cancer cell lines, including a total of four breast cancer lines (19, 22). Studies of the activity of a minimal human E-cad promoter in two prostate cancer cell lines have also suggested a correlation between endogenous E-cad expression and activation of E-cad promoter elements (21), although we have not found clear-cut a relationship in our preliminary studies in prostate cancer cell lines. Consistent with the results of the promoter activity studies, in vivo footprinting analyses have demonstrated protection of several distinct elements in the minimal E-cad promoter in E-cad-positive cancer cell lines but not in E-cad-negative lines (19).

Others have suggested that methylation of the E-cad promoter region may be responsible for the loss of E-cad expression in cancer, and increased methylation of the E-cad proximal promoter region has been identified in cancers and cancer cell lines lacking E-cad expression (18), an observation that we also confirmed in the lines studied here (data not shown). In a previous study, treatment of selected breast and prostate cancer cell lines with the demethylating agent AzAC was reported to reactivate E-cad expression in a minimal fraction of the treated cells (18). However, we failed to detect E-cad protein expression in any of five E-cad-negative breast cancer cell lines treated with AzAC, demonstrating that E-cad expression cannot be reactivated to the levels seen in E-cad-positive breast cancer cell lines by brief AzAC treatment. By studying E-cad promoter constructs in which CpG dinucleotides had been methylated in vitro by purified methylases, we did obtain support for the proposal that methylation of CpG dinucleotide sites in the E-cad promoter region might cooperate with transcriptional defects to further extinguish E-cad expression.

The binding of AP-2 or an AP-2-related factor to two tandem sites in the GC-rich region of the murine E-cad promoter has been suggested to regulate E-cad expression (23). However, we found that transfection of an AP-2 CDNA failed to activate the E-cad promoter in either of two E-cad-negative cell lines. Hence, loss of AP-2 is not likely to underlie the frequent loss of E-cad expression in breast cancer. Others have suggested that overexpression of HER-2/neu may cause a decrease in E-cad expression (25), although we failed to demonstrate a correlation between E-cad and HER-2/neu expression in breast cancer in our previous studies (16). Therefore, although the present evidence supports the proposal that defects in signaling and/or transcription factor pathways are the predominant mechanisms underlying altered E-cad expression in breast cancer, additional studies are clearly needed to elucidate the specific nature of these defects. Further characterization of these defects should provide new and important insights into the pathogenesis of breast cancer and the mechanisms underlying tumor cell heterogeneity and progression.

Materials and Methods

Plasmid Constructs. Genomic clones containing human E-cad exons 1 and 2, as well as 5’ flanking sequences, were isolated from a human genomic DNA library generously provided by Dr. Jeremy Nathans (Johns Hopkins University School of Medicine, Baltimore, MD) using a human E-cad CDNA probe and multiple rounds of hybridization selection. An approximately 2.5-kb Sall-Nico fragment extending 5’ from the initiating methionine codon in exon 1 was identified and subcloned into pBlue-scriptII (Stratagene, La Jolla, CA). Deletions of varying extent in the E-cad sequences were generated using exonuclease III and mung bean nuclease (Stratagene). A series of Luc reporter gene constructs containing E-cad 5’ flanking sequences of various extents was generated by subcloning the E-cad sequences into the Sacl and HindIII sites of the pGL2-Basic vector (Promega Corp., Madison, WI) immediately upstream of the coding region of the luciferase (Luc) gene. During the subcloning of each E-cad fragment, the E-cad initiating methionine codon was destroyed. The identities of the E-cad sequences present in the vectors were confirmed by sequence analysis. The control Luc vectors pHUC-13-3 and pMLP/Luc, containing CMV and adenovirus major late promoter elements, respectively, have been described previously (26, 27). The pCH110 plasmid, containing a functional Lec2 gene expressed under control of the SV40 early promoter, was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). The vector pcdNAS-AP2 was constructed by subcloning a 1.6-kb HindIII-EcoRl murine AP-2 CDNA fragment (kindly provided by Dr. Trevor Williams, Yale University) into the HindIII and EcoRI sites of the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). All plasmid DNAs were isolated using reagents from Qiagen, Inc. (Chatsworth, CA). In vitro methylation of the plasmid vector pcEad5/Luc was carried out on 20 μg of DNA using purified HhaI or HpaII methylase (New England Biolabs, Inc., Beverly, MA) and the manufacturer’s recommended reaction conditions.

Cell Cultures. All cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained in the recommended growth media. Cultures were incubated at 37°C with 5% CO2 except for those maintained in Lebovitz’s L-15 medium, which were grown at 37°C without CO2. Selected cell lines were treated with 1 or 3 μM AzAC (Sigma Chemical Co., St. Louis, MO) for 5 days.

Transfections and Luc and β-Galactosidase Reporter Assays. Cell lines growing at roughly 70% confluence were transfected in six-well plates using 1 ml of Opti-MEM reduced serum medium (Life Technologies, Inc.) supplemented with FBS (10%) and Polyethylenimine (PEI) (Polysciences, Inc., Warrington, PA) at a PEI:DNA ratio of 1:1 (v/v) as described previously (28).

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