Cadherin-11 Is Expressed in Invasive Breast Cancer Cell Lines

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ABSTRACT

In several cancers, including breast cancer, loss of E-cadherin expression is correlated with a loss of the epithelial phenotype and with a gain of invasiveness. Cells that have lost E-cadherin expression are either poorly invasive with a rounded phenotype, or highly invasive, with a mesenchymal phenotype. Most cells lacking E-cadherin still retain weak calcium-dependent adhesion, indicating the presence of another cadherin family member. We have now examined the expression of the mesenchymal cadherin, cadherin-11, in breast cancer cell lines. Cadherin-11 mRNA and protein, as well as a variant form, are expressed in the most invasive cell lines but not in any of the noninvasive cell lines. Cadherin-11 is localized to a detergent-soluble pool and is associated with both α- and β-catenin. Immunocytochemistry shows that cadherin-11 is localized to the cell membrane at sites of cell-cell contact as well as at lamellipodia-like projections, which do not interact with other cells. These results suggest that cadherin-11 expression may be well correlated with the invasive phenotype in cancer cells and may serve as a molecular marker for the more aggressive, invasive subset of tumors. Cadherin-11 may mediate the interaction between malignant tumor cells and other cell types that normally express cadherin-11, such as stromal cells or osteoblasts or perhaps even with the surrounding extracellular matrix, thus facilitating tumor cell invasion and metastasis.

INTRODUCTION

Cadherins are transmembrane adhesion molecules that mediate calcium dependent cell-cell adhesion. The cadherins are members of a superfamily of related proteins, the members of which include the “classical” cadherins, desmosomal cadherins, protocadherins, and products of tumor suppressor genes like c-ret and Fat (1). Members of the classical cadherin subgroup have been shown to be essential for strong cell-cell adhesion and maintenance of tissue integrity and cell polarity (2). They also facilitate, via homophilic adhesion, cell signaling functions such as the communication between malignant tumor cells and other cell types that normally express cadherin-11, such as stromal cells or osteoblasts or perhaps even with the surrounding extracellular matrix, thus facilitating tumor cell invasion and metastasis.

Previously, it was shown in a panel of breast cancer cell lines that E-cadherin expression is lost as cells become more invasive and less differentiated (11, 12). Loss of E-cadherin is also associated with the less differentiated, more invasive subset of breast tumors (1). However, exogenous expression of E-cadherin in invasive breast cancer cells did not inhibit their invasive phenotype (11). In addition, blockade of E-cadherin in noninvasive cells prevented cell-cell adhesion but did not result in an invasive phenotype (12). Instead, E-cadherin expression and the state of differentiation of these cells is representative of their place along a putative epithelial-mesenchymal transition (11, 12). As the cells become more fibroblastoid, they acquire the molecular characteristics of fibroblasts, including an increase in vimentin expression, and loss of known junctional proteins including E-cadherin, desmoplakin, and the tight junctional protein, ZO-1 (Table 1). Nevertheless, even the most invasive cell lines exhibited calcium-dependent cell-cell adhesion, suggesting the presence of another functional cadherin (12). Recent work has demonstrated the presence of other cadherins in mesenchymal and fibroblast cells (13, 14). One such mesenchymal cadherin is cadherin-11 (15). We now show that cadherin-11 mRNA and protein and a cadherin-11 variant mRNA are expressed in invasive and poorly differentiated breast cancer cell lines. Cadherin-11 is localized to the cell membrane in a detergent-soluble complex, where it associates with α-, and β-catenin, and may facilitate tumor cell invasion and metastasis.

MATERIALS AND METHODS

Cell Lines. All cell lines were obtained from American Type Culture Collection and grown in DMEM (Life Technologies, Inc.) plus 5% fetal bovine serum as described previously (11). A summary of the characteristics of the breast cancer cell lines has been published (11). An expanded table is included below (see Table 1). Please note in particular the level of invasiveness of each cell line, as well as the expression of the various adherens junction molecules. MCF-7/ADR cells are a variant of MCF-7 cells that are resistant to Adriamycin.

RT-PCR. RT-PCR was performed using 0.2 μg (β-actin) or 1.0 μg (cadherin-11) of total RNA, isolated using the guanidine isothiocyanate method (16). The following primers were used: β-actin upstream, 5'-TGACGGGGTCAACCCACTGTCGGCGCATCTA-3'; β-actin downstream, 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'; cadherin-11 wild type upstream, 5'-ACCAGATGCTGTGTCGA-3'; cadherin-11 wild-type downstream, 5'-GTCATCTTTCAGTCCTGCA-3'; cadherin-11 variant upstream, 5'-CCGCCGGAATTTGGAACCCCCCTC-3'; cadherin-11 variant downstream, 5'-CGGCCGGAATTTGGAACCCCCCTC-3'. First-strand synthesis with the downstream primer and MMLV-RT (Gibco/BRL) was followed by PCR using Taq polymerase (Life Technologies, Inc.) after adding the upstream primer. The following cycling parameters were used: cadherin-11 wild type, 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, 35 cycles; cadherin-11 variant, 94°C for 1.5 min, 55°C for 2 min, 72°C for 30 s, 35 cycles. Both parameters could be used for β-actin. The PCR product was run on a 1% agarose gel. The following fragments were amplified: β-actin, a 661-bp fragment that spans an intron to rule out genomic contamination; cadherin-11 wild type, a 742-bp fragment from a region encoding part of the extracellular domain; cadherin-11 variant, a 194-bp fragment that encodes most of the COOH-terminal 75 amino acids present only in the variant (17).

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The abbreviations used are: RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Northern Blot. Twenty μg of total RNA were separated on a 1% agarose gel and transferred to a nylon membrane (Boehringer Mannheim; Ref. 16). A 1.6-kb fragment of the cadherin-11 cDNA was labeled using 32P-labeled dCTP and used to probe the blot (DNA fragment donated by Colin MacCalman, University of British Columbia, Vancouver, British Columbia, Canada). The blot was hybridized at 50°C overnight, then washed three times in 2% SSC at 55°C and 65°C (last wash). The labeled bands were visualized using a phosphorimager. The nylon was then reprobed for GAPDH as a control.

Western Blot. Cells from confluent 10-cm dishes were isolated and homogenized in a hypotonic solution (10 mM Tris, 0.2 mM MgCl2, pH 7.5). The homogenate was centrifuged first for 10 min at 3000 g to remove nuclei. The supernatant was then centrifuged at 150,000 g for 1 h. The supernatant is the NP40 soluble membrane fraction. The resulting supernatant, defined as the cytoplasmic fraction, was added to four volumes of ethanol, and the proteins precipitated overnight. The proteins were then collected by ultracentrifugation and solubilized in sample buffer [2% SDS, 60 mM Tris (pH 6.8), and 10% glycerol] and sequentially reprobed, first with α-catenin polyclonal antibody (Boehringer Mannheim) and variant transcripts. MDA-MB-157 (not shown) and MCF-7ADR cells express much lower levels of mRNA than MDA-MB-231, BT549, or HS578T cells, despite the presence of similar levels of total RNA (polyclonal from David Rimm, Yale University). Although a variant cadherin-11 band was amplified from SK-BR-3 cell mRNA, we do not consider that all other results were negative (wild-type RT-PCR, Northern blot using a 1.6-kb fragment of the cadherin-11 cDNA as a probe). The probe identified a 4.4-kb band from the same five cell lines. Several larger bands can also be seen, but these have yet to be identified. The variant mRNA contains an insertion of 179 bp, but cadherin-11 wild-type (Fig. 1a) and variant (Fig. 1b) mRNA were expressed in five invasive cell lines, MDA-MB-157, MDA-MB-231, BT549, HS578T, and MCF-7ADR, MCF-7ADR cells, which are considerably less invasive than the others, the variant is expressed at very low levels. Two invasive cell lines, MDA-MB-436 and MDA-MB-435, did not express cadherin-11 nor did any of the noninvasive cell lines. Although a variant cadherin-11 band was amplified from SK-BR-3 cell mRNA, we do not believe that this result represents true expression of cadherin-11, considering that all other results were negative (wild-type RT-PCR, Northern and Western blots). The RT-PCR results were confirmed by Northern blot using a 1.6-kb fragment of the cadherin-11 cDNA as a probe (Fig. 1c). The probe identified a 4.4-kb band from the same five cell lines. Several larger bands can also be seen, but these have yet to be identified. The variant mRNA contains an insertion of 179 bp, but the Northern blot did not allow us to differentiate between wild-type and variant transcripts.

RESULTS

Cadherin-11 Wild-Type and Variant mRNA Expression in Human Breast Cancer Cell Lines. We first examined the expression of cadherin-11 in breast cancer cell lines by RT-PCR. Two sets of primers were used. The first amplifies a region that encodes the extracellular domain of cadherin-11 and thus would recognize both the wild-type and variant cadherin-11; the second set amplifies only a portion of the variant cadherin-11 (17). α-Catenin primers were used as a control. MRC-5, a human embryonic lung fibroblast cell line known to express cadherin-11, was used as a positive control (19). Cadherin-11 wild-type (Fig. 1a) and variant (Fig. 1b) mRNA were expressed in five invasive cell lines, MDA-MB-157, MDA-MB-436, MDA-MB-435, and MCF-7ADR cells, which are considerably less invasive than the others, the variant is expressed at very low levels. Two invasive cell lines, MDA-MB-436 and MDA-MB-435, did not express cadherin-11 nor did any of the noninvasive cell lines. Although a variant cadherin-11 band was amplified from SK-BR-3 cell mRNA, we do not believe that this result represents true expression of cadherin-11, considering that all other results were negative (wild-type RT-PCR, Northern and Western blots). The RT-PCR results were confirmed by Northern blot using a 1.6-kb fragment of the cadherin-11 cDNA as a probe (Fig. 1c). The probe identified a 4.4-kb band from the same five cell lines. Several larger bands can also be seen, but these have yet to be identified. The variant mRNA contains an insertion of 179 bp, but the Northern blot did not allow us to differentiate between wild-type and variant transcripts. MDA-MB-157 (not shown) and MCF-7ADR cells express much lower levels of mRNA than MDA-MB-231, BT549, HS578T cells, despite the presence of similar levels of total RNA and GAPDH expression.

Cadherin-11 Protein Expression. We next wanted to examine cadherin-11 protein expression. Ten μg of protein from a detergent lysate were run on a polyacrylamide gel, and the subsequent blot was probed with an anti-cadherin-11 monoclonal antibody (ICOS, Inc.; Fig. 2a). This monoclonal antibody recognizes the extracellular por-

Table 1 Molecular characteristics of several breast cancer cell lines

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* Plak, Plakoglobin; NcadProt, N-cadherin protein; NcadRNA, N-cadherin RNA; Cad11 WT, cadherin-11 wild-type; Cad11 var, cadherin-11 variant.
tion of cadherin-11 and should recognize both wild-type and variant protein. In Western blots, cadherin-11 runs as a Mr; 120,000 band. The cell lines that contained cadherin-11 mRNA also expressed cadherin-11 protein. MDA-MB-157 and MCF-7ADR cells express lower levels of cadherin-11 protein. Although the putative variant protein would be Mr; 7,000 smaller than the wild type, a band of this size could not be definitively identified on this Western blot (17). Several bands significantly smaller than the full-length cadherin-11 are present. One of these could represent the variant form if the protein migrated faster than expected. Alternatively, these bands could represent degradation products. The Western blot was reprobed several times to examine the expression of other adherens junction proteins. Probing with a pancadherin antibody revealed that a number of cell lines express another member of the cadherin family (Fig. 2b). The pancadherin antibody is immunoreactive to several members of the cadherin family (the arrow indicates the location of the cadherin-11 band) but is only weakly reactive to E-cadherin. Based upon the strong immunoreactivity and molecular weight (Mr; ~140,000), we believe the pancadherin antibody is identifying N-cadherin in most cell lines. This is consistent with results demonstrated by Hazan et al. (20). Interestingly, an anti-E-cadherin Western blot demonstrates that E-cadherin and cadherin-11 are never coexpressed (Fig. 2c). Finally, most cell lines express α-catenin (Fig. 2d) and β-catenin (Fig. 2e).

**Cadherin-11 Protein Is Expressed Primarily in an NP40 Soluble Pool.** We next wanted to determine the solubility of the cadherin-11 protein complex. Cultured cells were separated into three fractions (see “Materials and Methods”): cytoplasmic, NP40-soluble (membrane-bound, noncytoskeletally associated), and NP40-insoluble (cytoskeletally associated). Twenty-five μg of protein from each fraction were run on a polyacrylamide gel, and the blotted proteins were probed with the cadherin-11 monoclonal antibody. In the cells expressing cadherin-11, cadherin-11 protein is found mostly in the...
NP40-soluble pool. Some cadherin-11 could also be found in the NP40-insoluble pool (Fig. 3a). As expected, no cadherin-11 was found in the cytoplasmic pool. By contrast, significant amounts of another cadherin (probably N-cadherin) were found in both the soluble and insoluble pools (Fig. 3b). Also, β-catenin could be found in all three pools, although less was found in the insoluble pool (Fig. 3c).

Cadherin-11 Is Localized to the Cell Membrane. In epithelial cells, cadherins typically function as cell-cell adhesion proteins as part of an adherens junction (21). However, cadherin-11 mRNA has been found only in mesenchymal cells, which do not usually form adherens junctions (13, 15). Immunocytochemistry was performed on paraformaldehyde-fixed cells (Fig. 4). Cadherin-11 is expressed at the cell membrane in BT549 and HS578t cells (Fig. 4, a and b) but not in MCF-7 cells (Fig. 4d). Staining of BT549 with normal mouse IgG reveals no nonspecific staining (Fig. 4c). In BT549 and HS578t cells, cadherin-11 is localized to sites of cell-cell contact, as is typical of cadherin family members. Surprisingly, cadherin-11 is also found in lamellipodia-like extensions and possibly at regions of contact with the substrate.

α-Catenin and β-Catenin Immunoprecipitations. Previously, Shibata et al. (19) were able to isolate cadherin-11 by probing a cDNA library with radiolabeled β-catenin protein, thus establishing their interaction in vitro. We wanted to determine whether cadherin-11 interacts with α-catenin and β-catenin in vivo (Fig. 5). Confluent cells were lysed in an NP40 buffer, and the precleared lysate was immunoprecipitated with polyclonal antibodies to either α-catenin or β-catenin (both kindly donated by David Rimm, Yale University). Western blots were performed first with the cadherin-11 monoclonal antibody (Fig. 5a), then with a pancadherin polyclonal antibody (Fig. 5b), then with a monoclonal antibody to E-cadherin (Fig. 5c), and finally with antibodies to α-catenin (polyclonal) or β-catenin (monoclonal) (Fig. 5, d and e). Immunoprecipitation with nonimmune rabbit IgG revealed no immunoreactive bands in any of the Western blots (data not shown). The Western blots confirmed that the same five cell lines express cadherin-11, and that in all five cases cadherin-11 is associated with both α-catenin and β-catenin (except in MDA-MB-157 cells, which lack α-catenin). Pancadherin blotting and E-cadherin blotting revealed that several cell lines express other cadherins that are α-catenin and/or β-catenin associated. Several invasive cell lines express N-cadherin, whereas E-cadherin expression is restricted to the most differentiated, noninvasive cells (12, 20). A few of these cell lines lack α-catenin or β-catenin (See Table 1). One particularly striking example cell line is T47D. In these cells, both α-catenin and β-catenin are associated with E-cadherin but not with each other. Nevertheless, these cells are well differentiated and exhibit strong cell-cell adhesion, suggesting that compensatory mechanisms exist in the function of the adherens junction (12). In addition to confirming the results of the Western blots, the α-catenin and β-catenin immunoprecipitations identify other defects in the cadherin/catenin system in various cell lines, which are summarized in Table 1. Finally, some proteins that were undetectable by Western blot of cell lysates were present and detected by immunoprecipitation, presumably as a result of the selective concentration afforded by the immunoprecipitation.

Previously, we published a table that listed the molecular characteristics of several breast cancer cell lines (12). We have now updated that table to include the information on cadherin-11 as well as published results on N-cadherin protein and our preliminary results on N-cadherin mRNA. Note in particular that cadherin-11 is expressed only in the cell lines with a stellate morphology. These are the most invasive cell lines, and they all lack E-cadherin.

DISCUSSION

Cadherin-11 Is Expressed in Invasive, Fibroblastoid Breast Cell Lines. Loss of E-cadherin expression or function is associated with a more invasive, less differentiated phenotype in cancer cell lines.
and primary tumor samples (11, 12). In earlier studies, we established three subsets from a panel of breast cancer cell lines (11, 12). E-cadherin-expressing cell lines (which all lack vimentin) were poorly invasive in Boyden Chamber assays and well-differentiated, forming tight cell clusters, in Matrigel. Cell lines lacking E-cadherin and vimentin were also poorly invasive but only moderately differentiated, forming loose cell aggregates in Matrigel. Finally, cell lines lacking E-cadherin but expressing vimentin were highly invasive and poorly differentiated and had a stellate, fibroblastoid morphology. However, transfection of E-cadherin into these cells did not reverse the invasive phenotype (12). These results indicated that loss of E-cadherin expression or function may be necessary but is not sufficient for the establishment of invasive, highly malignant tumors.

Despite the lack of E-cadherin expression, most of the invasive cells exhibited calcium-dependent adhesion, indicating the presence of another member of the cadherin family (11). Recent work has demonstrated the expression of cadherins in fibroblastic and mesenchymal cells. N-cadherin is expressed in several types of mesenchymal tissue as well as in cultured fibroblasts (14). In addition, cadherin-11 expression is restricted to mesenchymal cells in the developing mouse (13, 15, 22) and is expressed in stromal cells in the adult (19). Hazan et al. (20) have shown that N-cadherin expression in breast cancer cell lines is limited to most of the invasive, fibroblastoid cells. They proposed that N-cadherin expression is restricted to less differentiated cells, and that the expression of N-cadherin facilitates the interaction of tumor cells with the underlying stroma. Our preliminary results indicate that N-cadherin mRNA (but not a pan-cadherin immunoreactive protein) is also present in several noninvasive cell lines (see Table 1; data not shown). Consequently, it may be interesting to explore the posttranscriptional regulation of N-cadherin and its possible relevance in breast cancer.

We now present evidence that cadherin-11 is expressed in breast cancer cell lines that lack E-cadherin but express vimentin and are highly invasive and poorly differentiated. RT-PCR, Northern blot, and Western blot analyses confirm that five of seven invasive breast cancer cell lines express cadherin-11. Cadherin-11 was found previously to be expressed only in embryonic mesenchymal tissues, osteoblasts, and invasive tumors of the stomach and the kidney (17, 19, 22, 23). Furthermore, cadherin-11 has never been shown to be expressed in E-cadherin-expressing cell lines or tissues (22, 23). This indicates that cadherin-11 is a very specific marker for only the most invasive subset of cancer cell lines. Although we have been unable to determine the level of expression of cadherin-11 in human tumor tissue, the results obtained using cancer cell lines suggest that cadherin-11 is a potential molecular marker and could be used to identify highly malignant tumors that would require more aggressive therapy. Moreover, our results, together with those of Hazan et al. (20), show that invasive cells express cadherin-11, N-cadherin, or both and indicate that detection of these molecules could identify with confidence highly malignant tumors.

The five cell lines that express cadherin-11 also express a variant of cadherin-11. This variant arises from a 179-bp insertion that results in a protein that lacks the majority of the wild-type cytoplasmic domain, including the catenin-binding regions (17). If the cadherin-11 variant is expressed as a functional protein, it could act as a dominant negative and reduce cadherin-mediated cell adhesion. In fact, expression of this variant mRNA has been associated with invasive tumors (17, 24). However, by using cell extracts, we were unable to definitively identify a protein product of a size anticipated from the variant mRNA sequence.

**Cadherin-11 Associates with α- and β-Catenin.** Cadherin-11 is a typical type II cadherin and can mediate calcium-dependent cell-cell adhesion. Previously, Shibata et al. (19) have shown that cadherin-11 interacts with β-catenin in vitro. We have shown here that cadherin-11 forms complexes containing both α- and β-catenin in vivo. However, in the invasive breast cancer cell, BT549 cadherin-11 is found predominantly in a detergent-soluble pool, indicating that it is not associated with the cytoskeleton. In the same cells, a pan-cadherin reactive protein (probably N-cadherin) is found in the detergent insoluble pool. Thus, cadherin-11 is not found in a typical adherens junction in which a cadherin is linked, through the catenins, to the actin cytoskeleton. Adherens junctional cadherins are also localized to sites of cell-cell contact. However, our immunocytochemistry shows that cadherin-11 is also found at lamellipodia-like extensions and possibly to regions of cell-substrate contact. These results suggest that cadherin-11 may have several functions. It may function in a transient form of cell-cell adhesion that involves the cytoplasmic catenins but is not associated with the cytoskeleton. The cadherin-11 complex may also have a role in contacting the cell-matrix, particularly in leading extensions of the cell. Such a complex would be much more transient and could facilitate the ability of a motile cell to interact with its surroundings, which could include both matrix proteins, as well as other mesenchymal proteins. These potential functions of cadherin-11 have implications with respect to the invasive and metastatic ability of tumor cells.

**Functional Significance of Cadherin-11.** Cadherin-11 could be involved in cell and matrix recognition that may facilitate cell motility and may also be essential for the loose aggregation of cell types that is necessary in tissue morphogenesis. These hypotheses are supported by several observations. First, cadherin-11 expression is associated with invasive cells, both during normal stages of embryogenesis and in invasive tumor cells (17, 19, 22, 25). This invasion may be facilitated by the association of the invasive cadherin-11 cells with the surrounding mesenchymal cells, which also express cadherin-11, as well as with the surrounding matrix. For example, during embryogenesis mesenchymal cells express cadherin-11 (15). In addition, cells that undergo an epithelial to mesenchymal transition also begin to express cadherin-11 as they invade the surrounding tissue to form new structures (22). This can be seen during branching morphogenesis of lungs and kidneys and also in the formation of the nasal septum, skin, vibrissae, teeth, and various glands (22). Shibata et al. (19) show that in a panel of gastric tumors (both primary tumors and tumor cell lines), cadherin-11 is only expressed in signet ring cell carcinomas, which are typically infiltrative. They suggest that the expression of cadherin-11 in the tumor cells may allow for interactions with the underlying stroma that would facilitate invasion. Thus, unlike other cadherins, such as E-cadherin and cadherin-6, which have invasion suppressor function, cadherin-11 may actually enhance tumor cell invasiveness and may be a new target for treatment (11).

Next, cadherin-11 may be essential for the loose aggregation of cell types. For example, during embryogenesis, the expression of cadherin-11 is increased dramatically in cells undergoing the epithelial-mesenchymal transition that precedes the formation of various structures and also in regions of mesenchymal condensation, such as occurs prior to chondrogenesis (13). The association and loose aggregation of cells may depend upon cadherin-11 and could be extremely significant in cancer. For example, it is possible that cadherin-11 may act to specifically target metastatic tumor cells to sites that express cadherin-11. Consequently, expression of cadherin-11 may facilitate association of metastatic cells with cadherin-11-expressing osteoblasts in the bone, thus establishing a bony metastasis.

These studies indicate that cadherin-11 expression is associated with invasive breast cancer and may play a significant role in facilitating tumor cell invasion and the formation of metastatic tumors. Elucidation of the functions and regulation of cadherin-11 may enhance our understanding of the roles of cadherins in invasive cancer and mesenchymal cells and may provide future targets for therapy.
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