Aberrant Expression of Novel and Previously Described Cell Membrane Markers in Human Breast Cancer Cell Lines and Tumors

Huayi Huang,1 Jeff Groth,1 Khalid Sossey-Alaoui,2 Lesleyann Hawthorn,2 Stephanie Beall,3 and Joseph Geradts1,2

Abstract

Purpose: In a previous gene expression array study, we identified some 300 genes that were differentially expressed in human epidermal growth factor receptor tyrosine kinase 2 (HER2)–positive versus HER2-negative breast cancer cells. We have now done validation experiments on a group of three cell membrane components that had previously not been implicated in breast cancer. We also studied the expression of three other cell membrane proteins known to play a role in mammary neoplasia.

Experimental Design: By immunohistochemistry, we examined up to 130 archival breast carcinomas for Celsr2, E-cadherin, Kai1, and CD9 expression. The expression levels of NET-6 and TROP-2 were determined by quantitative reverse transcription-PCR in a subset of frozen tumors. We also studied fresh pellets and paraffin-embedded cell buttons of nine human breast cell lines. The relationship between the expression of all six membrane proteins and a variety of pathologic and biological variables, including estrogen receptor, HER2, and epidermal growth factor receptor status, was also examined. The NET-6 gene was transfected into a low-expressing cell line, and the effect on cellular morphology, growth, and invasion in vitro was recorded.

Results: Celsr2 was down-regulated in one cell line and in 7% of breast cancers. E-cadherin, Kai1, and CD9 were down-regulated in 35%, 76%, and 79% of tumors, respectively, confirming the important role of these markers in human mammary neoplasia. In breast cancer cell lines and tissues, TROP-2 was generally expressed at low levels, although a few specimens showed relative overexpression. NET-6 levels were lower in HER2-negative breast carcinoma cells. In addition, NET-6 was markedly down-regulated in estrogen receptor–negative breast cancers, and expression was lowest in “basal-like” tumors. Ectopic expression of NET-6 in low-expressing MDA-MB-231 cells altered cellular morphology, inhibited growth in vitro, and decreased invasion in a Boyden chamber assay.

Conclusions: We have confirmed the expression of three new membrane markers that had previously not been implicated in human breast cancer, and one of them (NET-6) was correlated with HER2 and estrogen receptor status. NET-6 levels were decreased in estrogen receptor–negative and high-grade tumors, and ectopic expression of this gene had an inhibitory effect on proliferation and invasion. Thus, NET-6 may represent a novel breast cancer suppressor gene.

The human epidermal growth factor (EGF) receptor (EGFR) tyrosine kinase 2 (HER2) gene is amplified in 20% to 25% of breast cancers, and this amplification is associated with overexpression of the encoded protein in most cases (1–3). HER2 positivity is associated with an unfavorable disease phenotype and has been targeted for therapeutic intervention (2, 4–6). The molecular mechanisms by which HER2 overexpression confers aggressive tumor biological behavior are poorly understood, and not all HER2-positive tumors will culminate in poor outcome. This implies that additional genes must act in concert with HER2 overexpression to determine the tumor phenotype.

In an effort to elucidate the adverse prognostic significance of HER2 positivity, we previously used cDNA microarray technology to identify genes that were differentially expressed in HER2-positive and HER2-negative breast cancer cell lines and tissues (7). In that study, almost 300 genes (of 5,184) were found to be up-regulated or down-regulated in HER2-positive breast cancer cell lines and tumors. These included the bona fide metastasis suppressor gene Kai1 (CD82), which showed one of the lowest differential expression levels in HER2-positive breast carcinomas, and we previously confirmed its extensive down-regulation in the group of tumors studied by gene expression microarrays (7). Here, we report validation studies for three additional cell membrane markers that had not been described in human...
Materials and Methods

Cell culture. The HER2-negative breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-468, the HER2-positive breast cancer cell lines MDA-MB-361, BT474, and SKBR3, and the nontransformed human mammary epithelial cell line MCF-10A as well as a derivative cell line stably transfected with HER2 were originally obtained from the Imperial Cancer Research Fund Cell Culture Facility (London, United Kingdom) and were used in our previous gene expression profiling studies (7, 11). Cell lines were cultured in DMEM with 10% fetal bovine serum and 2 mmol/L glutamine (MDA-MB-231, MDA-MB-435); DMEM/F-12 with 5% fetal bovine serum (MDA-MB-468); DMEM with 20% fetal bovine serum (BT474). The cell lines were cultured in a humidified incubator. For immunocytochemistry, cells were plated at 80% confluence on Lab-Tek chamberslides or Lab-Tek 15-well plates (Nunc, Naperville, IL) and fixed in 4% formaldehyde for 10 minutes or frozen in isopentane at −20°C. The latter included both fixed and frozen tissues that were derived. In benign breast epithelial cells, typically moderate membrane reactivity. If there was no discernible membrane staining, Reduced expression (R) was present if <30% of cells showed weak staining intensity were scored at 2+. Cell pellets showing weak cell pellets were then xylene cleared and embedded in paraffin.

Immunohistochemistry. The primary antibodies were applied after an antigen retrieval step. The paraffin sections were heated in subboiling buffer for 20 minutes in a steamer. For Celsr2, we used the high pH buffer from DAKO (Carpinteria, CA). For the other antibodies, we used 0.01 mol/L citrate buffer (pH 6.0). The primary antibody incubation was done for 2 hours at room temperature for E-cadherin and overnight at 4°C for the other antibodies using a Shandon Sequenza rack. The detection reactions were done on the DAKO autostainer. For E-cadherin, we used the Vectastain Elite kit (Vector Laboratories, Burlingame, CA). For Kai1 and CD9, we followed the DAKO Envision protocol. For Celsr2, we employed a goat anti-chicken secondary antibody conjugated to horseradish peroxidase at 1:100 for 20 minutes (KPL, Gaithersburg, MD). The color reactions used 3,3′-diaminobenzidine and hematoxylin as counterstain. Positive control samples included benign breast tissue for Celsr2 and E-cadherin, HeLa cells for Celsr2, and tonsil and normal lung for Kai1 and CD9. Negative control reactions used PBS instead of the primary antibodies.

Interpretation of immunostains. It was established that there was no significant intratumor heterogeneity in the expression of any of the four immunohistochemical markers and that the reactivity of the tissue microarray cores was representative of the tumors from which they were derived. In benign breast epithelial cells, typically moderate to strong membrane staining was observed for all four markers (Celsr2, E-cadherin, Kai1, and CD9). Cell pellets with a similar staining intensity were scored at 2+. Cell pellets showing weak membrane staining in 30% to 70% of cells were scored as 1+. Reduced expression (R) was present if <30% of cells showed weak membrane reactivity. If there was no discernible membrane staining, the sample was scored as negative. The tissues were similarly scored but for statistical analysis were dichotomized as normal (1+, 2+) and abnormal (R, −).

NET-6 transfection. The whole full-length NET-6 gene cDNA insert was cloned into EcoRI and BglII sites of the pEGFP-C1 vector (Becton Dickinson Clontech, Palo Alto, CA) in frame with the enhanced green fluorescent protein. The DNA (8 μg per 60 mm dish) was transfected into subconfluent MDA-MB-231 cells using the LipofectAMINE 2000

breast carcinomas: NET-6, TROP-2 [also known as tumor-associated calcium signal transducer 2 (TACSTD2) or GA733-1], and Celsr2 [also known as multiple EGF-like domain 3 (MEGS3)], the mammalian homologue of the Drosophila flamingo 1 gene. We reasoned that deregulation of these markers in HER2-positive breast cancer cells may be associated with altered cell-cell or cell-matrix adhesion, stromal invasion, loss of contact inhibition, or altered signal transduction. Moreover, in an attempt to comprehensively define deregulation of cell surface proteins, we also studied the expression of E-cadherin, Kai1, and CD9 [also known as motility-related protein 1 (MRP-1)]. The latter two markers, along with NET-6, belong to the tetraspanin family of cell membrane (glyco)proteins that play an increasingly prominent role in the pathogenesis of human neoplasia (8–10). We showed the expression of the three novel membrane markers in human breast epithelial cells, albeit at highly variable levels, and more importantly obtained evidence that NET-6 may be a new breast cancer suppressor gene. Moreover, we confirmed the frequent down-regulation of E-cadherin, Kai1, and CD9 in breast carcinomas.

Reverse transcription and real-time quantitative PCR. Total RNA (5 μg) was used for cDNA synthesis using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). cDNA (100 ng) from each sample was used as real-time PCR template. Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) was used as amplification reaction mixture. The probes for the genes of interest were purchased from ABI (Foster City, CA; Assay-on-Demand primer-probe set). The Certified Lux Primer Set for human glycerdehyde-3-phosphate dehydrogenase was used as the endogenous standard (Invitrogen). PCR reactions were done on a PE-ABI PRISM 7700 Sequence Detection System. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Experiments were done in triplicate for each gene in a 96-well plate (25 μL/well). The Ct (threshold cycle) variable was used for quantitation and the ΔCt between the gene of interest and the endogenous standard (glycerdehyde-3-phosphate dehydrogenase) was determined and translated into copy number difference (14). A relative score of 1,000 indicates that the test gene and glycerdehyde-3-phosphate dehydrogenase are expressed at equal levels.

Antibodies. The anti-Celsr2 polyclonal chicken antibody was generated in Dr. Kamin Johnson’s laboratory at Brown University (Providence, RI) and was kindly provided by the collaborating author (15). For immunohistochemistry, it was used at a 1:75 dilution. The anti-E-cadherin mouse monoclonal antibody (clone HEC1D) was obtained from Zymed (South San Francisco, CA). It was used at a concentration of 0.2 μg/mL. The anti-Kai1 mouse monoclonal antibody (clone G-2) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and was used at 1 μg/mL. The anti-CD9 mouse monoclonal antibody (clone 9C01) was from NeoMarkers (Fremont, CA) and was used at 1:300 dilution.

Cell culture. The HER2-negative breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-468, the HER2-positive breast cancer cell lines MDA-MB-361, BT474, and SKBR3, and the nontransformed human mammary epithelial cell line MCF-10A (London, United Kingdom) and were used in our previous gene expression profiling studies (7, 11). Cell lines were cultured in DMEM with 10% fetal bovine serum and 2 mmol/L glutamine (MDA-MB-468); DMEM with 20% fetal bovine serum and 2 mmol/L glutamine (MDA-MB-361); DMEM/F-12 with 5% fetal bovine serum, 10 μg/mL insulin, 10 ng/mL EGF, 0.2% fungizone, 0.2% cholera enterotoxin, and 0.2% hydrocortisone (MCF-10A and MCF-10A transfected with HER2); and DMEM/F-12 with 10% fetal bovine serum, 10 ng/mL EGF, 10 μg/mL insulin, and 2 mmol/L glutamine (BT474). All media also contained 100 units/mL penicillin and 100 μg/mL streptomycin. All cell lines were maintained in an atmosphere of 5% CO2 in a 37°C humidified incubator. For immunocytochemistry, cells from subconfluent cultures were trypsinized and pelleted. Cell blocks were prepared by fixation of the pelleted cells in 10% buffered formalin for 20 minutes followed by a wash in 70% ethanol for 30 minutes and 100% ethanol overnight. The fixed cell pellets were then xylene cleared and embedded in paraffin.

Tissues. Formalin-fixed, paraffin-embedded primary breast cancers obtained from the Roswell Park Cancer Institute Paraffin Archive Resource Core Facility and from the Institute’s surgical pathology files were used to construct tissue microarrays (12). Two 1 mm cores from each tumor were transferred into the recipient blocks. We also included seven cases from our previous gene expression profiling study (7). Additional samples were from an ongoing study of premenopausal breast carcinomas (13). The latter included both fixed and frozen tissues that could be used for molecular analysis. All tumors were typed and graded by the study pathologist (J.G.). Estrogen receptor (ER), HER2, and EGFR expression had previously been determined for all tumors and cell lines.

RNA extraction. Cell line RNA was extracted using the Trizol method (Life Technologies, Grand Island, NY). Tissue sections were homogenized using a Tekmar Tissumizer in Trizol reagent. The RNA quantity and quality were determined by an Agilent 2100 bioanalyzer (RNA 6000 Nano LabChip kit for detection of 18S and 28S RNA bands) and spectrophotometer to assure the quality of the samples for intact total RNA and protein.
system (Invitrogen) following the manufacturer's instructions. pEGFP-C1 was used as a negative control for the transfections. The transfected cells were selected in medium containing G418 (maintenance concentration, 700 μg/mL, Invitrogen). To evaluate morphology, transfected and untransfected cells were grown on coverslips, briefly fixed in 10% neutral buffered formalin, and stained with H&E.

Cell proliferation (trypan blue exclusion) assay. Cell numbers were counted in triplicate daily for 1 week. Cells (n = 200,000; wild-type MDA-MB-231, mock-transfected, and two sublines transfected with NET-6) were seeded in 60 mm tissue culture dishes, grown in the appropriate medium, and harvested at the appropriate time points. Equal volumes of cell suspension and 0.4% trypan blue solution were mixed. Using a hemocytometer, the unstained (viable) cells were counted, and the total number of cells per dish was calculated.

Cell invasion (Boyden chamber) assay. Cell invasion was assayed using a kit from Chemicon International (Temecula, CA) following the manufacturer’s protocol. The experiment was done in quadruplicate. The invasive cells were examined after 3 days and photographed by a Nikon TE-300 inverted phase contrast microscope.

Statistics. Statistical analysis of correlations between variables was done using either χ² test or Fisher’s exact test for categorical data or Student’s t test for means.

Results

Celsr2 protein expression analysis

As we had access to an anti-Celsr2 antibody, we studied the expression of this protein by immunohistochemistry. Most cell lines showed weak to moderate staining, whereas only a small subset of MCF-7 cells were reactive, indicating a reduced expression level (Table 1). In benign breast tissue, nonneoplastic epithelium displayed distinct and generally strong membrane staining (Fig. 1A). The majority of carcinomas were characterized by similarly strong membrane reactivity (Fig. 1B). A small subset of tumors showed reduced (Fig. 1C) or absent (Fig. 1D) staining. This aberrant down-regulation was identified in 9 of 130 (7%) samples (Table 2). There was no statistically significant correlation with ER or HER2 status.

Immunohistochemical analysis of E-cadherin, CD9, and Kai1

We studied the expression of these well-characterized tumor suppressed proteins both in cell lines and in tumors. All nine cell lines tested failed to express E-cadherin. Conversely, all cell

<table>
<thead>
<tr>
<th>Table 1. Immunohistochemical expression pattern of membrane tumor suppressor and oncoproteins in breast cancer cell lines</th>
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<tr>
<td><strong>Cell line</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Nontransformed</td>
</tr>
<tr>
<td>MCF-10A/HER2-</td>
</tr>
<tr>
<td>HER2+ transformed</td>
</tr>
<tr>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>MDA-MB-435</td>
</tr>
<tr>
<td>MDA-MB-468</td>
</tr>
<tr>
<td>HER2+ transformed</td>
</tr>
<tr>
<td>BT474</td>
</tr>
<tr>
<td>SKBR3</td>
</tr>
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</table>

**NOTE:** - , negative; R, reduced expression level; +, weakly positive; 2+, positive; 3+, strongly positive.
lines were positive for CD9. Four of the cell lines were Kai1 negative. This down-regulation was not related to ER, HER2, or EGFR expression (Table 1). Benign breast epithelium and some tumors showed strong membrane staining for all three markers. In contrast, 30 of 85 (35%) breast cancers were characterized by markedly reduced or absent E-cadherin expression. A higher rate of down-regulation (79% and 76%, respectively) was noted for the two tetraspanins CD9 and Kai1 (Table 2). The number of data points varied from marker to marker because there were variable numbers of evaluable cores on the tissue microarrays and variable numbers of inconclusive stains.

Quantitative reverse transcription-PCR analysis of novel membrane markers

TROP-2. MCF-10A cells, with and without the HER2 transgene, expressed a very low amount of TROP-2 (0.06 and 0.03, respectively). In fully transformed breast cancer cell lines, TROP-2 levels were very low as well (0.02-0.29), and there was no correlation with HER2 status (Fig. 2A). In 32 breast cancer tissues, TROP-2 levels were higher. Three of the carcinomas showed relative overexpression (TROP-2/glyceraldehyde-3-phosphate dehydrogenase ratio, 25-33). However, there was no correlation with ER or HER2 expression (Fig. 2B). Further analysis revealed that ER-negative/HER2-positive tumors had significantly higher TROP-2 levels than ER-positive/HER2-negative cases (P = 0.03).

NET-6. We determined the relative NET-6 mRNA levels in 6 breast cancer cell lines and in 34 primary breast carcinomas and nodal metastases. In addition, we tested the nontransformed human mammary epithelial cell line MCF-10A as well as a derivative cell line stably transfected with HER2. The parental and transfected MCF-10A cells showed similar NET-6 expression (96.72 and 92.14, respectively). All three HER2-negative breast cancer cell lines showed lower NET-6 levels (mean, 71.16) compared with the three HER2-positive lines (mean, 402.67; Fig. 3A). There was a wide range in relative expression from 3.22 in MDA-MB-231 to 611.32 in BT474 cells. In the tumors, NET-6 expression was found to be dependent on both ER and HER2 status. NET-6 levels were markedly lower in ER-negative carcinomas and were also significantly reduced in HER2-negative cases (P = 0.03).

Table 2. Immunohistochemical expression of four membrane proteins in archival breast cancers

<table>
<thead>
<tr>
<th>Protein</th>
<th>Immunohistochemistry expression level (%)</th>
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<tbody>
<tr>
<td></td>
<td>+/2+</td>
</tr>
<tr>
<td>Celsr2</td>
<td>121 (93)</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>55 (65)</td>
</tr>
<tr>
<td>Kai1</td>
<td>24 (24)</td>
</tr>
<tr>
<td>CD9</td>
<td>20 (21)</td>
</tr>
</tbody>
</table>

NOTE: +/2+, positive expression (normal); R/-, significantly reduced or no expression (abnormal).
levels. The difference between ER-positive/HER2-negative and ER-negative/HER2-positive cases was not statistically significant ($P = 0.078$).

**Correlation between cell membrane marker expression and pathologic variables**

Statistical analysis was done to correlate the expression of NET-6, Celsr2, E-cadherin, CD9, and Kai1 with several morphologic and biological tumor characteristics, including tumor grade, tumor size, nodal status, and expression of ER, HER2, and EGFR. Only a few statistically significant associations were identified. Down-regulation of NET-6 and E-cadherin was more common in high-grade carcinomas (Table 3). Down-regulation of Kai1 occurred more frequently in ER-positive tumors ($P = 0.0052$). The relationship between NET-6 and ER/HER2 status was described above. NET-6 levels were not correlated with tumor size, lymph node involvement, or EGFR status.

**Effects of ectopic NET-6 expression in MDA-MB-231 breast cancer cells**

MDA-MB-231 cells had the lowest level of NET-6. Ectopic expression of the NET-6 gene in these cells induced a significantly simplified cell shape with markedly reduced filopodia formation (Fig. 4). It also had a marked growth inhibitory effect in vitro (Fig. 5). In contrast, transfection of the empty vector did not affect cellular proliferation. Ectopic expression of NET-6 also reduced the invasiveness of MDA-MB-231 cells in a Boyden chamber assay (Fig. 6).

**Discussion**

In an attempt to identify biomarkers that might mediate the aggressive phenotype of HER2-positive breast carcinomas, we previously did cDNA microarray analysis interrogating some 5,200 genes. About 110 genes were up-regulated and almost 180 genes were down-regulated in HER2-positive versus HER2-negative breast cancer cells (7). We decided to focus our further studies on genes that had previously not been implicated in mammary neoplasia and that, by inference, might be involved in important biochemical or physiologic processes. Specifically, we set out to investigate three genes encoding products that presumably were localized to the cytoplasmic membrane and that, by virtue of their subcellular localization, had the potential to play a role in cell-cell or cell-matrix adhesion, invasion through the basement membrane, or signal transduction pathways. Considering the possibility that these new membrane markers may interact with other biologically important cell membrane proteins, we also studied the expression of three additional tumor suppressor proteins that have a known role in mammary neoplasia as well as two oncogenic tyrosine kinase receptors. In aggregate, we wished to arrive at a more complete understanding of the deregulation of cell surface markers in breast cancer cells in vitro and in vivo.

Three of the markers studied (Kai1, CD9, and NET-6) belong to the tetraspanin superfAMILY comprising at least 26 molecules (16, 17). Members of this family are characterized by four transmembrane motifs, two extracellular loops, one intracellular loop, and cytoplasmic NH$_2$ and COOH termini (18). Tetraspanins have been implicated in a range of biological

<table>
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<th>Marker</th>
<th>Expression level</th>
<th>SBR grade*</th>
<th>$P$</th>
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<tbody>
<tr>
<td>NET-6</td>
<td>Low $^1$</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Low</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>34</td>
<td>20</td>
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*Modified Scarff-Bloom-Richardson.
$^1$s50 (relative mRNA expression level).
processes, including differentiation, motility, proliferation, and metastasis (18–20). Two of the tetraspanins included in our study are well-characterized metastasis suppressors. Kai1 (CD82) is a gene on chromosome 11p11.2 that encodes a highly glycosylated protein. We previously documented extensive loss of Kai1 expression in squamous neoplasia, lymphomas, and colorectal neoplasia (10, 21). In human breast cancer, too, Kai1 down-regulation was reported to be common and to correlate with aggressive clinical behavior (22–24), and ectopic expression suppressed the invasive and metastatic capability of breast cancer cells (25). In our previous gene expression profiling study, Kai1 had the third lowest expression ratio of down-regulated genes in HER2-positive breast cancer cell lines (7). In the current study, we identified markedly reduced or absent Kai1 expression in 4 of 7 breast cancer cell lines and in 74 of 98 archival tumors, confirming the important role of this marker in mammary neoplasia. Surprisingly, Kai1 down-regulation was more common in ER-positive tumors, but there was no association with other pathologic or immunophenotypic markers of tumor aggressiveness, such as grade, lymph node status, and HER2 or EGFR expression. A previous report suggested that Kai1 might attenuate the EGFR signaling pathway (26).

CD9 shares many similarities with Kai1. In experimental systems, CD9 overexpression inhibited motility and increased apoptosis (27–29). In breast cancer, CD9 down-regulation was correlated with nodal metastasis and decreased survival (8, 22, 30). We observed reduced or absent CD9 expression in 77 of 97 breast carcinomas, and this was not correlated with other tumor characteristics, including nodal involvement, nor was there a statistically significant relationship with Kai1 expression, which is in agreement with a previous report using quantitative reverse transcription-PCR (31). Although our CD9 down-regulation rate (79%) is higher than that in the Japanese series reported by Huang et al. (33), we are confident that our finding does not represent an artifact of paraffin section immunohistochemistry, because admixed benign cells always were strongly positive.

The third tetraspanin family member included in our study, NET-6, has not yet been characterized. The NET-6 gene is located on chromosome 7p21.1 and encodes a 204 amino acid protein with a predicted molecular weight of 24 kDa (32). NET-6 is part of a locus that was reported to be deleted in Wilms' tumors, but no mutations in this gene were identified (33). In our previous study, NET-6 was found to be expressed at high levels in HER2-positive breast cancer cell lines (7). We have now confirmed this observation by quantitative PCR (Fig. 3A). Furthermore, HER2-positive breast carcinomas also had higher levels of NET-6, particularly in the ER-negative subgroup (Fig. 3B). On the other hand, we observed a marked reduction of NET-6 expression in ER-negative compared with ER-positive breast carcinomas. The lowest levels were in ER-negative/HER2-negative tumors. This basal-like subset of breast cancers has been suggested to have a particularly unfavorable prognosis (34). In addition, NET-6 down-regulation was significantly more common in high-grade tumors. Our data show that NET-6 levels can vary widely in breast cancer cells and that down-regulation of the gene may be associated with a more aggressive tumor phenotype.
Importantly, we obtained experimental evidence that NET-6 may indeed have tumor suppressive activity. MDA-MB-231 cells are characterized by complex cell shape with prominent filopodia. They are highly invasive and display a moderately brisk growth rate in vitro. This cell line had the lowest level of NET-6. Transfection of the NET-6 gene into MDA-MB-231 cells was associated with simplification of cell shape and reduced invasiveness. Proliferation of the cells in culture was markedly decreased, and the mechanism of this growth inhibitory effect is currently under investigation.

The TROP-2 gene on chromosome 1p32 encodes a cell surface glycoprotein that is not or scarcely expressed in normal adult tissues but that is overexpressed in a significant subset of colorectal carcinomas (35, 36). TROP-2 was shown to form an oncogenic fusion protein with cyclin D1 (37). A recent study implicated TROP-2 for the first time in human breast cancer cells. By immunohistochemistry, Kluger et al. identified TROP-2 expression in four of six breast cancer cell lines (38). Our quantitative PCR data confirmed the previously described higher level of TROP-2 in BT474 cells and lower levels in SKBR3, MDA-MB-435, and MDA-MB-468 cells. However, we could not substantiate our previous observation of higher TROP-2 expression in HER2-positive breast cancer cell lines (Fig. 2A). TROP-2 levels in breast tumors were generally low, although a few cases showed relative overexpression. There was no significant correlation with HER2 or ER status; however, ER-negative/HER2-positive tumors had higher TROP-2 levels than ER-positive/HER2-negative cases (Fig. 2B), suggesting that TROP-2 overexpression may be associated with a less favorable breast cancer phenotype.

Located at 1p13.3-p21.1, Celsr2 is a nonclassic member of the cadherin family containing seven transmembrane and nine cadherin domains as well as seven EGF-like and two laminin AG-type repeats. It was suggested that Celsr2 may function as a receptor involved in contact-mediated communication (39). Previous studies largely focused on the role of Celsr2 in murine brain development (40–42), but this protein has also been implicated in rodent testicular function (15). In our previous gene expression study, Celsr2 was down-regulated in HER2-positive breast carcinomas (7). To the best of our knowledge, there is very limited information on Celsr2 expression in human cells and tissues. Using a custom-made polyclonal antibody, we examined Celsr2 protein expression in human breast cancer cells both in vitro and in vivo. We found strong membrane staining in benign breast epithelial cells and in most carcinomas and breast cancer cell lines. However, by immunohistochemistry, 1 of 9 cell lines and 9 of 130 tumors show markedly reduced or absent Celsr2 reactivity, suggesting that down-regulation of this protein may be important in a subset of human breast cancers.

The other cadherin family member included in our study, E-cadherin, has been well described in breast cancer. It plays an important role in epithelial cell-cell adhesion, migration, and signaling (43). In many breast cancers, the gene is inactivated by mutations or by promoter methylation (44, 45). Loss of E-cadherin expression is characteristic of in situ and invasive lobular neoplasia and has been linked to unfavorable outcome (46–48). In our study, we noted absence of E-cadherin reactivity in all nine cell lines, which is at variance with previous reports, indicating E-cadherin positivity in cell lines BT474, MCF-7, MDA-MB-361, and MDA-MB-468 (49, 50). This discrepancy may well be related to technical differences; however, inferior sensitivity of our assay is an unlikely culprit because we observed strong membrane staining in our positive controls and in normal breast epithelia. Among the carcinomas, 35% showed markedly reduced or absent E-cadherin expression, and this frequency is in agreement with other studies (47, 48). Significantly, we noted a higher rate of E-cadherin down-regulation in high-grade carcinomas, confirming previous reports (46, 51).

In summary, we studied the expression of eight cell surface markers (including HER2 and EGFR) in a significant number of breast cancer cell lines and tissues. This report for the first time documents the expression of the novel cell membrane markers NET-6, TROP-2, and Celsr2 in human breast carcinomas. The levels of these markers were not related to each other, to three other well-described cell surface proteins (Kai1, CD9, and E-cadherin), or to EGFR. Only NET-6 expression was significantly correlated with HER2 status. Low NET-6 levels were also associated with ER negativity and high grade and were lowest in basal-like carcinomas. Moreover, ectopic expression of NET-6 in MDA-MB-231 cells inhibited growth and invasion in vitro, indicating that this gene may function as a tumor suppressor. TROP-2 levels were significantly higher in ER-negative/HER2-positive versus ER-positive/HER2-negative breast carcinomas, suggesting that it might function as a mammary oncogene. Clearly, abnormal expression of cell surface markers is common in human breast cancer and likely has profound pathophysiologic consequences. Similar conclusions were reached by Sauer et al. who studied the expression of four tetraspanins (including Kai1 and CD9) in breast cancer cell lines (31). The current report introduces a few new players in the complex game of cell-cell and cell-matrix interactions, and some of them may well prove to play an important role in the development of mammary tumors.

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