Cleavable ErbB4 Isoform in Estrogen Receptor–Regulated Growth of Breast Cancer Cells

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Abstract

ErbB1 and ErbB2 receptors are well-characterized targets for anticancer drugs, but the clinical relevance of the related ErbB4 receptor is unknown. Here, we have assessed the clinical significance of the proteolytically cleavable ErbB4 isoforms in breast cancer patients and investigated their functions in vitro. The expression of transcripts encoding the cleavable ErbB4 isoforms associated with estrogen receptor–α (ER) expression (P < 0.001) and a high histologic grade of differentiation (P < 0.002) in real-time reverse transcription-PCR analysis of 62 breast cancer samples. Despite high ErbB4 mRNA expression levels in a subset of samples, ErbB4 gene amplification was not observed. High ErbB4 protein expression levels, as assessed by immunohistochemistry, associated with a favorable outcome in ER-positive cases from a series of 458 breast cancer patients (P = 0.01), whereas no association between ErbB4 expression and survival was found among women with ER-negative cancer (P = 0.86). However, nuclear ErbB4 immunoreactivity was associated with poor survival as compared with women whose cancer had membranous ErbB4 staining (P = 0.04). In vitro, overexpression of a cleavable ErbB4 isoform in ER-positive breast cancer cells resulted in translocation of a proteolytically released intracellular ErbB4 receptor fragment into the nucleus, as well as, enhanced proliferation, anchorage-independent growth, and estrogen response element–mediated transcriptional activity. These results suggest that the association of ErbB4 expression with clinical outcome is dependent on the subcellular localization of ErbB4 and that a proteinase-cleavable ErbB4 isoform promotes growth of ER-positive breast cancer and enhances ER-mediated gene transcription. (Cancer Res 2005; 65(4): 1384-93)

Introduction

ErbB receptor tyrosine kinase family consists of four receptors, ErbB1 (also known as epidermal growth factor receptor or HER1), ErbB2 (c-Neu, HER2), ErbB3 (HER3), and ErbB4 (HER4). ErbB receptors receive extracellular signals from epidermal growth factor–like ligands and transform them to a range of cellular responses, including cell proliferation, survival, migration, and differentiation (1).

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Aberrant ErbB signaling is a frequent phenomenon in various human cancers (1). ErbB1 and ErbB2 have well-characterized roles in tumorigenesis, and cancer drugs that specifically inhibit ErbB1 (such as gefitinib, erlotinib, and cetuximab) or ErbB2 (trastuzumab) have been introduced into clinical practice. Less is known about the tumor biology and clinical relevance of the most recently characterized ErbB family member, ErbB4 (2, 3). The presence of ErbB4 in breast cancer cells has been found to be associated with a well-differentiated phenotype (4, 5) and favorable prognosis (6–8), but contradictory results suggesting an oncogenic role for ErbB4 have also been reported both from cell biological (9–11) and clinical (12, 13) studies.

We have previously shown that ErbB4 is expressed as four alternatively spliced isoforms (14–16). Alternative splicing occurs within the intracellular cytoplasmic tail (isoforms CYT-1 and CYT-2) and within the extracellular juxtamembrane region (isoforms JM-a and JM-b), which generates receptors with different signaling capabilities. The CYT-1 isoform includes an additional exon with docking sites for SH2-domain and putatively WW-domain containing signaling molecules that is absent from the CYT-2 isoform (15, 17, 18). The JM isoforms differ in their sensitivity to proteinase cleavage. Tumor necrosis factor–α–converting enzyme (TACE) can cleave the JM-a isoform whereas the JM-b isoform is proteinase resistant (14, 19). This difference in susceptibility to receptor ectodomain shedding is also reflected in intracellular signaling via ErbB4: cleavage of ErbB4 at the JM domain by TACE-like enzymes triggers a secondary cleavage event by γ-secretase activity, which releases a soluble intracellular domain (ICD) fragment that can translocate into the nucleus and regulate gene transcription (18, 20, 21).

In this study, we investigated the expression of different ErbB4 isoforms in clinical breast cancer samples and determined their associations with breast cancer patient survival, cancer estrogen receptor (ER) expression, and a few other prognostic markers. Our findings suggest that nuclear translocation of the proteolytically released ErbB4 ICD fragments associates with adverse clinical outcome compared with women who have full-length ErbB4 present at the tumor cell surface. Results from in vitro experiments show that expression of the cleavable ErbB4 isoform in breast cancer cells results in nuclear localization of an intracellular receptor fragment, as well as, enhanced tumor cell growth and estrogen response element (ERE)–mediated transcriptional activity. These findings suggest a tumor-promoting role for the ErbB4 isoform capable of nuclear translocation.
Materials and Methods

Patients. Tumor tissue was obtained from 62 women diagnosed with primary breast carcinoma at the Tampere University Hospital (Tampere, Finland) for RNA and DNA analysis (22). Total RNA extracted from 20 different normal human tissues was obtained from BD Biosciences (Palo Alto, CA). Samples consisted of RNA extractions from single patients (cerebellum, brain, heart, liver, and lung) or of pooled RNA extractions from 2 to 84 patients (adrenal gland, bone marrow, kidney, placenta, prostate, salivary gland, skeletal muscle, spleen, thymus, thyroid gland, trachea, uterus, colon, small intestine, and mammary gland). A tissue microarray consisting of a series of 458 breast carcinomas was prepared as described elsewhere (23).

Cell Culture. MCF-7 breast cancer cells (24, 25) were maintained in RPMI supplemented with 10% FCS (Autogen Biokehr Ltd., Wiltsire, United Kingdom), 1% t-glutamine-pectinillin-streptomycin solution (Sigma, St. Louis, MO), 1 mmol/L 17-β-estradiol (Sigma), and for the transfected cell lines with 150 mg/ml Hygromycin B (Roche, Indianapolis, IN). For the experiments that addressed the effects of estradiol, the cells were grown for 48 hours in a phenol red- and estradiol-free medium supplemented with dextran-charcoal stripped FCS before treatment.

Quantitative Reverse Transcription-PCR Analysis of ErbB mRNA Expression. Total RNA was extracted from breast carcinoma tissues using a GeneElute mammalian total RNA purification kit (Sigma; ref. 22). cDNA synthesis and quantitative real-time reverse transcription-PCR (RT-PCR, Taqman) was done as previously described (16). β-Actin mRNA expression was used as a reference for ErbB mRNA quantification. Samples were analyzed in triplicate, and in each measurement, the SD of the Ct values was < 5% of the mean. ErbB mRNA expression was presented as a percentage of the β-actin mRNA expression measured from the same sample.

ErbB Gene Amplification Analysis. ErbB4 gene amplification was analyzed by PCR of genomic DNA extracted from 33 patients with known ErbB mRNA expression levels using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). PCR primers and probes were designed to measure the gene copy number of ErbB4 and the reference gene transforming growth factor-α (TGF-α). Relative copy number of the ErbB4 gene was determined as the ratio of PCR signals obtained for ErbB4 and TGF-α. TGF-α gene is located on the same chromosome as ErbB4 (2q33) but in the short arm (2p12; refs. 26, 27). Comparison of signals from two genes in opposing arms of the same chromosome allowed detection of isolated gene amplification, as opposed to aneuploidy or polyploidy. The oligonucleotide primers and probes used for analysis of ErbB4 were 5′-TCAAGCATTGGA-TATATCCGCA-3′, 5′-AGTTGCTGTATACATCTTCACT-3′, and 5′-FAM-TATCATACATGCTACTATCAACATTTG-3′. Real-time PCR was done as previously described (16). Samples were analyzed in duplicate, and in each measurement, the range of the Ct values was < 5% of the mean.

ErbB2 gene amplification was analyzed by fluorescence in situ hybridization as described (28). ErbB2 was considered to be amplified when the copy number was ≥ 20 in a minimum of 50 counted cells.

ErbB4 Immunohistochemistry. Immunostaining for ErbB4 was done using 1:50 dilution of a monoclonal HFR-1 antibody (NeoMarkers, Fremont, CA) that recognizes the intracellular tail of ErbB4, as described elsewhere (16). The tumor tissue array consisted of 0.6-mm-diameter samples from representative tumor regions obtained from 458 breast cancers patients with a median of 10 years of clinical follow-up after the diagnosis of breast cancer. The preparation of the tumor tissue microarrays, histopathology of tumors, and analysis of ER protein expression have been described (23).

The intensity of ErbB4 staining was scored from all 458 samples as negative, weak, or strong without knowledge of the clinicopathologic data. The cancer cell staining pattern was scored from 410 samples and classified either as membranous or nuclear. When 5% to 100% of the cancer cell membranes stained for ErbB4 (weak or strong intensity), the staining pattern was considered to be membranous. When 5% to 100% of the nuclei stained for ErbB4 (weak or strong intensity), the staining pattern was classified as nuclear. Cytosolic immunoreactivity was observed in conjunction with both the membranous and the nuclear staining, and was not considered a distinct defined staining pattern.

ErbB4 Expression Vectors. The expression vectors encoding the full-length ErbB4 isoform Jα-CYT-2 and Jβ-CYT-2 (pCEPHerbB4/Jβ-CYT-2 and pCEPHerbB4/Jα-CYT-2) were generated by cloning 4,345- and 4,315-bp XbaI-Pmel1 fragments from pcDNA3.1 pCEPHerbB4/Jα-CYT-2 and pDNA3.1 pCEPHerbB4/Jβ-CYT-2 plasmids, respectively, into XhoI and Xhol sites in the pCE4 vector (Invitrogen, Carlsbad, CA). The XhoI site was filled to blunt-end with Klenow before ligation. Construction of the vector encoding the ICD of ErbB4 of the CYT-2 type (pDNA3.1 pEHBHCYD/CYT2) will be described elsewhere (29).

Western Blot Analysis of ErbB4 Cleavage. MCF-7 cells were transfected with pCEPHerbB4/Jα-CYT-2 as previously described (25). Cells were starved overnight in the absence of FCS and stimulated for indicated periods of time with neuregulin-1 (NRG-1; 20 ng/mL; R&D systems, Minneapolis, MN) or phorbol 13-myristate 12-acetate (100 ng/mL; Sigma). Full-length ErbB4 and its cleaved intracellular domain were visualized by Western blotting using an antibody that recognizes the intracellular tail of ErbB4 (Santa Cruz Biotechnology, Santa Cruz, CA), as described (17).

Nuclear Translocation of ErbB4. MCF-7 breast cancer cells (24) were grown on coverslips in the presence of 1 mmol/L estradiol and transiently transfected with 0.5 µg of ErbB4 expression plasmids using FuGENE 6 Transfection Reagent (Roche). Twenty-four hours after transfection, the cells were treated for 1 hour at 37°C with or without leptomycin B (100 ng/mL; Sigma) and phorbol 12-myristate 13-acetate (100 µg/mL). The cells were fixed with methanol, stained with a mouse monoclonal anti-ErbB4 antibody (HFR-1; NeoMarkers; 1:50 dilution) followed by Alexa Fluor goat anti-mouse IgG (Molecular Probes, Eugene, OR). TO-PRO-3 iodide (Molecular Probes) was used to visualize the nuclei. Coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Images were obtained by LSM 510 Meta confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

Cell Proliferation Assay. MCF-7 breast cancer cell lines transfected with a vector encoding ErbB4 (pEBS7/ErbB4/Jα-CYT-2) or an empty vector (pEBS7; ref. 25) were plated at a density of 5 × 104 cells per well on 24-well plates and cultured in the presence or absence of 10 mmol/L estradiol as described above. The cell numbers were counted with hemocytometer.

Anchorage-Independent Growth Assay. Before analysis of growth in soft agar, MCF-7 breast cancer cell lines transfected with a vector encoding ErbB4 (pEBS7/ErbB4/Jα-CYT-2) or an empty vector (pEBS7; ref. 25) were plated at a density of 5 × 104 cells per well on 24-well plates and cultured in the presence or absence of 10 mmol/L estradiol as described above. The cell numbers were counted with hemocytometer.

Analysis of ERE-Mediated Transcription. MCF-7 cell lines (24) with and without ErbB4 overexpression were generated using an ErbB4 expression vector (pCEPHerbB4/Jα-CYT-2) and the empty vector (pCE4), as previously described (25). Cells were cultured in 24-well plates and transiently transfected with 0.5 µg of a firefly luciferase reporter construct

including two ERE and an SV40 promoter (pGL2-2xERE). A herpes simplex virus-TK promoter driven Renilla luciferase reporter plasmid (pRL-TK; Promega, Madison WI) was cotransfected for normalization of the ERE-regulated luciferase signal. Luminescence from firefly and Renilla luciferases was detected 24 hours after transient transfection and treatment with or without 10 nmol/L estradiol using a Dual-Luciferase Reporter Assay System kit (Promega).

**Statistical Analysis.** Associations of ErbB mRNA expression levels with other variables were analyzed using nonparametric, tie-corrected, Spearman’s rank test (SPSS for Windows; SPSS, Inc., Chicago, IL). Association of ErbB4 protein expression with survival was analyzed with Kaplan-Meier statistics, and survival between groups was compared using the log-rank test. Distant disease-free survival was used for statistical analyses and was defined as the time computed from the date of the diagnosis of breast cancer to the first detection of distant recurrence or to death from breast cancer. All Ps are two tailed.

**Results**

**Tissue-Specific Alternative Splicing of ErbB4 in Normal Human Tissues.** ErbB4 isoforms are generated from a single ErbB4 gene by alternative splicing (16). To assess the physiologic distribution of ErbB4 isoforms in nonneoplastic tissues, mRNAs representing 20 normal human tissues were analyzed using quantitative real-time RT-PCR (Fig. 1A). Ten of the 20 normal tissues expressed ErbB4 mRNA at levels exceeding 0.1% of the β-actin mRNA level measured from the same sample. Kidney, salivary gland, trachea, thyroid gland, prostate, and mammary gland expressed exclusively the JM-a type of JM domain, whereas brain, cerebellum, skeletal muscle, and heart expressed predominantly JM-b. Little or no ErbB4 was expressed in adrenal gland, bone marrow, liver, lung, placenta, spleen, thymus, uterus, colon, or the small intestine. All tissues where ErbB4 was expressed produced both the CYT-1 and the CYT-2 transcripts at approximately equal molar ratios, regardless of the JM isoform present. The sum of the expression levels of the two alternative extracellular JM isoforms (JM-a + JM-b) was reflected in a quantitatively similar sum of the two alternative intracellular CYT isoforms (CYT-1 + CYT-2), implying that no additional ErbB4 isoforms were expressed in normal human tissues at quantitatively significant levels.

**Transcripts Encoding Cleavable ErbB4 Isoforms Are Over-expressed in a Subset of Breast Cancers.** To obtain data about the relative expression of ErbB4 isoforms in breast carcinoma, 62 clinical breast cancer samples were analyzed by real-time RT-PCR (Fig. 1B). As previously reported using the same material,12 and similar to normal mammary gland (Fig. 1A), the only JM isoform expressed in breast cancer was JM-a, whereas both CYT variants were present. This indicates that only receptors susceptible to proteolysis and subsequent putative nuclear translocation (isoforms JM-a CYT-1 and JM-a CYT-2) were expressed in breast cancer. However, in contrast to normal tissues, the quantity of the JM-a signal generally seemed to exceed the sum of the signals from the two CYT variants, suggesting that some aberrant ErbB4 transcripts may be generated in the tumors. ErbB4 JM-a transcript was overexpressed in 19% of breast cancer patients when overexpression was defined as expression exceeding the mean expression level plus five SDs calculated from the 20 normal tissues.

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2.0. These data suggest that ErbB4 gene amplification did not contribute to ErbB4 mRNA overexpression in the series.

Overall ErbB4 Protein Expression Associates with Improved Survival in Breast Cancer. To assess the significance of ErbB4 protein expression, a breast cancer tissue array (n = 458) was analyzed by immunohistochemistry. ErbB4 protein was detected using a monoclonal antibody (HFR-1) recognizing an epitope in the intracellular tail of ErbB4. Strong ErbB4 staining was detected in 46%
of patients. Weak ErbB4 signal was observed in 34% of the patients, whereas 21% of patients were negative for ErbB4 immunoreactivity. In univariate survival analysis, breast cancer patients with strong ErbB4 expression tended to have better survival than patients with weak or no ErbB4 staining (Fig. 4A, left; \( P = 0.09 \)). Because ErbB4 mRNA expression clearly correlated with the presence of ERs (Fig. 1B), the analysis was repeated as stratified by the ER status. In women with ER-positive cancer, strong ErbB4 expression was associated with significantly longer survival than weak expression or no ErbB4 immunoreactivity (\( P = 0.01 \)). In turn, no significant association was present between ErbB4 protein expression and survival among patients with ER-negative cancer (\( P = 0.86 \)). These results suggest that ErbB4 protein expression associates with a favorable prognosis among patients with ER-positive breast cancer.

**Nuclear ErbB4 Immunoreactivity Associates with Shorter Survival than Cell Surface ErbB4 Immunoreactivity.** Proteolytic cleavage of ErbB4 releases a soluble ICD from the cell membrane, after which it may translocate into the nucleus and regulate transcription (18, 20, 21). It is therefore conceivable that full-length ErbB4 on the cell membrane would be functionally different from nuclear ICD. When subcellular localization of ErbB4 immunoreactivity was analyzed from the tissue array (\( n = 410 \)), distinct staining patterns were observed. For example, 69 of the microarray samples (17%) had ErbB4 immunoreactivity at the cell membrane, whereas 85 of the samples (21%) had ErbB4 immunoreactivity in the cancer cell nuclei (Fig. 4B). Staining at the cell membrane or in the nuclei was mutually exclusive, as no overlap between these two staining patterns was detected. On the other hand, cytosolic ErbB4 immunoreactivity did not appear as a clearly defined phenotype and was observed in connection with both the cell membrane and the nuclear staining patterns. Nuclear staining may reflect the degree of the receptor cleavage and nuclear translocation of the cleavable ErbB4 isoforms. Interestingly, nuclear ErbB4 staining pattern was associated with significantly shorter survival compared

### Table 1. Association of ErbB4 mRNA expression with clinical variables and expression of other ErbBs

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NOTE: For each variable, line 1 = correlation coefficient, line 2 = significance, line 3 = \( n \).
*Correlation is significant at the 0.01 level.

**Figure 3.** Analysis of ErbB4 gene amplification in breast cancer. Genomic DNA extracted from 33 breast cancer samples was analyzed by real-time PCR with primers and probes specific for ErbB4 and TGF-\( \alpha \), two genes located in opposite arms of the same chromosome 2. Ratio of signals obtained for ErbB4 over TGF-\( \alpha \) (y-axis). ErbB4 mRNA expression of the corresponding patients (x-axis). Ranges of two parallel analyses were <5% of the means.
with the pattern where ErbB4 was localized on the cell membrane ($P = 0.04$; Fig. 4A, right). These data indicate that the prognostic value of ErbB4 depends on the subcellular localization of the ErbB4 protein, and that nuclear ErbB4 staining is associated with worse clinical outcome than cell membrane-associated ErbB4 staining.

**An Intracellular Fragment of a Cleavable ErbB4 Isoform Translocates into the Nucleus of Breast Cancer Cells.** To directly assess whether a proteinase cleavable ErbB4 isoform overexpressed in breast cancer can be translocated to the nucleus, ER-positive MCF-7 breast cancer cells were transiently transfected with cDNAs encoding the proteinase cleavable JM-a CYT-2 isoform, or the noncleavable JM-b CYT-2 isoform. HFR-1 antibody that recognizes the intracellular tail of ErbB4 was used to visualize the ErbB4 ICD by confocal microscopy. Nuclear staining was observed in cells overexpressing JM-a CYT-2, but not in cells expressing JM-b CYT-2, when nuclear export of proteins was blocked by treatment with leptomycin and ErbB4 shedding was stimulated with phorbol 12-myristate 13-acetate (Fig. 5A). To eliminate the need for cellular proteinase activity for analyzing subsequent targeting of the proteolytically released ICD, the MCF-7 cells were transiently transfected with a plasmid encoding only the CYT-2 ICD. This CYT-2 fragment readily translocated and accumulated in the nuclei also in the absence of leptomycin (Fig. 5A). Basal, as well as...
NRG-1- and phorbol 12-myristate 13-acetate–stimulated cleavage of ErbB4 JM-a CYT-2 in MCF-7 cells was also shown by visualizing the cleavage product, 80-kDa ICD, by Western blotting (Fig. 5B). Taken together, these results indicate that the intracellular domain of ErbB4 can translocate into the nucleus of breast cancer cells.

Overexpression of a Cleavable ErbB4 Isoform Promotes Estrogen-Independent Growth and ERE-Mediated Transcription in Breast Cancer Cells. Our data indicate that overexpression of mRNAs encoding cleavable ErbB4 isoforms correlates with ER expression (Fig. 1; Table 1). Moreover, the intensity of overall ErbB4 protein expression associates with a favorable outcome among patients whose breast cancer is ER positive (Fig. 4A). To analyze the functional relevance of the expression of proteinase cleavable ErbB4 in ER-positive breast cancer cells, we examined MCF-7 cell lines overexpressing ErbB4 JM-a CYT-2 and control cells transfected with an empty expression vector. The cells were grown in the presence or absence of 10 nmol/L estradiol and their proliferation was measured by cell counting (Fig. 5C). In the absence of exogenous estrogens, the vector control cells did not significantly proliferate. However, the cells overexpressing ErbB4 JM-a CYT-2 proliferated both in the presence and absence of estradiol, although the rate was slower in the absence of estradiol. In the presence of estradiol, the cells that overexpressed ErbB4 JM-a CYT-2 proliferated slightly more than the vector control cells. Similar results were obtained when the MCF-7 cell lines were analyzed for anchorage-independent growth using soft agar colony formation assays (Fig. 5D). In the absence of estradiol, ErbB4 JM-a CYT-2 overexpression resulted in 2.8-fold enhanced colony formation. This was more than the enhancement seen in the vector control.

![Figure 5](image.png)

**Figure 5.** A cleavable ErbB4 isoform releases an intracellular domain to nucleus and promotes estrogen-independent cellular responses in breast cancer cells. A, ER-positive MCF-7 cells were transiently transfected with plasmids encoding the full-length ErbB4 isoforms JM-a CYT-2 or JM-b CYT-2, or only the ICD of type CYT-2. Localization of an intracellular epitope of ErbB4 was analyzed by confocal microscopy. Before analysis, cells were either treated or not with PMA and leptomycin B, that stimulate ErbB4 cleavage and inhibit nuclear export, respectively. TO-PRO-3 staining was used to visualize the nuclei. B, MCF-7 cells overexpressing ErbB4 JM-a CYT-2 were treated with NRG-1 or PMA for the indicated periods of time. ErbB4 cleavage was analyzed using anti-ErbB4 antibody detecting both full-length 180-kDa ErbB4 and cleaved 80-kDa ErbB4 ICD. n.s., nonspecific band. C–E, MCF-7 cells overexpressing ErbB4 JM-a CYT-2 and vector control cells were grown in the presence or absence of estradiol, and assessed for cell number (C), number of colonies formed in soft agar (D), and ERE-mediated transcriptional activity. For ERE-activity analyses (E), the cells were transiently transfected with plasmids encoding firefly luciferase under the regulation of ERE, and Renilla luciferase with constitutive expression. ERE-activity is presented as ERE-luciferase signal normalized by Renilla luciferase signal.
control cells after addition of estradiol to the culture medium (2.3-fold). Overexpression of ErbB4 JM-a CYT-2 also enhanced colony formation by 3.9-fold in the presence of estradiol, as compared with the vector control cells.

Expression of ErbB4 JM-a CYT-2 might promote ER-independent growth by interacting with the ER signaling pathway that regulates transcription of estrogen-responsive genes. The effect of ErbB4 JM-a CYT-2 overexpression on ER-mediated transcription was analyzed by transiently expressing anERE-driven luciferase reporter gene in MCF-7 cells. Renilla luciferase reporter plasmid was cotransfected to control the transfection efficiency. In the absence of estradiol, overexpression of ErbB4 JM-a CYT-2 increased the ERE-mediated transcriptional activity by 3.7-fold (Fig. 5E). ErbB4 overexpression in the presence of estradiol resulted in a 1.6-fold enhancement as compared with the vector transfected cells.

These results show that overexpression of a cleavable ErbB4 isoform can promote proliferation and colony formation of hormone-dependent breast cancer cells both in the presence and absence of estrogen. The findings further suggest that the mechanism of growth promotion by ErbB4 may include enhancement of the transcriptional regulation of ER-responsive genes. Taken together with data from the clinical series, our findings suggest that cleavable ErbB4 isoforms are present in ER-positive breast cancer, and that they are capable of stimulating cellular functions associated with malignant cell behavior.

Discussion

The prognostic and therapeutic significance of ErbB4 in breast cancer has remained controversial. For example, reports from clinical breast cancer series have indicated association of ErbB4 expression with either favorable (6–8) or adverse (12, 13) clinical outcome, and in vitro studies have suggested that ErbB4 mediates either breast cancer cell differentiation (32–34) or tumorigenic growth (10). However, it is not known whether the existence of functionally different isoforms of ErbB4 (14, 17, 21) might explain some of the seemingly controversial data, nor is it known whether the isoforms actually have different biological roles in breast cancer. We have specifically addressed the significance of the proteinase-cleavable ErbB4 isoforms in breast cancer. Our results show that only mRNAs encoding the proteinase-cleavable ErbB4 isoforms are present in breast cancer. Importantly, the association of ErbB4 expression with clinical outcome is dependent on whether the ErbB4 protein is cleaved or remains intact at the cell surface. Evidence is provided suggesting that localization of the ICD of ErbB4 in the nucleus associates with a worse clinical outcome than localization of ErbB4 at the cell surface. In addition, we show that overexpression of a cleavable ErbB4 isoform in vitro results in translocation of a soluble ICD into nuclei, and promotes breast cancer cell proliferation, anchorage-independent growth, and ERE-mediated gene transcription.

Expression of transcripts encoding ErbB receptors in human tissue samples was specifically quantitated by real-time RT-PCR, which we previously shown to be accurate in measuring ErbB mRNA expression, and to reflect ErbB expression in tumor cells at protein level (16). Our results show that only cleavable isoforms of ErbB4 are present in breast cancer. The expression of these isoforms also significantly associated with both the presence of ER and a high histologic grade of differentiation. In particular, the association of ErbB4 expression with ER expression is consistent with previous studies, none of which address the role of specific ErbB4 isoforms (4–6, 8).

Although no isoform-specific antibodies are available, subcellular localization of the ErbB4 ICD can provide information about the presence of the cleavable or noncleavable ErbB4 isoforms. Our immunocytochemistry data showed nuclear ErbB4 staining when a cleavable ErbB4 isoform or its sole ICD was overexpressed in MCF-7 breast cancer cells, but not in cells overexpressing a noncleavable isoform. Consistent with the expression of ErbB4 transcripts encoding cleavable ErbB4 isoforms, 21% of breast cancer tissue samples analyzed showed nuclear ErbB4 immunoreactivity, suggesting that the cleavable ErbB4 isoforms are indeed proteolytically cleaved in vivo. In accordance with our findings, nuclear ErbB4 staining in breast cancer cells has previously been reported from studies using antibodies that recognize the ICD of ErbB4 (13, 35, 36).

In survival analyses, overall ErbB4 protein expression intensity was associated with a high survival rate. Intriguingly, the association with survival was dependent on the subcellular localization of the receptor. Nuclear ErbB4 staining associated with inferior survival as compared with membranous staining. Hypothetically, this might be due to the tumor-promoting properties of the nuclear ErbB4 fragments, or from the loss of tumor-suppressing activity of the full-length ErbB4 at the cell membrane. Our in vitro studies showing increased cancer cell proliferation rate, growth in soft agar, and reduced estrogen dependency, suggest a growth-promoting role for the cleavable ErbB4 JM-a CYT-2 isoform. These data also imply that the presence and activity of the relevant ErbB4-cleaving proteinase, such as TACE (19), in the tumor tissue may be more biologically relevant than the actual ErbB4 isoform, as cleavable and noncleavable ErbB4 isoforms may function similarly in the absence of proteinases (14). The significance of proteinase-cleavage in the process of translocation of receptor fragments was supported by our in vitro assays, in which the ErbB4 ICD was readily seen in the nucleus. When transfecting the full-length receptor, ErbB4 cleavage and detection of ErbB4 in the nucleus was clearly enhanced by activation of proteinases by phorbol 12-myristate 13-acetate. Furthermore, reports have suggested that TACE expression is up-regulated both at the mRNA and protein levels in breast cancer (37).

ErbB4 cleavage in breast cancer cells could be stimulated by ligands, such as NRG-1 (38). Enhanced cleavage in response to NRG-1 supplied into culture medium was also shown in our experiments with MCF-7 transfectants. However, the MCF-7 cells did not express detectable levels of NRG-1 mRNA when assessed by real-time RT-PCR (data not shown), ruling out a NRG-1-mediated autocrine loop regulating ErbB4 cleavage in this cellular context. Our experiments with in vivo tissue samples have also indicated that nuclear ErbB4 ICD localization is frequently observed in breast cancer sections with relatively low levels of NRG-1 immunoreactivity, suggesting that NRG-1 may not function as a key regulator of ErbB4 processing in vivo. Analysis of NRG-1 mRNA expression levels in the series of 62 breast cancer RNA samples also failed to show significant NRG-1 overexpression when compared with the 20 normal samples, or association of NRG-1 expression levels with...
ErbB4 expression, hormone receptor status, or histologic grade of differentiation (data not shown). In line with the association of ErbB4 mRNA expression and ER expression, ErbB4 protein expression associated with survival in breast cancer patients who were diagnosed with ER-positive cancer. The connection between ErbB4 signaling and ER signaling is also supported by our in vitro experiments with the estrogen-dependent breast cancer cells, in which ErbB4 overexpression promoted cellular responses also in the absence of exogenous estrogens. Furthermore, the growth of another ER-positive breast cancer cell line T-47D was suppressed by down-regulating endogenous overexpression of cleavable ErbB4 with ErbB4-specific small interfering RNA (data not shown) or ribozymes (10). Interestingly, ribozyme-mediated ErbB4 down-regulation did not reduce the growth of an ER-negative MDA-MB-453 breast cancer cell line (10), in accordance with our unpublished findings of a lack of effect of ErbB4 JM-a CYT-2 overexpression on basal growth of some ER-negative nonneoplastic cell lines.

ErbB4 overexpression also enhanced ER-mediated transcription, indicating a functional connection between ErbB4 and ER signaling pathways. Several previous studies have addressed the interaction between the ErbB and ER pathways. For example, activation by NRG or epidermal growth factor, or overexpression of ErbB2 have been shown to lead to ER phosphorylation, modulation of ER-responsive transcription, and hormone-independent growth in ER-positive breast cancer cells (39–42). Although providing support for the general concept for functional ErbB-ER interaction, these previous studies have not specifically addressed whether ErbB4 is involved in the crosstalk or suggested a molecular mechanism by which ErbB4 promotes ER-dependent cellular responses. Taken together, our findings suggest that targeting ErbB4 in breast cancer could be favorable in cases where cleavable ErbB4 isoforms are overexpressed and proteolytically released ICD fragments have translocated to the nuclei, but not when ErbB4 is located at the cell surface. One approach for specific ErbB4 targeting could be compounds that block the activity of the known ErbB4 processing enzymes (i.e., TACE or γ-secretase). These results further suggest that ErbB4 inhibitors might enhance the efficacy of ER inhibitors and inactivators, such as tamoxifen and fulvestrant, but these hypotheses require confirmation in clinical trials.

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