Subject Review

Systems-Level Analysis of ErbB4 Signaling in Breast Cancer: A Laboratory to Clinical Perspective

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Abstract

Although expression of the ErbB4 receptor tyrosine kinase in breast cancer is generally regarded as a marker for favorable patient prognosis, controversial exceptions have been reported. Alternative splicing of ErbB4 pre-mRNAs results in the expression of distinct receptor isoforms with differential susceptibility to enzymatic cleavage and different downstream signaling protein recruitment potential that could affect tumor progression in different ways. ErbB4 protein expression from nontransfected cells is generally low compared with ErbB1 in most cell lines, and much of our knowledge of the role of ErbB4 in breast cancer is derived from the ectopic overexpression of the receptor in non-breast-derived cell lines. One of the primary functions of ErbB4 in vivo is in the maturation of mammary glands during pregnancy and lactation induction. Pregnancy and extended lactation durations have been correlated with reduced risk of breast cancer, and the role of ErbB4 in tumor suppression may therefore be linked with its role in lactation. Most reports are consistent with a role for ErbB4 in reversing growth stimuli triggered by other ErbB family members during puberty. In this report, we provide a systems-level examination of several reports highlighting the seemingly opposing roles of ErbB4 in breast cancer and potential explanations for the discrepancies and draw the conclusion that future studies examining the function of ErbB4 in breast cancer should also take into account the pregnancy history, lactation status, and hormone supplementation or ablation history of the patient from whom the tumor or tumor cells are derived. (Mol Cancer Res 2008;6(6):885–91)

Introduction

Four receptors comprise the ErbB receptor tyrosine kinase family: ErbB1 (epidermal growth factor receptor, HER1, c-erb-B1), ErbB2 (HER2, neu, c-erb-B2), ErbB3 (HER3, c-erb-B3), and ErbB4 (HER4, c-erb-B4). Whereas ErbB1 and ErbB2 are frequently overexpressed in breast cancer and correlate with a poor prognosis, ErbB4 expression in breast cancers actually correlates with a favorable prognosis. Comparison between amino acid sequences among the vertebrate ErbB receptors suggested that evolutionary gene duplication generated two ancestral receptors: the ErbB1/ErbB2 and the ErbB3/ErbB4 precursors. Subsequent gene duplications of these two precursors further gave rise to the present four ErbB receptors (1). ErbB receptors are type I transmembrane proteins that contain a heavily glycosylated and disulfide-bonded ectodomain containing the ligand-binding site, a transmembrane domain, and a cytoplasmic region that encodes a tyrosine kinase as well as multiple phosphorylation sites. ErbB2 is unable to bind to extracellular ligand, but it heterodimerizes with and is then activated by other ErbB family members during puberty. In this report, we provide a systems-level examination of several reports highlighting the seemingly opposing roles of ErbB4 in breast cancer and potential explanations for the discrepancies and draw the conclusion that future studies examining the function of ErbB4 in breast cancer should also take into account the pregnancy history, lactation status, and hormone supplementation or ablation history of the patient from whom the tumor or tumor cells are derived. (Mol Cancer Res 2008;6(6):885–91)

ErbB4 Receptor Role in Development of Brain, Breast, and Heart

ErbB4 responds to many different ligands, including neuregulin-1 (heregulin), neuregulin-2, neuregulin-3, neuregulin-4, betacellulin, epiuregulin, and heparin-binding epidermal...
growth factor—like growth factor. Neuregulin-1 and neuregulin-2 are also recognized by ErbB3, whereas heparin-binding epidermal growth factor, betacellulin, and epipeplurin are also recognized by ErbB1. ErbB4 is widely expressed in many tissues at the mRNA level, including the epithelial lining of the gastrointestinal and respiratory tracts as well as urinary, reproductive, skin, skeletal muscle, circulatory, endocrine, and nervous system tissues. The highest expression levels of ErbB4 receptor during development are observed in the brain and heart (3). Whereas ErbB1 plays an essential role in ductal differentiation of the breast (4), ErbB4 plays an essential role in alveoli maturation during pregnancy-induced epithelial differentiation and during the initiation of lactation at parturition in breast tissue (5, 6). ErbB4 is also essential for both cardiac muscle differentiation and axon guidance in the central nervous system. ErbB4+/− mice do not survive beyond mid-embryogenesis due to the aborted development of myocardial trabeculae in the heart ventricle and striking alterations in the hindbrain of the central nervous system, similar to the embryonically lethal phenotype observed in heregulin-defective mice (7).

ErbB4 Signaling Mechanism Is Different than Other ErbB Family Members

Activation of ErbB4 by phorbol ester 12-O-tetradecanoylphorbol-13-acetate or by heregulin elicits an ectodomain cleavage, which results in the release of a 120-kDa ectodomain fragment into the extracellular space, generating a membrane-associated 80-kDa fragment (8, 9). Two alternative extracellular juxtamembrane domain sequences seem to be either sensitive associated 80-kDa fragment (8, 9). Two alternative extracellular cleavage, which results in the release of a 120-kDa ectodomain

activation of signal transducer and activator of transcription 5A (STAT5A) in transfected HEK 293T, COS7, and MCF-7 cells (6). Chromatin immunoprecipitation experiments were done in T47D cells to semiquantitatively validate the co-occupancy of the β-casein promoter by STAT5A and the ErbB4 ICD following a 1-h heregulin treatment.

Two cytoplasmic domains of ErbB4 are produced by alternative pre-mRNA splicing, thus generating the isoforms CYT-1 and CYT-2 (Fig. 1A; ref. 12) that differ by 16 amino acids (13), and both JM-a CYT-1 and JM-a CYT-2 were observed to be overexpressed in a subset of primary human breast cancers (12, 14) based on quantitative immunostaining, but the pregnancy and lactation status of these cells was not reported and it is therefore unknown whether ErbB4 might still function as a tumor suppressor in the context of the breast following pregnancy- and lactation-induced maturation. ErbB4 may have different transcriptional coactivation potential in a nonparous versus a parous host where cycles of estrogen and progesterone likely cause a global reorganization of chromatin structure in preparation for milk protein production. ErbB4 CYT-1 contains several known protein interaction motifs: Y1056 exists as a YTPM motif, which is a known consensus (YXXM) for recruitment of the p85 regulatory subunit of PI3K. ErbB4 CYT-2 was shown not to recruit p85 when expressed and stimulated in an NIH3T3 cell line (13). However, it should be noted that ErbB4 is often coexpressed with ErbB3 in mammary cell lines and ErbB3 contains seven YXXM recruitment motifs (15). Additionally, ErbB4 contains two other YXXM motifs besides Y1056: one at Y950 and the other at Y1150 that could potentially serve as PI3K recruitment sites if phosphorylated in the appropriate cell type. In addition to PI3K recruitment motifs, there are three PPXY motifs on ICD that are potential recruitment sites for class I WW-domain-containing proteins, such as Yes-associated protein (YAP). These include the PPXY motif at position 1034 to 1040, the PPAY motif at position 1052 to 1056 that exists only in the CYT-1 isoform, and the PPYY motif at position 1282 to 1286. However, interaction between YAP and ErbB4 occurs mainly via the WW domain of YAP and the PPHY motif at position 1282 to 1286 (16). YAP has been shown to function as a transcriptional coregulator for the COOH-terminal fragment of ErbB4 (16, 17) in the context of transfected COS7 cells. The binding between YAP and the JM-b CYT-1 isoform was reduced by the phosphorylation of Y1056 in HEK 293 cells following cotransfection of a JM-b CYT-1 and single WW domain-containing YAP cDNA (17). The PPHY motif at position 1282 to 1286 is sufficient, however, for basal interaction between YAP and the ErbB4 CYT-2 isoform (17).

WWOX, a WW domain-containing oxidoreductase with tumor-suppressive capabilities, is frequently deleted or mutated in several cancer cells. WWOX competes with YAP2 for interaction with ErbB4 and thereby inhibits ErbB4 transcriptional activity (Fig. 1B) in HEK 293 cells (18). Overexpression of WWOX was shown to sequester ErbB4 ICD in the cytoplasm of several cancer cell lines, including MCF-7 cells, after stimulation with ErbB4 ligand (18). Analysis of WWOX expression by immunohistochemistry in a series of breast cancer samples revealed that WWOX was absent in 36% of the cancers, and loss of WWOX expression was associated with an unfavorable outcome (19). WWOX expression was strongly associated with membranous ErbB4 localization and with overall ErbB4 expression (19). Coexpression of membranous ErbB4 and WWOX was associated with a favorable outcome. Results from COS7 cells showed that WWOX was associated with both ErbB4 JM-a CYT-1 and JM-a CYT-2 isoforms. Expression of WWOX in COS7 cells and in vivo correlated with increased levels of full-length membrane-associated ErbB4 (19). Knockdown of endogenous WWOX in MCF-7 cells by small interfering RNA also led to reduced expression of membrane-bound ErbB4 consistent with a model where WWOX protein remains constitutively bound to ErbB4 and thus stabilizes it at the cell membrane and reduces the binding, nuclear trafficking, and transcriptional activity resulting from binding of ErbB4 and ICD to other WW domain-containing proteins (19).
Effects of ErbB4 Expression on the Proliferation of Breast and Other Cell Lines

Although Y1056 on ErbB4 is required for the tumor suppressor activity of ErbB4 in the context of a constitutively active receptor in human mammary cell lines (20), ErbB4 isoforms have been reported to promote proliferation and survival of NIH3T3 cells. Heregulin promoted phosphorylation of SHC and mitogen-activated protein kinase and stimulated proliferation of cells expressing either ErbB4 CYT-1 or ErbB4 CYT-2. However, Akt was only phosphorylated in cell expressing ErbB4 CYT-1. Additionally, heregulin only protected cells expressing ErbB4 CYT-1 from starvation-induced apoptosis, suggesting that although both ErbB4 CYT-1 and CYT-2 can promote cell proliferation, only ErbB4 CYT-1 can function to promote cell survival on ligand stimulation in NIH3T3 cells (21). ErbB4 JM-a CYT-1 and JM-a CYT-2 have differential subcellular localization. JM-a CYT-2, but not JM-a CYT-1, was localized to nuclei in MCF-7 cells under both nonstimulated and heregulin-stimulated condition (12). Overexpression of the ErbB4 JM-a CYT-2 isoform in MCF-7 breast cancer cells resulted in the constitutive production, phosphorylation, and nuclear translocation of the ICD, which enhanced cell proliferation, anchorage-independent growth, and estrogen response element-mediated transcriptional activity (12, 14). Overexpression of ErbB4 JM-a CYT-2, but not other isoforms, stimulated the ligand-independent proliferation of interleukin-3–independent myeloid 32D cells (12), a line that lacks detectable expression of ErbB receptor, suggesting that different isoforms may coordinate with different signaling pathways to elicit distinct biological outcomes.

In contrast, other lines of evidence have suggested that ErbB4 elicits a suppressive effect on proliferation and survival of breast cancer cells. Stimulation of ErbB4 in ErbB4-expressing breast cancer cells caused cell cycle arrest and induced differentiation. Furthermore, treatment with heregulin or an ErbB4-specific agonistic antibody stimulated BRCA1 and suppressed cell proliferation by causing both G2-M cell cycle arrest and induction of apoptosis only in ErbB4-positive breast cancer cells (22, 23). Introduction of a stably expressed ErbB4 into ErbB4-negative SUM102 cells resulted in the acquisition of a heregulin-dependent antiproliferative response associated with markers of differentiation (22). Ectopic expression of a mutant ErbB4 allele harboring an activating transmembrane mutation (ErbB4-CA) resulted in a 5-fold increase in receptor tyrosine phosphorylation, higher levels of nuclear translocation, and enhancement of the ErbB4-induced STAT5A stimulation of the β-casein promoter. ErbB4-CA induced apoptosis more efficiently compared with wild-type ErbB4 in HEK 293 kidney cells (5). Production of ErbB4 ICD via cleavage was essential for the antiproliferative activity of ErbB4 in cancer cells because inhibition of the γ-secretase cleavage event on ICD blocked heregulin-dependent growth inhibition of the T47D mammary carcinoma cells in addition to T47-L4 cells, an NIH3T3 cell line that ectopically expresses ErbB4 (11). ErbB4 kinase activity and phosphorylation of ErbB4 Y1056 were necessary and sufficient for constitutively active ErbB4 mutant ErbB4-Q646C to inhibit colony formation in cell culture of MCF-7 and MCF-10 cells (20). Interestingly, although fewer colonies formed, the rate of growth of the surviving colonies did not seem to be different between ErbB4-Q646C–infected and control-infected cells. Because only the ErbB4 CYT-1 isoform contains Y1056 (13), it was speculated that recruitment of a signaling molecule, such as STAT5, or the activation of the AKT signaling pathway (through recruitment of PI3K) might be responsible for ErbB4 activity in limiting colony formation.

ICD was found to be functionally similar to BH3-only proteins. Cytosolic but not membrane-bound ErbB4 ICD expression in primary human breast tumors correlated with apoptosis according to in situ tissue assays. ErbB4 induced apoptosis endogenously in 20% of T47D cells when activated by heregulin and in 60% of cells following transfection into several different breast cancer cell lines versus epidermal growth factor receptor transfection alone. Ligand activation and subsequent proteolytic processing of ErbB4 resulted in mitochondrial accumulation of the ICD and cytochrome c efflux through activation of BAX/BAK and BCL-2 was able to suppress apoptosis induced by ErbB4 (23). Heregulin activation of ErbB4 in breast cancer cells provoked tyrosine phosphorylation of Hdm2 in ZR-75-1 breast carcinoma cells, which was sensitive to inhibition of γ-secretase activity, indicating that release of the ICD fragment was required. Overexpression of the ICD fragment promoted ubiquitination of Hdm2 and induced expression of p53 as well as one of its primary transcriptional targets p2110, a transcriptional target of p53 and a cell cycle inhibitor (24) in H1299 human lung carcinoma cells.

ErbB4 Expression as a Prognostic Marker for Breast Cancer

Expression of ErbB4 receptor is typically low compared with ErbB1 and ErbB2 in primary breast tumors, although a subset of breast tumors ranging from 12% to 45% exhibits substantial expression of ErbB4 (14, 25–31). Higher expression levels of ErbB4 have been associated with a more differentiated histologic phenotype of breast cancer (27). It should be pointed out that in some cases much greater concentrations of ErbB3 and ErbB4 antibody were used at incubation times 20-fold longer than the time of ErbB1 and ErbB2 incubation, calling into question the ability to directly compare expression level of family members (28) and highlighting the nonsystematic nature of immunohistochemistry in defining “overexpression” of one protein versus another.

Some studies reported that expression of ErbB4 receptors did not distinctly correlate with the overall survival rate of patients (25, 26, 28, 31). However, high expression of ErbB4 correlated to longer disease-free survival, good histologic grade, and lower Nottingham Prognostic Indices of all (25, 27–30) estrogen receptor–positive breast cancer patients and served as a predictive factor for nonrecurrent ducal carcinoma in situ (26). Another report suggested, however, that ErbB4 expression was associated with an adverse clinical outcome (31). ErbB4 expression has been associated with higher grade, vascular invasion, and nodal metastases, and ErbB4 overexpression was observed to be inversely correlated with estrogen receptor status and local recurrence (25). ErbB4-positive/ErbB2-negative ductal carcinoma in situ, however,
exhibited lower cell proliferation, higher estrogen receptor expression, and better prognosis (26). Nuclear ErbB4 immunoreactivity was associated with poor survival and inversely correlated with the Nottingham Prognostic Index compared with membranous ErbB4 staining, suggesting that the cleaved and translocated receptors may couple to effector proteins and activate signaling pathways distinct from those at the cell surface (14, 27). The changes in cellular localization may also
reflect the interaction between the ErbB4 receptor and different SH2 domain-containing protein expression levels in different cellular contexts.

A comparison of homodimer and heterodimer expression of ErbB receptors in 1,500 cases of invasive breast carcinoma immunohistochemically using tissue microarray technology found that although total expression of ErbB4 was not associated with survival, patients with tumors that coexpressed ErbB1, ErbB2, and ErbB4 showed a poorer outcome compared with patients with tumors that coexpressed ErbB3 and ErbB4. Patients with tumors expressing only ErbB4 (homodimers) but not other ErbB receptors showed a better disease-free survival (25).

**Systematic Studies of ErbB4 Cellular Signaling Pathways**

A profile of downstream gene transcription following stimulation of ErbB2 or ErbB4 has been generated using a 16,755-gene oligonucleotide microarray from CEM4 lymphocytic cells, and Sum44 and T47D breast cancer cells (32). Stimulation of ErbB4 receptor by an ErbB4 antibody for 1 h at 37°C preferentially increased the steady-state mRNA levels of several genes, including HOXD12, RAB2, MAPK4, GATA4, and EPS15R, by ~2-fold.

Higher throughput systems-level approaches have also been applied to investigate the interaction between target proteins and the activated ErbB4 receptor. Interaction between 89 phosphotyrosine sites on ErbB receptors and potential partner proteins was assayed by pull-down experiments using synthetic peptide pairs in phosphorylated and nonphosphorylated states. The ErbB4 receptor was found to interact with SHC, STAT5, PTPT2C, Crk, P13K, Grb2, and Nck2. Phosphorylation kinetics of several tyrosine residues was measured by mass spectrometry and correlated with interaction partner preference (33). Another study using protein microarray technology to study the interaction between phosphoryrosine peptides derived from all four ErbB receptors revealed novel binding partners of the SH2 and PTB families for the ErbB4 receptor. These microarray analyses were used to study the binding interaction of 159 isolated human SH2 and PTB domains with 66 peptides representing physiologic sites of tyrosine phosphorylation. Seven of the predicted phosphotyrosine sites on ErbB4 were tested: four were unique to ErbB4 and three were peptide sequences that exist in ErbB4 and other ErbB family receptors. ABL-1, ABL-2, BLK, JAK1, SYK, SHC1, P1K3R1, P1K3R2, P1K3R3, MIST, E109111, PLCγ1, and RASA1 SH2 domains were found to be recruited to phosphorylated tyrosine peptide sequences of ErbB4 (Fig. 1C; ref. 15). Among these proteins, ABL-1, ABL-2, CRK, JAK1, SYK, p52-SHC, STAT5, P13K, GRB2, and PLCγ1 have been previously reported to be oncogenic, whereas RASA1 and the p66-SHC isoform are tumor suppressive. Cell type–dependent differences in receptor and downstream signaling protein expression level may result in differences in ErbB4 phosphorylation and recruitment, thus activating different signaling networks and ultimately affecting different phenotypic outcomes.

**Conclusion**

Many contradictory reports have described the role of ErbB4 in breast cancer and the prognostic value of ErbB4 expression in predicting outcomes in the clinic. To attempt to unify some of the apparent discrepancies, we have examined the details of a limited number of studies about the role of ErbB4 in breast cancer. Analyzing these reports from a systems level is difficult for several reasons. First, ErbB4 expression is generally low in most breast cancer cell lines and therefore most groups have relied on MCF-7 and T47D cells to show the effect of ErbB4 stimulation on growth and survival in tissue culture. Because most other breast cancer cell lines express very low levels of ErbB4, these two cell lines are not representative of most breast cancer lines. Additionally, most initial ErbB4 experiments done in MCF-7 and T47D are then generally followed up by the transfection/infection of ErbB4 into a non–breast cancer cell line, such as HEK 293, T47-14 (NIH3T3), or COS7 cells, to boost the amount of protein to appreciable levels to do localization, apoptotic index, and colony-forming assays. The wide range of signaling protein expression levels in these non–breast cancer cells coupled with exaggerated expression of ErbB4 makes the results very difficult to interpret relative to breast cancer cell lines expressing “normal” levels of ErbB4. Comparing the apoptotic or growth-arresting potential of ErbB4 with green fluorescent protein for example is very dangerous given that ErbB4 is a membrane-bound protein that shuttles through the cell to the nuclear compartment after cleavage: if any cellular membrane transport function were to become overwhelmed by the overexpression, cleavage, and trafficking of ErbB4, it could potentially result in other proteins being improperly shuttled and ultimately in toxicity to the cell and abnormal trafficking of ErbB4 and other proteins. Green fluorescent protein in contrast is a soluble protein that is not shuttled between membranes or cell compartments and has low relative toxic side effects.

With regard to the role of ErbB4 as a prognostic marker in the clinic, it would seem logical, from a systems-level perspective, to examine the role of ErbB4 in the context of its normal function in the maturation of the breast during pregnancy, parturition, and onset and maintenance of...
lactation. Combined with the known reduction in risk brought about by pregnancy and extended lactation (34, 35), the prognostic significance of ErbB4 would best be assessed with a prior knowledge of the pregnancy history and lactation status of the patient. Because of the global changes in chromatin rearrangement that are likely induced by cycles of progesterone and estrogen during pregnancy and lactation, ErbB4 ICD is likely to have access to very different transcriptional targets in the context of postpubescent nonparous breast tissue, which is in a suspended state of growth and development brought about during puberty by other ErbB family members (4) in concert with hormonal stimulation. Extending this logic further, one would hypothesize that the stimulation of ErbB family heterodimers in cells from a nonparous host might elicit a growth response such as would be required in the ductal and alveolar growth required during pregnancy but might elicit a milk production response in cells from a parous or lactating host. The classic overexpression of ErbB1 and ErbB2 seen in aggressive breast cancers might be therefore the result of incomplete breast maturation and lack of complete chromatin silencing of the ErbB1 and ErbB2 promoters. Estrogen-containing oral contraceptives or lactation suppressors might also introduce variability into clinical studies by modulating the transcriptional coactivation output of ErbB4 via chromatin remodeling (histone acetyltransferase, histone deacetylase, and SWI/SNF recruitment), resulting in differential accessibility to target genes. This would unify inconsistent reports wherein some investigators have reported the ability of ErbB4 to prohibit growth when transfected/infected into breast cancer cells overexpressing other ErbB family members (20), whereas others have reported no such growth-inhibitory effect (36).

It is clear from previous studies that ErbB4 expression is generally low (relative to ErbB1 and ErbB2 expression) in most common breast cancer cell lines and that there are two extracellular versions that are either susceptible or not to cleavage and translocation to the nucleus and two intracellular versions that either contain a 16–amino acid sequence or not that contains recruitment motifs for both SH2 domains and WW domains (Fig. 1C; ref. 11). Because Y1056 was shown to be both necessary and sufficient for the tumor suppressor activity of ErbB4 in several human mammary cell lines (20) and only exists in ErbB4 CYT-1, it is possible that phosphorylation of Y1056 may play an important role in determining the localization and oncogenicity/tumor suppressor activity of ErbB4. Because phosphorylation of other tyrosine sites on ErbB4 may allow the recruitment of distinct modules of SH2- or PTB-containing proteins harboring either oncopgenic or tumor suppressor activity, a comprehensive understanding of cell type–dependent signaling protein expression and recruitment potential to receptor phosphorylation sites is of critical importance. In summary, a comprehensive systematic study of ErbB receptor signaling in the breast that takes into account factors such as the cell type, parous and lactation status of the host, cell type–specific expression level of collections of signaling proteins, especially of SH2 and WW domain-containing proteins, and finally hormonal supplementation history of the host might provide a deeper understanding of the role of ErbB4 in breast cancer.

References

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