Epidermal Growth Factor Receptor Variant III Contributes to Cancer Stem Cell Phenotypes in Invasive Breast Carcinoma

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Abstract

EGFRvIII is a tumor-specific variant of the epidermal growth factor receptor (EGFR). Although EGFRvIII is most commonly found in glioblastoma, its expression in other tumor types remains controversial. In this study, we investigated EGFRvIII expression and amplification in primary breast carcinoma. Our analyses confirmed the presence of EGFRvIII, but in the absence of amplification or rearrangement of the EGFR locus. Nested reverse transcriptase PCR and flow cytometry were used to detect a higher percentage of positive cases. EGFRvIII-positive tumors showed increased expression of genes associated with self-renewal and epithelial–mesenchymal transition along with a higher percentage of stem-like cells. EGFRvIII also increased in vitro sphere formation and in vivo tumor formation. Mechanistically, EGFRvIII mediated its effects through the Wnt/β-catenin pathway, leading to increased β-catenin target gene expression. Inhibition of this pathway reversed the observed effects on cancer stem cell (CSC) phenotypes. Together, our findings show that EGFRvIII is expressed in primary breast tumors and contributes to CSC phenotypes in breast cancer cell lines through the Wnt pathway. These data suggest a novel function for EGFRvIII in breast tumorigenesis. Cancer Res; 72(10); 2657-71. ©2012 AACR.

Introduction

Invasive breast carcinoma is the most common cancer in women, and despite major advances in screening and treatment, it remains the second leading cause of death from cancer. Breast tumors are extremely heterogeneous, with no dominant pathway or histologic subtype prevailing, and thus there is no standardized treatment applicable to all types (1). Characterization of breast cancer has historically been done on the basis of histologic traits and size or spread of the tumor, along with various pathologic markers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2; ref. 2). However, more recently tumors have been classified on the basis of their gene expression profiles. Using these profiles, breast cancers have been divided into 5 different subtypes: basal-like, HER2⁺, luminal A, luminal B, and a normal breast-like group (3). Approximately 15% to 21% of human breast tumors are the basal-like subtype, and this group is generally regarded as the most aggressive subtype with the worst prognosis (4).

Expression of the epidermal growth factor receptor (EGFR) has been linked to the basal-like subtype, with overexpression in more than 50% of tumors (5). Although EGFR amplification is generally rare in breast cancer, basal-like tumors have a relatively high incidence of EGFR amplification (6). Variants of the EGFR are common across tumors, with one of the most prevalent being EGFR variant III (EGFRvIII). EGFRvIII is a naturally occurring constitutively active variant that was originally identified in a high percentage of glioblastoma multiforme (GBM) brain tumors. It is found in 15% to 60% of primary GBM and is mostly associated with amplification and rearrangement at the EGFR locus (7). The rearranged EGFR gene deletes exons 2 through 7 resulting in an in-frame 801 base pair deletion. This gives rise to a truncated receptor lacking a significant portion of the extracellular ligand-binding domain, rendering EGFRvIII ligand independent and constitutively active (8).

EGFRvIII expression in other tumor types has been documented, but remains controversial (9–13). Previous reports showed EGFRvIII expression in breast carcinoma by Western blotting and reverse transcriptase PCR (RT-PCR; refs. 9, 12), but subsequent studies have provided conflicting evidence depending on the method of detection (14, 15). However, EGFRvIII is posited to be involved in tumorigenicity, invasiveness, and metastasis in breast cancer (16–19). In this study, we investigated the expression of EGFRvIII and the presence of EGFR amplification and rearrangement in primary breast carcinomas, and generated inducible EGFRvIII expressing cell lines to elucidate novel roles for EGFRvIII in tumorigenesis.

Materials and Methods

Dissociation of primary human breast tumors

Freshly resected human breast tumor samples, tumor-adjacent normal tissue samples, corresponding metastatic
lymph nodes, and nontumor associated normal breast samples were obtained from the Stanford University tissue bank under Institutional Review Board–approved protocols. Tissue samples were dissociated using 1x Collagenase/Hyaluronidase (Stem Cell Technologies) and treated with ACK/RBC lysis buffer (0.15 mol/L NaCl, 1.0 mmol/L KHCO₃, and 0.1 mmol/L Na₂-EDTA). Resulting cells were either resuspended in sterile fluorescence-activated cell sorting (FACS) buffer for flow cytometry or used for RNA isolation.

**Nested RT-PCR**

RNA from primary human breast tumors was used for nested RT-PCR using a method adapted from Ge and colleagues (20). Reaction products were electrophoresed in 2% agarose gels. Products were excised and purified using Qiagen’s gel extraction kit and sequencing was carried out by Elim BioPharm.

**Flow cytometry**

Before flow cytometry analysis or sorting, cells were dissociated as above or washed and dissociated using TrypLE (Gibco). Cells were resuspended in a 100 μL staining volume of FACS buffer (HBSS + 0.1% bovine serum albumin) and kept on ice. The following antibodies were used in flow cytometry: monoclonal mouse anti-EGFRvIII antibody (G100; Zymed), CD44-APC (BD Biosciences), CD24-FITC (fluorescein isothiocyanate), or CD24-PE (BD Biosciences),CD31-PECy7 (eBioscience), and CD45-PeCy7 (BD Biosciences). Detection of aldehyde dehydrogenase (ALDH) activity was done using the Aldefluor assay (Stem Cell Technologies) according to the manufacturer’s instructions. 4’, 6-Diamidino-2-phenylindole (DAPI) was used to discriminate between live and dead cells. CD31 and CD45 antibodies were used for lineage depletion. All analyses were conducted on live, lineage-negative cells. Samples were analyzed on a LSR II FACS machine (BD Biosciences) or sorted on an ARIA-II (BD Biosciences) at the Stanford University FACS facility. Appropriate isotype controls were used to control for nonspecific isotype background. To validate the significance of the observed differences, we analyzed samples using 2-sided t tests.

**Immunohistochemistry**

Unstained sections from formalin-fixed paraffin-embedded (FFPE) samples of invasive breast carcinoma were acquired from the pathology division of Stanford Medical Center with all patient identification removed. In addition, a tissue microarray was obtained containing 60 cases of invasive breast carcinoma (US Biomax, catalog no. BR1503a). Slides were deparaffinized in xylene and then rehydrated with ethanol and double distilled water (ddH₂O). Hydrogen peroxide was used to block nonspecific sites and Diva Decloaker (BioCare Medical) solution and microwaving were used for antigen retrieval. Sections were incubated overnight with a 1 μg/mL solution of an anti-EGFRvIII antibody (G100, Zymed) that recognizes EGFRvIII but does not cross-react with wild type EGFR. Expression was detected using horseradish peroxidase anti-mouse secondary antibody (BioCare Medical) and betazoid 3,3’-diaminobenzidine (BioCare Medical). The slides were counterstained with hematoxylin. To compare EGFRvIII expression and ER/PR/HER2 phenotype, we used the χ² and Fisher exact tests.

**Quantitative RT-PCR**

RNA was isolated from adherent cell cultures using TRIzol reagent according to the manufacturer’s instructions (Invitrogen) or from sorted cells using the RNeasy Micro Kit (Qiagen). Complementary DNA synthesis and quantitative PCR were carried out with the Power SYBR Green RNA-to-CT 1-Step Kit (Applied Biosystems). Primer sequences are listed in Supplementary Table S3. PCR conditions were 48°C for 30 minutes, 95°C for 10 minutes, followed by 50 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. Each reaction was conducted in triplicate in 384-well optical plates on an ABI 7900HT instrument (Applied Biosystems). Relative copy number was determined using the comparative Ct method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the normalizing control.

**FISH**

FISH was carried out on FFPE sections using a probe for the EGFR locus (7p12, Vysis LSI EGFR, SpectrumOrange, Abbott Vascular) and CEP7 as the chromosome 7 control probe (7p1.1-q11.1 Alpha Satellite DNA, Vysis SEP7, SpectrumGreen, Abbott Vascular). Deparaffinization, hybridization, washing, and counterstaining were carried out according to the manufacturer’s instructions. Fluorescence was visualized with an Olympus BX51 microscope and CytoVision imaging software (Genetix Corp). EGFR and CEP7 signals were quantitated in 100 cells per region. Cases were considered positive for amplification of EGFR if the EGFR/CEP7 ratio was greater than or equal to 2.0.

**Long-range PCR**

Long-range PCR was carried out according to the methods described by Frederick and colleagues (21). Briefly, genomic PCR amplification was done using the Expand Long Template PCR system (Roche Applied Science). Reactions contained either 20 pmol each of intron 1 sense primer mixture A or B and exon 8 antisense primer for the detection of exon 2 to 7 deletions or each of exon 13 sense and exon 16 antisense primer. Reaction products were electrophoresed in 0.7% agarose gels, excised, and sequenced.

**Quantitative PCR**

FFPE sections that had been previously used for immunohistochemical analysis were used for DNA isolation. Real-time quantitative PCR was carried out using a previously published protocol (22). Briefly, 3 primer sets were used, 2 within the EGFR gene and a third internal control (β-actin). Of the 2 EGFR primers, one is within the EGFRvIII deleted region (exon 2) and the other is external to the EGFRvIII deleted region (intron 15). Each reaction was conducted in triplicate in 384-well optical plates on an ABI 7900HT instrument (Applied Biosystems). Relative copy number was determined...
using the comparative $C_i$ method. U87 served as the normal control DNA.

**Microarray analysis**

High-resolution array comparative genomic hybridization (aCGH) data characterizing copy number across 169 primary breast samples were acquired from 2 previous studies (23, 24). High-resolution aCGH data across 54 breast cancer cell lines was obtained from the Wellcome Trust Sanger Institute Cancer Genome Project. Lastly, aCGH data from select EGFVIII-positive samples were curated from The Cancer Genome Atlas (TCGA) public archives (25). These studies were carried out on 3 different array platforms: Affy SNP 5.0 (23), Affy SNP6.0 (Sanger, TCGA), and Agilent 244K (24). Probes from the more dense Affy SNP arrays were collapsed to their nearest neighbor on the Agilent 244K array to enable visualization on the same heatmap.

**Cell lines and expression plasmids used**

Parental SUM149 and SUM159 cell lines were a kind gift of Dr. Jonathan Pollack and cultured as previously described (26). Parental MCF10A and U87-MG cells were obtained and cultured according to American Type Culture Collection protocols. U87-MG cells expressing EGFVIII (U87-vIII) were a kind gift of Dr. Donald O’Rourke (Hospital of the University of Pennsylvania, Philadelphia, PA) and were maintained in geneticin (500 mg/mL). EGFRvIII-inducible cell lines were created using the Tet-Off Advanced system (Clontech), according to manufacturer’s instructions and maintained in puromycin (2 g/mL) and geneticin (500 μg/mL) selection. EGFVIII gene expression was regulated by the addition or withdrawal of 100 ng/μL doxycycline.

**Western blot**

Cells were lysed with cold PBS/TDS buffer (PBS with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, complete inhibitor cocktail; Roche Applied Science). The signal was detected using the ECL system (Amersham Biosciences). The following antibodies were used for immunoblotting: G100, β-actin (Chemicon), E-Cadherin (Santa Cruz Biotechnology, Inc.), Twist (Santa Cruz Biotechnology, Inc.), N-Cadherin (Santa Cruz Biotechnology, Inc.), ALDH (BD Biosciences), Slug (Santa Cruz Biotechnology, Inc.), and active β-catenin (ABC; Millipore).

**Sphere formation**

Single cells were plated in ultra-low attachment plates (Corning) at limiting dilutions or at a density of 3,000 cells per well. Cells were cultured in Mammocult media according to the manufacturer’s instructions (Stem Cell Technologies). For estimation of sphere initiating cell frequency, limiting dilution analysis (LDA) was done as described previously (27, 28). Cultures were carried out for 7 days and scored for wells that did not have spheres. An extreme LDA algorithm was used to determine the frequency of sphere initiating cells (29). Each experiment was done in triplicate and analyzed using 2-sided $t$ tests.

**Tumor formation**

Cells were resuspended in a 1:1 mixture of cold PBS and Matrigel (BD Biosciences) and adjusted to the appropriate concentration. One hundred microliters of the cell suspension was injected into the mammary fat pad of female 6- to 8-week-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (NIH). Tumor size after injection was measured by a dial-caliper and the volumes were calculated. Mice were sacrificed under approved Institutional Animal Care and Use Committee protocols.

**Wnt activity reporter assay**

To assay for induction of Wnt signaling in control or EGFVIII expressing cell lines, the pTopFLASH-GFP reporter plasmid was used. GFP expression of cells transiently transfected with pTopFLASH-GFP was analyzed on a LSR II FACS machine (BD Biosciences) at the Stanford University FACS facility. The pTopFLASH-GFP plasmid was a kind gift from Dr. Irv Weissman (Institute for Stem Cell Biology & Regenerative Medicine, Stanford, CA).

**Pathway inhibitors**

Human Recombinant DKK-1 (Invitrogen), β-Catenin/Tcf Inhibitor FH535 (EMD Millipore), and the EGFR tyrosine kinase inhibitor AG1478 (EMD Millipore) were used in inhibitor studies at the following concentrations for 48 hours: 100 ng/mL (DKK-1), 20 μmol/L (FH535), and 4 μmol/L (AG1478). MCF10A cells were treated with only 0.25 μmol/L AG1478. Typically, cells were incubated for 48 hours with each compound and then analyzed. For longer experiments, 50% of the medium was replaced every 3 days with fresh medium containing treatment compounds at the original concentrations. Controls were treated with 0.01 percent dimethyl sulfoxide (DMSO; Sigma).

**Results**

EGFRvIII is expressed in primary breast carcinoma using multiple methods of detection

To assay for the presence of EGFvIII in primary breast carcinoma, we first analyzed EGFvIII expression using immunohistochemical analysis with an antibody specific for EGFvIII. Immunohistochemistry (IHC) revealed that 5% (7 of 136) of cases were EGFvIII positive. Some displayed low-level expression in the majority of tumor epithelial cells, whereas others had more focal staining patterns (Fig. 1A). One case showed positivity in only a single duct, suggesting that EGFvIII expression may be extremely rare and sporadic in some tumors (Fig. 1A, Sample BR89). To confirm EGFvIII expression, we carried out nested RT-PCR and sequencing analysis of RNA isolated from these tumors. The resulting PCR product validated EGFvIII expression (Fig. 1B), and the band was confirmed as an EGFvIII product by sequencing (Fig. 1C).

Because of the high degree of heterogeneity in breast carcinoma and the differing sensitivity of antibodies, it is possible that if EGFvIII is expressed at a low level and/or in a minority population it may go undetected by IHC. Flow cytometry allows detection of EGFvIII expression on a cell-by-cell basis.
Figure 1. EGFRvIII is expressed in primary breast carcinoma using multiple methods of detection. A, primary human breast tumor FFPE sections were stained for EGFRvIII. Four EGFRvIII-positive samples are shown. Scale bar, 50 microns. B, RNA was isolated from primary dissociated tumors and analyzed for expression of EGFRvIII by nested RT-PCR. A series of dilutions of U87-vIII cells served as the positive control, and parental U87-MG cells were used as a negative control. Data shown are a representative sample. C, sequence analysis of the EGFRvIII nested RT-PCR band from BR7 confirms the presence of the EGFRvIII junction (black box). D, fresh primary human breast tumor samples were dissociated and analyzed for EGFRvIII (FITC labeled, x-axis) expression by flow cytometry. Representative plots from BR1, BR3, BR8, and BR18 samples are shown. Top panels show gating determined from the isotype control (mouse IgG1). RNA was isolated from primary dissociated tumors (E) or sorted EGFRvIII-positive and EGFRvIII-negative cells from BR18 (F) and analyzed for expression of EGFRvIII by nested RT-PCR. A series of dilutions of U87-vIII cells served as the positive control, and parental U87-MG cells were used as a negative control.
and analysis of more total cells. Surprisingly, flow cytometry analysis showed that 80% (16 of 20) of samples had at least 1% EGFRvIII-positive cells compared with an isotype control (Table 1, Fig. 1D). Similar to our immunohistochemical results, some tumors had EGFRvIII expression in a high percentage of cells (>50% positive cells), whereas others had staining in only a few percent. To reconcile the differences in overall percentages of positive cases and rule out antibody nonspecificity leading to false positives, we carried out nested RT-PCR on the matched samples that were analyzed by flow cytometry. Overall 69% of the samples (9 of 13) were positive by RT-PCR, and there was a high correlation between the 2 methods (Fig. 1E, Table 1). When the percentage of positive cells identified by flow cytometry was greater than 5%, there was a 100% correlation between flow cytometry and nested RT-PCR (7 of 7). Only when the percentage of positive cells dropped to between 1% and 5% did the correlation weaken, with 83% (5 of 6) of samples showing a correlation. This suggested that the sensitivity of the nested RT-PCR assay is not always sufficient to detect EGFRvIII when expressed in an extremely low number of cells. As an additional control for antibody specificity, we sorted EGFRvIII-positive and EGFRvIII-negative cells by fluorescence activated cell sorting (FACS) and carried out nested RT-PCR on the resulting populations. An EGFRvIII-specific RT-PCR product was identified in the positive, but not negative, sorted population (Fig. 1F). Lastly, we assayed for EGFRvIII expression using IHC in 4 of the tumors that were positive according to RT-PCR and/or flow cytometry (BR7, BR9, BR14, and BR16). Three of the 4 tumors had fewer than 10% EGFRvIII-positive cells by flow cytometry, and one had 29% EGFRvIII-positive cells (BR7). Only BR7 was also positive by IHC. From these analyses we concluded that flow cytometry and nested RT-PCR have higher sensitivity than IHC using our antibody.

Because breast tumor subtype and the expression of various receptors are important determinants of breast cancer prognosis and treatment, we investigated any correlation between EGFRvIII and expression of ER, PR, and HER2. Using data from 147 cases analyzed by IHC or flow cytometry and RT-PCR, we found 9 of 13 (69%) EGFRvIII-positive tumors were of the ER+/PR+/HER2− subset, whereas only 3 of 13 (23%) were ER+/PR−/HER2− and 1 of 13 (8%) was ER−/PR+/HER2− (Supplementary Table S1). EGFRvIII expression was significantly associated with the ER+/PR+/HER2− subtype (P < 0.01), but there was no significant association of EGFRvIII positivity with triple-negative breast cancer or the other subtypes.

We also observed the presence of EGFRvIII-positive cells in some samples of normal-like breast tissue adjacent to the primary tumor and in metastatic tumors of the lymph node using flow cytometry and nested RT-PCR analyses (Supplementary Table S2). In general the percentage of EGFRvIII-positive cells was lower in the adjacent normal tissue compared with the primary tumor, but higher in the metastatic tissue. In addition, we did not observe EGFRvIII expression in 6 samples from nontumor associated normal breast tissues (NTB), implying that EGFRvIII is not expressed in normal noncancerous breast cells (Supplementary Fig. S1). Although this is a small sample size, the data suggested...
that EGFVIII-positive cells may be present in what is thought to be extratumoral "normal" tissue and also in metastatic sites.

**EGFVIII expressing breast tumors do not show rearrangement or amplification of the EGF locus**

**EGF** locus amplification and rearrangement are common in GBM, but rare in other tumor types. EGFVIII was originally identified as a genomic rearrangement, but it has been postulated that other mechanisms, such as alternative splicing, could also give rise to the variant (9). In addition, the above analyses showed that EGFVIII is often expressed at a very low level compared with what we observed in GBM. We screened the tumors deemed EGFVIII-positive by IHC for EGF amplification and rearrangement using several methods. Long-range PCR was done to attempt to amplify a fragment corresponding to rearranged EGF using a method described by Frederick and colleagues (21). This method uses a single antisense primer located in exon 8, and 1 of 2 sense primer mixtures (primer sets A and B) containing oligonucleotides spaced at approximately 10-kb intervals throughout intron 1, the size of which is greater than 120 kb. If no rearrangement has occurred, the distance between the sense and antisense primers is too large to amplify, thus a positive band with either primer set A or B indicates that the EGFVIII rearrangement has occurred. PCR synthesis with a primer pair from exons 13 and 16 of EGF served as the positive control for the presence of functional DNA. This method was readily able to identify rearranged DNA corresponding to EGFVIII in a control primary GBM case, but no rearrangement existed in the primary breast carcinoma samples (Fig. 2A). FISH was carried out using an EGF-specific probe to assess amplification, which showed normal copy number of EGF and chromosome 7 in tumors with expression of EGFVIII protein (Fig. 2B). Lastly, qPCR was conducted to verify that no amplification and rearrangement occurred using a method adapted from Biernat and colleagues (22). This qPCR method uses 2 primer sets to calculate DNA copy number, one within the EGFVIII deleted region (exon 2) and the other external to the EGFVIII deleted region (Intron 15), with normalization to an internal control primer set (β-actin) and a control cell line containing a normal copy number of EGF and chromosome 7. This method readily detected EGF amplification and rearrangement in a control GBM cell line, whereas the primary breast carcinoma samples had no amplification or rearrangement of the EGF locus (Fig. 2C). Because our sample size was limited, we also looked for evidence of EGF rearrangement corresponding to EGFVIII in more than 200 primary breast and breast cancer cell line samples using aCGH. The EGFVIII rearrangement was not observed in any of these cases, even though EGFVIII was readily detected in GBM samples (Fig. 2D). This showed that although EGFVIII is expressed in primary breast carcinoma, it does not originate through amplification and rearrangement of the EGF locus.

**EGFVIII correlates with stem cell associated marker and gene expression**

Evidence from other tumor types showed that EGFVIII can contribute to chemotherapy and radiation resistance, suggesting that EGFVIII may play a role in a critical population of cells required for tumorigenesis or tumor progression (30, 31). Together with our results suggesting that EGFVIII is often expressed in a minority population in primary breast tumors, we hypothesized that EGFVIII may contribute to cancer stem cell (CSC) phenotypes in breast cancer. Several markers have been used to identify stem-like cells in primary breast cancer. Cells displaying a CD44+/CD24−/low phenotype or increased activity of the enzyme ALDH have been shown to display increased self-renewal and tumor initiating abilities (32, 33). To test our hypothesis, we used flow cytometry to probe for overlap between the EGFVIII+ and CD44+/CD24−/low populations in primary breast carcinoma samples. Our analysis was able to detect CD44+/CD24−/low populations at percentages in accordance with the literature (Table 1; ref. 32). All of the EGFVIII+ tumors examined displayed a subset of cells that were also EGFVIII+/CD44+/CD24−/low. The percentage of EGFVIII+ cells within the CD44+/CD24−/low population ranged from 2.5% to 58%, with an average of 26% of CD44+/CD24−/low cells coexpressing EGFVIII (Table 1, Fig. 3A and B). Although there was not a complete overlap between the 2 populations, this showed that EGFVIII is expressed within at least a subset of CD44+/CD24−/low cells.

Cell-surface marker expression does not necessarily imply induction of markers functionally associated with stem cell traits such as self-renewal. We probed for stem cell–associated gene expression in primary breast carcinoma. We used EGFVIII-positive and EGFVIII-negative sorted populations for quantitative RT-PCR (qRT-PCR) analyses and noted significant increases in ABCG1, ABCG2, BMI1, Oct4, and Sox2 gene expression in EGFVIII expressing cells compared with nonexpressing cells (Fig. 3C). Similarly, the epithelial–mesenchymal transition (EMT) is a process correlated with the acquisition of increased motility and other metastatic capabilities and has been associated with an expansion in the number of CSCs (34). This process involves the transformation of cells from an epithelial-like to a mesenchymal-like morphology and program, with concomitant losses in epithelial markers such as E-cadherin and gains in mesenchymal markers such as N-Cadherin, Twist, and Slug. Gene expression, assayed by qRT-PCR, of fibronectin, N-Cadherin, Slug, Snail, and Twist were increased in EGFVIII-positive sorted cells compared with the EGFVIII-negative fraction (Fig. 3C). Together this data suggested that EGFVIII correlates with increased expression of stem cell associated markers and genes in primary tumor samples.

**Inducible EGFVIII-expression in breast cancer cell lines modulates stem cell–associated marker and gene expression**

To further investigate a connection between EGFVIII and CSC phenotypes, we created 3 inducible EGFVIII expressing breast cancer cell lines. A tetracycline-off system was used in which EGFVIII is expressed in the absence of doxycycline. Control cell lines were generated in which luciferase (LUC) was expressed instead of EGFVIII. The EGFVIII-inducible constructs were stably transduced into 3 commonly used breast cancer cell lines: SUM159, SUM149, and MCF10A. SUM159 has...
Figure 2. EGFRvIII expressing primary breast cancers lack EGFR amplification and rearrangement. A, DNA was isolated from dissociated cells and long-range PCR was carried out using a method that uses a single antisense primer located in exon 8, and 1 of 2 sense primer mixtures (A and B) containing oligonucleotides spaced at approximately 10-kb intervals throughout intron 1, the size of which is greater than 120 kb. A positive band with either of the 2 sense primer sets indicates that the EGFRvIII rearrangement has occurred. A primary GBM with known EGFRvIII rearrangement and amplification served as a positive control, showing amplification of a band in A corresponding to rearranged EGFRvIII. A, primer mixture A; B, primer mixture B; +, EGFR positive control primer set (forward primer in exon 13, reverse primer in exon 16). The presence of an additional band in the (+) lane of the positive control likely corresponds to additional rearrangements in intron 13 to 15 of this sample. Data shown are a representative sample. B, EGFR FISH was carried out on FFPE sections from BR7 and BR21 using a probe for the EGFR locus (red) and CEP7 as the chromosome 7 control probe (green). C, quantitative PCR was carried out on DNA extracted from FFPE tissues. If EGFRvIII amplification and rearrangement have occurred, Intron 15 will be preferentially amplified relative to exon 2. Relative copy number was determined using the comparative Ct method. Known EGFRvIII rearranged and amplified DNA from a primary GBM served as the positive control and parental U87-MG cells were used as a negative control, having normal copy number of the EGFR gene. Data shown is a representative sample. D, high-resolution aCGH data showing copy number of 223 breast cancer samples (169 primary and 54 cell lines) is depicted at the EGFR locus (rank ordered by amplification). Select EGFRvIII-positive glioma samples are shown for comparison. Internal deletions of the EGFR gene generating EGFRvIII occur in glioma but do not appear across breast cancers. Samples are shown across columns and each row represents a probe on the array. Log2 ratios are depicted in a color scale in which red represents gain and blue loss.
a spindle-like fibroblastic morphology and contains more than 90% basal-like CD44+/CD24−/low cells, whereas SUM149 and MCF10A display epithelial morphology and are composed of a mixed population of basal and luminal-like cells (26, 35). Each inducible cell line robustly expressed high protein levels of EGFRvIII in the absence of doxycycline compared with the control cells by Western blot analysis (Fig. 4A). EGFRvIII expression was efficiently reduced through the addition of doxycycline (100 ng/mL) as evidenced by the reduction from 28% to 50% positive EGFRvIII cells to 0.25% to 1.21% using flow cytometry analysis (Fig. 4B and Supplementary Fig. S2). Because not all of the cells within the population expressed EGFRvIII, a pure EGFRvIII expressing population was created by sorting of the cells, and this population was used in subsequent experiments.

To determine whether EGFRvIII modulates the absolute number of stem-like cells, we measured the percentage of CD44+/CD24−/low and ALDH activity, using the Aldefluor assay, in the presence and absence of EGFRvIII. Expression of EGFRvIII produced a 2- to 4-fold increase in the percentage of CD44+/CD24−/low and Aldefluor+ cells in the 3 cell lines examined. This phenomenon was reversible upon the addition of doxycycline, reiterating the importance of EGFRvIII in maintaining the CSC-like subpopulation (Fig. 4C–E). In addition, we analyzed ALDH expression in SUM149-EGFRvIII cells by Western blot and confirmed a 2.5-fold increase in its expression (Fig. 4F).

We next investigated the expression of EMT markers in SUM149, SUM159, and MCF10A cells with and without the expression of EGFRvIII (Fig. 4F). A 45% to 58% decrease in E-cadherin was observed in EGFRvIII expressing cells. During EMT, the decrease in E-cadherin expression is usually accompanied by an increase in N-cadherin. We discovered a 2.5- to 4-fold increase in N-cadherin expression for all EGFRvIII expressing cell lines. In addition, we noted 1.5- to 3-fold increases in Twist and Slug expression. This suggested that EGFRvIII

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Figure 3. EGFRvIII correlates with increased stem cell–associated marker and gene expression. A and B, primary human breast tumor samples were dissociated and analyzed for CD44 (APC labeled), CD24 (PE labeled), and EGFRvIII (FITC labeled) expression by flow cytometry. Representative plots from BR1 and BR18 are shown. A, left panels show gating determined from the isotype control. Right panels show CD44 and CD24 expression. B, panels show the fraction of the CD44+/CD24− population from A that also expresses EGFRvIII. C, RNA was isolated from EGFRvIII-positive and EGFRvIII-negative sorted cells from BR18 and BR19 and analyzed for stem cell and EMT-associated gene expression by qRT-PCR. Graph shows fold change in gene expression (normalized to GAPDH) of the EGFRvIII− fraction over the EGFRvIII+ fraction, error bars indicate SD. Asterisk indicates statistically significant differences in gene expression between EGFRvIII expressing cells compared with nonexpressing cells (P < 0.05).
Figure 4. Breast cancer cell lines engineered to express EGFRvIII under an inducible promoter: EGFRvIII expression modulates stem cell associated marker and gene expression. A, SUM149, SUM159, and MCF10A breast cancer cell lines were stably transduced with retroviral vectors expressing EGFRvIII (vIII) or Luciferase (LUC) under control of the tetracycline response element (TRE) and grown in the absence of doxycycline. Whole-cell lysates were used in Western blot analysis with anti-EGFRvIII and anti-β-actin antibodies. B, SUM149-LUC and SUM149-vIII cells were cultured for 72 hours with or without the addition of doxycycline at a concentration of 100 ng/mL. Cells were harvested and analyzed by flow cytometry for EGFRvIII expression. Left panel shows background from the isotype control (mouse IgG1), middle panel shows EGFRvIII expression in the absence of doxycycline, and right panel shows EGFRvIII expression in the presence of 100 ng/mL doxycycline. C, representative flow cytometry plots showing percentages of CD44+/CD24−/low stem cell–like cells in SUM149-LUC and SUM149-vIII cells cultured for 72 hours with or without the addition of doxycycline at a concentration of 100 ng/mL. Top left panel shows background from the isotype control (mouse IgG2a and IgG2b). Quantification of percentages of CD44+/CD24−/low (D) and Aldefluor+ (E) stem-like cells in each cell line in the presence or absence of doxycycline (100 ng/mL). F, whole-cell lysates from SUM149-LUC or SUM149-vIII cells were used in Western blot analysis with the antibodies as indicated (ABC, anti-active-β-catenin). Quantification of blots relative to β-actin expression is shown. G, RNA was isolated from EGFRvIII expressing and nonexpressing cells and analyzed for stem cell and EMT-associated gene expression by qRT-PCR. Graph shows fold change in gene expression (normalized to GAPDH) of EGFRvIII expressing cells over nonexpressing cells. Error bars show SD; *, statistically significant differences between EGFRvIII expressing cells compared with nonexpressing cells (P < 0.05). dox, doxycycline.
induces the expression of markers associated with EMT. To establish a link between EGFRvIII and self-renewal, we used qRT-PCR to look for changes in stem cell–associated gene expression. We found 2- to 5-fold increases in ABCC1, ABCC2, BM11, Nestin, Oct4, and Sox2 and expression in EGFRvIII expressing cell lines compared with the control cells (Fig. 4G).

**EGFRvIII contributes to enhanced in vitro sphere and in vivo tumor formation**

Neurosphere culture has become widely accepted as a method for culturing cells capable of self-renewal and tumor initiation from primary GBM and has since been adapted for other tumor types (36). We analyzed in vitro sphere formation in breast carcinoma cells using the inducible EGFRvIII expressing breast cancer cell lines. EGFRvIII expressing cells displayed higher in vitro sphere forming ability in LDA (Fig. 5A). From this LDA, the frequency of sphere initiating cells can be calculated, and EGFRvIII expressing cells contained more than 6.5-fold higher frequency of sphere initiating cells compared with control cells (Fig. 5A). To confirm the importance of EGFRvIII in sphere formation, EGFRvIII expression was turned off through the addition of doxycycline and we observed a reduction in sphere formation to a level similar to that found in the LUC control cell lines (Fig. 5B).

We next assayed the ability of EGFRvIII expressing cells to increase in vivo tumor formation. Various doses of cells from $10^3$ to $10^6$ cells were injected into the mammary fat pad of mice and followed over the course of 60 days. For all doses of cells injected, the induction of EGFRvIII enhanced tumor formation to produce larger tumors with shorter latency (Fig. 5C). Importantly, EGFRvIII expressing populations also contained a higher frequency of tumor initiating cells (Fig. 5C). At the highest dose of cells injected, an equal number of mice formed tumors in both control and EGFRvIII expressing cell lines; however, a difference in the tumor initiating ability was observed at the lower doses. EGFRvIII expressing cells formed tumors in 4 of 4 mice at all doses tested, but control cells produced tumors in only 1 of 4 and 3 of 4 mice when injected with $10^3$ and $10^4$ cells, respectively. Therefore, expression of EGFRvIII enhances the tumorigenicity of breast carcinoma cells and mediates an increase in the number of tumor initiating cells.

**EGFRvIII mediates its effects through activation of Wnt signaling**

To begin to understand the mechanism by which EGFRvIII induces CSC-associated gene expression changes and upregulation of putative breast CSC markers, we assessed activation of the Wnt pathway, which is one of several signaling pathways thought to play a role in mammary stem cell phenotypes (37–39). When this pathway becomes activated, b-catenin is stabilized, translocates to the nucleus, and interacts with and activates the transcription factor TCF/LEF-1. Both SUM159-EGFRvIII and MCF10A-EGFRvIII cell lines displayed a 2- to 4-fold increase in activated b-catenin (ABC) expression (Fig. 4F). In addition, we determined whether this increase was functionally significant by using the pTopFLASH-GFP reporter system. This system places 3 copies of the TCF-response motif upstream of a minimal c-fos promoter to drive GFP expression in cells with activated Wnt signaling. Flow cytometry analysis of GFP expression detected more than 50% increase in GFP-positive cells in the SUM159-EGFRvIII expressing cell line compared with the control (Fig. 6A). We confirmed this finding by qRT-PCR and found 3- to 7-fold increases in Lef1 gene expression in EGFRvIII expressing cell lines (Fig. 6B). Importantly, we observed a 5- to 30-fold increase in Lef1 gene expression in EGFRvIII expressing primary tumor cells compared with nonexpressing cells (Fig. 6B).

To establish a stronger link between EGFRvIII signaling and the Wnt pathway, we used 2 inhibitors of the Wnt pathway, recombinant human DKK-1 and b-catenin/TCF inhibitor FH535, and an inhibitor of EGFR tyrosine kinase activity, AG1478. After incubation with each inhibitor for 48 hours, control and EGFRvIII expressing cells transfected with the pTopFLASH-GFP reporter were analyzed for GFP expression by flow cytometry. A decrease in GFP expression was observed in both the SUM159-LUC control cells and SUM159-EGFRvIII cells, confirming that the inhibitors were acting to downregulate the Wnt pathway (Supplementary Fig. S3). In addition, the EGFR tyrosine kinase inhibitor AG1478 was able to mediate a reduction in Wnt signaling to levels observed in the control cell line, indicating that EGFRvIII tyrosine kinase signaling was responsible for the induction of the Wnt pathway (Supplementary Fig. S3).

To determine whether EGFRvIII increased the percentage of CSC-like cells through Wnt activation, we incubated the inducible cell lines with each inhibitor for 48 hours and assayed the percentage of CD44+/CD24−low cells and ALDH activity by flow cytometry. Incubation with DKK-1 or FH535 significantly reduced the CD44+/CD24−low population or the percentage of Aldefluor+ cells (Fig. 6C and D, Supplementary Fig. S4). This reversion indicated that EGFRvIII mediated increases in these CSC-associated phenotypes through the Wnt pathway. Surprisingly, SUM149-EGFRvIII cells treated with the Wnt inhibitors decreased ALDH activity to a level lower than the control cells. This indicated that, in addition to EGFRvIII-specific effects, the Wnt pathway is being activated by other pathways in this cell line. A similar trend was noted in SUM159-EGFRvIII cells. In addition, incubation with AG1478 reverted the percentage of Aldefluor+ SUM149- and SUM159-EGFRvIII cells to levels comparable with control cells, confirming the importance of EGFRvIII in maintaining cells of this phenotype. However, when MCF10A-EGFRvIII cells were treated with the EGFR inhibitor, the percentage of CD44+/CD24−low cells was reduced significantly below the control cells or the MCF10A-EGFRvIII cells treated with DKK-1 or FH535. This finding suggested that there might be high endogenous wtEGFR activity in this cell line.

We have also shown that EGFRvIII leads to increased sphere formation. We tested the effect of the DKK-1, FH535, and AG1478 on sphere formation and each mediated a significant decrease in sphere forming ability (Fig. 6E–G). Treatment with DKK-1 and FH535 were able to reduce the frequency of sphere initiating cells by 5.5- to 13.8-fold compared with the frequency before treatment. Likewise, EGF inhibitor AG1478 mediated a 4.9- and 7-fold decrease in sphere forming ability in SUM149-
Figure 5. Breast cancer cells expressing EGFRvIII display enhanced in vitro sphere initiating ability and in vivo tumorigenicity. A, cells expressing inducible EGFRvIII (vIII) or LUC were plated in a series of limiting cell doses down to a single cell per well. The percentage of wells with no spheres was calculated across 3 replicates. An extreme LDA algorithm was used to determine the frequency of sphere initiating cells (29), which is shown for each cell line. B, cells expressing inducible vIII or LUC were plated at 3,000 cells per well in 6-well ultra low attachment culture dishes. Cells were cultured in the presence or absence of doxycycline (100 ng/mL). The mean number of spheres per well after 7 days was calculated across 3 replicates. Error bars show SD for each cell dose; 
 asterisk statistically significant differences between EGFRvIII expressing cells and the other groups (P < 0.05). C, tumor volume over time resulting from the injection of a limiting dilution of SUM159-LUC or SUM159-vIII cells into the mammary fat pad of female NOD/SCID mice. The number of tumor bearing mice over the total number of injected mice is indicated in the legend for each cell line and dose. dox, doxycycline.
Figure 6. EGFRvIII mediates its effects through activation of Wnt signaling. A, SUM159-LUC and SUM159-vIII cells were transfected with the pTopFLASH-GFP reporter system. Flow cytometry was used to quantify GFP expression, plots show percentages of GFP⁺ cells. Left panel shows background from noninfected cells, middle panel shows GFP expression in SUM159-LUC cells, and right panel shows GFP expression in SUM159-vIII cells. B, graph shows fold change in Lef1 gene expression (normalized to GAPDH) of EGFRvIII expressing cells over nonexpressing cells. Quantification of percentages of Aldefluor⁻/CD44⁻/CD24⁻/low (C) and CD44⁺/CD24⁻/low (D) stem-like cells in each cell line in the presence or absence of DMSO (vehicle control), doxycyline (100 ng/mL), human recombinant DKK1 (100 ng/mL), FH535 (20 µmol/L), or AG1478 (4 µmol/L). Cells expressing inducible vIII or LUC were plated in a series of limiting cell doses down to a single cell per well (E) or at 3,000 cells per well in 6-well low attachment culture dishes (F). Cells were cultured in the presence or absence of DMSO, DKK1, FH535, or AG1478. G, quantification of LDA from E. Error bars indicate SD. Asterisk indicates statistically significant differences (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
EGFRvIII and SUM159-EGFRvIII cell lines, respectively. As before, the MCF10A cell line displayed high sensitivity to EGFR inhibitor AG1478, showing a reduction in sphere forming ability of more than 60-fold. Together these findings suggested that EGFRVIII modulates its effects on stem cell-like phenotypes through the Wnt/β-catenin pathway.

Discussion

Our results show that EGFRvIII is expressed in primary breast carcinoma and contributes to CSC phenotypes in EGFRvIII expressing cell lines. This agrees with previous studies reporting EGFRvIII expression in breast cancer (9, 12, 20). However, several studies have been unable to verify this expression (14, 15). In general, there is no consistently used EGFRvIII-specific antibody or detection assay that could lead to varying sensitivities and specificities. We also noted a discrepancy between our immunohistochemical and flow cytometry/RT-PCR analyses. We believe this is likely due to a high false-negative rate in immunohistochemical analysis and the actual incidence is closer to that predicted by both flow cytometry and RT-PCR (69%). A potential explanation for the high false-negative rate in immunohistochemical analysis may be sampling bias, as our results suggest that EGFRvIII may be expressed focally and sporadically in some tumors. In cases with such low expression it is probable that a given section, especially if it is small in size, might not contain EGFRvIII-positive cells. Indeed, anecdotally we have found that in tumors with low percentages of positive cells, some sections will contain positive cells and others will not. Studies that have been able to successfully identify EGFRvIII in a high percentage of cases used a nested RT-PCR approach and/or microdissection of positive regions of the tumor before RT-PCR (15, 20). We also could only readily identify EGFRvIII in a high percentage of cases using a nested RT-PCR approach and additionally on a cell-by-cell basis using flow cytometry. Nested RT-PCR can carry the risk of false-positives; however, using flow cytometry we identified EGFRvIII mRNA in only EGFRvIII-positive, but not negative, sorted fractions, and we also confirmed the EGFRvIII junction using sequencing, indicating that our protocol has identified only true positives. Also flow cytometry may have the additional advantage of allowing for detection of proteins in their native states. Lastly, our results showed lower levels of EGFRvIII expression in breast cancer as compared with what is observed in GBM and lack of amplification or rearrangement of the EGFR locus. This suggested that another mechanism, such as alternative splicing, may account for its presence and the difficulty in detection compared with GBM.

We also observed a correlation between EGFRvIII expression and the ER+/PR+/HER2− subtype. These tumors are thought to fall within the Luminal A category, whereas wtEGFR expression and amplification is usually associated with triple-negative/basal breast cancer (3, 5). Given the lack of amplification we observed in EGFRvIII expressing tumors, it is perhaps not surprising that EGFRvIII expression would correlate with a different tumor subtype. It is also likely that EGFRvIII signaling would be most advantageous in a tumor type without redundancy from other ErbB family members, suggesting a potentially novel role for this oncogene in tumors typically lacking wtEGFR or HER2 expression.

Although EGFRvIII is rarely found in normal noncancerous tissue (9), we were surprised to see a low percentage of EGFRvIII-positive cells in the seemingly normal tissue adjacent to breast tumors, but not in nontumor associated normal breast tissue. This suggests that there could be additional residual EGFRvIII-positive cells in the surrounding tissue. We also noted EGFRvIII-positive cells in corresponding lymph node metastases, which would support the presence of infiltrating or invading EGFRvIII expressing cells. The presence of EGFRvIII-positive cells in infected lymph nodes agrees with a previous study, supporting this as a true observation (18). Furthermore, as EGFRvIII has been implicated in chemotherapeutic and radiation therapy (30, 31), these residual cells may be cause for particular concern. Currently an EGFRvIII-specific peptide vaccine directed against the unique epitope present at the exon 1–8 junction is being evaluated in phase III clinical trials for GBM. Preclinical and clinical data show increases in median time to tumor progression and overall survival compared with historical controls (40). Our data provides support for the use of EGFRvIII-targeted therapy in patients with EGFRvIII-positive breast cancer, and a vaccination approach could be successful in eliminating residual EGFRvIII-positive cells.

In addition to providing evidence for the presence of EGFRvIII in primary breast carcinoma, we show that EGFRvIII is expressed in only a subset of cells, including the putative CSC CD44+/CD24low population, and that EGFRvIII expression is associated with higher expression of EMT and stem cell–associated genes. Our study provides further impetus to pursue the role of EGFRvIII in CSC phenotypes. We present evidence that expression of EGFRvIII increases tumor sphere formation and tumor formation. In addition, we observed an increase in the percentages of CD44+/CD24low and Aldefluor− stem-like cells within heterogeneous cell lines, suggesting that EGFRvIII plays a role in stimulating expression patterns characteristic of these stem-like cells. However, EGFRvIII does not cause all cells to acquire these characteristics, indicating that there are additional factors that influence the ability of a cell to acquire stem-like properties. There is also not a complete overlap between the EGFRvIII+ and CD44+/CD24low populations, and we think it is unlikely that EGFRvIII marks an entirely pure population of CSCs. Likewise, the CD44+/CD24−/low population is not thought to exclusively mark CSCs, but instead enriches for a population containing CSCs. Most importantly though, EGFRvIII functionally contributes to CSC phenotypes and is not simply a cell-surface marker.

Interestingly, our results suggest a novel downstream target of EGFRvIII signaling: the Wnt pathway. The Wnt pathway and downstream activation of β-catenin play known roles in the self-renewal of CSCs and the upregulation of breast CSC markers (41). AKT has also been implicated in similar processes and leads to the activation of β-catenin (41). Unlike wtEGFR, EGFRvIII mediates little basal MAP kinase or ERK activity (8, 42, 43) and instead promotes cellular transformation via the PI3-kinase/AKT pathway (44). Our results show that EGFRvIII induces the Wnt pathway by upregulating the
transcriptional activity of β-catenin. This suggests that EGFRvIII plays a role in a critical population within breast cancer and provides a connection to the Wnt pathway, which has known involvement in CSC phenotypes. We are currently extending these findings to other tumor types and investigating additional connections to other stem cell–associated pathways.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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