Alterations of Smad Signaling in Human Breast Carcinoma Are Associated with Poor Outcome: A Tissue Microarray Study

Wen Xie, Joachim C. Mertens, Daniel J. Reiss, David L. Rimm, Robert L. Camp, Bruce G. Haffty, and Michael Reiss

ABSTRACT

Based largely on studies of cell lines in vitro and of transgenic mouse models, disruptions of transforming growth factor (TGF-β) signaling are thought to contribute to the development and progression of human breast cancer. However, whether and how TGF-β signaling becomes disrupted during human breast cancer development in vivo remains largely unknown. To address this question, we have compared the patterns of expression and activation of the postreceptor components of the TGF-β signaling pathway, the so-called Smads, in human breast cancer cell lines with those in breast carcinoma specimens. None of the breast carcinoma cell lines were growth arrested by TGF-β in vitro. Each of the tumor cell lines expressed normal levels of Smad2 and -3. Moreover, TGF-β treatment induced phosphorylation of Smad2 (Smad2P) in each of these lines, except those that lacked TGF-β type II receptors. Moreover, only one of the cell lines failed to express Smad4. Among 456 cases of human breast carcinoma assembled in tissue microarrays, the majority (92%) expressed Smad2, Smad2P, as well as Smad4, indicating their ability to proliferate within a microenvironment that contains bioactive TGF-β. Thirty cases (6.6%) failed to express Smad2P, suggesting the loss of TGF-β receptor signaling. Nine cases (2%) failed to express Smad4, and 3 of these also failed to express Smad2P. Thus, the phenotypes of breast tumors in vivo paralleled that of human breast cancer cell lines in terms of Smad2P and Smad4 expression. Loss of Smad signaling was not associated with any particular histological subtype, histological or nuclear grade, estrogen- or progesterone receptor expression, or HER2/neu expression. Loss of Smad4 was inversely correlated with the presence of axillary lymph node metastases. Most importantly, among patients with stage II breast cancer, lack of Smad2P expression in the tumor was strongly associated with shorter overall survival. Finally, analysis of a small cohort of hereditary breast cancer cases failed to reveal any association between BRCA1 or BRCA2 genotype and alterations in Smad signaling.

INTRODUCTION

The TGF-β superfamily of secreted polypeptides regulates cell proliferation, differentiation, motility, and apoptosis in a variety of different cell types (1, 2). TGF-β is a 25-kDa dimeric polypeptide that is the most potent known inhibitor of normal human mammary epithelial cell replication in vitro (3). In vivo, TGF-β appears to regulate normal ductal and alveolar development in the mammary gland (4, 5). Moreover, in the adult mammary gland, TGF-β probably mediates the massive cell death and restructuring that takes place during postlactational involution (6). Besides these physiological functions, there is considerable evidence that TGF-β plays an important role in mammary carcinogenesis (reviewed in Refs. 7, 8). First of all, a number of transgenic mouse studies have provided strong evidence that TGF-β is able to protect against mammary tumor formation in vivo (9–11), because either a relative lack of TGF-β or the inactivation of the TGF-β signaling pathway results in loss of tumor suppression and promotes carcinogenesis. On the other hand, virally transformed tumorigenic mammary epithelial cell lines as well as most of the cell lines derived from invasive human breast carcinomas are resistant to the antiproliferative effects of TGF-β in vitro and do not respond to treatment with TGF-β in vivo (7). In aggregate, these studies have suggested that, at some point during mammary tumor development, the epithelial cells escape from TGF-β-dependent cell cycle control. Several years ago, we proposed that this seemingly paradoxical switch in the responsiveness of tumor cells to TGF-β during progression is the consequence of the activation of the latent TGF-β in the tumor microenvironment, which then drives the selection and clonal expansion of TGF-β-resistant tumor cells (7). However, direct evidence in support of the idea that human breast cancer cells in vivo are refractory to TGF-β-mediated cell cycle arrest has been lacking.

The TGF-β signal is transduced by a pair of transmembrane serine-threonine kinase receptors (2). TGF-β binds primarily to TβR-II receptor homodimers, which then form heterotetrameric complexes with two TβR-I molecules. As a consequence, the TβR-II kinase phosphorylates TβR-I, thereby activating its serine-threonine kinase. In response to receptor activation, two cytosolic proteins, Smad2 and Smad3, become transiently associated with and phosphorylated by the TβR-I kinase. After their activation, Smad2 and -3 form heteromeric complexes with a third homologue, Smad4. These complexes are translocated to the nucleus, bind to DNA in a sequence-specific manner, and regulate gene transcription (2). The resulting repression of c-myC and induction of cyclin-dependent kinase inhibitors as well as cdc25A phosphatase lead to G1 phase cell cycle arrest.

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throughput of this type of analysis, we made use of tissue microarrays. Tissue microarrays can hold several hundred tissue specimens on a single slide and can be used for multiple studies, thereby conserving tissues and assuring the uniformity of experimental conditions across a large number of samples (17). Therefore, much larger numbers of cases can be analyzed to obtain a more accurate estimate of the frequency and mechanisms of inactivation of particular signaling intermediates.

MATERIALS AND METHODS

Cell Culture. The following nontumorigenic epithelial cell lines were used in these studies: HC-11, a cloned mammary epithelial cell line isolated from mid-pregnant mammary gland cells of BALB/c mice (18, 19); HBL-100, isolated from human breast milk (20); and BALB/MK spontaneously immortalized mouse keratinocytes (21). The following human breast carcinoma cell lines were used in these studies: BT-20 (22), BT-474, BT-549, and HS578T (23) derived from primary breast adenocarcinomas; MCF-7 (24), T47D (25), MDA-MB-231, MDA-MB-435, MDA-MB-453, and MDA-MB-468 (26) derived from malignant pleural effusions; and ZR-75-1 and ZR-75-30 (27) derived from malignant ascites. All of the cell lines were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum, epidermal growth factor (10 ng/ml; Upstate Biotechnology, Inc., Lake Placid, NY), insulin (5 μg/ml; Collaborative Research, Bedford, MA), transferrin (10 μg/ml; Collaborative Research), and gentamicin (10 μg/ml). All of the cell lines were routinely screened for and found to be free of Mycoplasma contamination using the method described by Chen (28).

Detection of Smad Expression by Western Blotting. Smad protein expression was detected by Western blot as described previously (29). Briefly, confluent cells were treated with 100 μM of TGF-β for 1 h and lysed in situ. Protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose filters, and stained using anti-Smad antibody. Smad2 and -3 were detected using a goat polyclonal dual specificity anti-Smad2/3 antibody (N-19; Santa Cruz Biotechnology). Smad4 was detected using a mouse monoclonal anti-Smad4 antibody (B-8, Santa Cruz Biotechnology). Smad2P was detected using a polyclonal rabbit antibody raised against a Smad2 COOH-terminal phosphopeptide (29). Blots were developed using a 1:2000 dilution of an appropriate horseradish peroxidase-tagged secondary IgG antibody (Calbiochem, San Diego, CA), and the bands were visualized using DuPont NEN Chemiluminescence Reagent as recommended by the manufacturer. Autoradiograms were photographed using an Eagle Eye II Imaging system (Stratagene), and the images subjected to densitometry using the NIH Image (v. 1.62) software package.

Construction and Processing of Breast Cancer Tissue Microarrays. Formalin-fixed paraffin-embedded tissue blocks containing invasive breast carcinomas and normal breast tissue were retrieved, along with the corresponding H&E-stained slides, from the archives of the Yale University School of Medicine Department of Pathology. Blocks were stored under ambient conditions. Formalin-fixed, paraffin-embedded tissue blocks containing invasive breast carcinomas and normal breast tissue elements, were identified by a researcher (R. L. C.) and subjected to 3 × 3 min high temperature microwave oven treatment followed by the treatment with 0.3% H2O2 in methanol for 30 min at 37°C to inactivate endogenous peroxidase. To eliminate nonspecific staining, sections were incubated with the appropriate preimmune serum for 20 min at room temperature, followed by incubation with primary antibodies at 4°C overnight. These included a polyclonal goat anti-Smad2 (S-20) antibody (1:100; Santa Cruz Biotechnology Inc.), a polyclonal rabbit anti-phospho-Smad2 (1:100; Smad2P) antibody (raised in our laboratory, see above), or a monoclonal mouse anti-Smad4 (B-8) antibody (1:150; Santa Cruz Biotechnology Inc.). Slides were washed three times in 0.01 M TBS and then incubated with a 1:2000 dilution of biotinylated secondary antibodies at 37°C for 30 min followed by three washes using TBS. After incubation with the avidin-biotin-horseradish peroxidase complex (Vectastain ABC Peroxidase kit, Standard Elite Series; Vector Laboratories, Inc.) for 30 min at room temperature, slides were washed three times in TBS, rinsed in 0.5% Triton-X-100/TBS for 30 s, and developed by immersion in 0.1% of freshly prepared solution of 3, 3′, 3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co. Fast; Sigma Chemical Co. Chemicals, St. Louis, MO) for 6–8 min. Sections were lightly counterstained with hematoxylin, dehydrated in graded alcohols, cleared in toluene, and mounted using Permount mounting medium (Fisher Scientific Company, Fair Lawn, NJ). To exclude nonspecific reactions of secondary antibodies or avidin-biotin complexes, control sections of each specimen were processed without primary antibodies. The intensity of positive staining was scored independently by two observers. Cases of disagreement were rereviewed jointly to arrive at a consensus score. Each tumor was represented on the arrays by at least two cores. Disc scores from the same tumor were averaged to produce a single score or expressed as a ratio of positive:negative cases, depending on the analysis. We have determined previously that analysis of two cores concurs with analysis of an entire tissue section with >95% accuracy (30).

The ratio of specimens with positive versus negative staining for either Smad2, Smad2P, or Smad4 did not differ significantly among cases in the second microarray obtained from successive decades between 1932 and 1999. This confirms our previous results using a number of different antibodies (30) and diminishes the likelihood that the current results might have been skewed by including samples that had been archived over a period of 7 decades.

Mutational Analysis. In select cases, full-size 5-μm sections were cut from the same tissue blocks used for microarray construction. Genomic DNA was extracted as described by Frank et al. (31). Briefly, 20-gauge Precision-Glide sterile single use injection needles (Becton Dickinson, Franklin Lakes, NJ) were used to scrape tissue from the slide into a mixture of 100 μl of xylene and 100 μl of 100% (v/v) ethanol. The samples were mixed gently and spun in a microcentrifuge at 12,000 rpm for 10 min. After decanting the supernatant, the tissue pellet was dried at room temperature and then resuspended in 50 μl of 50 mM Tris (pH 8.3) containing 200 ng/ml of proteinase K. The samples were incubated at 37°C overnight followed by a 10-min immersion in boiling water to inactivate the enzyme and then immediately placed on ice. Genomic DNA was examined for the presence of mutations by PCR-SSCP as described previously (14, 32).

Statistical Analysis. Survival curves were estimated according to the method of Kaplan-Meier (33). For each curve, the starting point was the date of diagnosis of breast cancer. Death from any cause was counted as an event in calculating survival time. For surviving patients, time was censored at the last available follow-up date. Follow-up data were updated as of September 20, 2000. The median follow-up time in this series was 69 months. The Breslow,Gehan-Wilcoxon rank test was used to compare outcomes of different groups.
RESULTS

To determine the sensitivity of human breast cancer cell lines to TGF-β-mediated growth inhibition, cells were incubated in the presence of TGF-β for 6 days (Fig. 1). As shown in Fig. 1, TGF-β1 strongly inhibited the growth of non-neoplastic HC-11 mouse mammary epithelial cells and BALB/MK mouse keratinocytes (IC₅₀: 6.25 and 5 pt, respectively). In contrast, HBL-100 cells (not shown) and each of the breast carcinoma cell lines were able to proliferate in the presence of as much as 400 pM TGF-β (Fig. 1). Growth rates were minimally affected by TGF-β, resulting in reductions in total cell numbers at the end of 6 days in culture by no more than 40%. Thus, these breast cancer cell lines were essentially refractory to TGF-β-mediated cell cycle arrest. Of note, although HBL-100 cells were derived from breast milk of a healthy woman and are not tumorigenic in nude mice, they express SV40 large T antigen, are aneuploid, and are capable of anchorage-independent growth (20, 34). Thus, it is perhaps not surprising that they fail to be growth arrested by TGF-β.

To determine at which level the TGF-β signaling pathway might be interrupted, we examined the expression and activation state of the Smad postreceptor signaling intermediates. The overall levels of expression of Smad2 and -3 were similar among the nine transformed breast epithelial cell lines examined (Fig. 2A) and did not appear to change appreciably in response to TGF-β treatment (data not shown). To test the functional integrity of the TGF-β receptor system, we took advantage of the fact that phosphorylation of Smad2 is exclusively dependent on activation of both TβR-I and -II in response to ligand binding. We have recently developed an antibody that selectively recognizes Smad2 in its phosphorylated form (Smad2P) and that can be used for immunohistochemical analyses of tumor specimens (29). Whereas Smad2P was not detectable in the absence of TGF-β, treatment with TGF-β1 clearly induced phosphorylation of Smad2 in most of the breast carcinoma cell lines (Fig. 2A). This finding indicates that both TGF-β receptors were capable of active signaling in the majority of the cell lines. As the one exception, TGF-β failed to induce Smad2 phosphorylation in T47D cells. This finding is entirely consistent with the fact that these cells do not express TβR-II receptors (Fig. 2A; Refs. 35, 36).

Recent studies have demonstrated that loss of immunostainable Smad4 protein as assayed by using the B-8 monoclonal antibody (Santa Cruz Biotechnology) is an extremely sensitive and specific surrogate marker for structural alterations of the Smad4 gene in tumor specimens (37, 38). Thus, we determined whether or not TGF-β resistance of any of the breast carcinoma cell lines might be attributable to loss of expression of Smad4 (Fig. 2B). Smad4 protein was clearly detectable in eight of the nine transformed breast epithelial cell lines by Western blotting, and its levels did not change appreciably in response to TGF-β treatment (Fig. 2B). However, one of the breast carcinoma lines (MDA-MB-468) failed to express any Smad4 protein (Fig. 2B), consistent with a previous report that this cell line has microbial characteristics.

Major departures from proportional hazard assumptions were excluded by graphic checks. Contingency table analyses using the Fisher’s exact test were used to determine the relationships between Smad status and known prognostic factors for breast carcinoma. These analyses were performed using Abacus Concepts, Statview 4.51 (Abacus Concepts, Inc., Berkeley, CA).
undergo a homozygous deletion of the Smad4 gene (16). Although the steady-state level of Smad4 appeared to be significantly lower in ZR-75–1 cells than in the other breast cancer lines, whether this decrease in Smad4 expression accounts for the ability of the cells to proliferate in the presence of TGF-β remains to be determined (Fig. 2B).

**Smads Expression Patterns in Normal Breast Tissue.** To evaluate the pattern of Smad2 expression and activation in normal mammary glandular tissue, we examined normal human breast tissue sections included in one of the tissue microarrays (Fig. 3). As expected, all of the normal tissue elements, including ducts and terminal lobular units, stromal cells, and capillaries, expressed Smad2 as well as Smad4. However, to our surprise, Smad2P was clearly detectable in the epithelial cells of normal ducts and lobules, as well as capillary endothelial cells (Fig. 3). In contrast, we found no evidence for Smad2 phosphorylation in interglandular stromal elements. These findings indicate that biologically active TGF-β is present within the microenvironment immediately surrounding normal mammary glandular structures and capillaries, resulting in activation of the TGF-β signaling pathway selectively in mammary epithelial and endothelial cells.

**Smad Expression Patterns in Breast Cancer Tissue Microarrays.** To determine the status of Smad signaling in invasive human breast carcinomas in vivo, Smad2, Smad2P, and Smad4 expression were examined by immunostaining of tissue microarrays (Table 1). These included a total of 367 (80%) primary invasive ductal carcinomas, 88 (20%) primary invasive lobular carcinomas, and a single metastatic focus of an invasive ductal carcinoma. All 456 evaluable cases expressed Smad2 (Fig. 4). Diffuse-positive staining for Smad2 was seen primarily in the cytoplasm of tumor epithelial cells. Phosphorylation of receptor-activated Smads by ligand-induced activation of the TGF-β receptor complex is the key step in the intracellular transduction of TGF-β signaling. Our anti-Smad2P antibody allowed us to assess the state of activation of receptor-associated Smad2 by the TβR-II/TβR-I receptor complex in tumor tissue in situ. Diffuse-positive staining for Smad2P was seen in 426 (93.3%; 95% CI: 91–96%) of breast cancer specimens (Table 1; Fig. 4). This is an important finding, because it indicates that the TGF-β receptor signaling is activated in the vast majority of invasive breast carcinomas. By inference, then, these tumor cells are capable of proliferation despite an activated TGF-β signaling pathway. This finding indicates not only that biologically active TGF-β is present within the microenvironment of these tumors but also that the carcinoma cells must have escaped from TGF-β-mediated cell cycle arrest. In the 30 remaining cases (6.6%; 95% CI: 4–9%), we were unable to detect Smad2P expression within the tumor cells, although it was present in stromal cells and endothelial cells of small blood vessels (Fig. 4). Thus, these cases are likely to have lost expression or to have acquired inactivating mutations of one or the other TGF-β receptor subtype (13, 14, 39).

To determine whether inactivating mutations were responsible for the loss of Smad2P expression in our tissue microarrays, we extracted genomic DNA from full-size 5-μm paraffin sections obtained from

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**Fig. 3.** Smad expression and activation in normal human breast tissue. Consecutive 5-μm sections of a tissue microarray that included 13 samples of normal breast glandular tissue were stained using a mouse monoclonal anti-Smad4 antibody (B-8; Santa Cruz Biotechnology), a polyclonal goat anti-Smad2 (S-20) antibody, or our polyclonal rabbit anti-Smad2P antibody to detect expression of Smad4, total Smad2, and Smad2P, respectively, as described in “Materials and Methods.” Control sections of each specimen were processed without primary antibodies. All sections were counterstained with hematoxylin. Representative 0.6-mm diameter areas of normal ductal (Du) and lobular (Lob) glandular elements of the breast are shown (×200 magnification). All tissue elements expressed Smad2 as well as Smad4. Interestingly, phospho-Smad2 (Smad2P) expression seems to be restricted to ductal and lobular epithelial cells and to endothelial cells (Endo).
the corresponding donor blocks of 10 of the Smad2P-negative cases. The entire coding sequences of the TβR-I and II genes were then screened for mutations by PCR-SSCP, as described previously (14). To our surprise, we failed to identify any mutations in either the TβR-I or II genes in any of these cases. To exclude the possibility that the loss of Smad2 phosphorylation was attributable to a mutation and/or deletion of the COOH-terminal phosphorylation sites of the Smad2 gene, we also examined exon 10 of the Smad2 gene by PCR-SSCP. No structural abnormality of this region was identified in any of the 10 cases.

Smad4 expression was observed in 447 (98%; 95% CI: 96–99%) of 456 breast cancer specimens (Fig. 4). Diffusely positive staining was predominantly confined to the cytoplasm of tumor epithelial cells with occasional associated nuclear staining. Smad4 was undetectable in the tumor cells in the remaining 9 cases (2%; 95% CI: 1–4%). Thus, based on their expression pattern of Smad2, Smad2P, and Smad4, breast cancers could be grouped into four different categories (Table 1). The majority (n = 420) of cases coexpressed Smad2, Smad2P, and Smad4. Among the remaining 36 cases, 27 were Smad2P-negative, 6 were Smad4-negative, and 3 failed to express either Smad2P or Smad4, suggesting the presence of a dual defect in TGF-β signaling.

**Associations between Smad2P and Smad4 Expression with Pathological and Biological Features of Breast Carcinoma.** To identify possible associations between the patterns of expression of Smad2P and Smad4 and other clinical and pathological features of the breast carcinomas, Smad expression patterns were compared with tumor histological type, histological grade, nuclear grade, estrogen-, progesterone-, and HER2/neu receptor expression, as well as lymph
node status (Table 2). This analysis failed to demonstrate any significant association between any of these parameters and the absence of either Smad2P or Smad4. The only exception was an apparent inverse association between loss of Smad4 expression and the presence of lymph node metastases (Relative risk: 0.62; 95% CI: 0.32–1.17; P = 0.012).

**Association between Loss of TGF-β Receptor Signaling and Patient Survival.** A number of recent studies have suggested that loss of TGF-β type II receptor expression defines a subset of breast cancer with a particularly aggressive phenotype (12, 13). To test this hypothesis, we examined the possible relationship between loss of receptor signaling (as measured by Smad2P negativity) and overall survival among 335 cases of lymph node positive breast cancers for which this information was available (Fig. 5). As shown in Fig. 5, the overall survival of patients whose cancers failed to express Smad2P was significantly shorter than that of those whose tumors expressed Smad2P (Median survival: 110.5 versus 306.5 weeks; P = 0.024 by Breslow-Gehan-Wilcoxon test). Moreover, our findings suggest that the negative impact of loss of TGF-β receptor signaling on prognosis is independent of other prognostic markers (Table 2). Thus, absence of Smad2P appears to define a previously unrecognized small but particularly aggressive subset of breast cancers.

**Lack of Association between Smad Expression and BRCA1 or BRCA2 Mutation Carrier State.** The vast majority of hereditary nonpolyposis colorectal carcinomas in patients with inherited DNA mismatch repair deficiencies display intragenic inactivating mutations of the TβR-II gene, whereas this gene is only rarely affected in sporadic colon cancer (40–42). To investigate the possibility that hereditary breast cancers associated with germ-line BRCA1 or -2 mutations might also be associated with a higher frequency of TGF-β receptor inactivation than sporadic cases, we analyzed a panel of primary breast cancers obtained from women <35 of age of which the BRCA1 and -2 gene status had been determined by direct sequencing. As summarized in Table 3, the frequency of Smad2P-negative cases (indictative of a TβR defect) was similar among cases bearing germ-line BRCA gene mutations and age-matched wild-type controls. Thus, in contrast to hereditary nonpolyposis colorectal cancers in which the frequency of TβR-II mutations is ≥80%, hereditary breast cancer does not appear to be associated with loss of TβR receptor signaling.

**DISCUSSION**

The first question this study addressed was whether TGF-β is activated within the microenvironment of human breast cancers. Immunostainable TGF-β has been detected in and around the malignant epithelial cells of breast carcinomas, and the intensity of this staining seems to correlate with advancing stages of tumor progression (43–46). However, interpretation of these studies is complicated by difficulties associated with distinguishing the biologically inactive, latent form of TGF-β from its activated form. To circumvent these problems, we used phosphorylation of the principal TβR-I substrate, Smad2, as a surrogate marker of activation of the TGF-β receptor system by TGF-β. Using cell lines in vitro, we showed that our Smad2P-specific antisem recognizes the phosphorylated form of Smad2 in a highly specific and sensitive manner (Fig. 2), and that Smad2 phosphorylation occurs in response to TGF-β treatment in a dose- and time-dependent fashion (29). Thus, the presence of Smad2P in cells can be used as an indicator of receptor signaling by biologically active TGF-β in the cellular microenvironment.

Our results indicate not only that the microenvironment of the normal mammary gland apparently contains biologically active TGF-β but that this is restricted to the epithelial glandular structures and capillary walls. This finding was somewhat unexpected, because TGF-β is generally believed to be deposited in the extracellular milieu in its latent form and not to become activated except in response to tissue injury (47, 48). Alternatively, Smad2 phosphorylation could be induced by activin-mediated activation of its cognate receptors. However, in vivo, the expression of activins appears to be restricted mainly to gonadal and neural tissues. Although activins have been detected in breast tissue, it is not clear which cells in the breast express activin receptors and how they might respond to activin signals (49, 50). Thus, it is less likely that the Smad2 phosphorylation we see in breast epithelium is induced by activin.

Perhaps our most surprising finding was the fact that in 90% of breast carcinomas the malignant epithelial cells continue to express Smad2P. This indicates that bioactive TGF-β is present in the microenvironment and is inducing receptor signaling in all of these cases. In addition, and perhaps more importantly, this observation provides the first direct evidence that the malignant tumor cells are, in fact, capable of proliferating even in the presence of biologically active TGF-β in vivo. In this respect, the phenotype of breast carcinomas in vivo parallels that of the majority of breast cancer cell lines in vitro. Moreover, this finding supports our prediction that TGF-β provides a selective pressure that favors the outgrowth of cell clones that are resistant to TGF-β-mediated cell cycle arrest.

Approximately 7% of the cancers failed to express Smad2P. This was clearly attributable to a lack of phosphorylation and not because of a loss of Smad2 expression. Although missense mutations in both the TβR-I and -II genes in breast cancers have been described (14, 39), we found no evidence for homozygous loss or intragenic muta-
tion of either the TβR-I or -II genes as the cause of Smad2P negativity.

It is possible that loss of TβR-I or -II receptor mRNA expression accounts for the absence of Smad2P in some cases. Several studies have attempted to quantify TβR receptor expression in breast cancer specimens, although the results have been conflicting (13, 51–53). These discrepancies may be attributable to technical difficulties associated with correctly quantifying cell surface receptor expression. For example, Gobbi et al. (13) recently reported that 40% of primary

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* Relative risk: 0.62 (95% CI: 0.32–1.17).

ER, Estrogen receptor; PgR, Progesterone receptor; LNN, Lymph nodes.
smad2 phosphorylation-negative breast cancers failed to express TβR-II protein and that most of the TβR-II-negative cases were high-grade lesions. Even if we assume that all of our cases of Smad2P negativity were attributable to loss of TβR-II expression, the true frequency of this event must be much lower than reported by Gobbi et al. (13) based on receptor immuno-staining. Moreover, in our series, Smad2P negativity did not correlate with histological or nuclear grade. Only complete absence of detectable TβR-II results in loss of TGF-β responsiveness (54). Thus, our results suggest that a reduction in TGF-β receptor expression to a degree that would result in complete loss of signaling occurs in certainly no more than 10% of breast carcinomas. Moreover, this conclusion is entirely consistent with the fact that complete loss of TβR expression has been found in only a very small number of breast carcinoma cell lines (35, 55, 56). It should be noted that only complete loss-of-function TβR mutations would result in Smad2P negativity. Thus, we cannot rule out the possibility that some of the Smad2P-negative breast cancers carry TβR mutations that do not completely abrogate signaling activity as we have described recently (14).

The predominant phenotype of breast cancers with regards to TGF-β receptor signaling clearly contrasts with that of several other tumor types. For example, the majority of hereditary nonpolyposis colorectal cancers carry inactivating mutations of the TβR-II gene (40, 41, 57). Similarly, we have recently found Smad2P immuno-staining to be weak or undetectable in the majority of in situ and invasive endometrial cancers compared with normal endometrium and glandular hyperplasias. In contrast, even among the hereditary breast cancers included in the present study, the frequency of Smad2P-negative was exceedingly low (Table 3).

In our series, 9 cases (2%) failed to express Smad4 protein. Wilzent et al. (37, 38) demonstrated recently that loss of Smad4 immuno-staining using the same antibody accurately reflects the presence of inactivating structural alterations of the Smad4 gene. It is likely that failure to express Smad4 confers resistance to TGF-β-mediated signals, including cell cycle arrest. This is illustrated by the breast cancer cell line MDA-MB-468, which has undergone a homozygous deletion of the Smad4 gene (16, 58). Not only is this cell line completely unresponsive to TGF-β (58–60), but transfection of wild-type Smad4 restores most transcriptional responses to TGF-β as well as TGF-β-mediated growth arrest (58–61).

Interestingly, we encountered two cases of breast cancer that failed to express both Smad4 and Smad2P. This finding suggests that these tumors carry a dual defect in the TGF-β signaling pathway, one that affects receptor signaling and one that affects transcriptional regulation of target genes. It is possible that inactivation of TGF-β receptors and of Smad4 confer partially nonoverlapping selective advantages during tumor development. This idea is supported by a number of recent reports of Smad4-negative human tumor cell lines in which some of the TGF-β-dependent transcriptional responses were retained, suggesting that these do not depend exclusively on Smad4 (61, 62).

Our study revealed remarkable parallels between the phenotypes of breast cancer cell lines in vitro and primary breast cancers in vivo. Across the board, the cell lines were capable of proliferation in the presence of TGF-β and appeared to have escaped from TGF-β-mediated cell cycle arrest, although most of them expressed Smad2P and Smad4. Thus, the mechanisms whereby the majority of breast cancers escape from TGF-β-mediated cell cycle control remain unclear. Because TGF-β specifically antagonizes the activity of a variety of mitogenic pathways, it is possible that the constitutive activation of such a mitogenic pathway could override the effects of TGF-β on cell

Table 3 Smad expression and activation in hereditary breast carcinomas

<table>
<thead>
<tr>
<th>BRCA status</th>
<th>Smad2P Positive</th>
<th>Smad2P Negative</th>
<th>Total</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>33 (89)</td>
<td>4 (11)</td>
<td>37</td>
</tr>
<tr>
<td>Mutant</td>
<td>13 (93)</td>
<td>1 (7)</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>5</td>
<td>51</td>
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5 W. Yan and M. Reiss, unpublished observations.
cycle. For example, overexpression of cyclin D1 causes nontransformed epithelial cells to be relatively resistant to growth inhibition by TGF-β1 compared with appropriate controls (63). Breast cancers commonly overexpress cyclin D1 (64, 65). Perhaps a more common scenario is that breast cancer cells selectively lose their ability to regulate c-myc (and perhaps other cell cycle regulators) in response to TGF-β as suggested recently by Chen et al. (66) based on a comparison of gene expression profiles of TGF-β-sensitive and -resistant mammary epithelial cell lines. Although these findings need to be confirmed in fresh tumor specimens, they are entirely consistent with our observation that most breast carcinomas have retained the ability to respond to TGF-β by activation of their receptors and phosphorylation of Smad2.

Finally, our finding that a small subset of breast cancers have lost the ability to phosphorylate Smad2 and/or have lost expression of Smad4 raised the question of whether this phenotype has prognostic and/or predictive clinical significance. Failure to express Smad2P or Smad4 raised the question of whether this phenotype has prognostic and/or predictive clinical significance. Failure to express Smad2P or Smad4 raised the question of whether this phenotype has prognostic and/or predictive clinical significance. Failure to express Smad2P or Smad4 raised the question of whether this phenotype has prognostic and/or predictive clinical significance. Failure to express Smad2P or Smad4 raised the question of whether this phenotype has prognostic and/or predictive clinical significance. Failure to express Smad2P or Smad4 raised the question of whether this phenotype has prognostic and/or predictive clinical significance. Failure to express Smad2P or Smad4 raised the question of whether this phenotype has prognostic and/or predictive clinical significance. Failure to express Smad2P or Smad4 raised the question of whether this phenotype has prognostic and/or predictive clinical significance.

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Alterations of Smad Signaling in Human Breast Carcinoma Are Associated with Poor Outcome: A Tissue Microarray Study

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