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SMAD4 is a potential prognostic marker in human breast carcinomas

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Abstract

SMAD4 is a downstream mediator of transforming growth factor beta. While its tumor suppressor function has been investigated as a prognostic biomarker in several human malignancies, its role as a prognostic marker in breast carcinoma is still undefined. We investigated SMAD4 expression in breast carcinoma samples of different histologic grades to evaluate the association between SMAD4 and outcome in breast cancer. We also investigated the role of SMAD4 expression status in MDA-MB-468 breast cancer cells in responding to TGF- β stimulation. SMAD4 expression was assessed in 53 breast ductal carcinoma samples and in the surrounding normal tissue from 50 of the samples using immunohistochemistry, Western blot, and real-time PCR. TGF- β -SMAD and non-SMAD signaling was assessed by Western blot in MDA-MB-468 cells with and without SMAD4 restoration. SMAD4 expression was reduced in ductal breast carcinoma as compared to surrounding uninvolved ductal breast epithelia ($p < 0.05$). SMAD4 expression levels decreased from Grade 1 to Grade 3 ductal breast carcinoma as assessed by immunohistochemistry ($p < 0.05$). Results were recapitulated by tissue array. In addition, immunohistochemistry results were further confirmed at the protein and mRNA level. We then found that non-SMAD MEK/MAPK signaling was significantly different between SMAD4 expressing MDA-MB-468 cells and SMAD4-null MDA-MB-468 cells. This is the first study indicating that SMAD4 plays a key role in shifting MAPK signaling. Further, we have demonstrated that SMAD4 has a potential role in the development of breast carcinoma and SMAD4 was a potential prognostic marker of breast carcinoma. Our findings further support the role of SMAD4 in breast carcinoma development. In addition, we observed an inverse relationship between SMAD4 levels and breast carcinoma histological grade. Our finding indicated that SMAD4 expression level in breast cancer cells played a role in responding non-SMAD signaling but not the canonic SMAD signaling. Further mechanistic studies are necessary to establish the role of SMAD4 in breast carcinoma prognosis and potential specific targeting.

Keywords

Ductal breast carcinoma; SMAD4; Immunohistochemistry; Tissue array; Quantitative real-time PCR; Western blot analysis; Prognosis; TGF- β ; Non-SMAD signaling

Introduction

Human breast cancers are the most frequent carcinomas in females and the second most common cause of cancer-related mortality in women [1]. In recent years, breast cancer incidence has increased globally; however, mortality has decreased, which has been attributed to earlier diagnosis and more efficient treatment options because of better understanding of the underlying molecular pathology [2]. Molecular medicine has facilitated

the identification of different tumor markers, which may increase the accuracy of classifying and grading tumors, as well as aid in prognostic assessment. Such an extensive understanding will assist clinical therapeutic decisions in order to provide optimal treatment [3].

Nearly 80 % of all diagnosed pre-invasive and invasive breast cancers worldwide are of the ductal subtype [4]. Ductal breast carcinoma is characterized by profound heterogeneity [4]. This complexity increases the difficulty of accurate histo-logic assessment and grading, which ultimately affects the choice of treatment regimens. Thus, identifying a molecular marker specific for ductal breast carcinoma, which also provides insight regarding tumor grade and prognosis, is of clinical importance. Previous studies have demonstrated an association between breast carcinomas and various alterations in gene expression, including *p53* [5], *PTEN* [6], *BRCA* [7], and *FHIT* [8]. In addition to their prognostic significance, specific molecular markers such as estrogen receptor status or HER2/Nu status have been used to tailor therapies in breast cancer.

Recently, we and other researcher have discussed that SMAD4 was implicated in tumor progression [9, 10]. SMAD4 is expressed ubiquitously and was initially isolated as a tumor suppressor gene on chromosome 18q21.1 in pancreatic ductal adenocarcinomas [11].

SMAD4 is a downstream mediator of transforming growth factor beta (TGF- β), an important multifunctional cytokine that regulates cell proliferation, differentiation, and extracellular matrix production [12]. As a tumor suppressor, TGF- β inhibits cell growth predominantly by signaling via the SMAD pathway. Upon binding to the type 2 TGF- β receptor (TGFBR2), TGF- β causes TGFBR2 and TGFBR1 to heterodimerize with two TGFBR1/TGFBR2 complexes. The ensuing TGFBR1 autophosphorylation induces phosphorylation of SMAD2/3, thereby allowing SMAD2/3 to bind to SMAD4. Although SMAD4 is not required for translocation into the nucleus, it is required for the SMAD complex to act as a transcription factor [12].

The relationship between SMAD4 gene inactivation and tumorigenesis has been extensively studied [13], especially in pancreatic cancer [14] and colorectal cancer [15], but there are limited data evaluating the relationship between SMAD4 expression and tumor progression and very little data in breast cancer. Given the central role played by TGF- β in breast cancer development and progression [16], studies of SMAD4 expression are potentially of great interest.

Similar to TGF- β , SMAD4 may play a dual role in breast tumorigenesis with varying roles during the development and progression of breast cancer. Wiercinska et al. demonstrated that SMAD4 is crucial for TGF- β -induced breast cancer cell invasion by inducing matrix metalloproteinase 2 and MMP9 [17]. Li et al. found that SMAD4 induced apoptosis in ER-positive breast cancer cells [18]. In SMAD4 mammary epithelium conditional knockout mice, nearly 100 % of female mice developed squamous cell carcinomas and mammary abscesses [19]. However, TGF- β -induced epithelial-mesenchymal transformation (EMT) and metastasis of breast cancer cells to bone were also critically dependent on SMAD4. Ren et al. concluded that aberrant expression of SMAD4 or disruption of SMAD4 activity is a

potential mechanism for the loss of TGF- β negative regulation on ER α transcriptional activity in breast cancer [20]. These studies highlight the complexity of SMAD4 interactions and functional activity in breast cancer tissues and cells.

TGF- β signaling is both enhanced by and runs in parallel to the mitogen-activated protein kinase (MAPK) signaling pathway [21, 22]. Mouse model data indicate that invasion and metastasis of MDA-MB-231 cells are mediated by MAPK/ERK kinase signaling, leading to the activation of MMP9 independent of SMAD4 [23]. ERK, JNK, and RhoA regulate TGF- β -induced migration in MCF-7 cells as well as in the SMAD4-deficient breast cancer cell line, MDA-MB-468 [24]. We have previously shown that *TGFBR1**6A, a common hypomorphic *TGFBR1* variant, enhances the migration and invasion of MCF-7 cells through RhoA and ERK pathway activation [25].

Currently, a definitive diagnosis for most tumors depends on clinical biopsy. Immunohistochemical (IHC) analysis is a less complicated technique and more widely available in routine clinical laboratories than genetic analysis. Therefore, IHC for SMAD4 is an ideal tool for examining the SMAD4 expression in breast carcinomas and could potentially be used as a diagnostic and prognostic indicator of disease type, prognosis, and response to specific therapeutics. In this study, we used IHC to assess the level of expression of SMAD4 in 53 breast tumor samples and 70 cases of tumor samples from IMGEX tissue arrays. Moreover, we have tested TGF- β signaling in MDA-MB-468 cells with SMAD4 and without SMAD4 in responding to TGF- β 1 stimulation. We also have evaluated a potential role of SMAD4 in breast carcinoma development.

Materials and methods

Patients and tissue samples

This study analyzed 53 tumor specimens, originally collected for standard pathological diagnosis, from patients undergoing potentially curative surgery for breast carcinoma between October 1995 and September 2011 (Department of Surgery, Beihua University Affiliated Hospital (Jilin, China). All patients were female, ranging from 35 to 73 years old (mean, 50.21 $\pm$ 7.43 years). Fifty of the 53 tumor samples had surrounding normal breast tissue, and these tissues were used as normal tissue control. This study was approved by both of the Research ethics Committee of the Department of Surgery, Beihua University Affiliated Hospital (Jilin, China) and the Research ethics committee of the First Clinic Hospital of Jilin University (Changchun, China). All specimens were handled and anonymized according to ethical and legal standards of the People's Republic of China.

None of the patients had received radiation or chemotherapy prior to surgery. Tumors were histopathologically classified according to the WHO classification. Patient data included age, sex, tumor size, lymph node metastasis, date and type of initial surgery, and details of the follow-up. A portion of each specimen was fixed in 10 % formaldehyde and imbedded in paraffin for histological sectioning. Another portion was placed in liquid nitrogen for 10 min, then into an -80 °C ultra-freezer for mRNA and protein isolation.

Tissue array slides IMH-371 (Human breast: cancer-normal adjacent) and IMH-364 (Human breast: cancer-metastasis-normal) were obtained from IMGENEX (San Diego, CA, USA). Detailed clinical information on all cases is available from the company's web (www.tissue-array.com).

Immunohistochemical assay

Immunohistochemical assays were performed using the conventional streptavidin–peroxidase method. In brief, 5–3m paraffin sections were dewaxed using routine techniques, and slides were incubated in antigen retrieval buffer (citric acid and sodium citrate preparation) and boiled (95 °C) for 15–20 min, followed by cooling to room temperature. After peroxidase blocking with 3 % H₂O₂ for 30 min, specimens were blocked with goat serum (Maixin Fuzhou, China) in phosphate-buffered saline (PBS) for 30 min. All incubations with anti-SMAD4 antibody (B-8, sc-7966, Santa Cruz, USA) at 1:200 dilutions were carried out overnight at 4 °C. Then, the specimens were briefly washed in PBS (3×5 min) and incubated at room temperature with anti-rabbit antibody and then streptavidin–biotin protein peroxidase (Maixin Fuzhou, China). The specimens were then washed in PBS and color-developed with DAB (diaminobenzidine solution, Dako Corporation, Carpinteria, CA, USA). After washing with water, specimens were counterstained with hematoxylin (Sigma Chemical Co., St Louis, MO, USA). Normal adjacent breast tissues were used as control tissues and non-immune IgG was also used as negative control antibody for immunohistochemical staining. IHC for tissue array IMH-371 (Human breast: cancer-normal adjacent) and IMH-364 (Human breast: cancer-metastasis-normal) was carried out with the same IHC procedure as for our clinical samples.

Semi-quantitative analysis by immunohistochemistry

All SMAD4 IHC stained sections were scored by two independent experienced pathologists who were blinded to the clinico-pathologic parameters and clinical outcomes of the patients. By microscopy, the distribution and intensity of SMAD4 staining were observed and at least five fields (×400) were analyzed for each sample. The number of immunopositive cells was semi-quantitatively estimated as follows: (a) scoring according to staining intensity: colorless scored 0, pallide-flavens scored 1; yellow scored 2; brown scored 3; (b) according to the percentage of positive cells: no positive cells scored 0; below 10 % positive cells scored 1; 11 to 50 % of positive cells scored 2; 51 to 75 % of positive cells scored 3, and >75 % positive cells scored 4. An immunoreactive score (IRS) was calculated by multiplying (a) and (b). IRS was stratified as - (0 score, absent), + (1–4 score, weak), ++ (5–8 score, moderate), and +++ (9–12 score, strong) according to the proportion and intensity of positively stained cancer cells. An IRS between 1 and 12 (1 IRS 12) was SMAD4-positive.

Quantitative real-time PCR

Total RNA was isolated from all 53 breast tumor specimen and the surrounding normal tissues from 50 of 53 cases using TRIzol reagents (Invitrogen, USA). The first-strand cDNA was synthesized using Thermoscript RT-PCR synthesis kit (Fermentas, USA) according to the manufacturer's instructions. Real-time PCR analyses of mRNA for *SMAD4* and *GAPDH*

were performed using the ABI 7900HT RT-PCR kits (Idaho Technology, Idaho Falls, USA) and the SYBR green I dye (Biogene), which binds preferentially to double-stranded DNA. The primers 5'-TATTAAGCATGCTATACTATGTG-3' and 5'-CTTCCACCCAGATTTCAATTC-3' were used to amplify *SMAD4* transcripts. The mRNA level of *GAPDH* was used as an internal control by using primers 5'-ACCACA GTCCATGCCATCAC-3' and 5'-TCCACCACCC TGTGCTGTA-3'. All primers were synthesized by Sangon Co. (Shanghai, China). The PCR profile consisted of an initial melting step of 1 min at 94 °C, followed by 30 cycles of 15 s at 94 °C, 15 s at 56 °C, and 45 s at 72 °C, and a final elongation step of 10 min at 72 °C. The fold change was calculated from the Cycle thresholds using the delta CT method.

Semi-quantitative-RT-PCR

Using the same RNA samples used for real-time RT-PCR, semi-quantitative RT-PCR was carried out under standard protocol using the following primers: *SMAD4*: (forward: 5'-CGCCTGTCTGAGCATTGTAC-3', reverse: 5'-CGAT TACTTGGTGGATGTTGG-3'), *β-actin*: (forward: 5'-TGAG CTGCGTGTGGCCCTGAG-3'; reverse: 5'-GGGG CATCGGAACCGCTCATTG-3'), PCR was performed at 94 °C for 5 min, followed by 30 cycles of amplification at 94 °C for 40 s, 51 °C for 40 s, and 72 °C for 1 min by using ABI9700. The band intensities were measured by a densitometer, and the results were normalized with *β-actin*. The results were repeated by at least three times independently.

Cell culture

All cell lines were obtained from the American Type Culture Collection and were maintained as following: MCF-10A cells were maintained in a 1:1 mixture of Dulbecco's Modified Eagles Medium (DMEM, Invitrogen, Carlsbad, USA) and Ham's F12 supplemented with 5 % horse serum, 10 µg/ml insulin (Sigma), 0.5 µg/ml hydrocortisone (Sigma), and 0.02 µg/ml epidermal growth factor (Sigma). MDA-MB-468 and MCF-7 cells were grown in DMEM supplemented with 10 % heat-inactivated fetal calf serum (FCS; Invitrogen). T47D cells were grown in RPMI-1640 medium supplemented with 10 % heat-inactivated FCS, 10 µg/ml insulin (Sigma). The cell cultures were maintained in a water humidified incubator with 5 % CO₂. TGF-β1 was obtained from R&D Systems (Minneapolis, MN, USA).

Plasmids pBabe(Hgro) and pBabe SMAD4 Rescue (Hygro) were from Addgene (Cambridge, MA02139, USA).

Generation of retrovirus and SMAD4 stable MDA-MB-468 cells

The retroviral packaging cell line 293 T was maintained in DMEM containing 10 % FCS supplemented penicillin, streptomycin, and fungizone. Transfections were done using standard protocols with Fugene HD (Invitrogen). After transfection, 293 T cells were cultured in DMEM containing 10 % FCS. Viruses were collected 48 and 72 h after transfection, filtered, and concentrated by ultracentrifugation. Concentrated retrovirus was used to infect cells in the presence of 5 µg/ml polybrene, typically resulting in a transduction rate of over 80 %. Infected cells were selected with hygromycin. The overexpressing

retrovirus vector, pBabe, was used to super-infect previously generated overexpressing MDA-MB-468 cells that were subsequently selected with hygromycin.

Assessment of TGF- β /SMAD and non-SMAD signaling

MDA-MB-468 cells stably expressing SMAD4 and vector control cells were plated at a density of 1.5×10^5 per well in a six-well plate (BD Falcon) a day before being serum-deprived for 16 h. The cells were treated with 100 pmol/L TGF- β 1 (R&D Systems) for indicated time points. Then, cells were collected for Western blot analysis.

Western blot analysis

Fifty-three of the surgical tumor tissue samples and matched normal breast specimens were homogenized in RIPA lysis buffer (Beyotime, China). Whole extracts were prepared, and protein concentration was determined according to Bradford's methods, with bovine serum albumin used as a standard. Protein samples (50 μ g) were separated by SDS-PAGE and blotted onto a PVDF membrane (Millipore Corp, Billerica, MA, USA). After blocking, PVDF blots were incubated overnight with primary antibodies diluted in TBS/Tween20 (0.075 %) containing 5 % non-fat dry milk. Mouse monoclonal antibody directed against SMAD4 (B-8, sc-7966, 1:400, Santa Cruz, USA) and AKT (B-1, sc-5298, 1:1,000), rabbit against p38 (c-20, sc-535, 1:1,000), rabbit anti-phosphorylated SMAD2 (ser465/467, Cell Signaling #3108, 1:500), rabbit anti-SMAD2 (D43B4, Cell signaling #5339, 1:1,000), rabbit anti-phosphorylated AKT (Ser473, D9E, Cell Signaling # 4060, 1:500), rabbit anti-AKT (11E7, Cell Signaling #4685, 1:1,000), rabbit anti-phosphorylated ERK1/2 (E10 Cell signaling #9106, 1:500), and rabbit anti-ERK1/2 (Cell Signaling #9102, 1:1,000) were used to probe the membrane. The membranes were re-probed with mouse anti-GAPDH (G-9, sc-365062, Santa Cruz, USA) or α -Tubulin (10D8, sc-53646, Santa Cruz, USA) as loading control. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were used as secondary antibodies correspondingly. After four washes in TBS/Tween-20, the membranes were developed with distilled water for detection of antigen using the enhanced chemiluminescence system. Proteins were visualized with ECL-chemiluminescent kit (ECL-plus, Thermo Scientific).

Statistical analysis

All computations were carried out using the SPSS software version 17.0 for Windows. IRS values calculated for breast carcinoma tissue and adjacent normal breast epithelia originating from the same slide were compared by paired two-sample sign test. We used Spearman's rank correlation test to assess the relationship between SMAD4 expression status and various clinicopathologic parameters, such as tumor size, and histologic grade. The significance level was set at 5 % for all analyses.

Results

IHC analysis of SMAD4 protein levels in breast carcinoma tissues by immunohistochemistry assay analysis

SMAD4 expression was analyzed in 53 specimens of which 8 (14.4 %) were grade I, 34 (64.4 %) were grade II, and 11 (21.2 %) were grade III breast carcinomas. Applying the IRS

criteria (see “Materials and methods” section), 8 (14.4 %) of the tumor tissues were SMAD4-negative (IRS=0), while 45 (85.6 %) stained positive (1 IRS 12). However, no negative staining was observed in the surrounding normal tissues of the 50 cases with adjacent normal tissues.

SMAD4 immunostaining intensity varied greatly in both tumor tissues (A of Table 1) and uninvolved breast epithelia (B of Table 1) from different patients; therefore, we compared SMAD4 expression of tumor tissues and the surrounding normal tissues. In general, overall SMAD4 expression was significantly lower in breast carcinoma (IRS, 4.726 ± 1.59 , $n = 53$) than in normal breast epithelia (IRS, 7.028 ± 1.743 , $p = 0.024$, $n = 50$, A of Table 1), but there was no significant difference between the uninvolved breast epithelia ($p = 0.152 > 0.05$, B of Table 1).

In addition, negative SMAD4 expression was only observed in moderately or poorly differentiated adenocarcinomas. Absence of SMAD4 expression was not observed in tissues with well-differentiated adenocarcinomas. Thus, SMAD4 expression levels were inversely related to histologic grade (Fig 1g; $p < 0.05$). In contrast, SMAD4 expression was not significantly affected by age, tumor size, lymph node invasion, and metastasis (Table 2).

In order to expand the number of clinical cases, we carried out immunohistochemical staining using the commercial tissue array slides (IMGENEX) IMH-371 (Human breast: cancer-normal adjacent) and IMH-364 (Human breast: cancer-metastasis-normal). There were 70 breast carcinoma cases and 39 matched normal breast tissues on the two slides. The tissue array results are consistent with the SMAD4 staining patterns seen in our patient samples. The two sets of breast cancer samples studied support the novel finding that SMAD4 expression is decreased in breast carcinoma when compared with normal adjacent tissues ($p < 0.05$; see supplementary data, S Fig.1B). The typical SMAD4 IHC staining pattern is shown in Fig. 2; SMAD4 staining intensity was greatest in the surrounding normal breast epithelia and most decreased in the sites of high-grade tumors (Fig. 2a, c). Moderate staining intensity was seen in low-grade tumors (Fig. 2b).

SMAD4 protein and RNA levels in breast carcinoma and surrounding normal tissue

To investigate whether decreased SMAD4 staining intensity in breast carcinomas is also observed at the protein and mRNA levels, we performed Western blot and quantitative real-time RT-PCR. Additionally, a semi-quantitative analysis of the expression of *SMAD4* in the tissues of 53 breast carcinomas and 50 surrounding normal breast epithelial tissues was performed. The Western blot results show that SMAD4 expression was higher in the surrounding normal tissues compared with the breast carcinoma tissues (Fig. 3a). Quantitative real-time PCR also show reduced *SMAD4* mRNA expression in breast carcinoma tissues when compared with their adjacent normal tissues (Fig. 3b, $p < 0.01$). This was also supported by semi-quantitative RT-PCR result (Fig. 3c). These results indicate that SMAD4 expression level was decreased in breast carcinoma at both the protein and mRNA levels.

Restored SMAD4 in MDA-MB-468 breast cancer cells

To study the functional relevance of SMAD4 in breast cancer cells, we measured SMAD4 protein expression in mammary epithelial and breast epithelial originated cancer cell lines. Western blot data show that SMAD4 expression varied among the tested cell lines. As shown in Fig. 4a, nonmalignant immortalized MCF10A cells have a higher level of SMAD4 expression than the malignant T47D and MCF7 breast epithelial cell lines. MDA-MB-468 cells did not express SMAD4 at all; a mutant *SMAD4* gene was identified in a previous report [26]. To investigate the role of SMAD4 in breast carcinoma development, we restored SMAD4 in MDA-MB-468 cells using a retrovirus. As shown in Fig. 4b, we successfully established a stable SMAD4 expressing MDA-MB-468 cell line and a SMAD4-null expressing pBabe vector control MDA-MB-468 cell line. We then interrogated the TGF- β /SMAD and non-SMAD signaling studies using this pair of modified MDA-MB-468 cell lines. Our results show that the phosphorylated SMAD2 expression pattern induced by TGF- β 1 remained the same in the two cell lines (Fig. 4c). This suggests that SMAD4 restoration in MDA-MB-468 cells did not interfere with canonic TGF- β /SMAD signaling pathway. What was significantly different between the two cell lines was the non-SMAD pathways induced in response to TGF- β stimulation. TGF- β induced higher levels of phosphorylated MAPK/ERK1/2 in MDA-MB-468 vector control cell compared to SMAD4 transfected MDA-MB-468 cells (Fig. 5a). ERK1/2 expression in both cell lines remained comparable (Fig. 5b). There was no difference in the phosphorylated PI3K/AKT (ser473) induction pattern between the two cell lines (Fig. 5c), and AKT remained the same in both cell lines. However, phosphorylated p38 was highly induced by TGF- β 1 in the SMAD4 restored MDA-MB-468 cells as compared to the MDA-MB-468 vector control cells (Fig. 5e), but the p38 level was comparable in both cell lines. Loading control α -Tubulin expression remained the same at each time point for both cell lines (Fig. 5g). These results suggest that in MDA-MB-468 cells with restored SMAD4, TGF- β 1 could inhibit cell growth by activating the MAPK/p38 pathway because p38 MAPK is usually activated by a variety of environmental stresses [27]. The function of TGF- β may shift from cell growth inhibition to cell growth stimulation by activating MAPK in cells with deficient or absent SMAD4, similar to the expression pattern identified in low- and high-grade breast carcinoma samples. The findings indicate that SMAD4 plays a key role in shifting the MAPK signaling in response to TGF- β stimulation.

Discussion

The product of the *SMAD4* gene belongs to the evolutionally conserved family of SMAD proteins. SMAD4 is a central component of the TGF- β /SMAD pathway and is expressed in various tissues. Deletion or degradation of *SMAD4* in tumors could specifically inhibit the tumor suppressor effect of TGF- β . *SMAD4* alteration has been associated with specific loss of TGF- β induced growth resulting in increased angiogenesis and loss of epithelial integrity [28]. There is extensive evidence indicating that *SMAD4* inactivation is associated with the advanced disease in several human cancers [29, 30]. Our results show that *SMAD4* expression is markedly decreased or even lost in breast ductal carcinoma compared to the surrounding normal breast epithelium in both analysis of our clinical samples and a breast cancer tissue array. Moreover, the decreased SMAD4 expression was further confirmed at

protein and mRNA level by using Western blot and quantitative PCR analysis. The data from our study are consistent with previous reports. Recent study showed that *SMAD4* mRNA expression was reduced in ductal carcinoma as compared to normal tissues [30]. Thus, these results combined with high *SMAD4* expression in normal mammary epithelia suggest that *SMAD4* gene expression may preserve phenotypic characteristics under normal conditions and control the malignant progression of ductal breast carcinomas. To identify possible associations between the expression of SMAD4 and other clinical and pathological features of the ductal breast carcinomas, SMAD4 expression was compared with the age, tumor size, lymphatic invasion, and other clinical parameters of the patients. Our data did not demonstrate any significant association between any of these parameters and the absence of SMAD4 ($p > 0.05$). This was consistent with previous studies [29, 30].

Previous study indicated that induced SMAD4-mediated transcriptional activation was attributable to a cell cycle arrest at the G1 phase and an induction of apoptosis in *SMAD4*-null breast cancer cell line MDA-MB-468 in vitro [31]. It was also reported that SMAD4 inhibits tumor growth by inducing apoptosis in estrogen receptor- α -positive breast cancer cells in vivo [18]. These findings suggest that the loss or reduction of *SMAD4* expression occurs in the neoplastic progression leading to the development of ductal breast carcinoma. It is reasonable to postulate that SMAD4 plays a pivotal role in regulating all TGF- β superfamily signal pathways. The abrogation or downregulation of SMAD4 functions might cause a breakdown or imbalance in this signaling pathway and loss of transcription of genes critical to cell-cycle control. Cells might therefore become TGF- β resistant and escape from TGF- β mediated growth control and apoptosis. Experimental evidence has indicated that SMAD4 is part of the TGF- β signaling cascade and mediates TGF- β induced growth inhibition in breast tumor cells [26].

In order to study the role of SMAD4 in breast cancer development, we restored SMAD4 in a *SMAD4* mutant MDA-MB-468 cells using retrovirus technique. We found the induction of phosphorylated SMAD2 was identical between the SMAD4 restored MDA-MB-468 cells and its vector control cells in responding to TGF- β stimulation. Unchanged canonical SMAD signaling was reasonable because the pair of cell lines was not altered at the receptor level. TGF- β also uses non-SMAD signaling pathways to convey its signals. The non-SMAD pathways are activated directly by ligand-occupied receptors to reinforce, attenuate, or otherwise modulate downstream cellular responses. These non-SMAD pathways include various branches of MAP kinase pathways, Rho-like GTPase signaling pathways, and PI3K/AKT pathways [32]. It was reported that TGF- β induces cell migration by activating AKT and extracellular signal-regulated kinase (ERK 1/2) [33]. SMAD4 has been shown to play a pivotal role in the crosstalk between canonical SMAD and non-SMAD TGF- β signaling pathways. Here, we investigated whether non-SMAD signaling induction is altered by SMAD4 status in the MDA-MB-468 cells. Our results show extracellular signal-regulated kinase (ERK) 1/2 is highly induced in the MDA-MB-468 cells without SMAD4 compared to cells with SMAD4 (Fig. 5 line A); the ERK1/2 were the same level in both cell lines (Fig. 5 line B). This result indicates that cells without SMAD4 could drive proliferation, and division through activation of non-SMAD signaling pathways. Our current result shows that another pro-cell survival non-SMAD signaling pathway, PI3K/AKT, was

not dramatically induced by TGF- β in either the SMAD4 restored or deficient MDA-MB-468 cell lines and even the induction pattern of PI3K/AKT remained the same (Fig. 5 line C). AKT expression was similar in both cell lines at each time point. This indicates that SMAD4 did not affect the non-SMAD signaling-PI3K/AKT in the MDA-MB-468 cells. Interestingly, non-SMAD signaling MAPK/p38 was highly induced in the SMAD4 restored MDA-MB-468 cells, and even at baseline (Fig. 5, line E, 0 time point) there was upregulation in phosphorylated p38 compared to the vector control cells. This result suggests that TGF- β might inhibit growth in the MDA-MB-468 cells with restored SMAD4 by induction of MAPK/ p38 pathway. More recently, it has become apparent that TGF- β signaling pathway may play an important role in driving EMT and cell migration in many type of cancers [18, 34-36]. New evidence demonstrated that SMAD4 [19, 37, 38] as well as SMAD2 and -3 [39] contribute to the formation of osteolytic bone metastases by breast MDA-MB-231 cells. These studies indicate that, even though human breast carcinoma cells are typically refractory to TGF- β -mediated growth suppression, the remaining intrinsic TGF- β signaling contributes to the formation of macrometastases in bones and lungs [37, 40, 41]. It is now recognized that the cooperation between SMAD and non-SMAD signaling pathways determines the final outcome of cellular response to TGF- β [32]. Recently, it was reported that expression level of SMAD2, 3, and 4 was regulated by Bridge-1 through Activin signaling pathway in breast cancer cell line [42]. We need to consider other related factors that may influence TGF- β signaling involved in the development of breast cancer. Thus, it is important to continue to advance our understanding of the TGF- β signaling network of and how subtle perturbation of this network can result in pathology. This will help us to consider practical and clinical approaches for accurate diagnosis as well as molecularly targeted pharmacological interventions utilizing this signaling network for the treatment of a variety of diseases.

In conclusion, our data show that SMAD4 expression in breast cancer is significantly lower than that in normal adjacent breast epithelial tissue and that SMAD4 expression levels were inversely related to histologic grade. Our data provide evidence that the reduced expression of SMAD4 may play an important role during the development of ductal breast carcinoma and may be a potential molecular marker for assessing the degree of differentiation of ductal breast carcinoma. SMAD4 may be an independent predictor of differentiation and prognosis in ductal breast carcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TGF β	Transforming growth factor beta
MAPK	Mitogen-activated protein kinase
PI3K	Phosphatidylinositol 3-kinase
ERK	Extracellular signal-regulated kinase
MMP	Matrix metalloproteinase
EMT	Epithelial–mesenchymal transformation
IHC	Immunohistochemistry
ERα	Estrogen receptor α
AKT	Protein kinase B
JNKs	c-Jun amino-terminal kinases

Fig. 1.

Detection of SMAD4 in tumor tissues and surrounding normal breast epithelia by immunohistochemistry. SMAD4 staining intensity varied both in the surrounding normal breast epithelia (**a** ×400) and breast carcinoma (**b–d** ×400), **a** brown, normal breast lobules; **b** yellow, well-differentiated ductal breast carcinoma; **c** pallid-flavens, moderately differentiated ductal breast carcinoma; **d** poorly differentiated ductal breast carcinoma, colorless. Within the same specimen, less tumor cells were stained than their surrounding normal epithelia (**e**, **f** ×200). **e** Lobular breast epithelium (*blue arrow*), breast carcinoma tissue (*black arrow*); **f** ductal breast epithelium (*blue arrow*), breast carcinoma tissue (*black arrow*). **g** Expression of SMAD4 as indicated by IRS in breast carcinoma ($n = 53$) compared to surrounding normal breast epithelial (50). ($p < 0.05$)

Fig. 2. Microarray SMAD4 immunohistochemistry staining of breast carcinoma and surrounding normal epithelia. Representative examples of array case with normal surrounding epithelia and the SMAD4 expression intensity surrounding normal epithelia (a)>well differentiated (b)>moderately differentiated (c)>poorly differentiated (c)

Fig. 3.

Detection of SMAD4 expression in breast carcinoma and their surrounding normal epithelia at protein and mRNA level. **a** SMAD4 expression in breast carcinoma tissues compared to their surrounding epithelia by Western blot: SMAD4 expression level among the normal breast epithelial tissues varied, but was much higher when compared to breast carcinoma tissues. **b** Quantitative analysis of SMAD4 gene expression in breast carcinoma and their surrounding tissue: mRNA expression of SMAD4 normalized to GAPDH by real-time PCR, there was a significant decreased in the expression SMAD4 mRNA from the control breast epithelial tissue group ($n = 50$) to breast carcinoma tissue group ($n = 53$), $p < 0.01$ (Student's t test). **c** Semi-quantitative RT-PCR analysis SMAD4 gene expression in breast carcinoma and their surrounding tissues: Low expression level of SMAD4 mRNA was found among the breast carcinoma tissue as compared to their surrounding epithelial tissues. The β -actin expression between two groups was the same

Fig. 4.

SMAD4 expression and restoration in breast carcinoma MDAMB- 468 cell line. **a** SMAD4 expression level among MCF10A, MDAMB- 468, T47D, and MCF7 cell lines varied by Western blot. Nonmalignant immortalized MCF10A expressed a high level of SMAD4. No SMAD4 was detected inMDA-MB-468 cells. **b** Restored SMAD4 in MDA-MB-468 cell by retrovirus transduction.MDA-MB-468 cells were transduced with pBabe-SMAD4-rescue or pBabe vector control retrovirus. The cells were selected with hygromycin for 2 weeks, and the resistant clones were pooled.Western blot shows SMAD4 was expressed in MDA-MB-468 SMAD4 cells but not in the vector control cells. **c** TGF- β /SMAD signaling in the MDA-MB-468 SMAD4 and the vector control cells, level of phosphorylated SMAD2 (pSMAD2) were assessed in MDA-MB-468 SMAD4 and vector control cells after exposure to TGF- β 1. For internal control, the membrane was stripped and re-probed with antibodies against SMAD2 or α -Tubulin

Fig. 5. Induction of non-SMAD signaling by TGF-β1 in MDA-MB- 468V and MDA-MB-468 SMAD4 cells. MDA-MB-468 vector and MDA-MB-468 SMAD4 cells were collected for Western blot analysis after the cells' exposure to TGF-β1 as indicated time points. Non-SMAD signaling, MAPK/ERK, PI3K/AKT, and MAPK/p38 were assessed. For internal control, the membrane was stripped and re-probed with antibodies against normal ERK, AKT, p38, or α-Tubulin, respectively

Table 1

Correlations between expression of SMAD4 and tumor histological grade in human breast ductal carcinoma

Histologic grade	No. of cases	SMAD4			r	p
		-	+	++		
A. Tumor samples						
G1	8	0	2	6	0	-0.309 0.024<0.05
G2	34	5	6	21	2	
G3	11	5	8	6	0	
Total no. of cases	53					
B. Uninvolved breast epithelia						
G1	8	0	0	8	0	-0.206 0.152>0.05
G2	33	0	5	23	5	
G3	9	0	3	6	0	
Total no. of cases	50					

R correlation coefficient

Table 2
Correlations between expression of SMAD4 and clinicopathologic parameters in human breast ductal carcinoma

Clinicopathologic parameters	No. of cases 53	SMAD4 (n)		r	p
		Negative	Positive		
Tumor size					
T1	18	5	13	0.210	0.131
T2	27	2	25		
T3	7	1	6		
T4	1	0	1		
Nodal statuses					
N0	30	5	25	0.006	0.967
N1	15	1	14		
N2	4	1	3		
N3	4	1	3		
Metastases					
M0	51	8	43	0.083	0.552
M1	2	0	2		
Age (years)					
<50	25	5	20	0.129	0.355
50	28	3	25		

NS not statistically significant at the 5 % level, R correlation coefficient