Abstract. Background: The transforming growth factor beta (TGFβ) signaling pathway has been shown to exert divergent effects and to cross-talk with estrogen pathways in mammary gland tumorigenesis. TGF signaling in early stage breast cancer was investigated by examining the expression of TGFβ-1 and the signaling mediators pSmad2/3 and Smad4. Their association with oestrogen and progesterone receptors, as well as with clinical and pathological features was also analyzed.

Patients and Methods: Sixty-one tumor specimens from surgically treated patients with primary T1-2,N0 breast cancer were examined. The expression of TGFβ-1, pSmad2 and Smad4 was assessed implementing immunohistochemical assays. Results: TGFβ-1, pSmad2 and Smad4 were expressed in 50.9%, 74.0% and 61.0% of specimens, respectively. The degree of expression of the three molecules was significantly associated with each other. Loss of pSmad2/3 expression indicated a shorter disease-free survival in all patients, including those with oestrogen receptor-positive tumors. Patients not expressing TGFβ-1 were 4.6 times more likely to experience distant recurrence. Conclusion: Our results demonstrate that pSmad2/3 and TGFβ-1 may be promising novel prognostic markers for T1-2,N0 breast carcinomas.

Several cellular functions such as proliferation, differentiation and apoptosis of different cell types are attributed to the transforming growth factor beta (TGFβ) pluripotent superfamily of secreted polypeptides (1). TGF is a 25-kDa dimeric polypeptide that appears to possess an important role in normal mammary gland epithelial cell replication (3). TGF regulates normal ductal and alveolar development and is also involved in postlactational involution (4).

Besides its physiological function, TGF is implicated in mammary gland tumorigenesis. Overexpression of TGFβ-1 in the mouse mammary gland inhibits malignant transformation, while blocking TGF receptor function enhances it. These findings are consistent with the tumour suppressor function of TGF (5, 6). In early stage breast cancer development, the transformed epithelial cells appear to be sensitive to TGF-mediated growth arrest and TGF can therefore act as an antitumor agent (7). In contrast, advanced breast cancers are in their great majority refractory to TGF-mediated growth inhibition and produce large concentrations of TGF, which may enhance tumour cell invasion and metastasis (8, 9).

Three TGF molecular isoforms encoded by the same gene and produced by altered splicing have been described (1); all three are expressed during mammary gland development, but not during lactation (10). The TGF signal is transduced to the nucleus by a pair of transmembrane serine-threonine kinase receptors, T'R-I and T'R-II (1). Following TGF activation and binding to T'R-II homodimers, signalling is initiated by the phosphorylation of T'R-I by T'R-II. In particular, T'R-II forms heterotetrameric complexes with two T'R-I molecules; the T'R-II kinase subsequently phosphorylates T'R-I, which results in the activation of serine threonine kinase. T'R-I kinase activation brings about phosphorylation of two cytosolic proteins, Smad2 and Smad3. Upon their activation, Smad2 and Smad3 form heteromeric complexes with a third homologue (Smad4) and translocate to the nucleus where they regulate gene transcription by means of binding to DNA in a sequence-specific manner (1, 11).

It has been demonstrated that Smad2, Smad3 and Smad4 exhibit physical interaction with oestrogen receptors (ER) and might be important for the TGF regulation of ER-α signalling (12). Furthermore, inhibition of breast cancer cell growth by tamoxifen is possibly mediated through TGF (13-15).
Moreover, Smad4 has been identified as an ER-α corepressor; antiestrogens enhance this endogenous interaction (16). Smad4 mutation or deletion in cancer cells renders TGF incapable of inhibiting ER-induced gene transcription (16). These data suggest that the interaction between ER and TGF signalling is critical in breast cancer development.

Tumours of the gastrointestinal tract, lung, ovaries and lymphomas carry delections or inactivating mutations of the TGF signalling components (17, 18). There is however little evidence for inactivation of the TpR and Smad genes in breast carcinomas (18, 19). In addition to the above, most breast carcinoma cell lines are refractory to TGF in vitro (7).

Aiming to investigate this phenominal discrepancy, we assessed the status of TGF signaling in primary T1,2 and node negative breast carcinomas by evaluating the expression of both TGFβ-1 (the most widely studied protein among the three TGF isoforms) and its signalling mediators pSmad2/3 and Smad4. The expression of TGFβ-1 and its mediators was evaluated by applying immunohistochemical methods. The expression profile of these molecules was tested for correlation with that of oestrogen and progesterone (PR) receptors, with the intention of exploring a possible cross-talk between these molecules in mammary gland tumourigenesis. The clinical prognostic significance of each molecule was also examined.

Patients and Methods

Specimens and clinicopathological data. Paraffin-embedded sections of 61 tumour specimens from an equivalent number of patients with T1-2N0 primary breast cancer were used (tumour size smaller than 5 cm with negative axillary lymph nodes). Patients were operated on at the Surgery Department of the University of Patras Hospital, Greece. All patients were surgically treated by mastectomy (partial or total) and axillary lymph node resection, while surgical margins were free of neoplastic disease in all cases. A mean of 13 lymph nodes were pathologically examined per patient (range 6 to 24). Regarding the histological type of carcinoma, 57 patients were found to suffer from classic ductal NOS (not otherwise specified), 3 from mucinous and 1 from medullary tumors. Fifteen patients (24.3%) were staged as T1 and 46 (75.7%) as T2. Moreover, 12 tumor specimens were of grade I, 36 of grade II and 11 of grade III. Ten breast tissue specimens from women with non-neoplastic disease were also used as controls, for investigating protein expression in the normal mammary gland.

The patients mean age was 59 years (range between 26 and 80 years) and the median follow up time was 8 years. During follow up, nine patients had recurrence of their disease and finally died from metastatic cancer.

Immunohistochemistry. De-waxed and hydrated 5 μm sections were quenched with H2O2 (0.6%) in 100% methanol for 20 min to inhibit endogenous peroxidase activity. Antigenic retrieval was required and for this purpose the slides were baked within a microwave oven (3 times, 5 minutes each, at 700 Watt). Non-specific binding was blocked by incubating the sections in Tris buffered solution (TBS) containing 3% bovine serum albumin (BSA). The slides were then incubated with each of the following antibodies for 25 minutes at room temperature: (a) anti-TGFβ-1 goat polyclonal antibody (V), (Santa Cruz Biotechnology Inc., UK) at a dilution of 1:100; (b) anti-Smad4 mouse monoclonal antibody (B-8) (Santa Cruz Biotechnology Inc., UK) at a dilution of 1:500 and (c) anti-pSmad2/3 rabbit polyclonal antibody (#3101) (Cell Signaling Technology, Inc., USA) at a dilution of 1:200.

pSmad2/3 antibody selectively recognizes Smad2/3 proteins in their activated (phosphorylated) form. Smad2/3 phosphorylation is exclusively dependent on TpR activation in response to TGF binding. Labeling was detected using the streptavidine-biotin complex method, while 3,3’-diaminobenzidine (DAB) was used as a chromogen. Sections were finally rinsed, counterstained with hematoxylin and mounted. Negative controls included substitution of the primary antibody with non-immune mouse IgG diluted at the same concentration of each antibody, or the omission of primary antibodies. As positive controls, the peritumoral non-neoplastic breast tissue was used.

The ER and PR status of the same specimens was also assessed by the use of anti-ER-α and anti-PR monoclonal antibodies (NCL-ER/PGR-Paraffin, Novocastra Laboratories, Ltd, UK), aiming to investigate a potential association between the hormone receptor expression and the TGFβ-1, pSmad2/3 and Smad4 expression profile.

Two skilled pathologists, blinded to the clinical profile of patients, independently assessed all immunostained sections. The sections were whole-mounted and viewed through a Zeiss light microscope.

Immunohistochemical staining was graded on a scale of 0 to 1 according to the following arbitrary assessment: 0 for <10% positive cells; 1 for >11% positive cells. Either a cytoplasmic or a nuclear staining pattern of tumour cells was considered positive; slides exhibiting no staining or diffuse (background) pattern were excluded from the study. For the expression of ER and PR an optimized cut off of 5% positivity of the tumour cells was determined.

Statistical analysis. The associations between TGFβ-1, pSmad2/3, Smad4, ER and PR expression, as well as the associations between these molecules and other clinicopathological markers were analysed using the Chi-square test. Distant disease-free survival (DDFS) was calculated from the date of the diagnosis to the occurrence of metastases outside the locoregional area or death from breast cancer, whichever came first. Life tables were calculated according to the Kaplan-Meier method. Survival of the groups was compared with the log-rank test. Multivariate survival analyses were performed with the Cox proportional hazards model, entering the following covariates: (a) TGFβ-1 expression (negative versus positive); (b) pSmad2/3 (negative versus positive); (c) Smad4 (negative versus positive); (d) age (continuous); (e) tumour size in centimetres (<2 cm, 2-5 cm, >5 cm); (f) histological grade (I, II, and III); (g) ER (negative versus positive); (h) PR (negative versus positive). Cox regression was performed using a backward stepwise selection of variables and a p-value of 0.05 was adopted as the limit for inclusion of a covariate. A binary logistic regression model was used in order to assess the relative importance of TGFβ-1, pSmad2/3 and Smad4 in predicting the recurrence of disease. The expressions of the above molecules were entered in the model as categorical predictors (0: no staining, 1: positive staining). All statistical tests were two-sided. All analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).
Results

TGFβ-1, pSmad2/3 and Smad4 expression pattern in normal breast tissue and breast cancer. To evaluate the pattern of TGFβ-1, pSmad2/3 and Smad4 expression in normal mammary gland tissue, we examined sections of normal breast as well as the breast parenchyma adjacent to the tumor present in some tumor specimens. As expected, all normal tissue elements, including large ducts, lobules, terminal duct-lobular units (TDLU), stromal cells and capillaries expressed TGFβ-1, Smad4 and pSmad2/3 (the phosphorylated and thus activated form of Smad2/3 proteins). Scoring of the TGFβ-1, Smad4 and pSmad2/3 immunohistochemical staining of the 61 primary breast carcinomas revealed that 49.1%, 26.0% and 39.0% of the breast tumours were negative for TGFβ-1, pSmad2/3 and Smad4, respectively, whereas 50.9%, 74.0% and 61.0% of the tumours were positive for TGFβ-1, pSmad2/3 and Smad4 expression. TGFβ-1 exhibited a cytoplasmic staining pattern (Figure 1), while Smad4 protein localization was unevenly distributed and was mainly cytoplasmic, even though nuclear staining was also observed in some cases (Figure 2). pSmad2/3 immunostaining was nuclear (Figure 3).

Association between TGFβ-1, pSmad2/3 and Smad4 expression. The expression levels of the three molecules were significantly interrelated. In detail, the odds ratio for TGFβ-1 – pSmad2/3 association was 25 [confidence interval (CI): 2.9-215], for TGFβ-1 – Smad4 association 49.5 (CI: 9.1-270) and for pSmad2/3 – Smad4 association 19.9 (CI: 3.6-108).

No significant association was detected between TGFβ-1, pSmad2/3 and Smad4 expression and tumor grade (p=0.927, 0.436 and 0.923 respectively). Similarly, no association was found between TGFβ-1, pSmad2/3 and Smad4 expression and tumour size (p=0.84, 0.12 and 0.924, respectively).

Association of TGFβ-1, pSmad2/3 and Smad4 with ER and PR. An inverse association between TGFβ-1 immunostaining and PR expression (p<0.032, odds ratio: 0.306, CI: 0.1-0.91) was noted (Table I). An inverse relationship was also recorded between Smad4 immunostaining and ER, as well as PR expression (p=0.039, odds ratio: 0.294, CI: 0.09-0.97 and p=0.012, odds ratio: 0.239, CI: 0.075-0.76, respectively) (Table I). However, no significant association was found between either TGFβ-1 or pSmad2/3 and ER (p=0.25 and p=0.11, respectively), while an inverse association of
pSmad2/3 with PR approached borderline significance (p=0.056 odds ratio: 0.254, CI: 0.06-1.09) (Table I).

Loss of pSmad2/3 expression is associated with shorter disease-free survival while loss of TGFβ-1 expression indicates an increased recurrence risk. To estimate the prognostic value of TGFβ-1, pSmad2/3 and Smad4 for the patients enrolled in the study, we analysed the duration of disease free survival (DDFS) of patients. Univariate analyses in all tumours showed that the loss of TGFβ-1 expression indicated a marginally statistically significant shorter disease-free survival (p=0.054) (Figure 4A). However, Cox multivariate analysis showed that pSmad2/3 was an independent prognostic factor for shorter disease-free survival (p=0.014, relative risk (RR) 6.55, 95% CI: 1.45-29.54).

Subgroup analysis showed that loss of pSmad2/3 expression indicated a statistically significant worse outcome in ER positive tumours (p=0.05, Figure 4B), while in such tumours there was a tendency for a shorter disease-free survival in patients with tumours lacking Smad4 expression (p=0.073, Figure 4C). For PR-negative tumours the expression of TGFβ-1 indicated a significantly better prognosis (p=0.014, Figure 4D). For tumours sized more than 2 cm, loss of TGFβ-1 expression indicated a shorter disease-free survival (p=0.047, Figure 4E), while in these tumours there was a tendency for worse outcome with loss of pSmad2/3 expression (p=0.077, Figure 4F).

To further assess the relative importance of TGFβ-1, pSmad2/3 and Smad4 in predicting the recurrence of disease (i.e. the occurrence of metastases outside the locoregional area or death from breast cancer), the expression of the studied proteins was entered as categorical predictors in a binary logistic regression analysis. Patients not expressing TGFβ-1 were 4.6 times more likely to have recurrence of their disease (p=0.04, odds ratio: 4.6, 95% CI: 1.169-19.8).

Discussion

The involvement of the TGF – Smad pathway in tumourigenesis has been discussed in several articles (15-17, 20, 21). Despite the common perception that cancer arises as the result of the accumulation of damage in critical regulatory
genes, it appears that TGF possess a tumour-suppression role by controlling cell growth and regulating cell differentiation.

To assess the status of the TGF signalling pathway in mediation of the TGF-resistant phenotype in breast cancer, we used immunocytochemistry to examine TGFβ-1 and Smad protein levels in 61 early stage human breast cancers and analyzed the association of TGFβ-1, pSmad2/3 and Smad4 expression with other clinical and pathological features. The

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**Table I. Associations between pSmad2/3, Smad4 and TGF expression with ER and PR expression, tumour grade and size in the evaluated specimens.**

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<th>ER</th>
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<tr>
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All presented numbers (excluding p-values) represent total numbers of specimens.
Figure 4. Distant disease-free survival analysis. Continuous lines represent patients expressing the corresponding molecules while intermittent lines indicate patients not expressing them. ▲ represents censored data. P-values determined using the log-rank test. A) DDFS analysis according to TGFβ-1 expression in all tumours (p=0.054); B) DDFS analysis according to pSmad2/3 expression in ER-positive tumours (p=0.05); C) DDFS analysis according to Smad4 expression in ER-positive tumours (p=0.073); D) DDFS analysis according to TGFβ-1 expression in PR-negative tumours (p=0.014); E) DDFS analysis according to TGFβ-1 expression in tumours with size more than 2 cm (p=0.047); F) DDFS analysis according to pSmad2/3 expression in tumours with size more than 2 cm (p=0.077).
prognostic significance of the above proteins was also investigated. The significant interrelation between TGFβ-1, Smad4 and pSmad2/3 expression in the evaluated breast cancer specimens implies that TGFβ-1 mainly signals through Smads in early stage mammary gland tumours. However, it has been recently suggested that other major cellular pathways may act independently of, or synergistically with Smad transducers in response to TGF (22-24). Further studies are needed to clarify the exact role of these distinct pathways in inhibiting or promoting cell growth and migration.

This study demonstrated an inverse association between Smad4 expression and ER immunostaining. It has been documented that oestrogen receptor-expressing breast cancer cells are refractory to TGF-mediated growth control because of their reduced expression of TGF receptors. A broad variety of human cancer cell lines, including estrogen receptor-expressing (ER+) breast cancer cell lines exhibit TGF resistance without detectable changes in the TβR-I and TβR-II genes (25, 26). These cells express low or reduced levels of RI and RII, suggesting that abnormalities in transcriptional regulation, alterations in mRNA processing, or mRNA instability might contribute to reduced receptor expression and hence reduced activation of the TGF signalling mediators such as Smad2/3 and Smad4. However, previous studies suggested that Smad4 is a corepressor for ER transcriptional activity (14). Smad4 is incorporated in the ER-α-DNA complex where it may recruit corepressors, resulting in inhibition of the transcription of several downstream genes of oestrogen signalling. Smad4 mutation or deletion in cancer cells results in suspension of inhibition of ER-mediated transcription, thus inducing tumor progression (14).

The hypothesis that TGF and oestrogen pathways cross-talk is also suggested by several studies which illustrate that tamoxifen induces TGF mRNA production and TGF secretion in breast cancer cells (27-30). TGFβ-1 activation functionally restrains ER-α-positive cells from proliferating into adult mammary gland (31). A recent study confirmed the participation of Smad2/3 and Smad4 in antioestrogen activity (32). Tamoxifen treatment might, therefore, inhibit proliferation of ER-positive tumours through TGF. Moreover, high levels of Smad4 or pSmad2/3 would maintain a positive feedback loop of TGF signalling and lead to inhibition of cell cycling. Our finding that loss of pSmad2/3 expression indicated a shorter overall survival in ER-positive tumours supports the aforementioned considerations.

An inverse association between TGF and PR expression as well as between Smad4 and PR was also documented, while a similar relationship between pSmad2/3 and PR approached borderline. Previous studies indicate a key role of PR in ER signalling (33); while it was proposed that PR is an indicator of ER pathway integrity (34). Furthermore, it was recently reported that oestrogen and progesterone together with TGF signalling are necessary for the maintenance of p53 activity and, thus, growth inhibition in mammary epithelium (35). In this context, a cross-talk between TGF signalling components and PR in parallel with ER may exist and further investigations are required to test this hypothesis.

Concerning survival analyses, loss of pSmad2/3 was associated with shorter disease-free survival in all patients, including those with ER positive T1-2,N0 cancer tumours. This finding corroborates the suggested TGF antitumor properties in T1-2,N0 breast carcinomas (7). Another study by Xie et al. also demonstrated that loss of pSmad2/3 expression in breast cancers indicated a shorter overall survival (36). Moreover, in our study TGFβ-1 expression seemed to correlate with shorter overall survival in patients with larger-sized tumors. Finally, loss of TGFβ-1 immunopositivity was a predictor of disease recurrence. As it has earlier been noted (8), in advanced breast tumours TGF increases motility and invasion in transformed cells. However, according to our experimental procedure and other in vivo studies (9), it becomes apparent that in vivo and in early stages of breast tumorigenesis TGF-dependent tumor growth inhibition may be dominant over stimulation of cell mobility and invasion. On the basis of the results of the current study, it could even be proposed that pSmad2/3 and TGFβ-1 may become novel prognostic markers for T1,2,N0 breast cancer.

**References**


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