The prognostic role of TGF-β signaling pathway in breast cancer patients


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Background: The transforming growth factor-β (TGF-β) pathway has dual effects on tumor growth. Seemingly, discordant results have been published on the relation between TGF-β signaling markers and prognosis in breast cancer. Improved prognostic information for breast cancer patients might be obtained by assessing interactions among TGF-β signaling biomarkers.

Patients and methods: The expression of nuclear Smad4, nuclear phosphorylated-Smad2 (p-Smad2), and the membranous expression of TGF-β receptors I and II (TβRI and TβRII) was determined on a tissue microarray of 574 breast carcinomas. Tumors were stratified according to the Smad4 expression in combination with p-Smad2 expression or Smad4 in combination with the expression of both TGF-β receptors.

Results: Tumors with high expression of TβRII, TβRI and TβRII, and p-Smad2 (P = 0.018, 0.005, and 0.022, respectively), and low expression of Smad4 (P = 0.005) had an unfavorable prognosis concerning progression-free survival. Low Smad4 expression combined with high p-Smad2 expression or low expression of Smad4 combined with high expression of both TGF-β receptors displayed an increased hazard ratio of 3.04 [95% confidence interval (CI) 1.390–6.658] and 2.20 (95% CI 1.464–3.307), respectively, for disease relapse.

Conclusions: Combining TGF-β biomarkers provides prognostic information for patients with stage I–III breast cancer. This can identify patients at increased risk for disease recurrence that might therefore be candidates for additional treatment.

Key words: breast cancer, prognosis, p-Smad2, Smad4, TGF-β receptor I, TGF-β receptor II

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**Introduction**

The transforming growth factor-β (TGF-β) superfamily is a family of 33 structurally similar cytokines (bone morphogenic proteins (BMPs), activins, and TGF-β ligands) that play an important role in developmental biology, including mammary gland development [1]. The TGF-β ligands have three described isoforms; TGF-β1, -2, and -3. TGF-β influences tissue homeostasis by affecting proliferation, migration, and apoptosis of a wide variety of cells [2]. TGF-β plays a dual role in cancer development as it displays both tumorogenic and tumor-suppressive effects. TGF-β has been reported to act as a tumor suppressor by inhibiting the cell proliferation of breast cancer cell lines [3]. In the early stages of breast cancer development, hyperplastic breast ducts that lack TβRII expression have been shown to display an increased risk of developing into invasive breast cancer [4]. In contrast, in later stages of cancer, TGF-β has direct pro-tumorigenic effects through the stimulation of invasion, the migration of tumor cells [5], and the activation of the tumor stroma [6]. It has been hypothesized that although TGF-β initially suppresses growth, this is lost as tumors develop by genetic and epigenetic mechanisms inactivating selective downstream TGF-β mediators [2, 7].

TGF-β elicits its biological effects by binding to a heteromeric complex of transmembrane TGF-β serine/threonine kinase type 1 and II receptors (TβRI and TβRII). Canonical intracellular TGF-β signal transduction occurs through the Smad pathway. This involves the type I receptor-induced phosphorylation of receptor-regulated Smads 2 and -3 (R-Smad2–3), which associate with common mediator Smad4 to form heteromeric complexes. These complexes subsequently translocate to the nucleus where they regulate transcriptional responses.

The prognostic significance of TGF-β ligands and downstream signaling mediators has been investigated in several studies. High TGF-β1 serum levels have been associated with advanced stages of breast cancer [8], while high tissue levels of TGF-β1 were associated with an unfavorable prognosis [9]. Paiva et al. found that the complete absence of TβRII tissue expression in breast cancers was substantially associated with the development of distant metastases and overall survival (OS) [10]. In contrast, Walker et al. found that positive TGF-β1 expression in breast tumors had an increased chance of lymph node metastases [11]. In another large patient series, TGF-β expression was correlated with favorable prognostic features, including tumor size <2 cm, estrogen receptor (ER) positivity, and good to moderate differentiation, while the presence of phosphorylated-Smad2 (p-Smad2, indicative of active canonical TGF-β signaling) was associated with positive nodal status [12].

These results are seemingly discordant and possibly represent the dual role of TGF-β in cancer. Therefore, to establish the relationship of TGF-β signaling with prognosis combining several TGF-β-related biomarkers might be superior to the analysis of a single component of the pathway. This might allow for the identification of tumors that have successfully shut down part of the tumor-suppressive arm of TGF-β, while leaving the tumor-promoting arm intact.

We investigated whether the Smad4 status of tumors in combination with the presence of TGF-β receptors I and II or active TGF-β signaling (p-Smad2) is associated with patient prognosis in a cohort of stage I–III breast cancer patients.

**Materials and Methods**

**Study Population**

In a retrospective cohort study, patients were included with non-metastatic invasive breast cancer who were primary treated with surgery in the Leiden University Medical Center between 1985 and 1994 (n = 677). Patients were excluded from this series if they had a prior history of malignancy other than basal cell carcinoma or in situ carcinomas, or if they presented with synchronous bilateral breast cancer. A tissue microarray (TMA) of available formalin-fixed paraffin-embedded (FFPE) tumors of the patients cohort (n = 574) was constructed. The construction and characteristics of the TMA from this patient cohort have been described elsewhere in more detail [13]. The following data were available: patient age, tumor grade, histological type, TNM stage, local and systemic therapy, locoregional/distant recurrence, second primaries, and OS. The expression of ER, progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) were determined according to the standard diagnostic procedure, using standard histological staining protocols. All samples were handled in a coded fashion, according to the national ethical guidelines (‘Code for Proper Secondary Use of Human Tissue,’ Dutch Federation of Medical Scientific Societies).

**Immunohistochemistry**

Antibodies against Smad4 (sc-7966; Santa Cruz), TβRI (ab49575; Abcam), TβRII (sc-400; Santa Cruz), and p-Smad2 (Ser465/467; cell signaling technology) were used for immunohistochemical stainings. TMA sections of 4 μm were cut, deparaffinized, and rehydrated. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide methanol for 20 min. Heat-induced antigen retrieval at 10-min maximum microwave power was allowing performing using EDTA for Smad4 and TβRII staining and citrate for TβRI and p-Smad2 staining. Sections were incubated overnight with primary antibodies using predetermined optimal dilutions. Slides were incubated with secondary mouse or rabbit Envision (Dako) for 30 min. Staining was visualized using a diaminobenzidine solution, and sections were counterstained with hematoxylin, dehydrated, and finally mounted in malinol. For each antibody, all slides were stained simultaneously to avoid inter-assay variation.

**Evaluation of Immunostaining**

TMAs were scored for positivity for TβRI, TβRII, Smad4, and p-Smad2 by two observers. For TβRI and TβRII, the percentage of positive cells with membranous staining was estimated. For Smad4 and p-Smad2, the percentage of positive nuclear stained cells was determined. The mean score from these three cores was considered the final score. Each tumor was classified as to either low expression or high expression, using the median score of all tumors as cut-off point for all markers. Combination variables were created by combining Smad4 expression (low versus high expression) with p-Smad2 (low versus high expression) and TGF-β receptor expression (low expression of TβRI or TβRII versus high expression of both receptors).

**Statistical Analysis**

Statistical analyses were carried out using the statistical package SPSS (version 16.0 for Windows, SPSS Inc., Chicago, IL). Cohen’s κ coefficient...
was used to evaluate an inter-observer agreement in quantification. This revealed a substantial agreement in classification for Smad4 (k = 0.723) and an almost perfect agreement for p-Smad2 (k = 0.824), TβRI (k = 0.816), and TβRII (k = 0.904). The χ² test was used to evaluate associations between various clinicopathological parameters and p-Smad2, Smad4, TβRI, and TβRII expression. Relapse-free period (RFP) was defined as the time period from the date of surgery until locoregional recurrence and/or a distance recurrence, whichever came first. OS was defined as date of surgery until death. The Kaplan–Meier method was used for survival plotting and log-rank test for the comparison of survival curves. RFP is reported as cumulative incidence function, after accounting for death as competing risk. Cox regression was used for univariate and multivariate analyses for RFP and OS. Significant variables (P < 0.1) in univariate analysis were included in multivariate analysis.

results

patient and tumor characteristics
An FFPE material was available for 574 of the 677 patients (85%). The remaining 103 patients (15%) were excluded due to either the unavailability of an FFPE material from our archives or the quality of the material. No substantial differences in clinicopathological parameters were found among the tumors that were included in the TMA, and tumors that were left out. The clinicopathological characteristics of these patients are shown in supplementary Table S1, available at Annals of Oncology online.

discussion

expression of smad4, TβRI, TβRII, and p-smad2
Representative images from TMA cores stained for all biomarkers are presented in Figure 1. Immunoreactivity for TβRI and TβRII was evaluated in 555 and 474 patients, respectively. For TβRII, cut-off was 56.7%. A number of 282 (50.8%) tumors displayed low (Figure 1A) and 273 (49.2%) displayed high expression (Figure 1B). For TβRII, the cut-off used was 63.3% which resulted in 236 (49.7%) tumors with low expression (Figure 1C) and 239 (50.3%) tumors with high expression (Figure 1D). A total of 505 tumors had assessable Smad4 staining with a cut-off of 43.3%; expression was low in 240 tumors (47.5%) (Figure 1E) and high in 265 tumors (52.5%) (Figure 1F). For p-Smad2, the median score and cut-off value used was 0. The low nuclear expression of p-Smad2 was observed in 351 tumors (73.1%) (Figure 1G), whereas 129 tumors (26.9%) had high nuclear expression (Figure 1H). A positive association was found between TβRI and TβRII expression (P < 0.001). High TβRI and high Smad4 expression were also substantially positively associated (P = 0.037). A trend towards significance was found between high p-Smad2 and high Smad4 expression (P = 0.073).

association with prognostic parameters
To further examine the prognostic effect of the TGF-β-related biomarkers, the relationship between these markers and traditional prognostic markers (age, tumor grade, histological type, T-status, N-status, ER/PR/HER2 expression) was examined (supplementary Table S2, available at Annals of Oncology online). Statistically significant relations were found between increasing tumor grade and high TβRI and TβRII expression (P = 0.015 and 0.043, respectively). Smad4 low-expressing tumors were more often of the ductal subtype (P = 0.009). High expression of TβRII was associated with more advanced T-stage (P = 0.025). High expression of TβRII, Smad4, and p-Smad2 was associated with ER positivity (P = 0.046, <0.001, and <0.001, respectively). High p-Smad2 was associated with PgR positivity (P = 0.026). In addition, high Smad4 and high p-Smad2 expression were associated with HER2 negativity (P = 0.002 and 0.003, respectively).

survival analysis
In accordance with our hypothesis, low expression of Smad4 was associated with an unfavorable prognosis concerning RFP (P = 0.005, supplementary Figure S1A, available at Annals of Oncology online). An elevated expression of TβRII, combination of both TGF-βRI and -RII, and p-Smad2 were substantially associated with an unfavorable RFP (P = 0.018, 0.005, and 0.022, respectively; supplementary Figure S1C–E, available at Annals of Oncology online, respectively). An elevated expression of TβRI showed a trend towards decreased RFP (P = 0.061, supplementary Figure S1B, available at Annals of Oncology online). Since Smad4 was able to stratify tumors by favorable and unfavorable prognosis, we examined the expression of TβRI, TβRII, and p-Smad2 in Smad4 low- and Smad4 high-expressing tumors. For all tumors with high Smad4 expression, no progression-free survival differences were detected for low and high expression of TβRI, TβRII, both TGF-β receptors, and p-Smad2 (P = 0.450, 0.743, 0.345, and 0.657, respectively; supplementary Figure S1F–I, available at Annals of Oncology online, respectively). In the subgroup of patients with low Smad4 expression, statistically significant relations were found between progression-free survival and expression of TβRI (P = 0.009), TβRII (P = 0.036), a combination of both TGF-β receptors (P = 0.001) and p-Smad2 (P = 0.004, supplementary Figure S1J–M, available at Annals of Oncology online, respectively). To analyze the interplay among different components of the TGF-β signaling pathway, combination variables were created by combining the expression of Smad4 with Smad2 and TGF-β receptors I and II. The combination variable of Smad4 and p-Smad2 in particular was able to distinguish between patients with disease recurrence and those without with high power (P < 0.001, Figure 2). We also examined the prognostic power of a combination variable consisting of Smad4 and high expression of both TGF-β receptors concerning RFP (P < 0.001, Figure 3).

All variables were also investigated for their ability to stratify patients to good and poor prognosis regarding OS. In the overall population, high expression of Smad4 showed a trend towards better prognosis (P = 0.057, supplementary Figure S2A, available at Annals of Oncology online). High expression of p-Smad2 was substantially associated with a worse prognosis (P = 0.042, supplementary Figure S2E, available at Annals of Oncology online). Stratification for TβRII showed a trend for worse prognosis when this receptor was highly expressed (P = 0.099, supplementary Figure S2C, available at Annals of Oncology online). In the population of Smad4 high-expressing tumors, no statistically significant relations were found between OS and TβRI, TβRII, TβRII and
II, and p-Smad2 (P = 0.431, 0.364, 0.410, and 0.904, respectively; supplementary Figure S2F–I, available at Annals of Oncology online, respectively). When solely considering Smad4 low-expressing tumors, p-Smad2 was associated with worse prognosis concerning OS (P = 0.005, supplementary Figure S2M, available at Annals of Oncology online). A trend towards significance was found between OS and TβRI and TβRII and II (P = 0.061 and 0.054, supplementary Figure S2K and L, available at Annals of Oncology online, respectively). The combination variables consisting of Smad4/p-Smad2, and Smad4/TGF-β receptors were also both able to distinguish between patients with poor and good prognosis concerning OS (P = 0.001 and 0.028, respectively; supplementary Figures S3 and S4, available at Annals of Oncology online, respectively).

**Figure 1.** Representative images of TβRI, TβRII, Smad4, and p-Smad2 stainings on tissue microarray.

**univariate and multivariate analyses**

To further assess the relationship of the Smad4/p-Smad2 and Smad4/TβRI + TβRII combination biomarkers with RFP and OS, separate univariate and multivariate COX regression analyses were carried out. For RFP, substantially associated variables included tumor grade (P = 0.001), tumor stage (P < 0.001) and nodal stage (P < 0.001), and both our combination TGF-β variables (P < 0.001 for Smad4/TβRI + RII and P = 0.003 for Smad4/p-Smad2). The three variables that remained independently substantially associated with RFP were nodal stage (P < 0.001), Smad4/TβRI + TβRII (P = 0.001, hazard ratio (HR) 2.20, 95% confidence interval (CI) 1.464–3.307, supplementary Table S3, available at Annals of Oncology...
interaction among TGF-β pathway biomarkers. Our data indeed show that combining TGF-β variables can be used as powerful predictors of breast cancer patient outcome. Several other studies have previously addressed the prognostic implications of this signaling pathway. Conflicting results have been published in the literature. For instance, the data in one study revealed the absence of TβRII as an adverse prognostic factor [10], while our study has shown that high TβRII expression was associated with adverse outcome. These differences might be explained by several factors. First, there can be differences in the characteristics of the patient population (regarding breast cancer subtypes, tumor stage, tumor size etc.). Secondly, methodological choices regarding cut-off values (negative/positive versus low/high expression) affect the study results. Which downstream mediators are activated might differ, dependent on the level of receptor expression. Finally, as we have shown in our study, the combination of different downstream mediators is also relevant for patient outcome (while Smad4 and p-Smad2 are both downstream of TβRI and TβRII, our study has shown that Smad4 and p-Smad2 are associated with a relatively favorable and unfavorable prognosis, respectively). These observations indicate that the presence of certain downstream signaling molecules is important for the functionality and the prognostic implications of this signaling pathway.

The role of Smad4 as a tumor suppressor is consistent with the observation that high expression of this protein is associated with a favorable prognosis. Smad4 has been previously identified as a possible tumor suppressor since Smad4 mutations have been reported with high frequency in solid tumors including breast cancers [14, 15]. Smad4 expression was also found to be lower in breast tumor cells compared with normal epithelium [16]. While Smad4 is central to the TGF-β and BMP pathway, experimental data have shown that TGF-β regulates the expression levels of many proteins even when Smad4 is knocked down [17]. Other in vitro studies have shown that the expression of Smad4 is essential for the epithelial-to-mesenchymal transition (EMT) and is strongly involved in the TGF-β-induced anti-proliferative effects [18]. Previous pre-clinical studies regarding Smad3 have indicated that the intracellular levels are determinants for response to TGF-β [19]. This could be similar for Smad4; high levels of Smad4 might be a prerequisite for an effective inhibition of proliferation, which is why tumors with high Smad4 levels have a relatively favorable prognosis (Figure 4A). In contrast, low levels of Smad4 might be insufficient for the anti-proliferative effect of TGF-β, but allow for the EMT and thus increasing cell mobility (Figure 4B). This would result in a relatively poor prognosis, which is concordant with the results of our study.

In the case of tumors with low Smad4 levels, signaling might also be more geared towards Smad4-independent signaling. Smad-independent signaling can occur through either non-canonical TGF-β signaling (like extracellular signal-regulated kinase, c-Jun N-terminal kinase etc. [20], or through Smad4-independent, R-Smad-dependent signaling). The observation that Smad4 low/p-Smad2 high-expressing tumors have an unfavorable prognosis compared with Smad4 high/p-Smad2 high-expressing tumors indicates that the former possibility is an important pathway for breast tumors. R-Smads are capable

discussion

The TGF-β pathway has dual effects on the growth and progression of breast tumors. Because of this dual nature, determining a single biomarker (e.g. TβRII) might not be sufficient to distinguish patients at high risk of developing metastatic disease or locoregional recurrence. We hypothesized that the prognostic power could be improved by analyzing the interaction among TGF-β pathway biomarkers. Our data
of binding DNA and regulate gene transcription even in the absence of Smad4. Another possibility is that another molecule functions as co-Smad instead of Smad4 and functions to improve DNA binding. Which protein might be responsible for this, is an interesting question for future research. The non-canonical TGF-β pathways are thought to contribute to pro-tumorigenic TGF-β effects, like EMT [19, 21] and can also contribute to the relatively poor prognosis seen in Smad4 low-expressing tumors.

Several studies have reported on the interplay between ER signaling and TGF-β. In vitro studies have shown that canonical TGF-β signaling is suppressed by the ER [22]. Additionally, ER positivity in breast tumors is associated with the upregulation of several negative TGF-β regulators in cell lines [22, 23]. However, instead of a negative relationship between ER status and expression of TGF-β biomarkers, we found a substantial association between high expression of TβRII, p-Smad2, and Smad4 expression and ER positivity in our patient series. This might suggest that previous in vitro reports only partly describe the interaction between ER and TGF-β signaling in breast cancer cells and that alternative pathways exist that reverse this ER-mediated TGF-β suppression in advanced breast cancers. For example, poly(ADP-ribose) polymerase (PARP) is involved in TβRII transcription levels in ER-positive breast cancer cells and is also known to interact with Smad3 and 4 to regulate Smad signaling [24]. This protein might be one of the factors contributing to the re-expression of TβRII [25]. In addition to the interplay between ER and TGF-β, there was a strong negative association between HER2 and Smad4 and p-Smad2 expression in our study. HER2 has been shown to cooperate with TGF-β in cell culture models to increase migration [26]. However, Smad4 and Smad2 are negatively regulated by HER2 signaling [27], possibly through inhibitory Smad7 [28], which is concordant with the results from our study.

In conclusion, we have demonstrated that the combination of TGF-β pathway biomarkers can provide valuable prognostic value for breast cancer patients. Stratifying tumors according to the low or high expression of TGF-β biomarkers had strong prognostic implications in our patient population. Our results highlight the importance of accounting for protein expression levels and the complex interactions taking place between components with the TGF-β pathway.
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**disclosure**

The authors have declared no conflicts of interest.

**references**