Optimizing breast cancer survival models based on conventional biomarkers and stromal parameters

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

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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 tumorigenic 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 I 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 patient 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 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 diaminobezidine 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 (κ=0.723) and an almost perfect agreement for p-Smad2 (κ=0.824), TβRI (κ=0.816), and TβRII (κ=0.904). The $\chi 2$ 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.

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 TβRI, 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). 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). An elevated expression of TβRI showed a trend towards decreased RFP (*P*=0.061, supplementary Figure S1B). 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). 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). 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). High expression of p-Smad2 was substantially associated with a worse prognosis (*P*=0.042, supplementary Figure S2E). Stratification for TβRII showed a trend for worse prognosis when this receptor was highly expressed (*P*=0.099, supplementary Figure S2C). In the population of Smad4 high-expressing tumors, no statistically significant relations were found between OS and TβRI, TβRII, TβRI 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). A trend towards significance was found between OS and TβRII and TβRI and II (*P*=0.061 and 0.054, supplementary Figure S2K and L). 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).

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 online), and Smad4/p-Smad2 (*P*=0.002, HR 3.04, 95% CI 1.390-6.658, supplementary Table S4). In multivariate analysis for OS, both Smad4/TβRI + TβRII (*P*=0.010, HR 1.79, 95% CI 1.233-2.605, supplementary Table S5) and Smad4/p-Smad2 (*P*=0.005, HR 1.84, 95% CI 0.985-3.445, supplementary Table S6) were again substantially associated with survival independent of other parameters.

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 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 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.

Disclosure

The authors have declared no conflicts of interest.

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Table S1. The clinicopathological characteristics of the patients in this study.

Patient and tumor characteristics	
	N
< 40	48
40-50	145
50-60	132
> 60	249
Grade I	80
Grade II	282
Grade III	203
Ductal	513
Other	53
pT1	211
pT2	272
pT3/4	72
pN-	307
pN+	250
ER-Negative	203
ER-Positive	337
PR-Negative	223
PR-Positive	313
No HER2 overexpression	378
HER2 Overexpression	44
- Endocrine therapy	481
+ Endocrine therapy	93
- Chemotherapy	444
+ Chemotherapy	130
Mastectomy + radiotherapy	108
Mastectomy – radiotherapy	223
Lumpectomy + radiotherapy	238
Lumpectomy – radiotherapy	5

Table S2. Association of clinicopathological parameters with the TGF-β markers.

Chi-squared *p*-values	TβRI	TβRII	Smad4	p-Smad2
Age	0.369	0.059†	0.863	0.441
< 40				
40-50				
50-60				
> =60				
Grade	0.015*	0.043*	0.533	0.874
I				
II				
III				
Histological type	0.087†	0.576	0.009*	0.367
Ductal				
Lobular				
T-status	0.143	0.025*	0.297	0.057
T1				
T2				
T3/4				
N-status	0.104	0.101	0.086†	0.797
N0				
N1-3				
ER-status	0.277	0.046*	< 0.001*	< 0.001*
Negative				
Positive				
PgR-status	0.212	0.767	0.052†	0.026*
Negative				
Positive				
Her2-status	0.537	0.748	0.002*	0.003*
Overexpression -				
Overexpression +				

* *P*-value below 0.05, † *P*-value below 0.1

Table S3. Multivariate analysis to investigate the effect of the Smad4/TβRI&II expression and traditional clinico-pathological features to relapse-free period (RFP).

Relapse Free Period		UNIVARIATE			MULTIVARIATE		
	N	HR	95% CI	*p*-value	HR	95% CI	*p*-value
< 40	48	1.00		0.422			
40-50	145	0.97	0.612-1.539				
50-60	132	1.17	0.734-1.853				
> 60	249	0.90	0.574-1.408				
Grade I	80	1.00		0.001	1.00		0.339
Grade II	282	1.43	0.945-2.172		1.07	0.659-1.739	
Grade III	203	2.02	1.326-3.078		1.31	0.801-2.138	
Ductal	513	1.00		0.291			
Other	53	1.24	0.832-1.846				
pT1	211	1.00		< 0.001	1.00		0.064
pT2	272	1.59	1.205-2.093		1.12	0.801-1.571	
pT3/4	72	2.49	1.706-3.635		1.70	1.073-2.693	
pN-	307	1.00		< 0.001	1.00		< 0.001
pN+	250	3.06	2.379-3.945		2.85	2.088-3.880	
ER-negative	203	1.00		0.725			
ER-positive	337	1.05	0.808-1.359				
PgR-negative	223	1.00		0.744			
PgR-positive	313	0.96	0.743-1.236				
No HER2 overexpression	378	1.00		0.401			
HER2 Overexpression	44	1.21	0.776-1.883				
- Endocrine therapy	481	1.00		0.197			
+ Endocrine therapy	93	1.24	0.896-1.705				
- Chemotherapy	444	1.00		0.839			
+ Chemotherapy	130	0.97	0.730-1.291				
Smad4+ TβRI&II –	188	1.00		< 0.001	1.00		0.001
Smad4+ TβRI&II +	77	1.24	0.829-1.858		1.02	0.665-1.563	
Smad4- TβRI&II –	148	1.24	0.889-1.730		1.26	0.890-1.769	
Smad4- TβRI&II +	59	2.47	1.679-3.638		2.20	1.464-3.307	

Table S4. Multivariate analysis to investigate the effect of the Smad4/p-Smad2 expression and traditional clinico-pathological features to relapse-free period (RFP).

Relapse Free Period		UNIVARIATE			MULTIVARIATE		
	N	HR	95% CI	*p*-value	HR	95% CI	*p*-value
< 40	48	1.00		0.422			
40-50	145	0.97	0.612-1.539				
50-60	132	1.17	0.734-1.853				
> 60	249	0.90	0.574-1.408				
Grade I	80	1.00		0.001	1.00		0.269
Grade II	282	1.43	0.945-2.172		1.07	0.662-1.729	
Grade III	203	2.02	1.326-3.078		1.34	0.819-2.196	
Ductal	513	1.00		0.291			
Other	53	1.24	0.832-1.846				
pT1	211	1.00		< 0.001	1.00		0.018
pT2	272	1.59	1.205-2.093		1.29	0.920-1.807	
pT3/4	72	2.49	1.706-3.635		1.95	1.230-3.095	
pN-	307	1.00		< 0.001	1.00		< 0.001
pN+	250	3.06	2.379-3.945		2.55	1.881-3.467	
ER-negative	203	1.00		0.725			
ER-positive	337	1.05	0.808-1.359				
PgR-negative	223	1.00		0.744			
PgR-positive	313	0.96	0.743-1.236				
No HER2 overexpression	378	1.00		0.401			
HER2 overexpression	44	1.21	0.776-1.883				
- Endocrine therapy	481	1.00		0.197			
+ Endocrine therapy	93	1.24	0.896-1.705				
- Chemotherapy	444	1.00		0.839			
+ Chemotherapy	130	0.97	0.730-1.291				
Smad4+ p-Smad2 +	29	1.00		0.003	1.00		0.002
Smad4+ p-Smad2 –	197	1.52	0.763-3.013		1.44	0.724-2.871	
Smad4 - p-Smad2 –	175	1.98	0.995-3.925		2.09	1.050-4.176	
Smad4 - p-Smad2 +	34	3.21	1.485-6.946		3.04	1.390-6.658	

Table S5. Multivariate analysis to investigate the effect of the Smad4/TβRI&II expression and traditional clinico-pathological features to overall survival (OS).

Overall Survival		UNIVARIATE			MULTIVARIATE		
	N	HR	95% CI	*p*-value	HR	95% CI	*p*-value
< 40	48	1.00		< 0.001	1.00		< 0.001
40-50	145	0.94	0.579-1.526		0.83	0.483-1.436	
50-60	132	1.43	0.888-2.295		1.22	0.701-2.119	
> 60	249	2.71	1.741-4.221		2.07	1.219-3.515	
Grade I	80	1.00		0.050	1.00		0.43
Grade II	282	1.23	0.890-1.701		1.05	0.692-1.598	
Grade III	203	1.48	1.060-2.066		1.24	0.805-1.897	
Ductal	513	1.00		0.079	1.00		0.126
Other	53	1.35	0.966-1.879		1.41	0.909-2.175	
pT1	211	1.00		< 0.001	1.00		< 0.001
pT2	272	1.69	1.336-2.149		1.40	1.044-1.881	
pT3/4	72	2.95	2.144-4.057		2.33	1.566-3.478	
pN-	307	1.00		< 0.001	1.00		< 0.001
pN+	250	2.07	1.674-2.549		2.28	1.721-3.024	
ER-negative	203	1.00		0.815			
ER-positive	337	0.97	0.784-1.211				
PgR-negative	223	1.00		0.228			
PgR-positive	313	0.88	0.710-1.085				
No HER2 overexpression	378	1.00		0.404			
HER2 overexpression	44	1.18	0.805-1.717				
- Endocrine therapy	481	1.00		0.001	1.00		0.093
+ Endocrine therapy	93	1.55	1.191-2.012		0.74	0.523-1.051	
- Chemotherapy	444	1.00		0.007	1.00		0.006
+ Chemotherapy	130	0.69	0.533-0.903		0.62	0.434-0.871	
Smad4+ TβRI&II –	188	1.00		0.030	1.00		0.010
Smad4+ TβRI&II +	77	1.22	0.878-1.689		0.97	0.676-1.379	
Smad4- TβRI&II –	148	1.16	0.886-1.528		1.26	0.941-1.686	
Smad4- TβRI&II +	59	1.71	1.200-2.426		1.79	1.233-2.605	

Table S6. Multivariate analysis to investigate the effect of the Smad4/p-Smad2 expression and traditional clinico-pathological features to overall survival (OS).

Overall Survival		UNIVARIATE			MULTIVARIATE		
	N	HR	95% CI	*p*-value	HR	95% CI	*p*-value
< 40	48	1.00		< 0.001	1.00		< 0.001
40-50	145	0.94	0.579-1.526		0.76	0.435-1.313	
50-60	132	1.43	0.888-2.295		0.98	0.563-1.707	
> 60	249	2.71	1.741-4.221		1.70	0.991-2.896	
Grade I	80	1.00		0.050	1.00		0.322
Grade II	282	1.23	0.890-1.701		1.04	0.685-1.577	
Grade III	203	1.48	1.060-2.066		1.26	0.820-1.946	
Ductal	513	1.00		0.079	1.00		0.118
Other	53	1.35	0.966-1.879		1.42	0.915-2.199	
pT1	211	1.00		< 0.001	1.00		< 0.001
pT2	272	1.69	1.336-2.149		1.51	1.112-2.043	
pT3/4	72	2.95	2.144-4.057		2.47	1.653-3.678	
pN-	307	1.00		< 0.001	1.00		< 0.001
pN+	250	2.07	1.674-2.549		2.25	1.687-2.990	
ER-negative	203	1.00		0.815			
ER-positive	337	0.97	0.784-1.211				
PgR-negative	223	1.00		0.228			
PgR-positive	313	0.88	0.710-1.085				
No HER2 overexpression	378	1.00		0.404			
HER2 overexpression	44	1.18	0.805-1.717				
- Endocrine therapy	481	1.00		0.001	1.00		0.180
+ Endocrine therapy	93	1.55	1.191-2.012		0.78	0.549-1.119	
- Chemotherapy	444	1.00		0.007	1.00		0.004
+ Chemotherapy	130	0.69	0.533-0.903		0.60	0.423-0.845	
Smad4+ p-Smad2 +	29	1.00		0.091	1.00		0.005
Smad4+ p-Smad2 –	197	0.88	0.547-1.409		1.01	0.605-1.677	
Smad4 - p-Smad2 –	175	1.13	0.701-1.808		1.50	0.904-2.488	
Smad4 - p-Smad2 +	34	1.40	0.779-2.530		1.84	0.985-3.445	

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

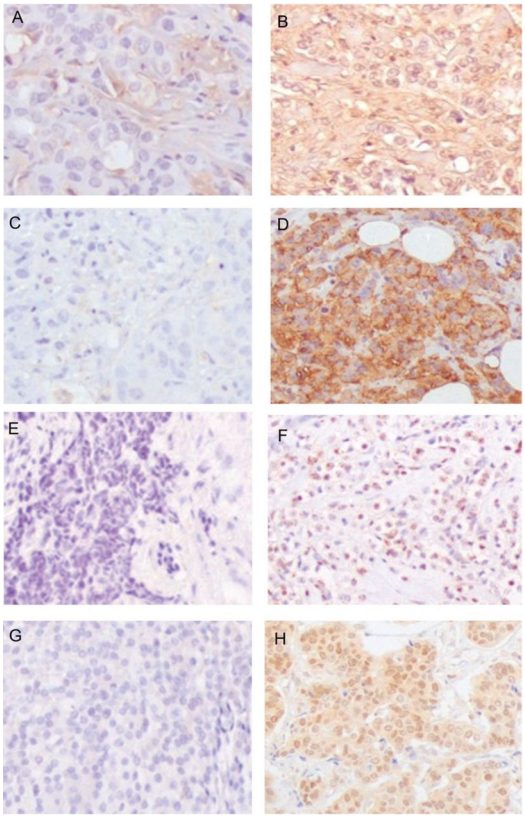

Figure 2. Relapse-free period of patients stratified according to the expression of both Smad4 and p-Smad2.

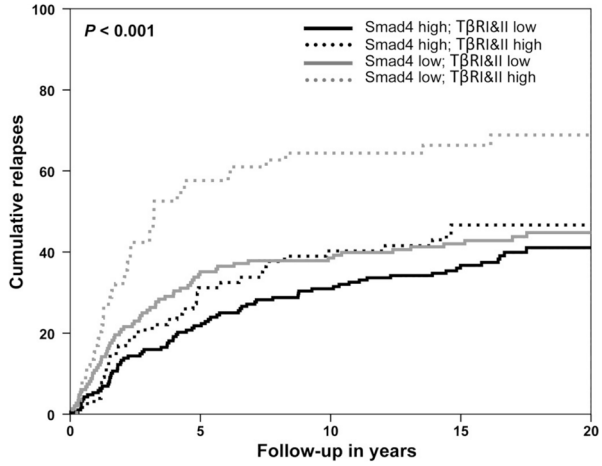

Figure 3. Relapse-free period of patients stratified according to the expression of both Smad4 and transforming growth factor-β receptors.

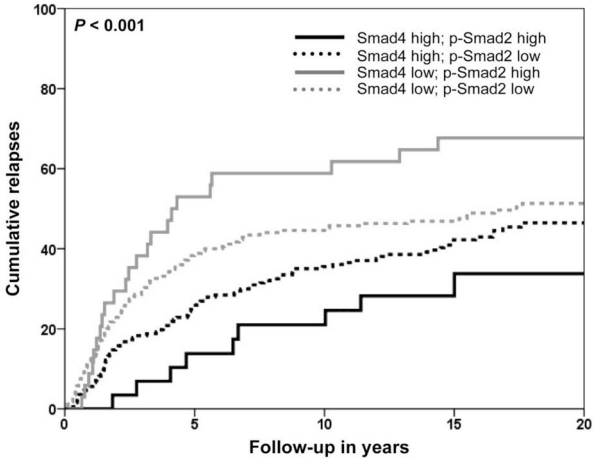

Figure 4. Hypothetical representation of the effects of Smad4, p-Smad2, TβRI, and TβRII levels on the functionality of the transforming growth factor (TGF)-β pathway and prognosis of the patient. In the case of high expression of Smad4, (A) the cytostatic response is intact, and the patient has a relatively favorable prognosis. In the case of low expression of Smad4, (B) the cytostatic response is inactive, and the patient has a relatively unfavorable prognosis. In the case of low expression of the TGF-β receptors, (C) there is low activity of the TGF-β signaling pathway, and the patient has a relatively favorable prognosis.

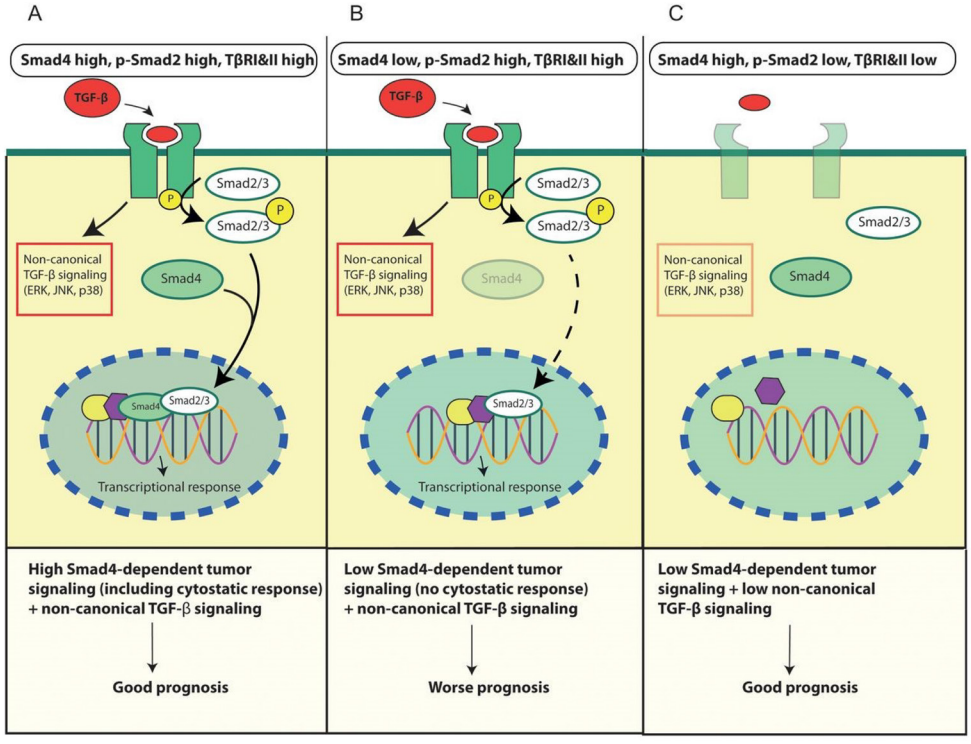

Figure S1. Relationship between progression-free survival and expression of Smad4, TβRI, TβRII and p-Smad2

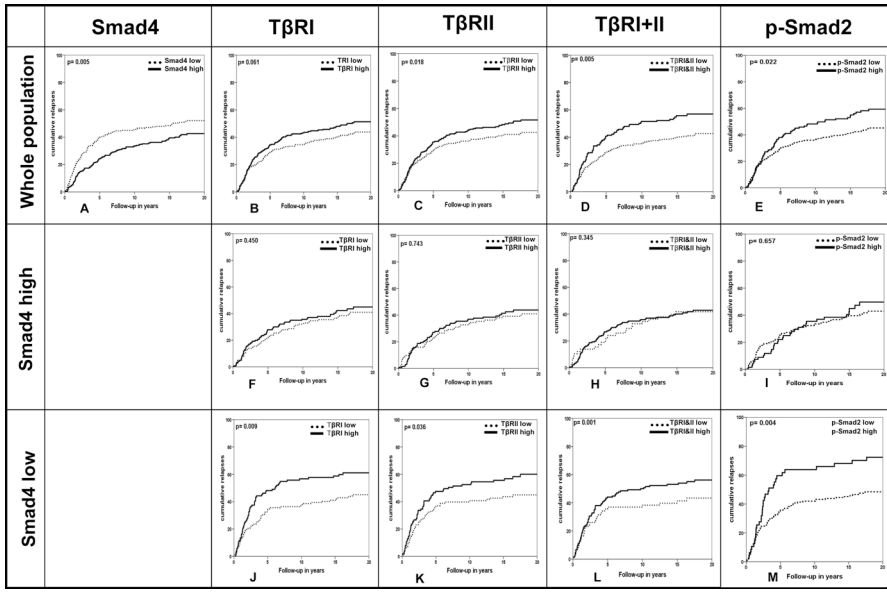

Figure S2. Relationship between overall survival and expression of Smad4, TβRI, TβRII and p-Smad2

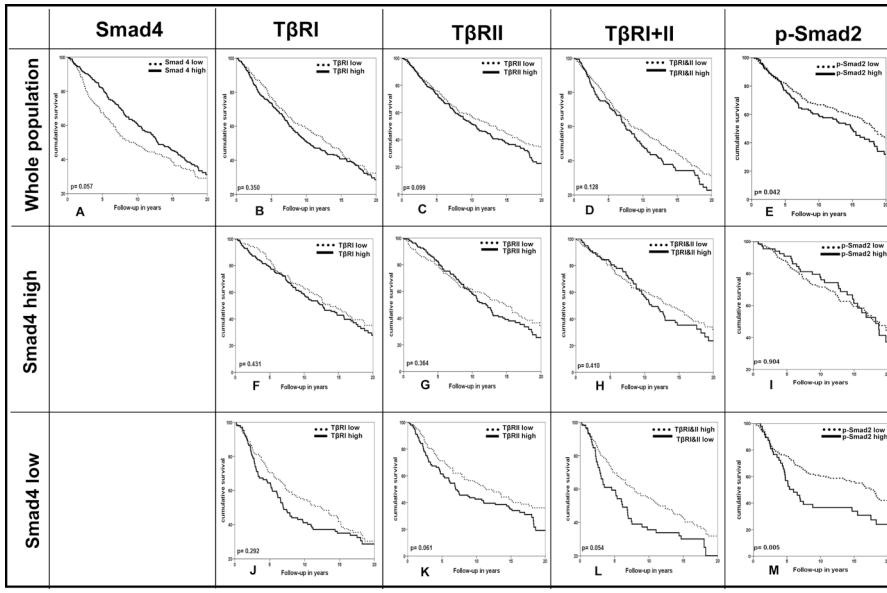

Figure S3. Overall survival of patients stratified according to expression of both Smad4 and p-Smad2.

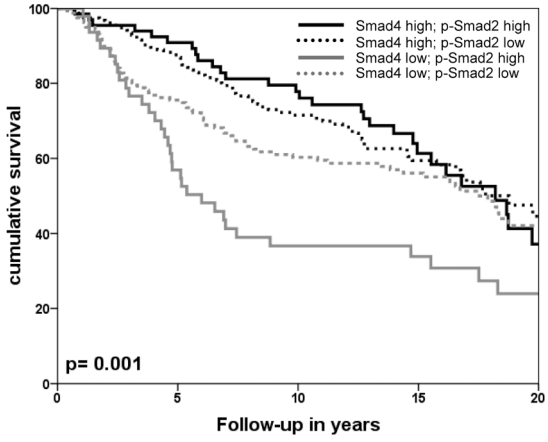

Figure S4. Overall survival of patients stratified according to expression of both Smad4 and TGF-β receptors.