

Clinicopathological Significance of SMAD4 Expression in Breast Cancer

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Purpose: SMAD4 is a member of the SMAD family and acts as a central mediator of transforming growth factor beta signaling. Little is known about SMAD4 expression and its prognostic significance in breast cancer. We evaluated the clinicopathological and prognostic significance of SMAD4 expression in breast cancer. **Methods:** Two hundred and fifty-five patients with invasive ductal carcinoma of the breast from 2000 to 2008 were retrospectively analyzed. We investigated SMAD4 expression using a tissue microarray-based immunohistochemical assay and evaluated the association between SMAD4 and prognosis of breast cancer. **Results:** High SMAD4 expression was positively associated with early stage ($p=0.009$), estrogen receptor positivity ($p=0.026$), and human epidermal growth factor receptor 2 negativity ($p=0.001$). A significant difference in overall survival (OS) was associated with high SMAD4 expression in patients with T1 stage tumors (hazard ratio: 0.459, $p=0.024$). **Conclusion:** High SMAD4 expression was correlated with several favorable prognostic factors and was associated with favorable OS in T1 stage breast cancer. SMAD4 in breast cancer has potential prognostic significance, and further investigations and understanding about SMAD4 expression are needed.

Key Words: Breast neoplasms, Prognosis, SMAD4 protein, Transforming growth factor beta

INTRODUCTION

Breast cancer is among the most common malignancies and a leading cause of death in women [1]. However, advances in surgical techniques and systemic therapies have improved the survival of breast cancer patients in recent decades [2]. Breast cancer is a heterogeneous disease with a variety of pathologic and molecular features. The prognosis of breast cancer and therapeutic decision-making are known to depend on classic immunohistochemistry (IHC) markers, namely estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Recently, genomic assays have been used to provide information and to help in decision-making for adjuvant treatment [3]. Differential expression of various proteins has been researched to develop more efficient options for diagnosis and treatment.

SMAD4 is a major downstream mediator in the signaling of the transforming growth factor beta (TGF- β) pathway. The TGF- β signal-

ing process is initiated when TGF- β binds to the homodimers of the TGF- β type II receptor (T β RII) on the cell surface. T β RII recruits and activates the TGF- β type I receptor (T β RI). The activated T β RI phosphorylates SMAD2 or SMAD3, which heterodimerizes with SMAD4. These complexes translocate into the nucleus where they bind to DNA and regulate TGF- β dependent gene expression [4]. TGF- β is crucial in supporting tissue homeostasis through its ability to regulate cell proliferation, differentiation, migration, apoptosis, and development. The loss of SMAD4 expression results in the deprivation of a major factor of cell growth inhibition, contributing to carcinogenesis. SMAD4 has been previously identified as a possible tumor suppressor since SMAD4 mutations have been reported with high frequency in solid tumors [5,6]. The SMAD4 expression level has been reported to correlate with prognosis in many types of cancers, including colon [7], pancreatic [8], and esophageal [9] cancers. However, very few studies on SMAD4 expression and prognosis in breast cancer have been performed, and the results have been conflicting [10-13]. In the present study, we investigated SMAD4 expression using a tissue microarray (TMA)-based IHC assay and evaluated its clinicopathological and prognostic significance in breast cancer.

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METHODS

Patients and tissue samples

Four hundred and seventy-one patients treated for invasive ductal carcinoma (IDC) of breast at Hanyang University Medical Center between December 2000 and December 2008 were considered. The present study was approved by the Institutional Review Board of Hanyang University Medical Center (No. 2019-08-029-004). Patients with incomplete clinical data sets, patients without sufficient archived tissues, and patients with stage IV cancers were excluded. Two hundred and fifty-five patients were selected for the study after exclusions. We examined patients' age at diagnosis, tumor size, lymph node status, and pathological findings according to the American Joint Committee on Cancer (AJCC, 7th edition) classification for stage, tumor size, lymph node metastasis stage, and hormone receptor status. The hormone receptor (ER and PR) statuses were assessed using IHC and were scored using the Allred score [14]. Patients with a score >1 were defined as ER/PR positive. Testing for HER2 was performed using either IHC staining or fluorescent in situ hybridization (FISH). HER2 expression was classified into levels 0 to 3+ according to the guidelines by American Society of Clinical Oncology/College of American Pathologists [15]. A score of 3+ was given to specimens showing uniform and intense membrane staining in $>30\%$ of invasive tumor cells, and these specimens were rated as HER2 positive. The cases with a score of 2+ that were equivocal for HER2 protein expression were evaluated by FISH analysis to measure HER2 amplification using an original paraffin block.

TMA construction

Slides stained with hematoxylin and eosin were used to define the most morphologically representative, well fixed, and non-necrotic areas. Single tissue cores (2 mm in diameter) were sampled from each paraffin block and were assembled into a recipient paraffin block using a TMA instrument (AccuMax Array, ISU ABXIS, Seoul, Korea).

IHC staining and interpretation of SMAD4 expression

Multiple 4- μm sections were cut using Leica microtome (Leica Biosystems, Wetzlar, Germany) and transferred to adhesive-coated slides. One section was routinely deparaffinized with standard xylene and hydrated using graded ethanol in water. It was stained with hematox-

ylins and eosin and covered with a coverslip. For IHC, the TMA slides were dewaxed by heating at 55°C for 30 minutes and by three 5-minute xylene washes.

Primary monoclonal mouse anti-Smad4 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) was diluted (1:200) in goat serum and incubated at room temperature for 1 hour. After three 2-minute washes with phosphate-buffered saline, the sections were incubated with a biotinylated goat anti-mouse secondary antibody for 30 minutes (DAKO, Carpinteria, USA). The slides were then dehydrated following standard procedure and sealed with coverslips. Negative controls were performed by omitting Smad4 antibody during the primary antibody incubation.

To analyze SMAD4 expression in breast cancer, we used the IHC assay with the application of immunoreactivity score (IRS) criteria [16]. The extensional standards were the "fraction of positive cells" (0: $<5\%$, 1: 6%–25%, 2: 26%–50%, 3: 51%–75%, and 4: $>75\%$) and the "staining intensity score" (0: colorless, 1: pallid-flavens, 2: yellow, and 3: brown). The IRS was calculated by multiplying the "staining intensity score" and the "fraction of positive cells" [17]. The staining score was stratified as absent (–, score 0), weak positive (+, score 1–4), moderate positive (++, score 5–8), and strong positive (+++, score 9–12) (Figure 1). To evaluate SMAD4 expression, specimens with absent and weak positive ($0 \leq \text{IRS} \leq 4$) scores were classified into the low SMAD4 expression (SMAD4 low) group. Specimens with moderate and strong positive scores ($5 \leq \text{IRS} \leq 12$) were classified into the high SMAD4 expression (SMAD4 high) group (Figure 1). The interpretation of IHC staining in this study was performed by a single pathologist (SS Paik).

Statistical analysis

Fisher's exact test, chi-squared test, and logistic regression analysis were used to evaluate the correlations between the clinicopathological features and SMAD4 expression. In the survival analyses, the plots were generated using the Kaplan-Meier curve and were compared using the log-rank test. A multivariate analysis was performed to identify the independent prognostic markers for disease-free survival (DFS) and overall survival (OS) using the Cox multistep regression model. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, USA) and R package version 3.5.1 (RStudio, Boston, USA).

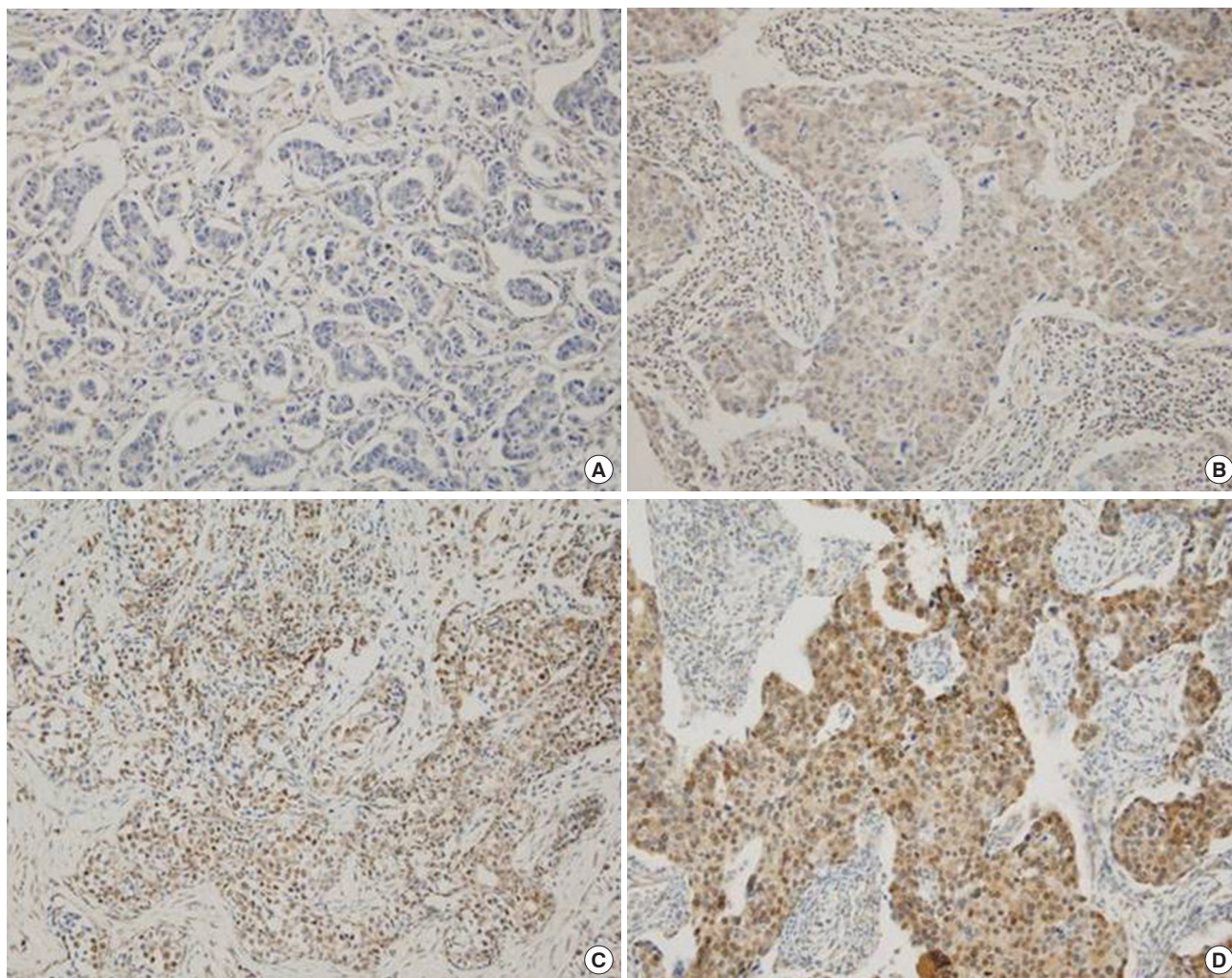


Figure 1. Representative sections of the immunohistochemistry of SMAD4 in breast cancer tissue (magnification, $\times 200$). (A) Negative SMAD4 staining. (B) Weak SMAD4 staining. (C) Intermediate SMAD4 staining. (D) Strong SMAD4 staining.

RESULTS

The median follow-up period was 66.3 months (range 3.9 to 134.2 months) and the median age was 49 years (range 27 to 79 years). Among the 255 cases, 239 (93.7%) patients had primary tumor size of less than 2 cm and 16 (6.3%) patients had primary tumor size of more than 2 cm. Regional lymph node metastasis was present in 131 cases (51.4%). According to the AJCC classification scheme, 77 (30.2%) patients had stage I cancer, while 178 (69.8%) had stage II and stage III cancer. ER expression was positive in 142 cases (55.7%) and PR expression was positive in 134 cases (52.5%). Out of all the cases, 67 (26.3%) were positive for HER2 expression on IHC analysis and/or positive for HER2 gene amplification detected by the FISH analysis. One hun-

dred sixty-six specimens (65.1%) were categorized into the SMAD4 low group and 89 specimens (34.9%) were categorized into the SMAD4 high group (Table 1, Figure 1).

Relationship between SMAD4 expression and clinicopathological parameters

High SMAD4 expression was positively associated with good clinical phenotypes of breast cancer such as low early AJCC stage ($p = 0.009$), ER positivity ($p = 0.026$), or HER2 negativity ($p = 0.001$). No significant correlation was detected between SMAD4 expression and the other clinicopathological parameters such as histological grade, age, lymph node metastasis, PR status, and triple-negative breast cancer (TNBC) (Table 1).

Table 1. Association between SMAD4 expression and clinicopathological parameters in breast cancer patients (n=255)

Characteristic	No. (%)	SMAD4		p-value
		Low expression (n = 166)	High expression (n = 89)	
Age(yr)				
≤ 50	139 (54.5)	91 (54.8)	48 (53.9)	0.892
> 50	116 (45.5)	75 (45.2)	41 (46.1)	
Histologic grade				
Grade 1	38 (14.9)	23 (13.9)	15 (16.9)	0.749
Grade 2 and 3	217 (85.1)	143 (86.1)	74 (83.1)	
Primary tumor size				
≤ 2 cm	239 (93.7)	152 (91.6)	87 (97.8)	0.052
> 2 cm	16 (6.3)	14 (8.4)	2 (2.2)	
Lymph node metastasis				
Negative	124 (48.6)	74 (44.6)	50 (56.2)	0.077
Positive	131 (51.4)	92 (55.4)	39 (43.8)	
Stage				
Stage I	77 (30.2)	41 (24.7)	36 (40.5)	0.009
Stage II & III	178 (69.8)	125 (75.3)	53 (59.5)	
Lymphovascular invasion				
Negative	111 (43.5)	66 (39.8)	45 (50.6)	0.097
Positive	144 (56.5)	100 (60.2)	44 (49.4)	
Perineural invasion				
Negative	194 (76.1)	128 (77.1)	66 (74.2)	0.599
Positive	61 (23.9)	38 (22.9)	23 (25.8)	
ER expression				
Negative	113 (44.3)	82 (49.4)	31 (34.8)	0.026
Positive	142 (55.7)	84 (50.6)	58 (65.2)	
PR expression				
Negative	121 (47.5)	86 (51.8)	35 (39.3)	0.057
Positive	134 (52.5)	80 (48.2)	54 (60.7)	
HER2 amplification				
Negative	188 (73.7)	111 (66.9)	77 (86.5)	0.001
Positive	67 (26.3)	55 (33.1)	12 (13.5)	
TNBC				
Non-TNBC	201 (78.8)	131 (78.9)	70 (78.7)	0.961
TNBC	54 (21.2)	35 (21.1)	19 (21.3)	

ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; TNBC = triple negative breast cancer.

Comparison between survival outcome and SMAD4 expression

During the follow-up period, there were 64 (25.1%) recurrences among the 255 patients. Out of these, 47 cases were SMAD4 low and 17 were SMAD4 high (73.4% and 26.6%, respectively). In overall survival, 55 events (21.6%) occurred. Out of these, 42 patients were SMAD4 low and 13 were SMAD4 high (76.4% and 23.6%, respectively). No difference was observed in DFS between the SMAD4 low group and the SMAD4 high group ($p = 0.115$), but the SMAD4 high

group showed significantly better OS ($p = 0.045$) than the SMAD4 low group (Figure 2A, 2B). In the subgroup analysis, high SMAD4 expression was correlated with better OS in T1 stage tumor (size ≤ 2 cm) ($p = 0.034$). However, it did not show significant correlation with DFS (Figure 2C, 2D). In the multivariate analysis, a significant association was observed between OS and SMAD4. High SMAD4 expression was observed to be an independent prognostic factor for better OS among patients with T1 stage tumor in the multivariate analysis (hazard ratio: 0.459, $p = 0.024$) (Table 2).

DISCUSSION

SMAD4 is a key mediator of the TGF- β pathway. In the present study, the expression of SMAD4 was investigated using IHC analysis in 255 cases of IDC. The present data showed that high SMAD4 expression was associated with favorable clinicopathological parameters (stage, ER positivity, and HER2 negativity) and OS. There was a significant association between SMAD4 expression and OS in patients with T1 stage tumors (tumor size ≤ 2 cm).

SMAD4 expression was found to be lower in breast tumor cells when compared with normal breast epithelium [11]. Two previous reports applied the IRS criteria with respect to the expression of SMAD4 in breast cancer. Lui et al. [13] reported that 43.1% of breast cancer cases were SMAD4 low ($0 \leq \text{IRS} \leq 4$) and 56.9% were SMAD4 high ($5 \leq \text{IRS} \leq 2$), whereas Stuelten et al. [11] reported the figures of 36.3% and 64%, respectively. Our results are closely analogous to those of these studies, with 34.9% SMAD4 low cases and 65.1% SMAD4 high cases. There are inconsistent reports on the correlation between SMAD4 expression and prognostic markers in breast cancer. In previous studies, SMAD4 showed no significant correlation with tumor size, metastases, nodal status, histological grade, histological type, or estrogen receptor expression [11]. However, Lui et al. [13] reported that SMAD4 expression was negatively associated with histological grade.

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. Recent studies have indicated that inactivation of SMAD4 is related to progression of disease in various cancers [18-21]. In colon cancer, SMAD4 inactivation promotes malignancy and drug resistance [22]. In pancreatic cancer, low expression of SMAD4 is associated with malignant progression [23]. Similarly, in non-small-cell

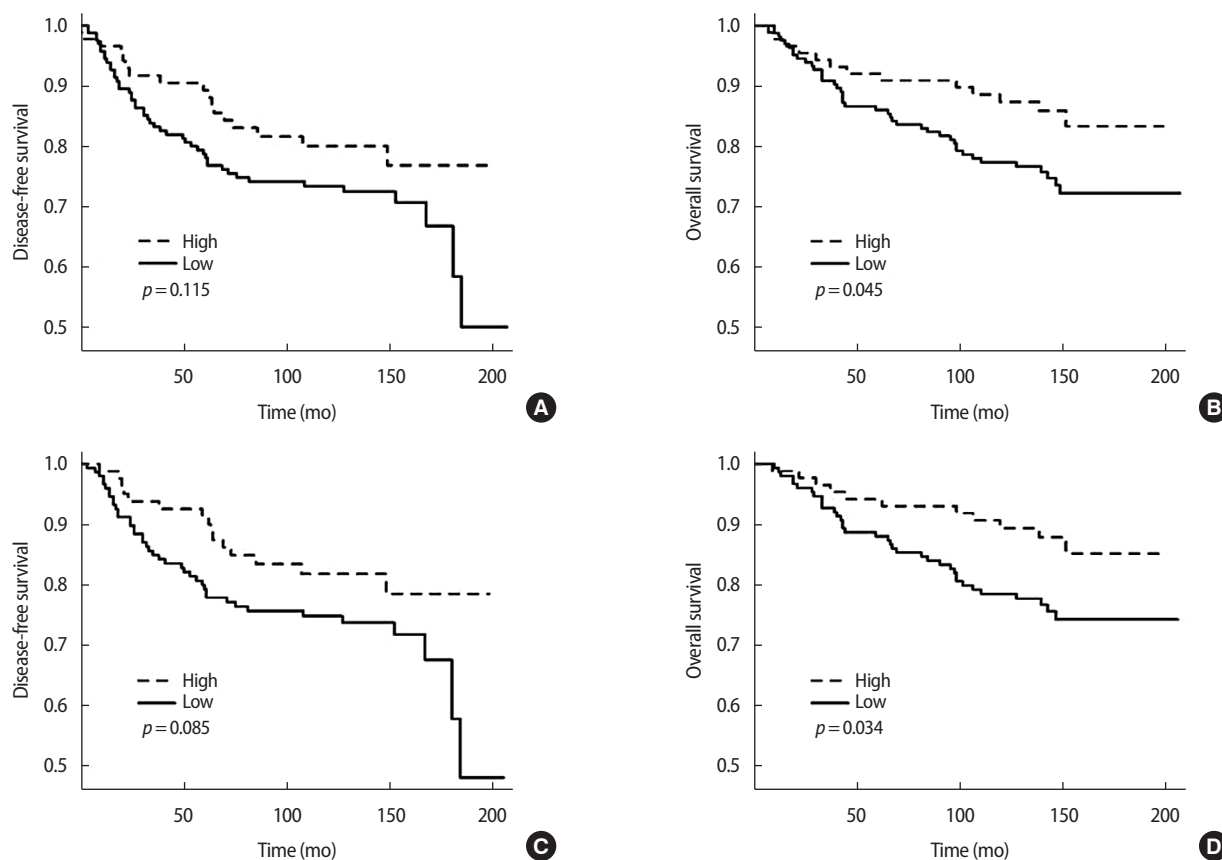


Figure 2. Kaplan-Meier curves for disease-free survival (DFS) and overall survival (OS) for breast cancer according to SMAD4 expression. (A) All patients –DFS. (B) All patient – OS. (C) T1 staged tumor (≤ 2 cm) – DFS. (D) T1 staged tumor (≤ 2 cm) – OS.

Table 2. Univariate and multivariate analysis of various prognostic parameters for survival in cancer patients with primary tumor size ≤ 2 cm

Characteristic	Disease free survival					Overall survival				
	Univariate		Multivariate			Univariate		Multivariate		
	HR	p-value	HR	95% CI	p-value	HR	p-value	HR	95% CI	p-value
Age (≤ 50 vs. > 50)	1.554	0.101				2.042	0.017	2.158	1.196–3.893	0.011
Histologic grade (G1 vs. G2 & G3)	3.410	0.002	2.995	1.346–6.665	0.007	1.645	0.149			
Lymph node metastasis (absent vs. present)	2.295	0.004				1.472	0.191			
Lymphovascular invasion (absent vs. present)	1.781	0.044				1.274	0.415			
Perineural invasion (absent vs. present)	1.811	0.034	1.749	1.003–3.048	0.049	1.760	0.063	1.918	1.054–3.489	0.033
SMAD4 expression (low vs. high)	0.598	0.089	0.653	0.358–1.191	0.165	0.488	0.038	0.459	0.233–0.903	0.024
ER expression (negative vs. positive)	0.670	0.135				0.537	0.035			
PR expression (negative vs. positive)	0.563	0.035	0.504	0.288–0.882	0.016	0.540	0.038			
HER2 amplification (absent vs. present)	0.809	0.514	0.585	0.299–1.145	0.118	0.940	0.853			
Non TNBC vs. TNBC	1.481	0.204				1.999	0.030	2.298	1.223–4.317	0.010

HR = hazard ratio; CI = confidence interval; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; TNBC = triple negative breast cancer.

lung carcinoma, SMAD4 expression was higher in the normal broncho-tracheal epithelium, but lower in the tumor tissues and closely correlated with lymph node metastasis [24]. Thus, low expression of

SMAD4 shows an unfavorable outcome in other types of cancers. Our study demonstrated similar results in breast cancer.

Very few studies have reported the correlation between SMAD4

and prognosis in breast cancer. Lui et al. [13] reported that SMAD4 expression appeared to be decreased in breast cancer when compared with normal tissues and reduced SMAD4 expression levels tended to exhibit more poorly differentiated tumors, a higher risk of recurrence, and shorter OS. Kruijt et al. [12] also reported that low expression of SMAD4 had an unfavorable prognosis regarding progression-free survival. These results are in agreement with the present study. However, one study suggested that a SMAD4-negative tumor showed marginally better overall 5-year survival than a SMAD4-positive tumor, though this difference was not statistically significant [11]. Another study demonstrated that loss of SMAD4 was correlated with a decrease in axillary lymph node metastasis [25]. Since there are conflicting reports in terms of prognosis, recent studies have attempted to determine whether SMAD4 as a prognostic factor combines with other genes or protein expressions [10,12].

TGF- β is considered to play 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 in early stage [26,27]. In contrast, in later stages of cancer, TGF- β has direct pro-tumorigenic effects through the stimulation of invasion, the migration of tumor cells, and the activation of the tumor stroma [28,29]. Positive association of high expression of SMAD4 in T1 stage early breast cancer with better survival outcome in the present study could be explained by the fact that SMAD4 is a key mediator of TGF- β pathway and TGF- β acts as a tumor suppressor in the early stage.

The present study has several limitations. No stage IV patients were included in the TMA data. The IHC analysis using TMA may not reflect the intratumoral heterogeneity. Moreover, the proportion of small-sized tumors was unintentionally higher than the large-sized tumors in our data. However, our results could provide data for SMAD4 expression and prognosis in the early stage breast cancer.

In conclusion, high SMAD4 expression was correlated with several favorable prognostic factors including tumor size, regional lymph node metastasis, AJCC stage, ER positivity, and HER2 negativity. Additionally, high SMAD4 expression was associated with favorable OS in T1 stage breast cancer. Therefore, SMAD4 in breast cancer has potential prognostic significance and further investigation and understanding are needed to elucidate it.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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