

Sox17 Promoter Methylation in Plasma DNA Is Associated With Poor Survival and Can Be Used as a Prognostic Factor in Breast Cancer

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Abstract: Aberrant DNA methylation that leads to the inactivation of tumor suppressor genes is known to play an important role in the development and progression of breast cancer. Methylation status of cancer-related genes is considered to be a promising biomarker for the early diagnosis and prognosis of tumors. This study investigated the methylation status of the *Sox17* gene in breast cancer tissue and its corresponding plasma DNA to evaluate the association of methylation levels with clinicopathological parameters and prognosis.

The methylation status of the *Sox17* gene promoter was evaluated with methylation-specific polymerase chain reaction (MSP) in 155 paired breast cancer tissue and plasma samples and in 60 paired normal breast tissue and plasma samples. Association of *Sox17* methylation status with clinicopathological parameters was analyzed by χ^2 tests. Overall and disease-free survival (DFS) curves were calculated using Kaplan–Meier analysis, and the differences between curves were analyzed by log-rank tests.

The frequency of *Sox17* gene methylation was 72.9% (113/155) in breast cancer tissues and 58.1% (90/155) in plasma DNA. *Sox17* gene methylation was not found in normal breast tissues or in their paired plasma DNA. There was a significant correlation of *Sox17* methylation between corresponding tumor tissues and paired plasma DNA ($r = 0.688$, $P < 0.001$). Aberrant *Sox17* methylation in cancer tissues and in plasma DNA was significantly associated with the tumor node metastasis stage ($P = 0.035$ and $P = 0.001$, respectively) and with lymph node metastasis ($P < 0.001$ and $P = 0.001$, respectively). Kaplan–Meier survival curves showed that aberrant *Sox17* promoter methylation in cancer tissues and plasma DNA was associated with poor DFS

($P < 0.005$) and overall survival (OS) ($P < 0.005$). Multivariate analysis showed that *Sox17* methylation in plasma DNA was an independent prognostic factor in breast cancer for both DFS ($P = 0.020$; hazard ratio [HR] = 2.142; 95% confidence interval [CI]: 1.128–4.067) and for OS ($P = 0.001$; HR = 4.737; 95% CI: 2.088–10.747).

Sox17 gene promoter methylation may play an important role in breast cancer progression and could be used as a prognostic biomarker to identify patients at risk of developing metastasis or recurrence after mastectomy.

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Abbreviations: AJCC = American Joint Committee on Cancer, CI = confidence interval, DFS = disease-free survival, HR = hazard ratio, MSP = methylation-specific polymerase chain reaction, OS = overall survival.

INTRODUCTION

Breast cancer is the most prevalent cancer and is a major cause of cancer-related death in women worldwide.¹ Over the past 3 decades, despite numerous advances having been made in breast cancer early detection and comprehensive therapies, a great many patients still finally die of cancer recurrence and metastasis. The detailed mechanisms underlying this malignancy remain largely unknown and current detection and treatment measures do not adequately improve the survival chances of women with this disease. Thus, the identification of markers for the early detection and effective therapeutic targets for breast cancer patients is urgent and necessary.

It is now recognized that solid malignant tumors can release a significant amount of genomic DNA into circulation in the blood,² and such DNA can account for >90% of total circulating cell-free DNA^{3–6} and can be characteristic of the overall heterogeneity of the tumor from which this DNA was released.⁷ The presence of abnormally high DNA concentrations in plasma has been reported in breast cancer, and the association of changes in the levels of circulating DNA with tumor burden and progression has been repeatedly confirmed.^{8,9} To date, almost all of the markers associated with genetic alterations, including epigenetic alterations, have been described in circulating DNA.¹⁰ Epigenetic silencing due to hypermethylation of tumor-related genes is known to play critical roles in the initiation and progression of breast cancer; this has been demonstrated in DNA damage repair genes, cell cycle regulation genes, and cell signal transduction genes, among others.^{11–13} Increasing amounts of data strongly suggest that DNA methylation can be a useful biomarker in risk assessment,¹⁴ early diagnosis,¹⁵ prognosis,^{16–20} and treatment^{18,21} for breast cancer patients. Furthermore, some studies have also shown that methylation patterns found in circulating cell-free DNA were similar to the patterns in

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primary tumors,^{22–24} indicating the potential utility of minimally invasive blood-based methods for breast cancer detection.

Sox17, a member of the Sry-related high-mobility group box gene family, is a high-mobility group box transcription factor that is known to function as a key regulator in various developmental and disease contexts, including endoderm organ development,^{25,26} vascular development,²⁷ oligodendrocyte development,^{28,29} and stem cell function regulation.^{30,31} In addition, *Sox17* can act as a negative regulation factor of β -catenin/TCF transcription activity in the Wnt/ β -catenin signal transduction pathway.^{25,29,32–36} Recently, growing evidence has indicated that *Sox17* also plays an important role in human carcinogenesis. The downregulated expression of *Sox17* has been detected in colorectal cancer, hepatocellular carcinoma, gastric cancer, and esophageal carcinoma, among other cancers.^{32–41} Further studies have revealed that *Sox17* gene silencing is associated with hypermethylation of the *Sox17* promoter. Hypermethylation of the *Sox17* promoter is correlated with poor prognosis in several cancers.^{34,37–38,40} In a previous study, we demonstrated that *Sox17* is often hypermethylated and provides important prognostic information in breast cancer patients.³⁴ Recent studies have shown that *Sox17* is also epigenetically silenced in circulating tumor cells isolated from the peripheral blood of patients with breast or gastric cancer,^{41–43} and that such silencing can be used as a molecular diagnostic marker in early-stage gastric cancer.⁴³ Therefore, the aim of this study was to evaluate the prognostic significance of *Sox17* promoter methylation in breast cancer patients.

MATERIALS AND METHODS

Patients and Samples

From January 2007 to June 2008, 155 patients with breast cancer, from Northern Jiangsu People's Hospital, Yangzhou, China, were enrolled in this study. All tissue specimens were flash frozen in liquid nitrogen and stored at -80°C for DNA extraction immediately after resectioning. Pathological information was obtained for the following: histological tumor type, primary tumor size, axillary lymph nodal status, histological grade, estrogen and progesterone receptors status, and HER2/neu status. The disease stage of the breast cancer cases was classified according to the American Joint Committee on Cancer-7 tumor node metastasis (TNM) staging system. Meanwhile, paired blood samples from all recruited individuals were collected before surgery. Sixty normal tissues adjacent to benign breast tumors and paired plasma samples were collected as controls. All patients gave written informed consent for the use of their samples in this research, and the study was approved by the Ethical Committee and Institutional Review Board of Northern Jiangsu People's Hospital.

Collection and Processing of Samples and DNA Preparation

Ten milliliters of blood samples were collected in BD Vacutainer[®] EDTA Tubes (Becton Dickinson, Franklin Lakes, NJ, USA) tubes before any invasive procedures or any treatment had been performed. Plasma was immediately separated from the cellular fraction by 2 rounds of centrifugation at 3000 rpm for 10 minutes at room temperature and then stored at -80°C for later use. Genomic DNA from breast cancer tissues was extracted with a DNeasy Tissue Kit (Qiagen, Hilden, Germany), and plasma DNA was isolated with a QIAamp DNA Blood Mini

Kit (Qiagen) according to the manufacturer's instructions. The extracted DNA was quantified spectrophotometrically and stored at -20°C .

Sodium Bisulfite Modification and MSP

DNA was modified with an EZ DNA Methylation-Gold Kit (ZYMO Research Co., Orange, CA) as previously described.³⁴ The methylation status of *Sox17* in breast cancer tissues and in circulating cell-free DNA was detected with the methylation-specific polymerase chain reaction (MSP) method. The primer pairs for both the methylated and the unmethylated sequences and the thermocycling conditions of MSP were to those reported in our previous study.³⁴ Each MSP reaction included 2 μL of DNA template, 0.18 μL of each primer, 0.45 μL of 10 mM dNTP Mix (Promega Corp., Madison, WI, USA) 1.5 μL 10 \times PCR buffer, and 0.12 μL of HotStart Taq DNA Polymerase (Sigma, Germany) in a final reaction volume of 15 μL . MSP products (4 μL) were loaded onto 2% agarose gels and visualized by ethidium bromide staining. SssI-methylated DNA was used as a positive control; whole-genome amplification DNA of normal peripheral lymphocytes was used as a negative control.

Follow-Up

Patients were tracked until September 30, 2014. For every patient enrolled, a complete diagnostic evaluation consisting of chest x-rays, mammography, ultrasounds of the liver, and a whole-body bone scan before surgery was performed to exclude the presence of distant metastasis. Patients were given a physical examination every 3 months for the first 2 years postoperatively and were subsequently examined every 6 months. Disease-free survival (DFS) was defined as the duration from the date of surgery to the date of first evidence of local recurrence, distant metastasis, or last contact. Overall survival (OS) was defined as the time from the date of surgery to the date of death or the date of last contact if the patient was still alive.

Statistical Analyses

Statistical analyses were performed with SPSS statistical software, version 16.0, for Windows (SPSS Inc., Chicago, IL). Categorical data were analyzed by χ^2 or Fisher exact tests. Correlations between the methylation statuses of both plasma DNA and tumor tissues were analyzed with Spearman correlation coefficient analysis. DFS and OS curves were calculated using the Kaplan–Meier method and comparisons were performed using the log-rank tests. A univariate Cox regression analysis was used to determine identified prognostic factors, and multivariate Cox regression analysis was used to explore combined effects. All *P* values presented are 2-sided; a *P* value <0.05 was considered to indicate statistical significance.

RESULTS

Patient Characteristics

A total of 155 patients with breast cancer were enrolled in this study. The median age was 48.4 years (range 26–75 years); 117 patients were of stages I and II, and 38 patients were of stage III. Patient demographics and pathological features are summarized in Table 2. Control tissue samples adjacent to benign breast tumors, along with paired plasma samples were obtained from 60 patients; these samples included 6 intraductal papilloma, 24 mastopathies, 20 fibroadenoma, and 10 breast adipomas.

TABLE 1. Correlation Analysis of Sox17 Gene Methylation in Breast Cancer Tissues and Paired Plasma DNA

		Tumor Tissues		P* Value
		Unmethylated	Methylated	
Plasma circulating DNA	Unmethylated	41 (48.5%)	24 (51.5%)	<i>r</i> = 0.688, <i>P</i> < 0.001
	Methylated	1 (5.7%)	89 (94.3%)	

* Spearman Correlation Analysis.

Frequency of Sox17 Promoter Methylation in Breast Cancer Tissues and Paired Plasma DNA

Aberrent Sox17 promoter methylation was present in 113 of 155 (72.9%) primary tumors, whereas 0/60 (0%) of the normal breast specimens exhibited this. There was a significant difference in Sox17 promoter methylation between the breast cancer and the normal tissues (*P* < 0.001). Sox17 promoter methylation was found in 90 of 155 (58.1%) plasma DNA from patients with breast cancer; it was not found in any of the control plasma samples. There was a significant difference between the 2 groups (*P* < 0.001). Representative results of MSP assays for Sox17 methylation are shown in Figure 1.

Correlation of Sox17 Methylation in Breast Cancer Tissues and Paired Plasma DNA

We further analyzed the relationship of the methylation status of Sox17 in breast cancer tissues with the methylation status of Sox17 in paired plasma DNA samples in these patients. Eighty-nine cases (57.42%) had Sox17 promoter methylation in both tumor tissues and in circulating DNA. Nonmethylated Sox17 in both tumor tissues and plasma DNA was found in 41 (33.3%) of the cases; 78.8% (89/113) methylated Sox17 in tumor tissues could be detected in plasma DNA, only 1 case showed Sox17 methylation in plasma DNA but not in tumor tissue. The consistency between tissues and plasma samples was

TABLE 2. Clinical Characteristics of 155 Patients With and Without Sox17 Methylation in Cancer Tissues and/or Plasma DNA

Clinical Parameter	No.	Tumor Tissues		P† Value	Plasma Circulating DNA		P† Value
		Methylated, n = 113 (72.9%)	Unmethylated, n = 42 (27.1%)		Methylated, n = 90 (58.1%)	Unmethylated, n = 65 (41.9%)	
Age, y				0.857			0.871
<50	84	62 (73.8%)	22 (26.2%)	0.035*	48 (57.1%)	36 (42.9%)	0.001*
≥50	71	51 (71.8%)	20 (28.2%)		42 (59.2%)	29 (40.8%)	
Stage				0.035*			0.001*
I–II	117	80 (68.4%)	37 (31.6%)	0.845	59 (50.4%)	58 (49.6%)	0.295
III	38	33 (86.8%)	5 (13.2%)		31 (81.6%)	7 (18.4%)	
Tumor grade				0.845			0.295
I–II	107	77 (72.0%)	30 (28.0%)	0.095	59 (55.1%)	48 (44.9%)	0.076
III	48	36 (75.0%)	12 (25.0%)		31 (64.6%)	17 (35.4%)	
Tumor diameter, cm				0.095			0.076
<2	57	37 (64.9%)	20 (35.1%)	0.000*	25 (43.9%)	32 (56.1%)	0.001*
≥2	98	76 (78.0%)	22 (22.0%)		65 (60.0%)	33 (40.0%)	
Lymph node status				0.000*			0.001*
Negative	84	47 (56.0%)	37 (44.0%)	0.708	24 (28.6%)	60 (71.4%)	0.395
Positive	71	66 (93.0%)	5 (7.0%)		66 (93.0%)	5 (7.0%)	
ER status				0.708			0.395
Negative	55	39 (70.9%)	16 (29.1%)	1.000	29 (52.7%)	26 (47.3%)	0.870
Positive	100	74 (74.0%)	26 (26.0%)		61 (61.0%)	39 (39.0%)	
PR status				1.000			0.870
Negative	65	47 (72.3%)	18 (27.7%)	0.525	37 (56.9%)	28 (43.1%)	0.252
Positive	90	66 (73.3%)	24 (26.7%)		53 (58.9%)	37 (41.1%)	
HER-2/neu status				0.525			0.252
Negative	118	84 (71.2%)	34 (28.8%)		65 (55.1%)	53 (44.9%)	
Positive	37	29 (78.4%)	8 (21.6%)		25 (67.6%)	12 (32.4%)	

* Statistically significant, *P* < 0.05.

† *P* values are obtained from χ^2 test.

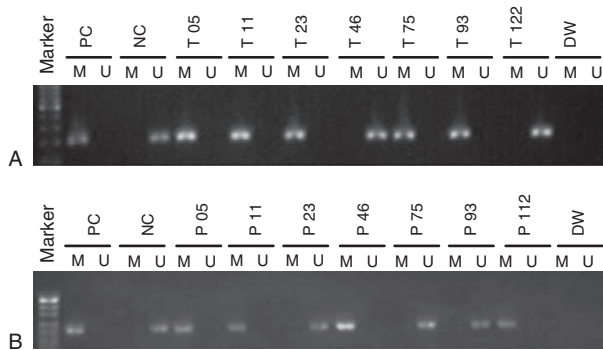


FIGURE 1. Methylation status of the *Sox17* promoter in breast cancer tissues and paired plasma DNA. (A) Representative results of MSP assays of *Sox17* methylation in primary breast cancer tissues; B. Representative results of MSP of *Sox17* methylation in paired plasma DNA. DW = distilled water, M = methylated, MSP = methylation-specific polymerase chain reaction, NC = negative control, P = plasma DNA, PC = positive control, T = breast cancer tissue, U = unmethylated.

130 of 155 (83.9%), and methylation in plasma was closely correlated with the *Sox17* methylation in tumor tissues ($r=0.688$, $P<0.001$; Table 1).

Association of *Sox17* Methylation Status in Breast Tissues and Plasma DNA With Clinicopathological Parameters of Breast Cancer Patients

Table 2 summarizes the association of *Sox17* methylation status in tumors and in plasma DNA with various clinicopathological parameters of breast cancer patients. The aberrant methylation status of *Sox17* in both cancer tissues and in plasma DNA was significantly associated with TNM stage III status and with lymph node metastasis ($P<0.05$). There were no statistically significant associations between *Sox17* promoter methylation status in both tumors and plasma samples with age, histological grade, tumor diameter, estrogen receptor status, progesterone receptor status, or HER2 status ($P>0.05$).

Sox17 Promoter Methylation and Survival in Patients With Breast Cancer

After a median follow-up period of 66 months (range 3–91 months), 45 of 155 (29.0%) patients relapsed, and 32 of 155 (20.6%) died of the disease. Relapse was detected in 33.6% (38/113) of patients with *Sox17* methylation in tumor tissues, and 24.8% (28/113) of patients with *Sox17* methylation in tumor

tissues died. Both of these rates were significantly higher than those of patients without *Sox17* methylation in tumor tissues ($P=0.039$ and 0.037 , respectively) (Table 3). Patients with methylated *Sox17* had worse DFS and OS than did those with nonmethylated *Sox17* (log-rank test, $P=0.033$ and 0.037 , respectively) (Figure 2).

In the paired plasma DNA, 40.0% (36/90) of patients with *Sox17* methylation experienced relapse and 28.9% (26/90) of patients with *Sox17* methylation died of breast cancer. In contrast, the rates of relapse and death were only 13.8% (9/65) and 9.2% (6/65), respectively, in those patients without *Sox17* methylation. Both the rate of relapse and death differed significantly between the patients with or without *Sox17* methylation in plasma DNA ($P=0.001$ and 0.003 , respectively) (Table 3). Worse DFS and OS were also observed for patients in the *Sox17* methylation group ($P<0.001$ and 0.002 , respectively) (Figure 3).

Univariate analysis indicated that TNM stage ($P<0.001$), histological grade ($P=0.030$), lymph node metastasis ($P<0.001$), and HER-2/neu status ($P=0.029$) were associated with a decreased DFS. TNM stage ($P<0.001$), lymph node status ($P<0.001$), and HER2 status ($P=0.005$) were significantly associated with worse OS (Table 4). Using a Cox regression model, all factors that determined to be significant in the univariate analysis were tested with multivariate analysis for association with DFS and OS. The results of this analysis are shown in Table 4. Multivariate analysis demonstrated that *Sox17* methylation (hazard ratio [HR] = 2.142; 95% confidence interval [CI]: 1.128–4.067; $P=0.020$), lymph node status (HR = 3.073; 95% CI: 1.099–8.594; $P=0.032$), and TNM stage (HR = 2.360; 95% CI: 1.145–4.865; $P=0.027$) were independently associated with a decreased DFS. *Sox17* methylation (HR = 4.737; 95% CI: 2.088–10.747; $P=0.001$), lymph node status (HR = 2.246; 95% CI: 1.049–4.811; $P=0.037$), and tumor stage (HR = 2.813; 95% CI: 1.145–4.865; $P=0.021$) were independently associated with a shorter OS. Similarly, results were also observed from *Sox17* promoter methylation in breast cancer tissues (Supplement Table 1, <http://links.lww.com/MD/A230>).

DISCUSSION

Although multiple epigenetic and genetic changes have been associated with breast cancer, the precise molecular mechanisms in breast cancer carcinogenesis and progression remain unknown. Therefore, sensitive and specific prognostic indicators that can reflect specific alterations in tumors are needed for use in clinical settings. Cell-free DNA in plasma is a type of blood-based biomarker; most of this DNA is released from cancer cells, and it can be used to glean important

TABLE 3. Incidence of Disease-Relapse and Disease-Related Death According to the Methylation Status of the *Sox17* Promoter

Breast Cancer	<i>Sox17</i> Methylation Status	Relapses (%)	P^*	Deaths (%)	P^*
Tumor tissues	M (n = 113)	38 (33.6%)	0.039	28 (24.8%)	0.037
	U (n = 42)	7 (16.7%)		4 (9.5%)	
Plasma DNA	M (n = 90)	36 (40.0%)	0.001	26 (28.9%)	0.003
	U (n = 65)	9 (13.8%)		6 (9.2%)	

DFS = disease-free survival, OS = overall survival.
* χ^2 test.

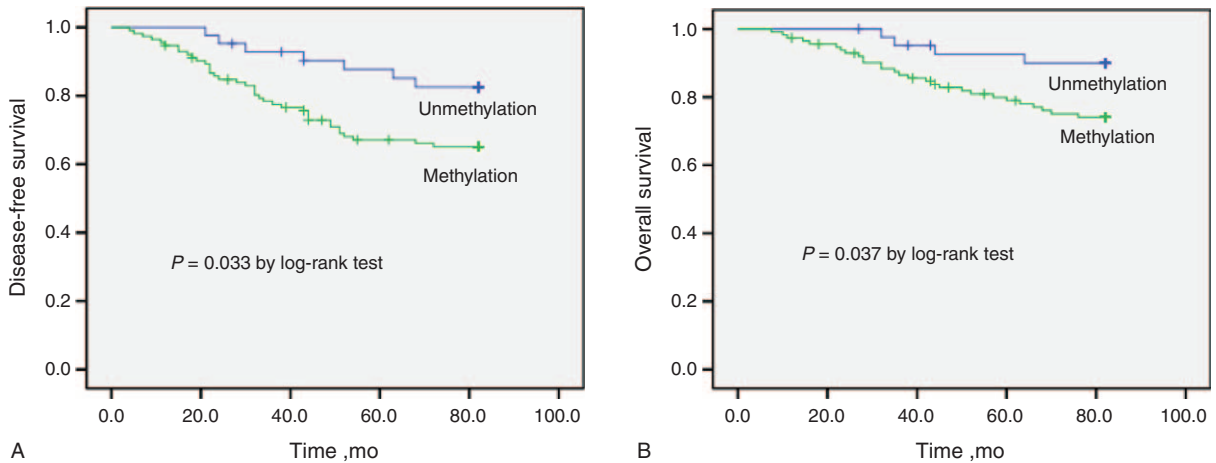


FIGURE 2. Kaplan–Meier estimates of (A) disease-free survival and (B) overall survival for breast cancer patients with or without *Sox17* promoter methylation in tumor tissues.

information about the tumor(s) that released it.^{44,45} Cell-free DNA in plasma is therefore considered to be a potentially useful noninvasive biomarker in breast cancer diagnosis and prognosis.

In a previous study, we found that the mRNA expression level of the *Sox17* gene was significantly decreased in both breast cancer cell lines and in the majority of breast cancer tissues, and observed that the expression level of *Sox17* was closely related to the methylation status of its promoter. Furthermore, *Sox17* methylation was found to be significantly related to breast cancer staging and lymph node metastasis.³⁴ In the present study, the *Sox17* promoter was methylated in 72.9% of cancer tissues and was not methylated in any of the control samples. This high rate of positivity in cancer tissues indicates that *Sox17* methylation may represent not only a frequent event in human breast cancer but may also be a useful marker in distinguishing malignant from nonmalignant breast lesions. The fact that *Sox17* methylation correlated with TNM stage and lymph node metastasis suggested that epigenetic silencing of *Sox17* may also accelerate the spread of cancer through

influencing the development of an invasive and biologically aggressive phenotype and thus expedite the progression of breast cancer.

Compared with tumor tissue, plasma samples are near ideal clinical specimen that are readily available, convenient, noninvasive, and can be sampled repeatedly over time. This study shows that *Sox17* promoter methylation was found in 58.1% of paired plasma samples, but not detected in any of the control plasma samples. Furthermore, we observed a high degree of consistency between *Sox17* methylation in plasma DNA and tumor tissue, suggesting that *Sox17* methylation in peripheral blood samples may be a good tumor marker for the diagnosis of breast cancer. Further analysis showed that aberrant *Sox17* promoter methylation in plasma DNA was associated with poor DFS and shorter OS, and it was an independent prognostic factor in breast cancer for both DFS and OS by analyzing with multivariate analysis. Thus, *Sox17* promoter methylation in plasma DNA is highly specific and can provide important prognostic information for patients with breast cancer.

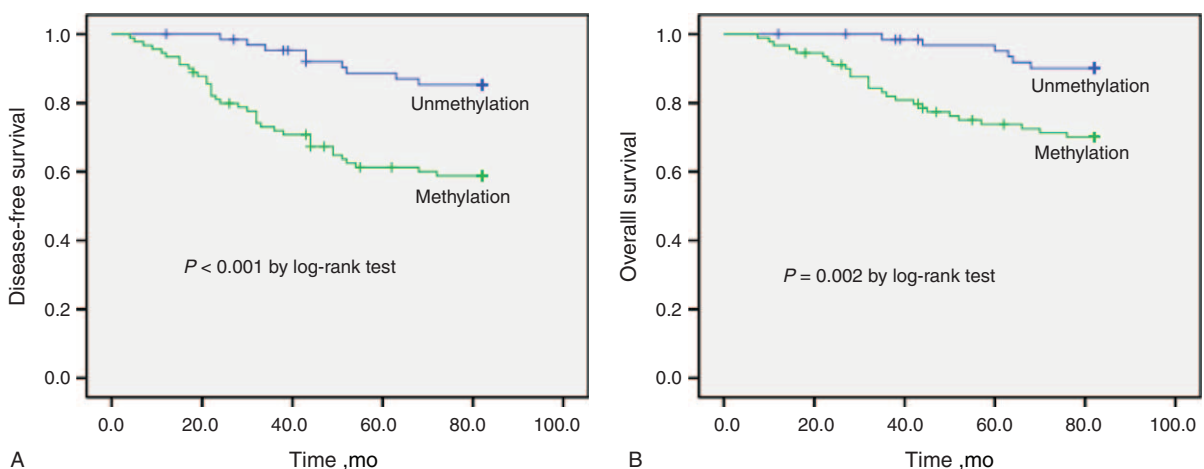


FIGURE 3. Kaplan–Meier estimates of (A) disease-free survival and (B) overall survival for breast cancer patients with or without *Sox17* promoter methylation in plasma DNA.

TABLE 4. *Sox17* Promoter Methylation in Plasma DNA of Patients With Breast Cancer: Univariate and Multivariate Analysis for DFS and OS

	Univariate Analysis*			Multivariate Analysis*		
	HR	95% CI	P	HR	95% CI	P
Disease-free survival						
Age (<50 vs ≥50 y)	0.925	0.461–1.859	0.828	2.158	1.030–4.517	0.041
Stage (III vs I–II)	7.238	3.253–16.104	0.000	2.360	1.145–4.865	0.027
Tumor grade (III vs I–II)	2.728	1.202–4.972	0.030	1.341	0.476–3.778	0.579
Tumor diameter (≥2 vs 0–2 cm)	1.977	0.938–4.170	0.071			
Lymph node status (negative vs positive)	7.194	3.208–16.137	0.000	3.073	1.099–8.594	0.032
ER status (negative vs positive)	1.495	0.730–3.062	0.272			
PR status (negative vs positive)	0.641	0.214–1.920	0.427			
HER-2/neu status (positive vs negative)	2.338	1.079–5.069	0.029	1.320	0.668–2.608	0.424
Methylation of <i>Sox17</i> (M vs U)	4.148	1.826–9.423	0.001	2.142	1.128–4.067	0.020
Overall survival						
Age (<50 vs ≥50 y)	1.236	0.567–2.694	0.691	3.008	1.295–6.989	0.010
Stage (III vs I–II)	5.203	2.261–11.974	0.000	2.813	1.171–6.753	0.021
Tumor grade (III vs I–II)	1.508	0.448–5.081	0.507			
Tumor diameter (≥2 vs 0–2 cm)	1.935	0.828–4.524	0.157			
Lymph node status (negative vs positive)	4.851	2.014–11.683	0.000	2.246	1.049–4.811	0.037
ER status (negative vs positive)	0.463	0.209–1.026	0.058			
PR status (negative vs positive)	1.908	0.865–4.206	0.109			
HER-2/neu status (positive vs negative)	3.382	1.471–7.775	0.005	2.148	0.997–4.630	0.151
Methylation of <i>Sox17</i> (M vs U)	3.994	1.536–10.387	0.003	4.737	2.088–10.747	0.001

CI = confidence interval, ER = estrogen receptor, HR = hazard ratio, M = methylation, PR = progesterone receptor, U = unmethylation.

* Cox-regression analysis.

Many studies have demonstrated that the *Sox17* gene can perform tumor suppression functions; it is known to be an important antagonist in the Wnt/ β -catenin signaling pathway.^{40–43} Thus, *Sox17* gene silencing due to promoter methylation may deactivate its tumor suppressor role and thereby contribute to poorer outcomes in breast cancer patients. The tumor suppressing function of nonmethylated *Sox17* may be partially maintained in patients without *Sox17* methylation, and this may slow the progress of tumor development. Furthermore, the occurrence of methylation of *Sox17* in plasma DNA provides additional information with clinical relevance. Namely, the situation is an indication that tumor cells may be circulating in blood.⁴⁶ In this light, detection of *Sox17* methylation in plasma DNA may also be useful in detecting the development of distant metastasis. MSP of *Sox17* methylation in plasma may therefore prove effective for the early detection of residual and/or recurrent tumors. Such detection could motivate successful salvage treatments for these patients in a timely manner. Moreover, *Sox17* methylation can be detected in some early-stage patients, which implies that it may be useful in the clinical application of screening and diagnosing breast cancer.

There were some limitations in this study. First, the MSP method is only a qualitative method to identify the presence of methylation and is thus not highly informative. Quantitative MSP, by contrast, is a highly sensitive assay that can detect 1 copy of the methylated gene among 10 000 unmethylated copies. This technology has the potential to screen hundreds to thousands of samples rapidly.⁴⁷ However, the main aim of this study was to identify whether or not *Sox17* promoter methylation in plasma DNA could be a useful and noninvasive biomarker for breast cancer diagnosis and prognosis.

Furthermore, MSP can be performed easily and economically in most clinical laboratories. Second, this was only a retrospective study and the number of cases in the study was limited. Further studies with larger sample sizes from multiple clinical centers will be needed to prove the clinical value of *Sox17* promoter methylation in plasma DNA.

In summary, our results clearly indicate that methylation of the *Sox17* promoter provides important prognostic information for breast cancer patients. *Sox17* methylation in plasma DNA can serve as a valuable noninvasive biomarker in breast cancer diagnosis and prognosis. This promising finding deserves further evaluation and validation in a larger patient cohort.

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