

Original Article

Running Title: TM6SF1 Methylation in Breast Cancer

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The Validation of Aberrant *TM6SF1* Gene Methylation in the Diagnosis of Breast Cancer Patients

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Abstract

Background: The limitations of breast cancer (BC) diagnosis and prognosis strategies are well-established. The present study aimed to evaluate transmembrane 6 superfamily member 1 (*TM6SF1*) DNA methylation as non-invasive biomarker for differentiating BC from non-cancer individuals (with benign breast diseases and healthy controls). Also, this study aimed to evaluate the association between *TM6SF1* methylation and clinicopathological BC features.

Method: A total of 200 women were enrolled in this retrospective study. The study participants were divided into three groups of 120 women with primary BC, 40 women with benign breast diseases, and 40 normal healthy controls. Blood sample were withdrawn and DNA was extracted. Then, *TM6SF1* DNA methylation level was detected using a quantitative polymerase chain reaction assay. Data were analyzed using GraphPad prism (version 6) and SPSS (version 20) programs. The statistical differences between the three groups were determined using ANOVA and Kruskal-Wallis tests. A *P* value < 0.05 was considered to be statistically significant.

Results: The study findings revealed that hyper methylation of *TM6SF1* level was reported higher in BC patients [82.2 (56.8-87.7)] as compared with benign [28.9 (27.9-64.8)] or healthy [27.8 (13-28.8)] controls at *P* = 0.0001. *TM6SF1* methylation had superior diagnostic efficacy as compared

with established BC markers [carcinoembryonic antigen (CEA) and cancer antigen (CA15.3)]. Also, methylation level showed significant difference among clinical stages, histological grading and lymph-node invasion.

Conclusion: The present study reveals that aberrant *TM6SF1* methylation is associated with BC tumorigenesis. It could facilitate early BC detection from benign breast diseases. Hypermethylated *TM6SF1* is associated with disease progression suggesting its potential role as diagnostic and prognostic biomarker for BC.

Keywords: Breast cancer; Early diagnosis; *TM6SF1*; DNA methylation; Circulating molecular markers

Introduction

Currently, breast cancer (BC) is the most frequent malignant tumor in females worldwide.¹ In developing countries, reduced survival rates are explained by insufficient early detection programs, leading to high percentage of women presenting with late-stage BC, along with delays related to treatment and the lack of adequate treatment and diagnosis facilities.^{2,3} Despite varied approaches that have the potential to detect BC, especially mammography, there is a challenge in early detection due to some significant limitations, including low sensitivity and high false positive rates.⁴ Therefore, there is an urgent need to identify and discover easy, non-invasive and specific tool that may improve BC detection.⁴

In normal tissues development, diseases and tumor development, epigenetic processes, including miRNAs, histone modifications and DNA methylation are important gene expression regulators.^{5,6} DNA methylation includes methyl group addition to cytosine pyrimidine ring in CpG island (dinucleotides) by DNA methyltransferases.⁷ In BC, differences in DNA methylation panels between normal and cancerous breast tissue have the potential to serve as prognostic and/or diagnostic approach.^{7,8}

On chromosome 15, transmembrane 6 superfamily member 1 (*TM6SF1*) was firstly isolated and characterized by Carim-

Todd et al.⁹ It encodes 370-amino acid protein with enhanced expression in peripheral blood leukocytes, testis and spleen.⁹ *TM6SF1* function is still unknown.¹⁰ In hepatitis B virus-related hepatocellular carcinomas, higher *TM6SF1* methylation levels were reported in single cancerous hepatocytes compared with adjacent and normal hepatocytes.¹¹ In both BC tissues and serum samples, *TM6SF1* gene promoter hypermethylation was reported.¹⁰ Other studies identified the role of *TM6SF1* in prognosis of acute myeloid leukemia (AML),¹² lung adenocarcinoma (LUAD).^{13,14} These studies demonstrated that the decrease in *TM6SF1* gene expression was associated with tumor progression. Hypermethylation may correspond to low gene expression and this may suggest that *TM6SF1* hypermethylation is associated with poor BC features and outcomes.

To date, most studies have evaluated small number of genes in BC.⁷ Very limited studies have focused on the association between *TM6SF1* promoter gene methylation and BC.^{10,15} Moreover, these studies did not take the tumor features and clinicopathological data into account including tumor subtypes, stage, grade, lymph node invasion, estrogen receptor, progesterone receptor and HER-2 protein status. Therefore, we aimed to investigate methylation patterns of *TM6SF1* promoter

gene methylation among Egyptian women with primary BC compared with other non-cancer individuals (with benign breast diseases and healthy controls). Also, we aimed to assess its diagnostic accuracy compared with established tumor markers (CEA and CA15.3) and to evaluate the association between these patterns and clinicopathological BC features.

Material and Methods

Patients and samples collection

After obtaining ethical approval from Institutional Research Board of Mansoura University and National Research Centre Medical Ethical Committee (ID: 15170), this hospital-based retrospective study was performed. The study participants were 120 women with newly diagnosed BC, 40 women with breast benign disorders and 40 healthy women. The participants were pathologically, radiologically and clinically diagnosed at Mansoura University Oncology Center, Mansoura, Egypt during Feb-Aug/2021 with primary BC. The exclusion criteria were patients with other malignancies or received any type of therapy, either chemo- or radio-therapy. The medical reports of BC patients were reviewed and their clinicopathological data including age, Tumor, Node, Metastasis (TNM) stage,¹⁶ histological grade, progesterone and estrogen receptors status and HER-2 expression were obtained. From each participant, before any surgical/medicinal interventions, venous blood (3 mL) was collected into sterile plain tubes. Serum was separated after centrifugation and stored for measuring tumor markers as well as quantifying *TM6SF1* promoter gene methylation.

Extraction of DNA

According to manufacturer's recommendations, DNA was extracted using commercial DNA Min kit (Cat 51104, Qiagen, Hilden, Germany) based on spin column. Before further investigation, extracted DNA concentration and purity was

detected using Nano-drop spectrophotometer (Quawell, Scribner, USA) then stored at – 20°C till further analysis.

TM6SF1 methylation pattern

TM6SF1 methylation pattern was detected using EpiTect Methyl II quantitative polymerase chain reaction (qPCR) System (Qiagen, Germany). In phase I, four genomic DNA equal aliquots were subjected to four different tubes, mock (M0), methylation-sensitive dependent (Msd), methylation-dependent (Md) and methylation-sensitive (Ms) enzymes. Using thermal cycler (SureCycler 8800, Agilent, Santa Clara, CA, USA), all reactions tubes were incubated for six hours at 37°C and for 20 minutes at 65°C. In phase II, using Max3005P QPCR system (Stratagene, Agilent Technologies, CA, USA), the enzyme reactions were mixed directly with qPCR master mix (RT2 qPCR SYBR Green/ROX Master Mix) and were dispensed into PCR plate containing pre-aliquoted primer mixes (Cat EPHS104761-1A, EpiTect Methyl II qPCR Primer Assay). PCR cycling conditions were 95°C for ten minutes (1 cycle), 99 °C for 30 second and 72°C for 1 minute (3 cycles), and finally 97°C for 15 second and 72°C for 1 minute (40 cycles). Unmethylated human sperm DNA and fully methylated *SssI* methylase treated MDA-MB-231 gDNA were included as controls. The relative amount of unmethylated and methylated DNA fractions were automatically calculated by pasting raw Δ CT values into pre-performed data analysis spreadsheet (EpiTect Methyl II PCR Array Microsoft Excel based data analysis template).

Serum tumor markers

After diagnosis and sample collection, all participants were screened for CA 15.3 and CEA using commercial enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical analyses

Data were analyzed using GraphPad prism and SPSS (SPSS, Inc., Chicago, USA) programs. Based on variable normality distribution, data were expressed as mean \pm SD or median (interquartile range), appropriately. Qualitative variables were represented as absolute numbers. The statistically significant differences were determined using ANOVA and Kruskal-Wallis for normally and non-normally distributed values and Fisher's LSD as post-hoc test, respectively. Two-tailed P value <0.05 was considered to be statistically significant. To identify the diagnostic power for the investigated gene, receiver operating characteristic (ROC) curve was constructed among studied groups.¹⁷ Spearman's rank correlation was assessed for determining the association between different parameters and the target gene methylation degree.

Results

Characteristics of the study population

The data of the study participants (patients and controls) are summarized in Table 1. At the time of diagnosis, there was no significant difference in age between the three groups. Benign breast diseases were pathologically divided into fibrocystic changes, intraductal papillomatosis and follicular hyperplasia. Most of included women were premenopausal. Tumor invasion, depth, histological grade, lymph node invasion, estrogen and progesterone receptors status and detection of HER-2 protein are summarized in Table 1.

TM6SF1 DNA methylation in BC

As compared with CEA and CA15.3 (Table 1), *TM6SF1* methylation degree was significantly ($P=0.0001$) associated with BC patients. Given cancer-specificity, BC [82.2 (56.8-87.7)] samples displayed significantly ($P=0.0001$) higher *TM6SF1* methylation degree compared with benign [28.9 (27.9-

64.8)] and healthy [27.8 (13-28.8)] controls (Figure 1A).

Diagnostic performances

ROC curve analysis was performed to determine the diagnostic ability and the optimal cutoff value was assessed for *TM6SF1* DNA methylation degree. In contrast to CEA (area under curve (AUC) = 0.538; $P = 0.467$) and CA 15-3 (AUC = 0.686; $P = 0.001$) (Table 2), *TM6SF1* DNA methylation had superior (AUC = 0.893; $P = 0.0001$; Figure 1B) diagnostic power for differentiating BC from all non-cancers (benign and healthy combined). This power did not significantly change when comparing BC to only benign breast disease (Figure 1C) indicating cancer-specification of *TM6SF1* DNA methylation. Moreover, this ability rises to AUC = 1.00 ($P = 0.0001$) when comparing BC with only healthy females (Figure 1D). In the detection of early BC stages and grades, the performance of *TM6SF1* DNA methylation reported to be significant (Table 2).

TM6SF1 methylation correlated with disease progression

High percentage of *TM6SF1* DNA methylation were associated with tumor late stages (Figure 2A), high grades (Figure 2B) and lymph-node invasion (Figure 2C) which is independent of hormonal status and HER-2 expression (Figure 2D-F). Also, *TM6SF1* DNA hypermethylation was significantly correlated with the increase in CA-15.3 and tumor stages and grades (Table 3).

Discussion

In this study, *TM6SF1* methylation was significantly ($P = 0.0001$) associated with BC [82.2 (56.8-87.7)] as it displayed higher methylation degree compared with benign [28.9 (27.9-64.8)] and healthy [27.8 (13-28.8)] controls. In contrast to CEA (AUC = 0.538; $P = 0.467$) and CA 15-3 (AUC = 0.686; $P = 0.001$), *TM6SF1* DNA methylation had superior (AUC = 0.893; $P =$

0.0001) BC diagnostic ability that rises to AUC = 1.00 ($P = 0.0001$) when comparing BC with only healthy females. These good performances did not significantly alter in the detection of early BC stages (AUC = 0.884) and grades (AUC = 0.882).

Although gene methylation profiles in blood are found to be related to BC incidence, they have not been frequently used in BC risk assessment.¹⁸ In different studies, genome-wide CpG island methylation analysis implicated *TM6SF1* promoter methylation in the pathogenesis different tumors including renal cell,¹⁹ gastric²⁰ and breast¹⁰ carcinomas. Using semi-quantitative methylation-specific PCR, de Groot et al. determined DNA promoter methylation status of 19 selected genes.¹⁰ In comparison with normal breast tissues, they found that absolute methylation levels of *TM6SF1* gene were higher in BC tissues. Among these genes, they concluded that only *TM6SF1* and *AKR1B1* may serve as candidate methylation BC biomarkers and they recommended its evaluation in different body fluids including blood.¹⁰ In metastatic BC sera and after careful filtration process, *TM6SF1* was included as one of hypermethylated genes panel that were frequently and specifically methylated in BC.¹⁵ Given other tumors, Kranenbarg et al. recently found, compared with normal colon tissues, that *TM6SF1* DNA displayed differential methylation in colon adenocarcinoma.²¹

Independent of hormonal status and HER-2 expression, we found that high percentage of *TM6SF1* methylation were associated with and significantly correlated to tumor late stages, high grades, lymph-node invasion and elevated CA-15.3 levels. To predict survival outcomes in metastatic BC, Visvanathan et al. examined the ability of a panel of cell-free DNA methylation markers. After testing ten genes, a cumulative methylation index (CMI) was generated on

the basis of 6 genes including *TM6SF1*.²² Median progression-free survival (PFS) and overall survival (OS) were significantly longer in patients with a low CMI (PFS, 5.8 months; OS, 21.7 months) compared with a high CMI (PFS, 2.1 months; OS, 12.3 months).²² A high versus low CMI was independently related to worse OS (hazard ratio, 1.75; 95% CI, 1.21 to 2.54; $P = 0.003$) and PFS (hazard ratio, 1.79; 95% CI, 1.23 to 2.60; $P = 0.002$). They concluded that CMI (including *TM6SF1* methylation) was a strong predictor of PFS, even in the presence of circulating tumor cells and thus it is a strong survival outcomes predictor in metastatic BC and may have clinical usefulness in disease monitoring and risk stratification.²² This is suggesting *TM6SF1* methylation potential prognostic use in BC which may need further investigations.

Although the exact role of *TM6SF1* hypermethylation (down-regulation) in modulating the tumor immune microenvironment of BC remains unclear, the results obtained by some recent studies suggested some potential mechanisms related to *TM6SF1* great protective impact against such tumors.^{12,13} Zhang et al. investigated the mechanistic role of such genes in hepatocellular carcinoma (HCC). In orthotopic mouse and transgenic mice models, they found that hepatocyte-specific *TM6SF2* knockout stimulated HCC development.²³ An opposite effect obtained by *TM6SF2* overexpression. T cell proliferation and cytotoxic CD8⁺ T cells were reduced in *TM6SF2* knockout mice.²³ Mechanistically, *TM6SF2* inhibited the nuclear factor kappa-B (NF- κ B) signaling pathway by its direct binding to inhibitor of NF- κ B subunit beta (IKK β), which in turn, decreased IL-6 releasing and activating CD8⁺ T cells. In the same line, CD8⁺ T cells depletion abolished *TM6SF2* tumor-inhibitory effect.²³ Another interesting study by Huang et al. demonstrated that *TM6SF1*

inhibits M2 macrophage polarization and LUAD progression by suppressing the PI3K/AKT/mTOR pathway.¹³ In LUAD cells, *TM6SF1* overexpression inhibited M2 macrophages polarization, epithelial–mesenchymal transition (EMT), migration, invasion, proliferation and cell viability.¹³ *TM6SF1* was correlated with the tumor microenvironment as its overexpression decreased expression levels of AKT, mTOR, p-mTOR, p-AKT and p-PI3K. PI3K activator in LUAD cells reversed *TM6SF1* effects.¹³ Such further well-designed studies are recommended and needed to clarify the underlying mechanisms of hypermethylated *TM6SF1* and BC development and progression.

The methylation of *M6SF1* in this study affords comparable diagnostic performance (AUC = 0.893) with other methylation markers, including good sensitivity (95%) and specificity (81.3%) in BC detection, which supports *M6SF1* clinical potential for BC diagnosis. In serum circulating free DNA (cfDNA), Shan et al. examined the promoter methylation of 6 genes (*RASSF1a*, *PCDHGB7*, *HOXD13*, *hMLH1*, *P16* and *SFN*). This 6-gene methylation panel revealed specificity of 78.1% and sensitivity of 82.4% in BC diagnosis compared benign disease controls.²⁴ In comparison with benign tissues, Downs et al. selected 10 from 25 gene methylation markers that were highly methylated in BC. They found that this panel revealed great diagnostic utility (AUC = 0.937) with achieved specificity of 88% and sensitivity of 87%.²⁵ Fackler et al. developed a quantitative multiplex methylation-specific PCR technique using a panel of 9 genes that revealed a specificity of 83% and a sensitivity of 71% in BC detection.²⁶ In a meta-analysis focused on diagnostic power of *RASSF1A* methylation in BC, they reported AUC of 0.89, 0.64 sensitivity and 0.95 specificity

using methylation-specific PCR (MSP) method.²⁷

The retrospective nature of the study and focusing on a single-centered cohort are the main limitations of the present study. Moreover, studies that include bigger sample size and more BC patients with early disease stage would be more informative. Therefore, future more multicentric comprehensive studies are urgently required to evaluate the specific role of *TM6SF1* DNA methylation in the pathogenesis of BC.

Conclusion

The study results reveal that aberrant *TM6SF1* DNA methylation is associated with BC development. High methylation percentages could accurately predict BC from benign breast diseases. Therefore, it could facilitate early BC screening so as to give timely treatments.

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Authors' Contributions

ME: Study design; BMF: Experimental work; MS: Study design, experimental work, data development; AA: Pathological diagnosis, samples collection; MAA: Study design, data development, statistical analysis. All authors read and approved the final manuscript.

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Conflict of Interest

None declared.

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Table 1. Characteristics of breast cancer patients and patients with benign disorders and healthy controls

Parameter	Breast cancer	Benign	Healthy	P value
Number	120	40	40	—
Age (years)	52.41 ± 9.20	52.04 ± 9.31	51.51 ± 8.91	0.920
Menopause (pre-/post-menopausal)	75/45	27/13	26/14	0.915
CEA (ng/mL)	12.00 (8-16.7)	14.21 (8.8-15.3)	8.02 (5.5-10.8)	0.015
CA-15.3 (U/mL)	22.10 (14-24)	17.02 (12.8-22)	12.02 (11-14.7)	0.002
Methylated <i>TM6SF1</i>	82.21 (56.8-87.7)	28.90 (27.9-64.8)	27.83 (13-28.8)	0.0001
Tumor invasion (in situ/invasive)	48/72	—	—	—

Tumor T stage (T≤2/T>2)	51/69	—	—	—
Histological grade (G1/G2-3)	45/75	—	—	—
Lymph node invasion	59/61	—	—	—
(negative/positive)		—	—	—
Estrogen receptor (negative/positive)	60/60	—	—	—
Progesterone receptor	42/78	—	—	—
(negative/positive)		—	—	—
HER-2 (negative/positive)	42/78	—	—	—

Differences between groups were established by ANOVA test or chi-squared (X^2) test appropriately. $P < 0.05$ is significant. CEA: Carcinoembryonic antigen; CA15.3: Cancer antigen; *TM6SF1*: Transmembrane 6 superfamily member 1; HER-2: Human epidermal growth factor receptor 2

Table 2. Diagnostic performance of different markers to discriminate breast cancer patients from controls to discriminate patients with early stages and grades from controls

Marker	AUC (95% CI)	P value	Cutoff	Sen. (%)	Sp. (%)	PPV (%)	NPV (%)	Accuracy (%)
Breast cancer vs. all non-cancers								
CEA	0.538 0.44-0.64	0.467	>9	70.00	40.00	63.60	47.10	58.00
CA-15.3	0.686 0.60-0.78	<0.001	>13	75.00	47.50	68.20	55.90	64.00
TM6SF1 methylation	0.893 0.82-0.96	<0.0001	>49	95.00	81.30	88.40	91.50	89.50
Breast cancer vs. healthy females								
CEA	0.669 0.56-0.78	0.020	>9	70.00	65.00	85.70	41.90	68.75
CA-15.3	0.686 0.60-0.78	<0.001	>13	75.00	65.00	86.50	46.40	72.50
TM6SF1 methylation	1.00 1.0-1.0	<0.0001	>49	95.00	100	100	87.00	96.25
Early stages from all non-cancers								
CEA	0.469 0.33-0.60	0.632	>9	58.80	40.00	38.50	60.40	47.33
CA-15.3	0.671 0.58-0.76	0.003	>13	68.60	47.50	45.50	70.40	55.73
TM6SF1 methylation	0.884 0.81-0.96	<0.0001	>49	90.20	81.30	75.40	92.90	84.73
Low grades from all non-cancers								
CEA	0.418 0.28-0.56	0.222	>9	60.00	40.00	36.00	64.00	47.20
CA-15.3	0.675 0.58-0.77	0.003	>13	68.90	47.50	42.50	73.10	55.20
TM6SF1 methylation	0.882 0.81-0.96	<0.0001	>49	88.90	81.30	72.70	92.90	84.00

AUC: Area under curve; Sen.: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; CEA: Carcinoembryonic antigen; CA15.3: Cancer antigen; TM6SF1: Transmembrane 6 superfamily member 1

Table 3. Correlation between methylated *TM6SF1* and other parameters

Factor correlated with <i>TM6SF1</i>	Correlation coefficient (<i>r</i>)	<i>P</i> value
Age	-0.03	0.736
Menopause	-0.02	0.885
CEA	0.13	0.143
CA-15.3	0.318	0.0001
Tumor stage	0.198	0.046
Tumor grade	0.216	0.045
Lymph node	0.183	0.105
Estrogen receptor	0.075	0.511
Progesterone receptor	0.156	0.167
HER-2	0.110	0.329

Pearson correlation was used for variables with interval scale, while Spearman correlation was used for variables with ordinal scales. CEA: Carcinoembryonic antigen; CA15.3: Cancer antigen; *TM6SF1*: Transmembrane 6 superfamily member 1; HER-2: Human epidermal growth factor receptor 2

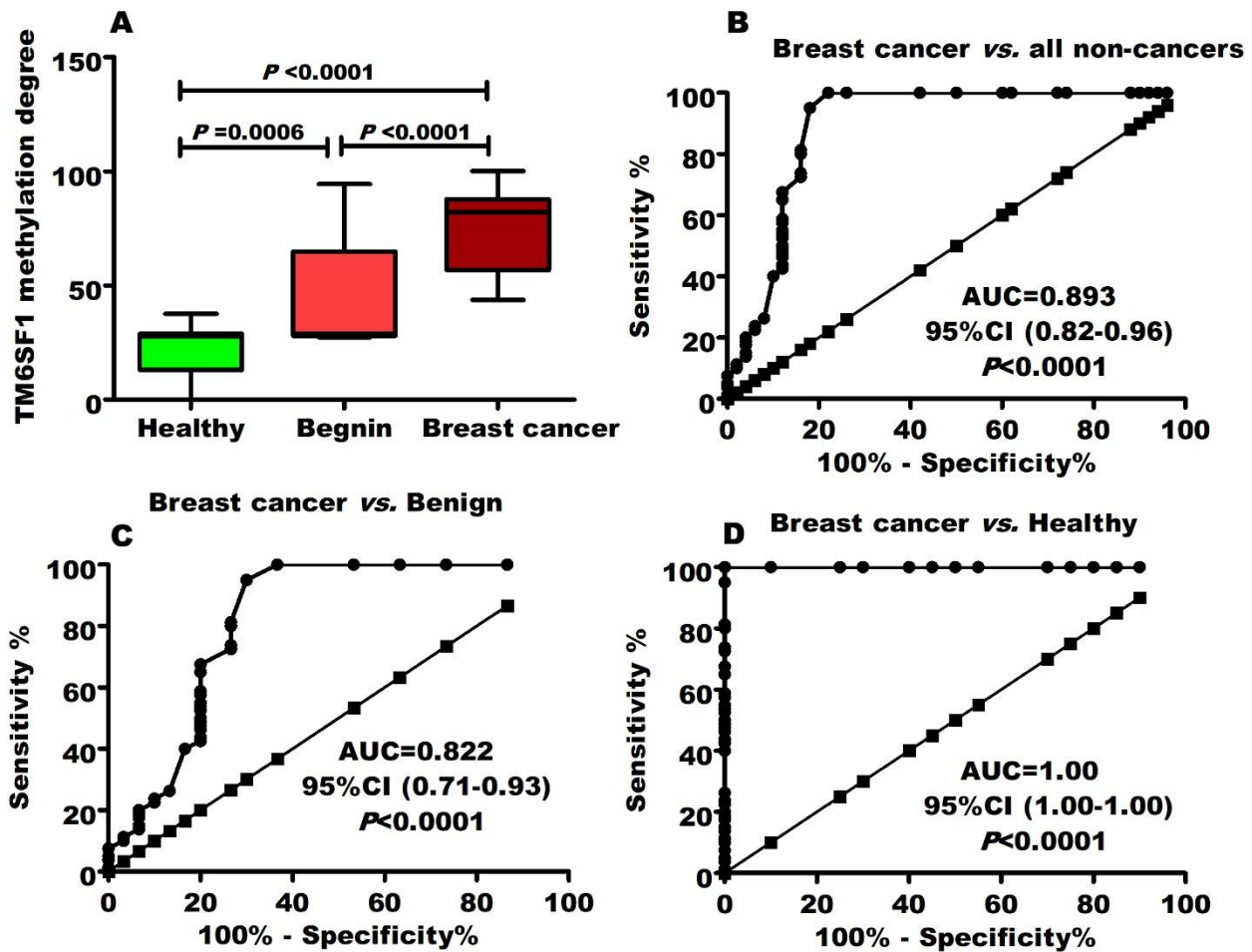


Figure 1. *TM6SF1* DNA methylation in breast cancer. (A) Breast cancer patients were significantly associated with hypermethylated *TM6SF1*. ROC curve of *TM6SF1* DNA methylation for separating breast cancer patients from (B) all non-cancer individuals (benign, healthy combined), (C) benign diseases and (D) healthy controls revealed good diagnostic abilities.

TM6SF1: Transmembrane 6 superfamily member 1; AUC: Area under curve; ROC: Receiver operating characteristic; CI: Confidence interval

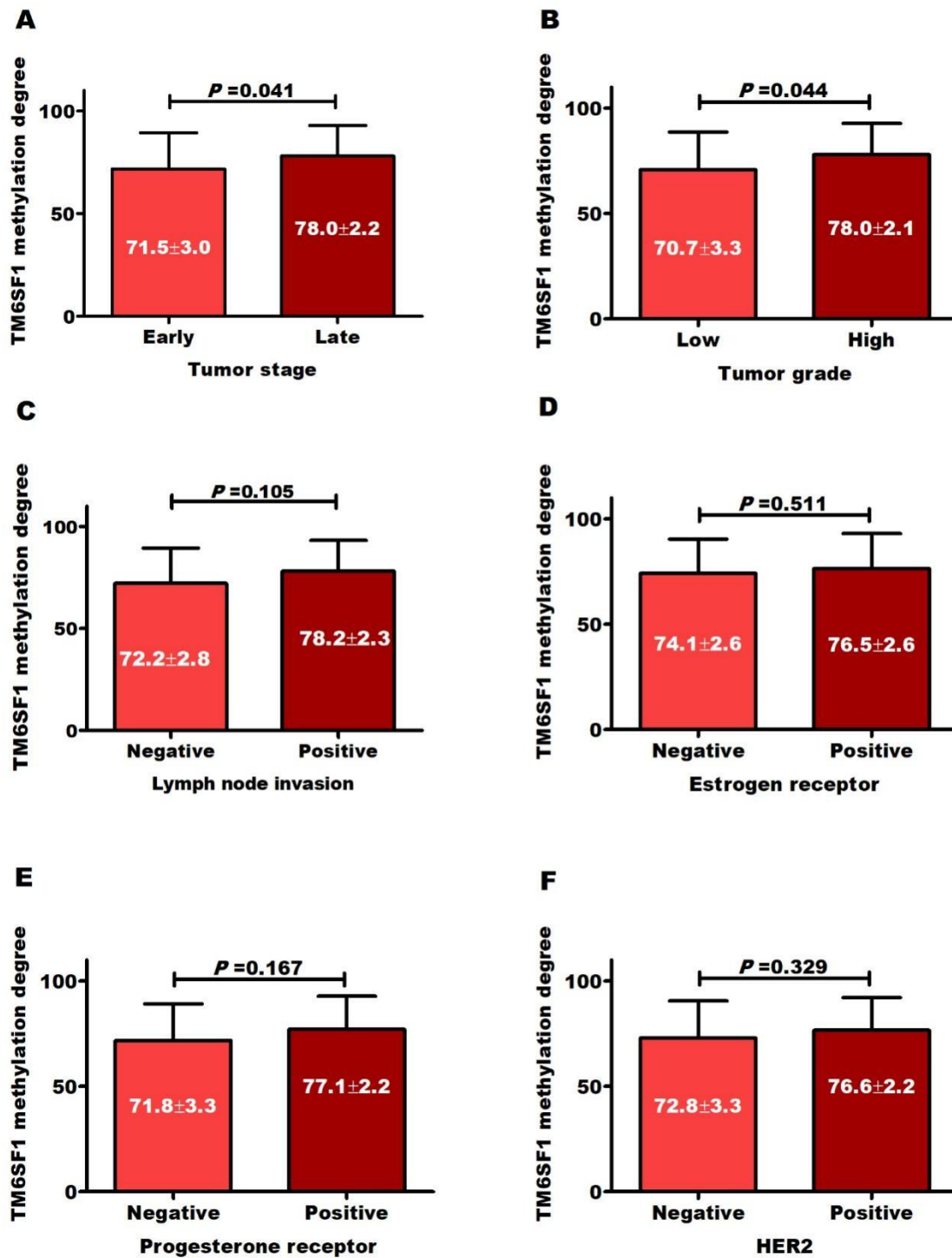


Figure 2. Distribution of *TM6SF1* methylation degree according to tumor (A) stages, (B) grades, (C) lymph node invasion, (D) estrogen and (E) progesterone receptors, and (f) HER-2 expression. *TM6SF1*: Transmembrane 6 superfamily member 1; HER-2: Human epidermal growth factor receptor 2