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ORIGINAL RESEARCH

The Clinical Significance of *PPEF1* as a Promising Biomarker and Its Potential Mechanism in Breast Cancer

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Background: Breast cancer (BC) is the leading cause of malignancy death in females worldwide. While intense efforts have been made to elucidate the pathogeny, the molecular mechanism of BC remains elusive. Thus, this study aimed to investigate the role of *PPEF1* in the progression of BC and further explore the better clinical significance.

Methods: The diagnostic and prognostic values of elevated *PPEF1* expression in BC were unveiled via public databases analysis. In addition, Gene Ontology (GO), Gene Set Enrichment Analysis (GSEA) and Protein–protein interaction (PPI) analysis were performed to explore the potential functions and molecular mechanisms of *PPEF1* in BC progression. Experimentally, transwell and CCK-8 assays were carried out to estimate the effects of *PPEF1* on the BC metastasis. Meanwhile, the differential expressions of *PPEF1* in paraffin-embedded tissues and serum samples were, respectively, analyzed by Immunohistochemical (IHC) analysis and enzyme-linked immunosorbent assay (ELISA) kit.

Results: The transcriptional levels of *PPEF1* were higher in BC than in normal breast tissues or adjacent normal tissues. Moreover, survival analysis revealed that higher *PPEF1* expression was negatively associated with overall survival (OS), all events-free (AE-free) and metastatic recurrence-free (MR-free) survival, and further was an independent risk factor of unfavorable prognosis in BC patients. Additionally, the present study provided the first evidence that *PPEF1* participated in multiple biological processes and underly signaling pathways involving in tumorigenesis and development of BC. Furthermore, *PPEF1* promotes the BC progression and can be used as a noninvasive diagnostic marker. Noteworthy, the combined determination of serum *PPEF1* and traditional tumor markers can enhance diagnostic accuracy thus is of vital importance in the early diagnosis of BC.

Conclusion: *PPEF1* exerted a tumorigenic role and involved in molecular mechanism of tumorigenesis in BC which served as a promising biomarker for prognosis and diagnosis.

Keywords: *PPEF1*, breast cancer, biomarker, molecular function, signaling pathway

Introduction

Breast cancer (BC) is the most common malignancy with high morbidity in females and remains a major cause of cancer-associated mortality worldwide.[1,2] The risk factors for BC have been indicated to be associated with the complex effects involving heterogeneity of reproductive, hormonal, obesity, drinking and many other potential factors.[3,4] In recent years, research on BC has progressed in advancement of surgical techniques, development of anticancer drugs, and targeted therapeutic strategies, but the long-term survival of patients is still very poor due to

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frequent recurrence and metastasis.[5,6] Therefore, there is an urgent need to identify new diagnostic biomarkers and/or therapeutic targets for the BC management.

PPEF1 gene encoding protein serine/threonine protein phosphatase was localized on chromosome Xp22, which was shown to be associated with excessive cell proliferation, cell growth and signal transduction.[7–11] In addition, *PPEF1* overexpression increased the tumorigenic growth of A549 cells, suggesting that *PPEF1* can act as an oncogene in lung cancer development by preventing cancer cell death.[12] A recent research has also shown that *PPEF1* could be a potential target for diagnosis or therapy of lymphoma.[7] However, the role of *PPEF1* in BC has not been previously reported.

In the present study, through bioinformatics analyses of Oncomine, GEPIA and TCGA database, we revealed that *PPEF1* was elevated in BC tissues compared with normal counterparts. Meanwhile, *PPEF1* overexpression was proved to be an independent prognostic factor and a good diagnostic index for patients with BC. Furthermore, we explored the potential functions, signal pathways and co-expression genes network of *PPEF1* in BC using GO, GSEA and PPI analysis, which suggested the potential molecular mechanisms underlying the oncogenic activity of *PPEF1*. And lastly, we provide the convincing experimental evidence supporting a rather definitive role of *PPEF1* in metastasis of BC as well as the vital clinical implications. This is the first research to identify key genes and pathways related with *PPEF1* which were in the occurrence of BC and explored the potential biomarker for prognosis, diagnosis and drug targets.

Materials and Methods

Oncomine Database Analysis

The expression levels of *PPEF1* gene in the selected cancers were analyzed using Oncomine database (https://www.oncomine.org/). In order to reduce our false discovery rate, we selected $P < 0.0001$, Fold Change > 2 and gene rank with the top 10% as a threshold.[13,14]

GEPIA Database Analysis

GEPIA (http://gepia.cancer-pku.cn/) is a visualization site based on GTEx and TCGA data integration.[15] Searching for keywords such as 'PPEF1' and "BRCA" in GEPIA to retrieve differential expression data for *PPEF1* in BC and normal breast tissue.

Analysis of Breast Cancer Data in TCGA Public Database

The breast cancer dataset, including mRNA expression and overall survival times, was obtained from TCGA (https://portal.gdc.cancer.gov/). The original data from TCGA was normalized and analyzed by the edgeR analysis method.

The Prognostic and Diagnostic Analysis of *PPEF1*

Kaplan-Meier analysis of TCGA-BRCA data was used to analyze the 10-year overall survival of BC patients. Log-rank test was used to calculate *P* value.[16] Additionally, online analysis database bc-GenExMiner v4.2 including 36 annotated genomic datasets (http://bcgenex.centregauducheau.fr/) was used to assess the association between *PPEF1* and survival of BC patients.[17,18]

Gene Ontology Function Enrichment Analysis of *PPEF1*

After normalizing the TCGA-BRCA dataset, we distinguished between *PPEF1* high group and *PPEF1* low group according to the mRNA expression level. The differences of GO functional enrichment between the two groups were obtained by Gene Set Variation Analysis (GSVA).[19]

Gene Set Enrichment Analysis of *PPEF1*

GSEA was performed to annotate the Hallmark effector gene sets and the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway associated with *PPEF1* mRNA expression in the TCGA-BRCA dataset.[20] GSEA software was obtained from the Broad Institute (http://www.broad.mit.edu/gsea).

Screening Co-Expressed Genes of *PPEF1*

Co-expressed genes of *PPEF1* in BC were analyzed from TCGA-BRCA expression data. Using Pearson correlation analysis, the correlation coefficient ≥ 0.4 was used as the threshold to find the genes associated with higher levels of *PPEF1*.

Construction of Protein Interaction Network of *PPEF1* Co-Expressed Genes

PPI network was constructed for *PPEF1* co-expressed genes using the STRING v11.0 database (https://string-db.org/), and the protein interaction score ≥ 0.4, ie,

medium and above reliability. To further analyze and screen the core genes in the PPI network.[21]

Cell Culture

MCF 10A, MCF7 and SK-BR-3 cells lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MCF7 and SK-BR-3 were cultured in DMEM high glucose medium (Hyclone, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA). MCF 10A were maintained in special culture medium (Procell, China).

RNA Extraction and RT-qPCR Analysis

RNA was extracted by TRIZOL (Takara, Japan) and reverse-transcribed into cDNA using PrimeScript RT reagent Kit (Takara, Japan). RT-PCR was conducted using SYBR Premix Ex Taq Kit (Takara, Japan) according to the manufacturer's protocol with the following primers. PPEF1: forward, 5′-GAAAGCGAACAGGACATGAGGGATAG-3′ and reverse, 5′-GTGAGAGGAAATTGTAGCCGAGGAC-3′; TBP: forward, 5′-CCGGAATCCCTATCTTTAGTCC-3′ and reverse, 5′-GCCTTTGTTGCTCTTCCAAAAT-3′.

siRNA transient interference assay

The siRNAs were purchased from GeneBio (GeneBio, China). 1×10^6 MCF7 and SK-BR-3 cells were, respectively, seeded in each well of a six-well culture plate (KIRGEN, USA). 7.5μL of siRNA combined with 7.5μL of EndoFectin^{TM}-Max transfection reagent (GeneCopoeia, USA) were diluted in 250μL MEM medium (Gibco, USA) respectively, and incubated for 5 min. The mixture was then used for transient interference assay following the manufacturer's protocol. The sequences of siRNAs are as follows:

5′-UUCUCCGAACGUGUCACGUTT-3′ (siN.C.)
5′-GCAUUAGUACCUACAUAUUTT-3′ (siPPEF1)

Cell Migration and Invasion Assays

Transwell chamber with a pore size of 8μm (Corning, USA) was used to perform cell invasion assay with Matrigel (100 μL, 1:8 dilution in serum-free medium) (Corning, USA) and migration assay without Matrigel. 5×10^4 MCF7 or SK-BR-3 cells in 200 μL serum-free medium were seeded into the upper chamber and 600 μL medium with 10% FBS was added in lower chamber. After 24 h of incubation, cells on the upper surface of the membrane were removed with a cotton swab and cells on the lower surface of the inserts were fixed with methyl alcohol and stained with 0.1% crystal violet (Solarbio, China). Then, the stained cells were observed under light microscopy and 10 visual fields of each insert were randomly selected.

CCK-8 Cell Proliferation Assay

Cellular proliferation was determined with the cell counting kit-8 (CCK-8) assay (Beyotime, China). MCF7 or SK-BR-3 cells (2×10^3/well) were seeded in 96-well plates (KIRGEN, USA). The absorbance of each well at 450nm was measured with an EnSpire multimode microplate reader (PerkinElmer, Singapore). Cell proliferation activity was examined for successive 6 days.

Immunohistochemical (IHC) Analysis

Paraffin-embedded BC tissues were obtained from the Pathology Department of the Affiliated Hospital of Southwest Medical University. Paraffin-embedded tissue slides were dewaxed and placed in normal goat serum (ZSGB-BIO, China) for nonspecific staining treatment, followed by incubation with the primary antibody anti-PPEF1 (1:250) (GeneTex, USA) overnight at 4°C. After that, the slides were treated with biotinylated secondary antibody and next with horseradish peroxidase-conjugated streptavidin complex (ZSGB-BIO, China). Finally, there were incubated with dimethylbenzidine and counterstained with hematoxylin. Immunostaining was expressed as the percentage of stained cells out of total number of tumor cells, and assigned to one of five categories: 0, <5%; 1, 5% ~ 25%; 2, 25% ~ 50%; 3, 50% ~ 75% and 4, >75%. The intensity of immunostaining was scored as: 1 weak (+); 2 moderate (++); 3 intense (+++). The two scores were multiplied and the product was defined as immunohistochemical score. The assessment of immunostaining was done without knowing results of other experiments.

Clinical Samples

A total of 114 serum samples were collected for *PPEF1* analysis, among which 57 cases were healthy control and 57 cases were BC patients without therapy, respectively. All the clinical samples were obtained from the Laboratory Medicine Department of the Affiliated Hospital of Southwest Medical University. Patient Consent Forms were obtained according to protocols approved by the Institutional Review Board of the Affiliated Hospital of Southwest Medical University. Moreover, this study obtained written informed consent from the participants. *PPEF1* were detected by commercial enzyme-linked immunosorbent assay (ELISA) kit according to the

instructions (Zeye Bio, China). CEA, CA125 and CA153 in serum samples were detected by Roche Cobas e601 Electrochemiluminescence System (Roche, Switzerland).

Statistical Analysis

Student's independent *t* test was utilized for normal distribution variables. Mann–Whitney *U*-test and K–S test were utilized for non-normally distributed variables. Data were analyzed by GraphPad Prism software and presented as mean ± SEM indicated in figure legends. Differences were considered statistically significant when $P < 0.05$.

Results

The Clinicopathologic Importance of High *PPEF1* Expression in BC

We first compared the transcriptional levels of *PPEF1* in 20 types of cancers with their normal counterparts by using Oncomine database. As shown in Figure 1A, the *PPEF1* expression was markedly upregulated only in two types of cancers, breast cancer and colorectal cancer. Further analysis revealed that *PPEF1* mRNA expression was significantly increased in 5 types of BC, such as breast carcinoma in situ stroma, invasive breast carcinoma,

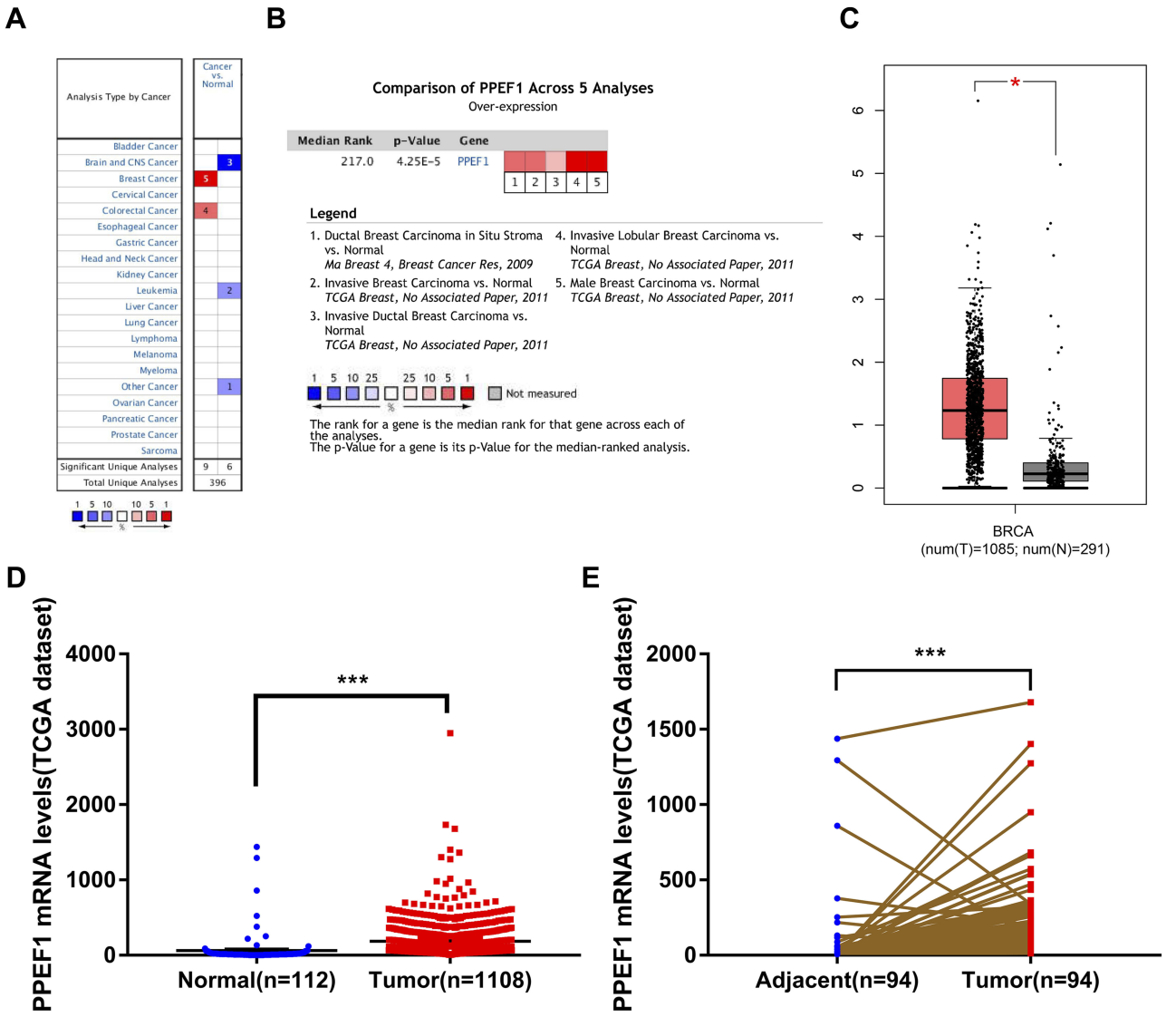

Figure 1 *PPEF1* expression is increased in human breast cancer tissues. (**A**) Oncomine analysis of *PPEF1* expression in human cancers. (**B**) The differential expression of *PPEF1* between tumor and normal tissues in multiple types of breast cancer. (**C**) Boxplot of *PPEF1* expression in BC tissues vs non-cancerous tissues from GEPIA analysis. (**D, E**) *PPEF1* expression level in BC tissues is higher than that in the normal or pericarcinomatous tissues. The mRNA level of *PPEF1* in unmatched BC and matched BC were download from TCGA datasets. *: $P < 0.05$, ***: $P < 0.001$.

invasive ductal breast carcinoma, invasive lobular breast carcinoma and male breast carcinoma (Figure 1B). To verify the findings of the Oncomine analysis, we conducted *PPEF1* expression analysis using the GEPIA database and TCGA-BRCA datasets. *PPEF1* was remarkably overexpressed due to increased mRNA in BC compared to normal individuals (Figure 1C and D). And the TCGA-BRCA data also demonstrated the mRNA expression levels of *PPEF1* in BC tissues were significantly higher than that in the adjacent normal tissues (Figure 1E). These results indicated that *PPEF1* expression is elevated in BC.

In addition, we compared the transcription levels of *PPEF1* among groups of patients, according to different clinicopathological characteristics (Table 1). The results demonstrated that there was no significant difference in age, stage, tumor invasion and distant metastasis status. Notably, high *PPEF1* expression was positive associated with lymphoid nodal(N) status (P = 0.009), estrogen receptor (ER) (P = 0.006), progesterone receptor (PR) (P = 0.012) and human epidermal growth factor receptor 2 (HER2) (P < 0.001).

Table 1 Correlations Between mRNA Expression of *PPEF1* and Clinicopathological Parameters of BC Patients

Variables	All Cases	*PPEF1* mRNA Expression		*p*-value
		Low (n=357)	High (n=356)	
Age at Surgery				0.915
<51	211	105	106	
≥51	502	252	250	
Stage				0.124
Ⅰ+Ⅱ	534	271	263	
Ⅲ+Ⅳ	175	86	89	
X	4	0	4	
Tumor Invasion				0.407
T1+T2	599	296	303	
T3+T4	113	61	52	
TX	1	0	1	
Lymphoid Nodal Status				0.009*
-	346	187	159	
+	361	170	191	
NX	6	0	6	
Distant Metastasis Status				
M0	701	355	346	0.053
M1	9	2	7	
MX	3	0	3	
ER				0.006*
-	161	96	65	
+	552	261	291	
PR				0.012*
-	238	135	103	
+	475	222	253	
HER2				<0.001*
-	551	302	249	
+	162	55	107	

Note: *Significantly different.

The High Prognostic and Diagnostic Significances of *PPEF1* in BC Patients

Kaplan-Meier Plotter tool was used to analyze the correlation between the *PPEF1* expression and BC survival. High expression of *PPEF1* was a poor prognostic marker for 10-year overall survival (OS) in TCGA cohort (HR = 1.45, 95% *CI* = 1.02–2.05, P = 0.037; Figure 2A). And integration analysis of multiple gene chip results from bcGenExMiner between 2002 and 2019 confirmed higher *PPEF1* expression negatively correlated with all events-free (AE-free) survival (HR = 1.12, 95% CI = 1.01–1.23, P = 0.0301) and metastatic recurrence-free (MR-free) survival (HR = 1.15, 95% CI = 1.00–1.31, P = 0.043) (Figure 2B and C). In addition, Cox univariate and multivariate survival analyses were performed including the *PPEF1* expression levels and patients' clinicopathological parameters. Univariate survival analysis suggested that differential expression levels, age and TNM stage were the important parameters affecting the survival time of BC patients. Furthermore, Cox multivariate revealed that *PPEF1* over-expression was an independent risk factor of unfavorable prognosis in BC patients (HR = 1.001, 95% CI = 1.000–1.001, P = 0.012, Figure 2D, Table 2). To assess the diagnostic importance of *PPEF1*, we generated ROC curve using the mRNA expression data of BC patients and healthy individuals from TCGA-BRCA datasets (Figure 2E). The area under the ROC curve was 0.903 (AUC = 0.903, 95% CI = 0.858–0.947, P < 0.001), with a sensitivity of 93.1% and a specificity of 82.3%, which indicated that *PPEF1* conferred well-diagnostic value.

The Gene Ontology Functions Enrichment Analysis of *PPEF1*

In order to explore the biological significance and function of *PPEF1*, we used GSVA to perform GO functional enrichment

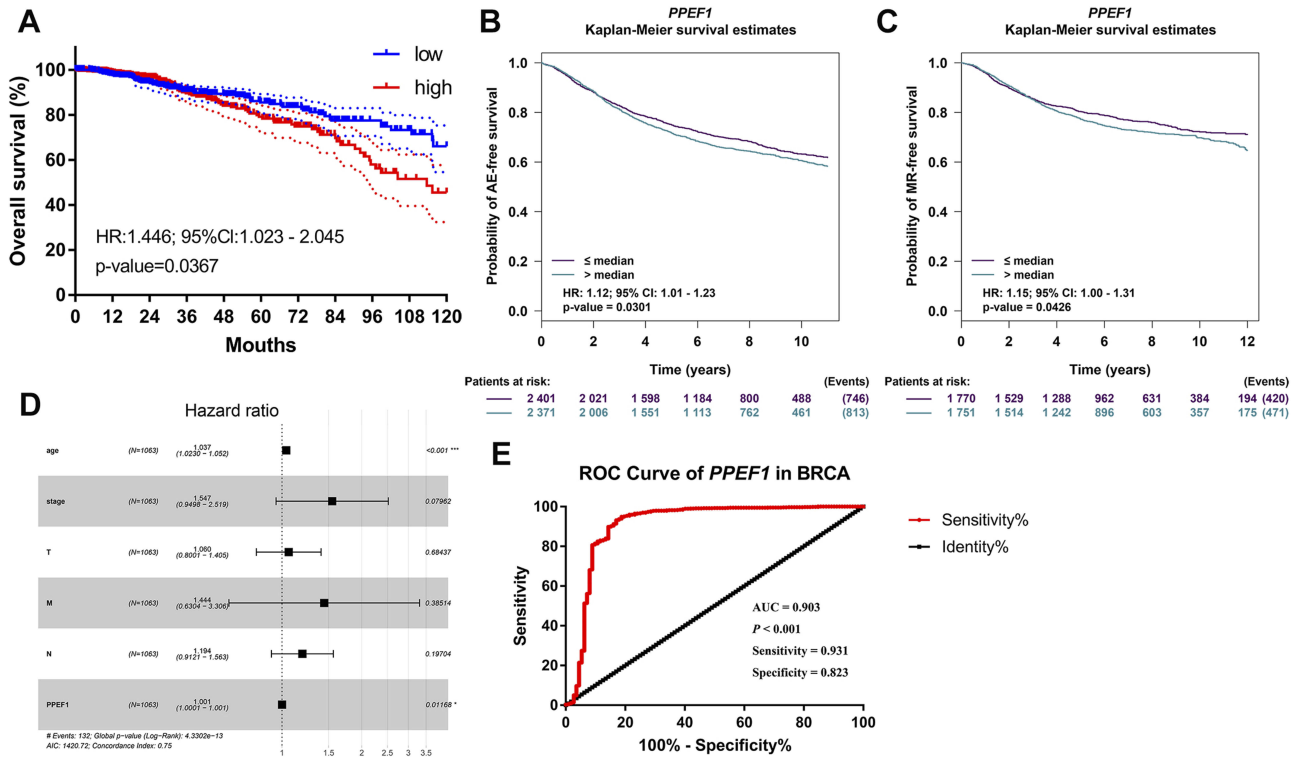

Figure 2 The prognostic and diagnostic significances of *PPEF1* in BC. Elevated expression of *PPEF1* indicated poor clinical outcome for BC patients; (**A**) Analysis of overall survival rates based on TCGA-BRCA data; (**B, C**) Analysis of events-free and metastatic recurrence-free survival rates based on bc-GenExMiner database. (**D**) Multivariate analysis of the correlation of *PPEF1* expression with OS among breast cancer patients. (**E**) ROC curve of *PPEF1* mRNA expression in BC patients.

analysis in TCGA-BRCA patients. GO enrichment analysis predicted the functional roles of *PPEF1* on the basis of three aspects, including biological process (BP), molecular function (MF), and cellular components (CC). As illustrated in biological process functional enrichment, upregulated *PPEF1* was significantly enriched in Autophagy, Negative Regulation of Response to DNA Damage Stimulus, Negative Regulation of Signal Transduction by P53 Class Mediator, Regulation of Response to Extracellular Stimulus and Post Translational Protein Modification, etc. (Figure 3A). For molecular function, upregulated *PPEF1* was enriched in Arylsulfatase Activity, Sulfuric Ester Hydrolase Activity, Racemase and Epimerase Activity, Ubiquitin Like Protein Ligase Activity, Snap Receptor Activity, and participated in Platelet-Derived Growth Factor Binding, Ascorbic Acid Binding, Collagen Binding, etc. (Figure 3B). Furthermore, the cellular components associated with high expression of *PPEF1* are located in Golgi-associated vesicle, Vesicle Coat, cis-Golgi network, β-catenin Destruction Complex, Ubiquitin Ligase Complex, etc. (Figure 3C).

Table 2 Univariate and Multivariate OS Analysis of *PPEF1* in Patients with BC

Parameter	Univariate Analysis			Multivariate Analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Age	1.036	1.021–1.050	**3.30E-07**	1.037	1.023–1.052	**2.75E-07**
Gender	0.888	0.124–6.365	0.874	-	-	-
Stage	1.981	1.594–2.461	**2.14E-11**	2.176	2.176–2.711	0.080
T classification	1.528	1.236–1.890	**9.09E-05**	1.060	0.800–1.405	0.684
N classification	1.623	1.357–1.942	**1.12E-07**	1.194	0.912–1.563	0.197
M classification	4.426	2.555–7.668	**1.18E-07**	1.444	0.630–3.306	0.385
PPEF1	1.001	1.000–1.001	**0.010**	1.001	1.000–1.001	**0.012**

Notes: Bold values indicate $P < 0.05$. HR, hazard ratio; CI, confidence interval. -, not assessed due to an insignificant result in the univariate analysis ($P > 0.05$).

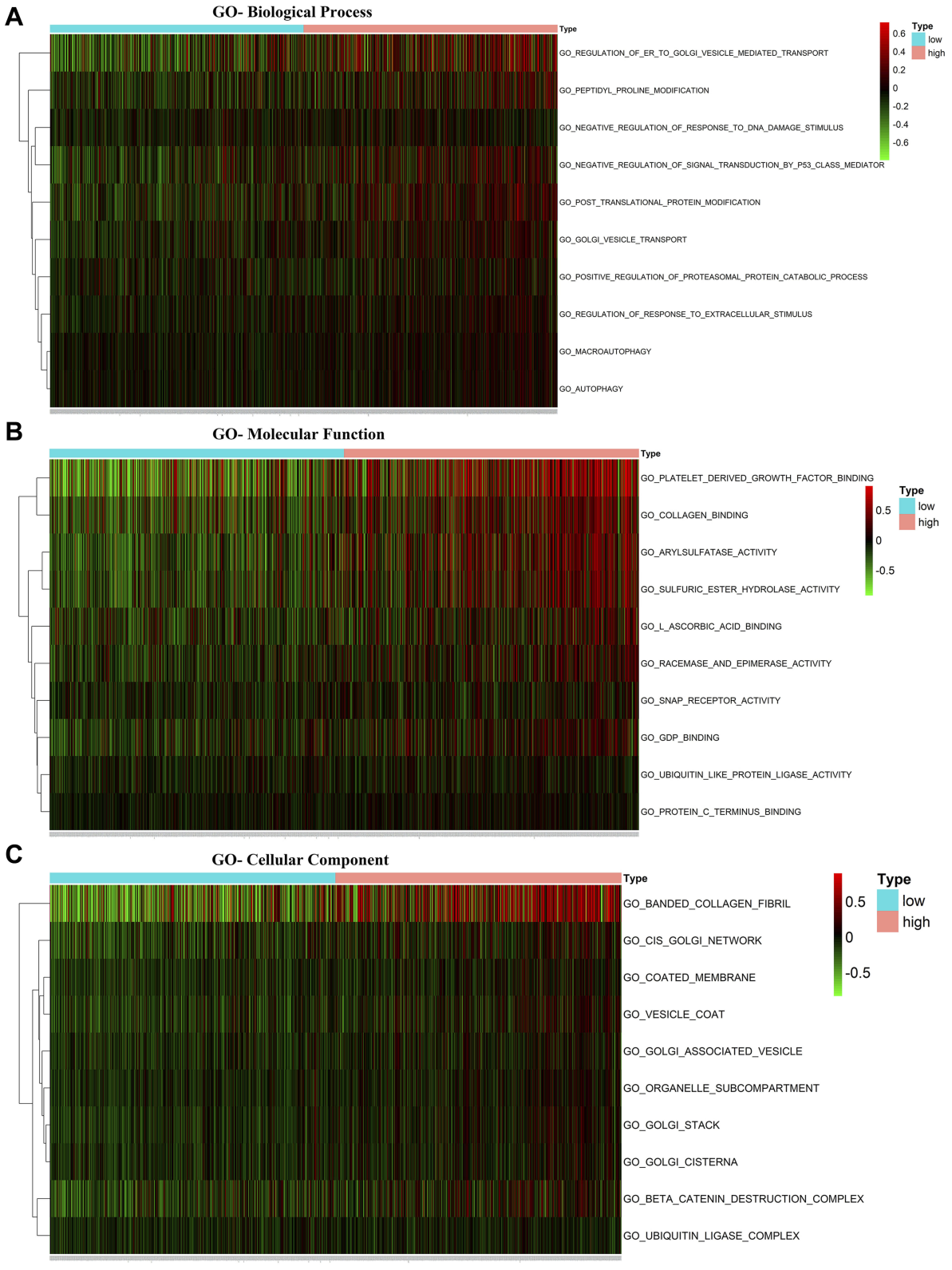

Figure 3 The Gene Ontology functions enrichment analysis of *PPEF1*. (**A**) Biological Process (BP); (**B**) Molecular Function (MF); (**C**) Cellular Components (CC).

The Gene Set Enrichment Analysis of *PPEF1* in BC

GSEA was performed to evaluate Hallmark effect gene sets and KEGG signaling pathway gene sets, which were associated with upregulated *PPEF1* in the TCGA BC samples. The most valuable 10 pathways of each category were presented, respectively, in Figure 4A and B. For Hallmark gene sets, the most valuable top three were epithelial-mesenchymal transition (EMT), angiogenesis and TGF-β signaling, whereas the top three KEGG signaling pathways were ECM receptor interaction, TGF-β signaling pathway and Focal adhesion (Table 3). Notably, our data support that *PPEF1* plays a vital role by regulating above classic cancer-related signaling pathways.

PPI Network Analysis of Genes Co-Expressed in *PPEF1* in BC

Given the complex procedures of BC progression involving multiple genes and proteins, we first acquired 96 genes associated with *PPEF1* expression in TCGA-BRCA datasets identified using Pearson correlation analysis. Then, PPI analyses of these co-expression genes were performed in STRING database to predict molecular regulation of *PPEF1* in BC. The 20 nodes and 35 lines in the map of PPI network illustrated the interaction between *PPEF1* related genes (Figure 5A). The node degree ≥ 2 was selected as the standard, and 9 of the top 10 core genes included *COL6A1, COL6A2, ITGA5, MMP9, ADAMTS14, BMP1, RUNX2, TGFBI* and *THY1* were significantly up-regulated in breast cancer tissues compared with non-cancerous breast tissues, which is consistent with *PPEF1* expression (Figure 5B and C). Noticeably, *COL6A2, ITGA5, ADAMTS14, BMP1, TGFBI* and *THY1* associated with the EMT pathway were flagged by Hallmark pathway analysis, whereas *COL6A1* and *ITGA5* are key genes in the ECM Receptor Interaction pathway flagged by KEGG pathway analysis. Taken together, these core genes might also similarly interact with *PPEF1* via various signaling pathways to perform the same function in BC.

PPEF1 Promotes the Migration and Invasion of BC Cells

Prior to the present study, the role of *PPEF1* as an oncogene in regulating BC migration and invasion has not been established. Using RT-qPCR, we found that the MCF7 and SK-BR-3 cells had elevated *PPEF1* mRNA expression in comparison with the normal breast MCF 10A cells (Figure 6A). To investigate the relationship between elevated *PPEF1*and metastasis in BC cells, we first inhibited *PPEF1* expression by transient siRNA interference and confirmed its effective silencing at the transcriptional level (Figure 6B). The effect of *PPEF1* silencing on BC cells metastatic potential was measured by cell migration and invasion assays. Transient silencing of *PPEF1* expression in MCF7-siPPEF1 and SK-BR-3- siPPEF1 cells significantly hindered the migration and invasion capabilities compared to control cells (Figure 6C and D). Notably,

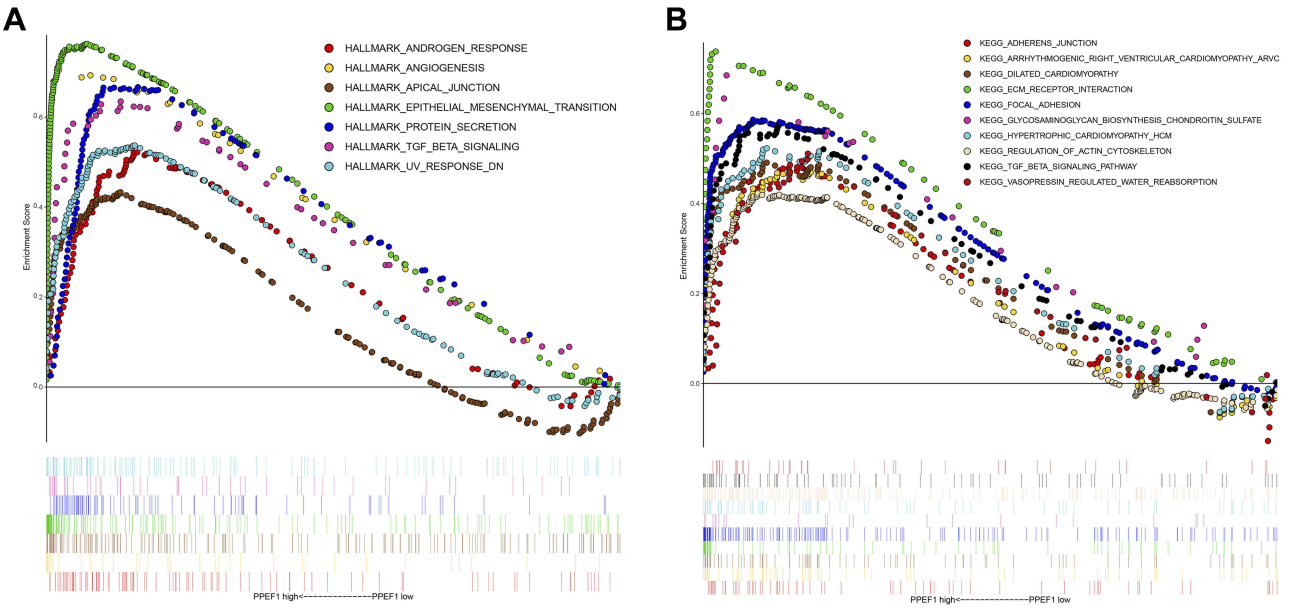

Figure 4 The GSEA analysis of *PPEF1*. (**A**) Hallmark effect gene sets; (**B**) KEGG signaling pathway gene sets.

Table 3 Gene Sets Enriched in the High *PPEF1* Expression Phenotype

Gene Set Name	NES	NOM p-value	FDR q-value
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	2.324	<0.001	<0.001
HALLMARK_ANGIOGENESIS	2.191	<0.001	8.44E-04
HALLMARK_TGF_BETA_SIGNALING	2.075	<0.001	0.005
HALLMARK_PROTEIN_SECRETION	2.045	0.004	0.006
HALLMARK_UV_RESPONSE_DN	1.953	0.006	0.013
HALLMARK_ANDROGEN_RESPONSE	1.891	0.017	0.023
HALLMARK_APICAL_JUNCTION	1.680	0.018	0.098
KEGG_ECM_RECEPTOR_INTERACTION	2.312	<0.001	7.38E-04
KEGG_TGF_BETA_SIGNALING_PATHWAY	2.083	0.002	0.012
KEGG_FOCAL_ADHESION	2.080	<0.001	0.009
KEGG_GLYCOSAMINOGLYCAN_BIOSYNTHESIS_CHONDROITIN_SULFATE	1.869	0.010	0.090
KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM	1.822	0.010	0.113
KEGG_DILATED_CARDIOMYOPATHY	1.747	0.015	0.180
KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC	1.731	0.025	0.175
KEGG_REGULATION_OF_ACTIN_CYTOSKELETON	1.683	0.018	0.216
KEGG_ADHERENS_JUNCTION	1.670	0.017	0.210
KEGG_VASOPRESSIN_REGULATED_WATER_REABSORPTION	1.653	0.022	0.212

Notes: Gene sets with NOM P-value < 0.05 and FDR q-value < 0.25 were considered as significantly enriched.
Abbreviations: NES, normalized enrichment score; NOM, nominal; FDR, false discovery rate.

there is no marked difference of proliferation activities between the MCF7-siPPEF1 or SK-BR-3- siPPEF1 cells and control cells (Figure 6E), confirming the differences in cell migration and invasion were not due to alterations in cell proliferation. These observations collectively indicated that *PPEF1* enhanced the metastasis of BC cells.

The Expression of *PPEF1* Could Be Regarded as a Promising Evaluation Index for Clinical Diagnosis

To further verify the clinical implications, we examined the protein expressions of *PPEF1* in BC paraffin-embedded tissues by the immunohistochemistry (IHC). A total of 88 clinical samples were collected for *PPEF1* analysis, including 44 BC cases and their matched adjacent normal tissues. The results indicated that the *PPEF1* was upregulated and localized in the cytoplasmic and membranous of breast tumor cells, which was matched with the GO enrichment analysis of cellular component (Figure 7A). And it also showed a positive association between *PPEF1* increased protein and N status ($P = 0.033$) and HER2 ($P = 0.033$) (Table 4).

Additionally, the aberrant expression of *PPEF1* in the serum was identified by the ELISA method and quantized on a standard curve. The immunoreactive *PPEF1* amount in the serum of 57 healthy individuals was (179.6 ± 11.4) pg/mL (Mean ± SEM), whereas the average serum *PPEF1* concentration in the 57 breast cancer patients was (366.5 ± 24.25) pg/mL (Mean ± SEM). Thus, the expression of *PPEF1* was much higher in the serum of BC patients compared with that in healthy individuals (Figure 7B). Moreover, correlation analysis showed that high *PPEF1* in serum was associated with HER2 ($P = 0.025$) (Table 5), which was consistent with database and IHC analysis.

And the area under the ROC curve (AUC) of *PPEF1* was 0.876 with a sensitivity of 86.0% and a specificity of 73.7%, which also demonstrates that *PPEF1* has higher clinical value as a non-invasive biomarker in BC. Meanwhile, CEA, CA125 and CA153 in ELISA tested patient serum were analyzed by chemiluminescence measuring instrument. Astonishingly, the combined detection of *PPEF1, CEA, CA125* and *CA153* can improve the diagnosis efficiency of BC with both better sensitivity (93.0%) and specificity (82.5%) (Figure 7D, Table 6).

Discussion

Breast cancer management remains a challenge due to its characteristics of high metastasis and high mortality rates.[22] It is known that phosphatases, as an oncogene or tumor suppressor, can modulate a variety of signaling pathways and dysregulation results in abnormal processes including uncontrolled proliferation, differentiation, angiogenesis, and metastasis.[23] *PPEF1*, a member of the protein serine/threonine phosphatase gene family, can inhibit apoptosis in

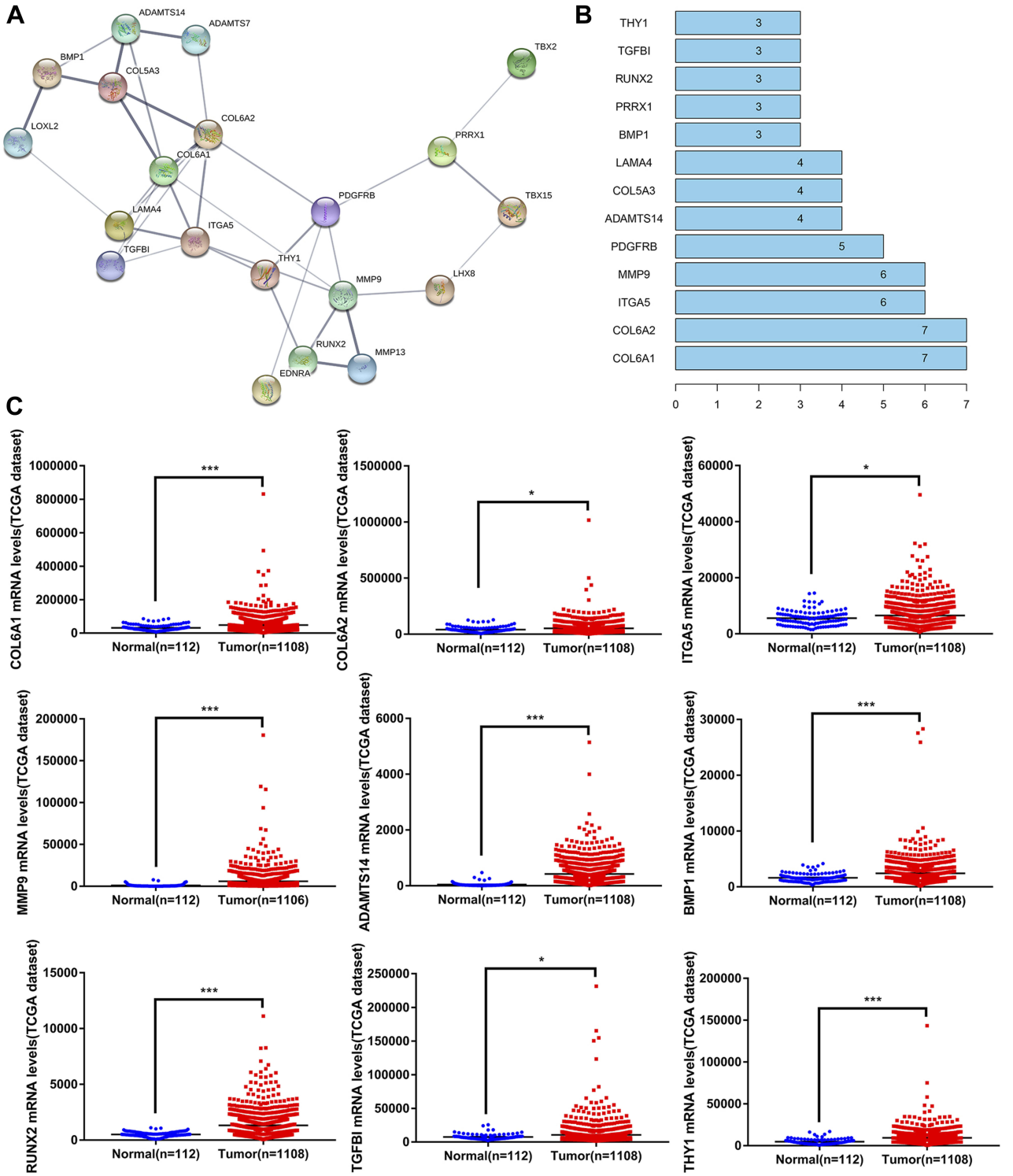

Figure 5 PPI network analysis of *PPEF1* co-expressed genes in BC. (**A**) The PPI network of the co-expressed genes of *PPEF1*. (**B**) The top 10 core proteins of PPI network, the ordinate coordinates represent the name of the gene, and the abscissa represents the number of gene connections. (**C**) Analysis of core gene affecting breast cancer progression. *: $P < 0.05$, ***: $P < 0.001$.

genotoxic stress, leading to uncontrolled overgrowth of cells,[24] which may be involved in the progression of some malignancies.[7,12] However, the potential role of *PPEF1* in BC has not been established prior to the present study.

This study has made the following novel findings that contribute to provide an evidence for *PPEF1* as an oncogene, which lay the foundation for further clinical and mechanistic understanding in BC:

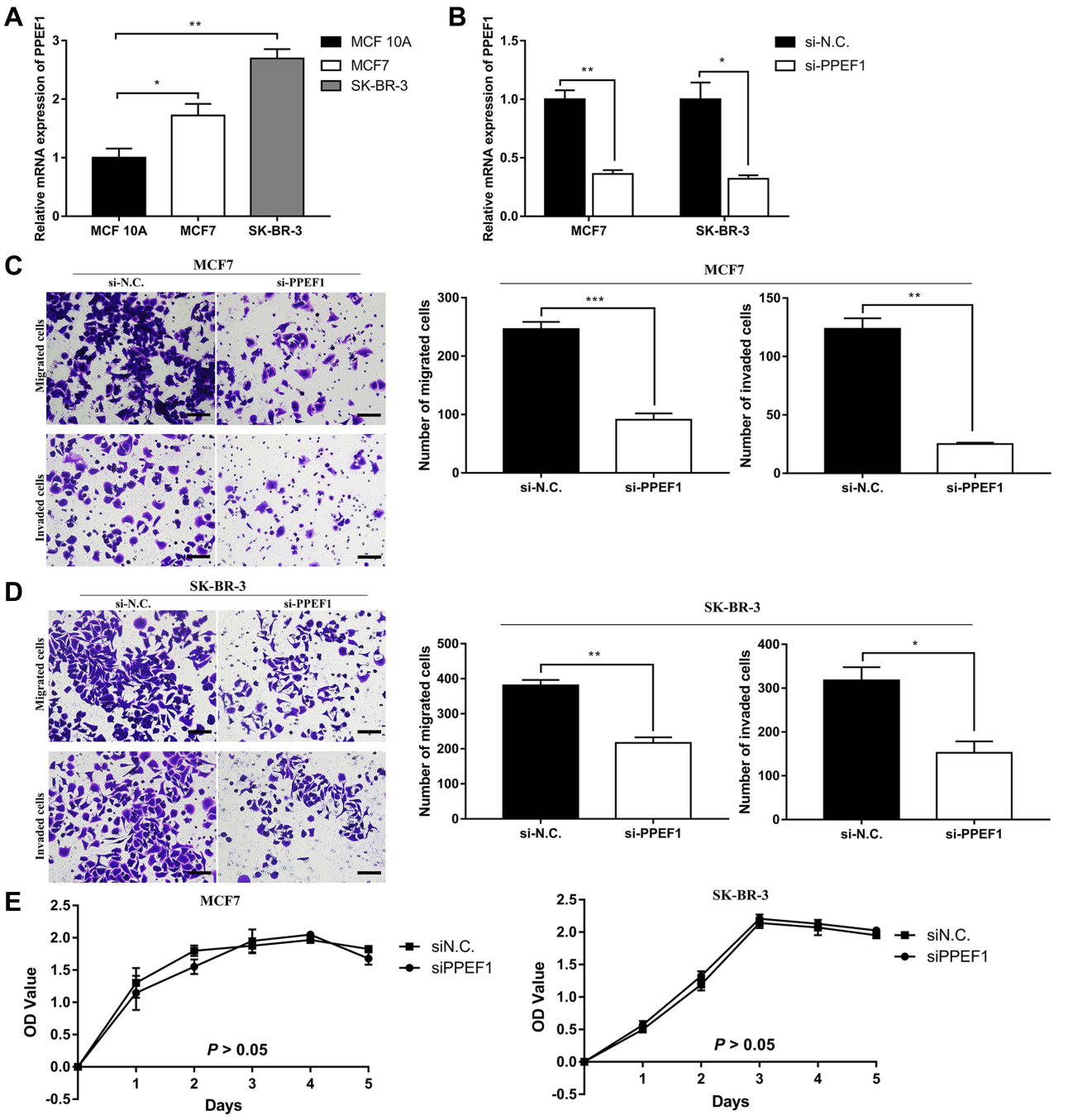

Figure 6 Functional verification of *PPEF1* in representative BC cells. (**A**) The relative mRNA expression levels of *PPEF1* in non-malignant MCF 10A and BC cells (MCF7 and SK-BR-3) were determined by RT-qPCR. (**B**) Efficiency of *PPEF1* siRNA interference determined by RT-qPCR in MCF7 and SK-BR-3 cells, respectively. (**C**) Transwell migration and invasion assays in MCF7. Scale bar = 60μm. (**D**) Transwell migration and invasion assays in SK-BR-3. Scale bar = 60μm. (**E**) Proliferation activities of MCF7-siN.C. (SK-BR-3-siN.C.) and MCF7-siPPEF1 (SK-BR-3-siPPEF1) cells measured by the CCK-8 assay. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

First, the oncogene function of *PPEF1* in BC has been uncovered by data mining of publicly available cancer clinical databases, including Oncomine, GEPIA and TCGA (Figure 1). Furthermore, high *PPEF1* expression was positively associated with N^+, ER^+, PR^+ and $HER2^+$ in BC patients (Table 1). Prior to this study, the clinical relevance of *PPEF1* has not been well explored and established. This study is the first to explore that *PPEF1* could be a promising prognostic biomarker for BC thus is of high clinical relevance. We also found that the high *PPEF1* expression was an independent predicted factor of unfavorable prognosis in patients with BC (Figure 2A-D, Table 2). Moreover, ROC

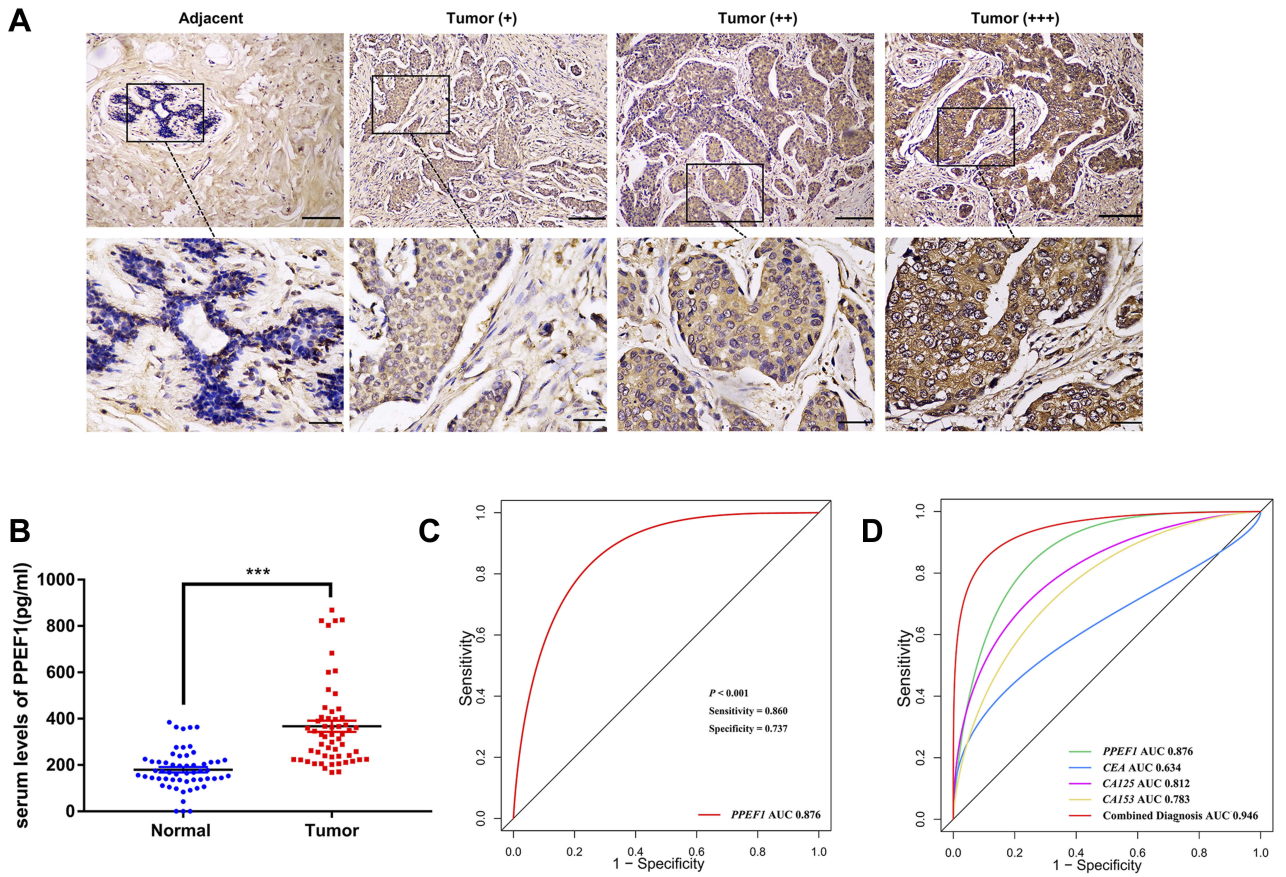

Figure 7 Clinical validation of *PPEF1* in BC patients. (**A**) Representative immunohistochemical staining of *PPEF1* expression. Scale bar = 60μm (upper panels), Scale bar = 30μm (lower panels). (**B**) Serum levels of *PPEF1* in BC patients and healthy people. ***: $P < 0.001$. (**C**) ROC analysis for the *PPEF1* in BC patient serum. (**D**) ROC curves for diagnostic models of the combination of *PPEF1*, *CEA*, *CA125* and *CA153*.

analysis confirmed that the high expression of *PPEF1* has the potential diagnostic value in patients with BC (Figure 2E).

Second, the present study provided the first evidence that *PPEF1* participated in multiple biological processes involving in tumorigenesis and development (Figure 3). The GO analysis suggested that the high expression of *PPEF1* could be involved in the negative feedback regulation of P53 signaling, leading to the development of BC. Consistent with our findings, Ho et al have found that *PPEF1* can inhibit P53-mediated apoptosis through dephosphorylation, and increase tumorigenicity and drug resistance of human lung cancer cells.[7] Meanwhile, accumulating evidence has suggested the participation of *PPEF1* in negative feedback regulation of P53 signaling, autophagy, regulation of DNA damage, which play important roles in growth, invasion and metastasis of BC.[25–27] Furthermore, *PPEF1* is involved in a variety of molecular functions related to tumor microenvironment, such as proteases,[28] platelet-derived growth factors and collagen.[29,30]

Third, we have innovatively predicted the potential signaling pathways underlying the oncogenic activity of *PPEF1* in BC by GSEA. The high expression of *PPEF1* is mainly enriched in EMT, angiogenesis, TGF-β pathway, Focal adhesion and ECM receptor interaction (Figure 4, Table 3). Among the above pathways, angiogenesis is the basic factor of tumor growth and metastasis.[31] EMT, ECM and Focal adhesion pathways are involved in biological processes such as tumor adhesion, invasion and metastasis.[32,33] Noteworthy, ECM can activate the Focal adhesion signaling pathway[34] and RTK downstream signaling pathway, resulting in tumor growth, invasion, migration, and angiogenesis.[35,36] TGF-β signaling pathway is one of the important pathways involved in the cancer cell proliferation, invasion, migration, angiogenesis, apoptosis, as well as in metastasis by agitation or invasion of metastasis-related factors, including epithelial-mesenchymal transition (EMT).[37–39] The relationship between *PPEF1* and these specific signaling pathways was not previously reported in the literature, thus and merits further studies.

Fourth, PPI analysis revealed that potential synergy or interaction of nine core genes with *PPEF1* contributed to the progression and prognosis of BC (Figure 5). Among

Table 4 Correlations Between *PPEF1* in IHC and Clinicopathological Parameters of BC Patients

Variables	All Cases	*PPEF1* Protein Expression		*p*-value
		Low (n=22)	High (n=22)	
Age at Surgery				0.761
<51	19	10	9	
≥51	25	12	13	
Stage				0.122
Ⅰ+Ⅱ	27	16	11	
Ⅲ+Ⅳ	17	6	11	
Tumor Invasion				0.099
T1+T2	31	18	13	
T3+T4	13	4	9	
Lymphoid Nodal Status				0.033*
-	25	16	9	
+	19	6	13	
Distant Metastasis Status				0.147
M0	42	22	20	
M1	2	0	2	
ER				0.131
-	21	13	8	
+	23	9	14	
PR				0.131
-	21	13	8	
+	23	9	14	
Her2				0.033*
-	19	13	6	
+	25	9	16	

Note: *Significantly different.

Table 5 Correlations Between *PPEF1* Expression in Serum and Clinicopathological Parameters of BC Patients

Variables	All Cases	*PPEF1* Expression		*p*-value
		Low (n=28)	High (n=29)	
Age at Surgery				0.083
<51	27	10	17	
≥51	30	18	12	
Stage				0.934
Ⅰ+Ⅱ	41	20	21	
Ⅲ+Ⅳ	16	8	8	
Tumor Invasion				0.964
T1+T2	46	25	26	
T3+T4	11	3	3	
Lymphoid Nodal Status				0.696
-	17	14	13	
+	40	14	16	
Distant Metastasis Status				0.322
M0	56	28	28	
M1	1	0	1	
ER				0.146
-	27	16	11	
+	30	12	18	
PR				0.696
-	17	14	13	
+	40	14	16	
HER2				0.025*
-	26	17	9	
+	31	11	20	

Note: *Significantly different.

the core genes, *COL6A1* can promote the metastasis of various tumors,[40,41] and *COL6A2* is a cancer-driven gene with a mutation rate of more than 5% in BC.[42] *ITGA5* promoted tumor invasion which was highly expressed in BC with migration, invasiveness and lung metastases.[43] *MMP9* overexpression hydrolyzes the basement membrane and promotes cancer cell invasion and migration.[44] *ADAMTS14* encodes a combination of integrin and metalloproteinases, which is linked to the progression of liver cancer.[45] There are also reported that *BMP1* associated with poor prognosis of renal clear cell carcinoma,[46] and *RUNX2* can promote breast cancer proliferation, migration, invasion and epithelial-mesenchymal transition.[47] Moreover, *THY1* encodes cell surface glycoprotein and protein immunoglobulin superfamily *CD90*, which is associated with breast cancer stem cells and EMT processes.[48]

Noteworthy, in our analysis, *TGFBI* was found highly expressed in BC as the co-expression gene of *PPEF1*. Inconsistent with our findings, some previous studies had shown that *TGFBI* can reduce the metastatic potential of lung and breast tumor cells in vitro and in vivo.[49,50] This merits further investigation.

Fifth, *PPEF1* expression is a promising biomarker for BC thus is of high clinical relevance. Firstly, we characterized the migration and invasion promoting effect of *PPEF1* in BC cells, providing more experimental evidences for the role of *PPEF1* in BC metastasis (Figure 6). Using IHC analysis, we evaluated

Table 6 Diagnostic Accuracy Based on Serum *PPEF1* Levels Combined Detection of Tumor Markers CEA, CA125 and CA153

Index	AUC	Sensitivity (%)	Specificity (%)	*P* value	Youden Index	+LR/-LR
PPEF1	0.876	0.860	0.737	<0.001	0.597	17.214
CEA	0.634	0.509	0.772	0.014	0.281	3.510
CA125	0.812	0.772	0.754	<0.001	0.526	10.378
CA153	0.783	0.754	0.790	<0.001	0.544	11.530
Combined Diagnosis	0.946	0.930	0.825	<0.001	0.755	62.633

Abbreviations: AUC, Area Under Curve, + LR, Positive Likelihood Ratio, - LR, Negative Likelihood Ratio.

the over-expression of *PPEF1* protein, which was localized in the cytoplasmic and membranous. Further, we found that *PPEF1* could be explored as non-invasive screening for BC patients with a better sensitivity and specificity (Figure 7). Noteworthy, the combined determination of serum *PPEF1* and traditional tumor markers (CEA, CA125 and CA153) can enhance diagnostic accuracy thus is of vital importance in the early diagnosis of BC (Table 6). And lastly, we found that increased *PPEF1* expression may play a role in BC aggressiveness following the positive expression of HER2 (Tables 4 and 5), which laid the foundation for future mechanism research.

In conclusion, to the best of our knowledge, our study is the first to provide convincing evidence that *PPEF1* exerts its novel function on driving breast tumorigenesis and progression. Meanwhile, high *PPEF1* expression could also serve as a biomarker for diagnosis and prognosis in BC patients.

Ethics Approval and Consent to Participate

All the clinical samples were obtained from the Laboratory Medicine Department of the Affiliated Hospital of Southwest Medical University. Patient Consent Forms were obtained according to protocols approved by the Institutional Review Board of the Affiliated Hospital of Southwest Medical University. Moreover, this study obtained written informed consent from the participants.

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Disclosure

The authors declare no conflicts of interest in this work.

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