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MiR-466 as a poor prognostic predictor suppresses cell proliferation and EMT in breast cancer cells by targeting PSMA7

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Abstract. – OBJECTIVE: MiR-466 has been reported to exert a tumor-suppressive role in several cancers, including colorectal cancer and osteosarcoma, but its clinical significance and functional mechanisms in breast cancer (BC) pathogenesis still remain elusive.

PATIENTS AND METHODS: The expression of miR-466 was determined using reverse transcription quantitative PCR. The clinical significance of miR-466 in BC patients was assessed by Chi-square test, Kaplan-Meier method and Cox regression analyses. Functional experiments, including CCK-8 and transwell assays, we[illegible] formed to analyze cell proliferation, mi[illegible] and invasion ability. The association be[illegible]n miR-466 and proteasome subunit α7 (PS[illegible] was confirmed by Luciferase reporter assay.

RESULTS: Here, we first obse[illegible] that the e[illegible] pression of miR-466 was signi[illegible]wnregu[illegible] lated in BC tissues and cell [illegible]. The[illegible]creased miR-466 expression was [illegible]ificant[illegible]ssociat-ed with tumor size (p = [illegible] lym[illegible] tastasis (p = 0.008), [illegible] [illegible]0.052), [illegible]d poor survival rate[illegible] addit[illegible]iR-466 was identified as an i[illegible]pendent pr[illegible]stic factor for BC patients[illegible]ther found t[illegible]e over-expression [illegible]iR-[illegible]ignificantly inhibited cell prolife[illegible]tion, migra[illegible]nd invasion. Mech-anistically[illegible]SMA7 was a[illegible]ntial target gene of miR[illegible] and negatively re[illegible]ated miR-466 in BC c[illegible] Onco[illegible]e database and Kaplan-Meier ov[illegible]surviv[illegible]nalysis indicated that upregu-latio[illegible]S[illegible] was a[illegible]ciated with poor prog-nosis o[illegible]patient[illegible]he rescue experiments demonstr[illegible]tha[illegible]SMA7 overexpression re-ve[illegible]l the e[illegible]f miR-466 on cell proliferation[illegible]igration[illegible]vasion and EMT transcription fa[illegible]adherin, N-cadherin, and vimentin).

CONCLUSIONS: Collectively, these results suggest that the miR-466/PSMA7 axis might have potential as a therapeutic target for BC [illegible]ent.

Key Words:
Breast cancer, MiR-466, Prognosis, PSMA7, Migration, Invasion.

Introduct[illegible]

Breast [illegible]er ([illegible] one of the most commonly diagnosed malign[illegible] in females, with an annual [illegible]ce of appro[illegible]tely 2.1 million and 0[illegible] million mortality world[illegible]ide[1]. Despite the fact [illegible] great progres[illegible]as been made in diverse ther-a[illegible]tic strategie[illegible]cluding surgery, chemothera-p[illegible] radiother[illegible] the prognosis of BC patients stil[illegible]ins[illegible]isfactory largely due to tumor metasta[illegible]dvanced stage when it is initially diagnosed[2,3]. Therefore, there is an urgent need [illegible]r understand the molecular mechanisms [illegible]in BC pathogenesis to improve the prognosis and treatment.

MicroRNAs (miRNAs/miRs) are a class of small (approximately 20-25 nucleotides) and non-coding single-stranded RNAs that negatively regulate gene expression by binding to the 3'UTR of their target genes[4]. By regulating various biological functions, such as proliferation, differentiation, migration and apoptosis, miRs function as tumor suppressors or oncogenes involved in the tumor progression and development[5,6]. Some studies[7-9] indicate that aberrantly expressed miRNAs exert as a prognostic factor involved in the pathogenesis of BC, including miR-505, miR-132-3p and miR-99a. In recent years, miR-466 has been reported to act as a tumor suppressor in several types of human cancer. For example, Colden et al[10] demonstrated that miR-466-mediated downregulation of RUNX2 could effectively inhibit tumor growth and bone metastasis in prostate cancer. Similarly, the suppressive role of miR-466 was also illustrated in colorectal cancer by Tong et al[11] and in osteosarcoma by Cao et al[12]. However, the clinical significance and functional mechanisms of miR-466 in BC remain yet to be fully investigated.

Proteasome subunit α7 (PSMA7) located on the chromosomal anomaly 20q13.33 region is fre-

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quently amplified in tumor[13]. PSMA7 is identified as an α-type subunit of the 20S proteasome core complex with a molecular mass of ~2,000 kDa, which comprises an associated 20S proteolytic core and one or two 19S regulatory complexes[14,15]. Most studies have reported the overexpressed expression of PSMA7 and its oncogenic role in different tumor cells. For instance, Romanuik et al[16] observed the higher or increased PSMA7 expression in castration-recurrent prostate cancer. Shi et al[17], Scotto et al[18] and Hu et al[14] consistently found the overexpression of PSMA7 in liver cancer, cervical cancer and colorectal cancer, respectively. Functionally, genetic or pharmacological inhibition of PSMA7 could significantly inhibit the cell growth and migration *in vitro*, as well as the *in vivo* tumorigenic ability of colorectal cancer cells[19]. The shRNA-mediating silencing of PSMA7 decreased cell proliferation, induced cell cycle G0/G1 phase arrest and apoptosis in cervical cancer cells[20]. Xia et al[21] also manifested that PSMA7 knockdown suppresses the proliferation, migration, invasion and subcutaneous tumorigenesis of gastric cancer cells in nude mice. Interestingly, Richardson et al[22] revealed that PSMA7 expression was overexpr d in testicular and BC. Based on these facts, speculated that PSMA7 might promote the g- nant cellular behaviors in BC cells by functio as an oncogene.

In our study, we investigated t ssion le els of miR-466 and PSMA7, ir prog- nostic values in BC patient g ava e tumor tissue samples or onlin form Through functional ex im aluat e regulatory effects of -466 o proliferation, migration and in . We furth lored the association be 466 and P 7 using Luciferase re ter as Importantly, whether PSMA7 a downstrea lator involved in miR-46 odulating cell fu s was addition- ally onstra by performing rescue experi- me hese ings might help identify another promi peutic t for BC.

Pat s and Methods

ue Specimens

A total of 75 paired tumor tissues and matched cent tissues were collected from BC patients w derwent surgical resection from the People's Liberation Army Medical College (Beijing, China). According to the inclusion criteria, patients did not have other systemic diseases or cancer at the time of their initial diagnosis and receive any preoperative chemotherapy/radiotherapy or death in the perioperative period and had basic clinical data. All tissue specimen e stored in liquid nitrogen for further a ter clinical diagnosis, the basic pati nforma tion, including age, tumor size a mph node metastasis are summarized in T Before surgery, all patients signed t writte med consents and were confir not to rec anti-tumor treatments cludin chemo therapy or immunot y. Af urgery, each patient was perfo ne follow and the correspondi urviv rmati as obtained by tele e commu is study was condu cordance e Helsinki Declarati d ap d by the Research Ethics Committee of the Pe Liberation Army Medical .

Culture Co ditions

ur BC cel es (MDA-MB-231, MCF-7, T and ZR-7) and a normal human breast epit MCF-10A were purchased from the A Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecodified Eagle's Medium (DMEM; Gibco, e, MD, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in humidified incubator containing 5% CO_2.

Cell Transfection

The miR-466 mimics and its negative control (miR-NC) were synthesized by GenePharma Co., Ltd. (Shanghai, China). The PSMA7 overexpression plasmids (pcDNA3.1-PSMA7) and the empty plasmid pcDNA3.1 were provided by Bioworld Biotech Co., Ltd. (Shanghai, China). For miR-466 overexpression, T-47D and ZR-75-30 cells were cultured in 6-well plates at a density of 1×10^5 cells per well and transfected with 20 nM miR-466 mimics or miR-NC for 48 h. In the rescue experiments, T-47D cells were transfected with miR-466 mimics or miR-NC together with pcDNA3.1 or pcDNA3.1-PSMA7 for 48 h. Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was utilized to conduct all transfections in accordance with the manufacturer's guidelines.

Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA was isolated from tissues or cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For miR-466 determination, cDNA was

Table I. Association between miR-466 and clinicopathological features of patients with breast cancer..

Variables	Cases (n = 75)	miR-466 expression		*p*-value
		Low (n = 48)	High (n = 27)	(chi-square
Age				
< 50	30	20	10	
≥ 50	45	28	17	
Tumor size (cm)				0.0
< 3	42	32	10	
≥ 3	33	16	17	
Lymph node metastasis				0.008
Negative	45	37		
Positive	30	11		
TNM stage				
I-II	47	26		
III-IV	28	22		
Estrogen receptor status				0.603
Negative	36	22	14	
Positive	39	26	13	
Progesterone receptor status				0.432
Negative	38	25		
Positive	37	23		
Epidermal growth factor receptor 2 status				0.715
Negative	56		20	
Positive	19	12	7	

miR, microRNA; TNM, tumor-node-metastasis classifi[illegible] s[illegible].

synthesized using miScript [illegible] kit (T[illegible]Ra, Dalian, China). Using the [illegible]A-spe[illegible] miRNA assay kit (A[illegible]ied [illegible]s, T[illegible]o Fisher Scientific, Ca[illegible]ad, CA, [illegible] the expression of miR-466 [illegible]uantified wi[illegible]as the internal control. [illegible]7 quantifica[illegible], reverse transcription w[illegible] perfo[illegible]using M-MLV cDNA synthesis k[illegible]romega Co[illegible]ion, Madison, WI, USA) a[illegible]e gene expressio[illegible]vels were examined [illegible] SYBR[illegible]emix Ex Taq II (TaKaRa Bio, To[illegible]apan) [illegible] GAPDH as the internal control. The t[illegible]ing co[illegible]ons for RT-qPCR were [illegible]follow[illegible]C for[illegible], followed by 40 cycles of [illegible]ration [illegible] for 15 s, annealing at 55°C for [illegible] and ext[illegible]on at 72°C for 30 s. The primer se[illegible]used in this study were as follows: miR-[illegible]for[illegible]5′-CTACCACGTGGGTCCCCTC-3′ [illegible]d reverse, 5′-CACCTCAAAGGAGCGTAG-3′; [illegible]orward, 3′-GCTTCGGCAGCACATATACTA-A[illegible]-5′ and reverse, 3′-CGCTTCAC-GAATTTGCGTGTCAT-5′; PSMA7 forward, 5′-TCAACAAGAGGCGACCAC-3′ and reverse, 5′-GATTGGCCTTTTCTTTTCCA-3′; and GAPDH forward, 5′-GACGGCCGCATCTTCTTGT-3′ and reverse, 3′-CACACCGACCTTACATTTT-5′. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were repeated three times.

Cell Proliferation Analysis

After 48 h transfection, BC cells were seeded onto 96-well plates at a density of 3,000 cells per well and incubated for 24, 48 and 72 h, respectively. At each incubation time point, each well was incubated with 10 µl Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) reagent for 2 h at 37°C. Then, the absorbance in each well was measured using a microplate reader at the wavelength of 450 nm. All experiments were repeated three times.

Cell Migration and Invasion Analysis

The migration and invasion abilities of BC cells were evaluated using transwell chambers (8 µm pore size; Corning, Inc., Corning, NY, USA) uncoated and coated with Matrigel, respectively. In brief, approximately 5×10^4 transfected cells sus-

Table II. Univariate and multivariate analysis of the prognostic variables influencing overall survival in breast cancer patients..

	Univariate analysis		Multivariate analysis	
Variables	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-v**
Age	0.654 (0.512-1.789)	0.601	NA	NA
Tumor size (cm)	1.212 (0.785-2.012)	0.032	1.182 (0.851-2.142)	0.038
Lymph node metastasis	1.065 (0.684-1.892)	0.013	1.619 (0.732-1.668)	0.019
TNM stage	1.885 (1.237-3.625)	0.027	1.923 (1.047-3.421)	60
Estrogen receptor status	0.843 (0.660-2.001)	0.435	NA	
Progesterone receptor status	1.243 (0.898-1.997)	0.381	NA	
Epidermal growth factor receptor 2 status	1.489 (0.998-3.021)	0.751		NA
miR-466 expression	1.453 (0.585-1.978)	0.014	1.063 -2.642)	0.012

HR: hazard ratio; CI: confidence interval; TNM, tumor-node-metastasis classification sy NA yzed.

pended in 150 µl serum-free DMEM were seeded in the upper chambers of the transwell chambers. Meanwhile, 600 µl of DMEM containing 10% FBS was added to the lower chambers. After 48 h incubation, the cells that migrated on the lower chambers were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% violet crystal for 10 min. The number of migratory or invasive cells was counted by averaging the cells i randomly selected fields under a light mic at a magnification of ×200. All experiment re repeated three times.

Luciferase Reporter Assay

PSMA7 was predicted as arget o miR-466 through TargetSc .1 (htt ww.targetscan.org/vert_71/), wh s furt by performing Lucife ay. B , the predicted wild-t 3'-UT SMA7 and a mutated sequenc in the pred target site were subclone pGL3 repo Lucifer- ase vector (P ega C ation, Madison, WI, USA) to erate WT a UT PSMA7 plasmids, r ctively. Subsequ BC cells were co-t ected w 50 nM of miR-466 mimics or mi with mg of the WT or MUT PSMA7 plasm Lipofec ne 2000 reagent (Invit- n, C d, CA SA) for 48 h. Next, Luciff- ctivit ermined using Dual-Luciferase orter as system (Promega Corporation, M WI, USA) the ratio of *Renilla* and firefly the relative Luciferase activity. All periments were repeated three times.

Western Blot Analysis

Total protein was extracted using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and corre ding entration was determined by Bicinchoninic A CA) assay (Beyotime Insti Biotechnol Equal amounts of p n samples (30 µg) we separated using 12% s um dodecyl sulphate-polyacrylamide gel e rophoresis (-PAGE) gels and transferred o olyethyler fluoride (PVDF) membranes. Aft kin non-fat powdered milk for 1 h at roo ature, the membranes were incubated with primary antibodies against PSMA7, E erin, N-cadherin, Vimentin and GAPDH v t at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. The target protein bands were visualized by enhanced chemiluminescence detection reagent kit (Tanon, Shanghai, China).

Meta-Analysis Based on Oncomine Microarray Database

We searched the online Oncomine database (www.onocomine.org) using the following terms: "PSMA7", "Cancer *vs.* Normal Analysis", "Breast Cancer" and "mRNA" to conduct a meta-analysis of PSMA7 expression in BC tissue *vs.* normal tissue. All data are reported as Log2 Median-Centered intensity in the Oncomine database.

Kaplan-Meier Overall Survival Analysis

The prognostic value of PSMA7 expression in BC patients was evaluated using Kaplan-Meier Plotter database (http://kmplot.com/analysis/). All BC patients were divided into two groups by median PSMA7 expression (high and low PSMA7 expression). The overall survival information was extracted and applied to analyze the effect of PSMA7 expression on the survival rate of BC patients by a Kaplan-Meier survival plot *via* dis-

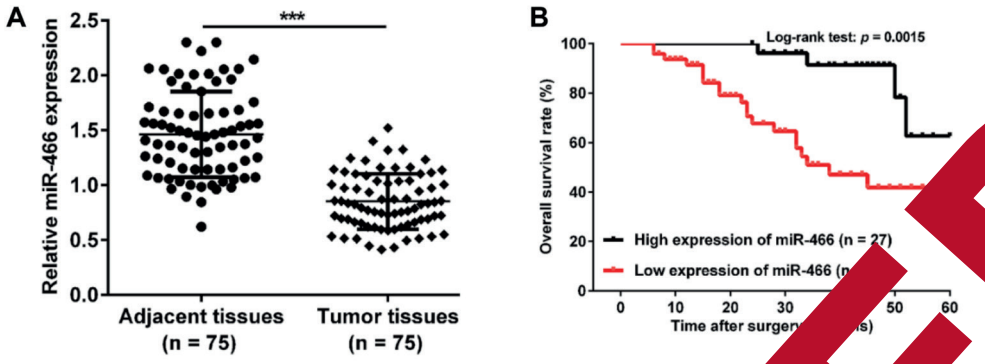


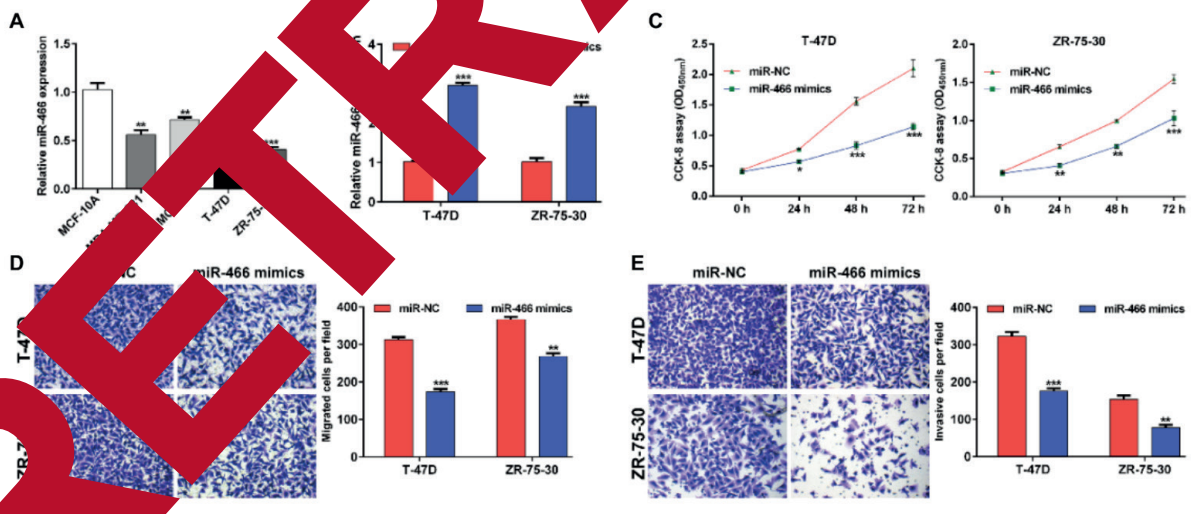
Figure 1. Decreased miR-466 expression predicted poor prognosis in BC patients. **A**, R ativ of miR- nor-mal adjacent tissues and tumors from BC patients were detected by RT-qPCR (n = 75 $p < 0.$ mpared adjacent tissues. **B**, Kaplan-Meier analysis of the overall survival rate of BC patients with high mi xpr h. Patients with high (n = 27) miR-466 expression levels were associated with higher surviv han those wit 48) miR-466 expression (log-rank test: $p = 0.0015$).

playing the hazard ratio (HR), 95% confidence intervals (CI) and log-rank *p*-value.

Statistical Analysis

All experiments were performed three times and data were expressed as mean±SD. Statistical analysis was carried out using SPSS version software (IBM, Armonk, NY, USA) or Gr Prism 6.0 software (GraphPad Software Inc, e Chi-square test was used to analyze the cor

tion n miR-466 ssion and the clini-c ological characteristics of BC. The overall ival rate wa assessed using Kaplan-Meier log-rank test he prognostic significance of m 66 was ev ted using univariate and multiva Cox ssion analyses. The correlation betwee 66 and PSMA7 expression in BC issues was analyzed using Spearman's correla-efficient. Statistical differences between oup were assessed using Student's *t*-tests or



ure 2. Overexpression of miR-466 significantly suppressed cell proliferation, migration and invasion in BC cells. **A**, PCR analysis of miR-466 expression in MCF-10A and four BC cell lines, including MDA-MB-231, MCF-7, T-47D and Z 0. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with MCF-10A. **B**, Quantification of miR-466 expression levels following miR-466 mics or miR-NC transfection in T-47D and ZR-75-30 cells by RT-qPCR analysis. **C**, Cell proliferation rate was determined by CCK-8 assay in transfected T-47D and ZR-75-30 cells. The effects of miR-466 overexpression on cell migration (**D**) and invasion (**E**) were evaluated using transwell assay in T-47D and ZR-75-30 cells (magnification × 200). Data were expressed as mean±SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with miR-NC.

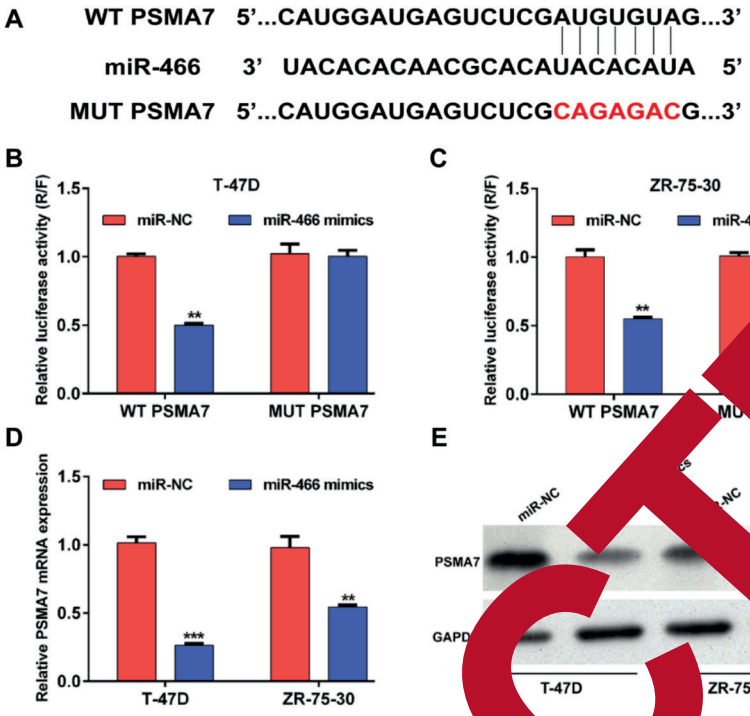


Figure 3. Identification of PSMA7 as a target gene of miR-466 in B[illegible]cted miR-466 target wild type (WT) sequence in the 3′-UTR of PSMA7 and positions of [illegible]d nucleotides ([illegible] corresponding 3′-UTR. **B**, **C**, Luciferase reporter assay of T-47D and ZR-75-30 cells transf[illegible]pGL3-WT PSMA7-3′-UTR or pGL3-MUT PSMA7-3′-UTR reporter plasmid and miR-466 mimics or miR-NC. ([illegible] Western blot analysis of PSMA7 expression levels in T-47D and ZR-75-30 cells transfected with miR-466 [illegible]s or [illegible] were expressed as mean±SD of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, compared [illegible].

one-way analysis of varia[illegible]follow[illegible]'s post-hoc test and sig[illegible]ces w[illegible]e accepted when the [illegible]ue is le[illegible]n 0.05.

Re[illegible]

Decre[illegible]d MiR-466 Exp[illegible]on Predicted Poo[illegible]ognos[illegible]n BC Patients

[illegible]luat[illegible] clinical significance of miR-466 in BC[illegible] we firs[illegible]ermined the expression [illegible]ern o[illegible]-466[illegible]g RT-qPCR. As shown in [illegible]1A, t[illegible]sion of miR-466 was significan[illegible]downreg[illegible]ted in 75 paired tumor tissues co[illegible]d with matched adjacent tissues derived [illegible]nts. Next, all patients were divided into high-expression group and low-expression group based on the median level of miR-466 e[illegible]sion to analyze the clinical significance of miR-466. As illustrated in Table I, Chi-square test showed that decreased miR-466 expression was associated with tumor size ($p = 0.003$), lymph node metastasis ($p = 0.008$) and TNM stage ($p = 0.032$). Kaplan-Meier analyses revealed that low miR-466 expression group has poorer survival than in high miR-466 expression group (Figure 1B). Moreover, we performed univariate and multivariate Cox regression analyses to identify potential risk factors that might affect the prognostic of BC patients. The results indicated that tumor size, lymph node metastasis and miR-466 expression significantly affected the overall survival of BC patients (Table II). Therefore, miR-466 might serve as an independent prognostic factor in BC patients.

Overexpression of MiR-466 Significantly Suppressed Cell Proliferation, Migration and Invasion in BC Cells

Consistent with the decreased miR-466 expression in BC tissues, we additionally observed that miR-466 expression was significantly downregulated in all investigated four BC cell lines compared with normal human breast epithelial cell

line MCF-10A (Figure 2A). To further investigate the biological function of miR-466 in BC, T-47D and ZR-75-30 cells, which had relatively lower expression of miR-466, we used to construct miR-466-overexpressed cell lines using miR-466 mimics transfection. As demonstrated by RT-qPCR analysis, miR-466 mimics transfection remarkably elevated the expression of miR-466 in both T-47D and ZR-75-30 cells, when compared with miR-NC transfection (Figure 2B). Cell proliferation analysis by CCK-8 assay showed that the overexpression of miR-466 significantly suppressed cell growth trends and proliferative rate in both T-47D and ZR-75-30 cells (Figure 2C). In addition, transwell assay displayed that the number of migratory cells (T-47D: 174.3 ± 6.8 *vs.* 313.3 ± 6.1; ZR-75-30: 268.0 ± 8.9 *vs.* 366.0 ± 7.5) (Figure 2D) and invasive cells (T-47D: 176.0 ± 7.2 *vs.* 322.3 ± 11.7; ZR-75-30: 78.0 ± 7.5 *vs.* 153.7 ± 10.7) (Figure 2E) was significantly decreased in miR-466 mimics group compared with miR-NC group in these two BC cell lines.

Identification of PSMA7 as a T[illegible]et Gene of MiR-466 in BC Cells

To better understand the mech[illegible] underlying the suppressive effects [illegible] miR-[illegible] cell proliferation and metasta[illegible]e target g[illegible] miR-466 were predicted[illegible]ne, of [illegible]hich PS[illegible] was selected as a p[illegible]al ta[illegible] of miR-466 and the predicted i[illegible]ween t[illegible] was illustrated in F[illegible] 3A[illegible] sequen[illegible] Luciferase reporte[illegible]say was [illegible]d [illegible]examine whether mi[illegible]rectly targe[illegible]e predicted binding s[illegible]n t[illegible]MA7 3'UTR. The results showed that the Luc[illegible]e activity was signifi-

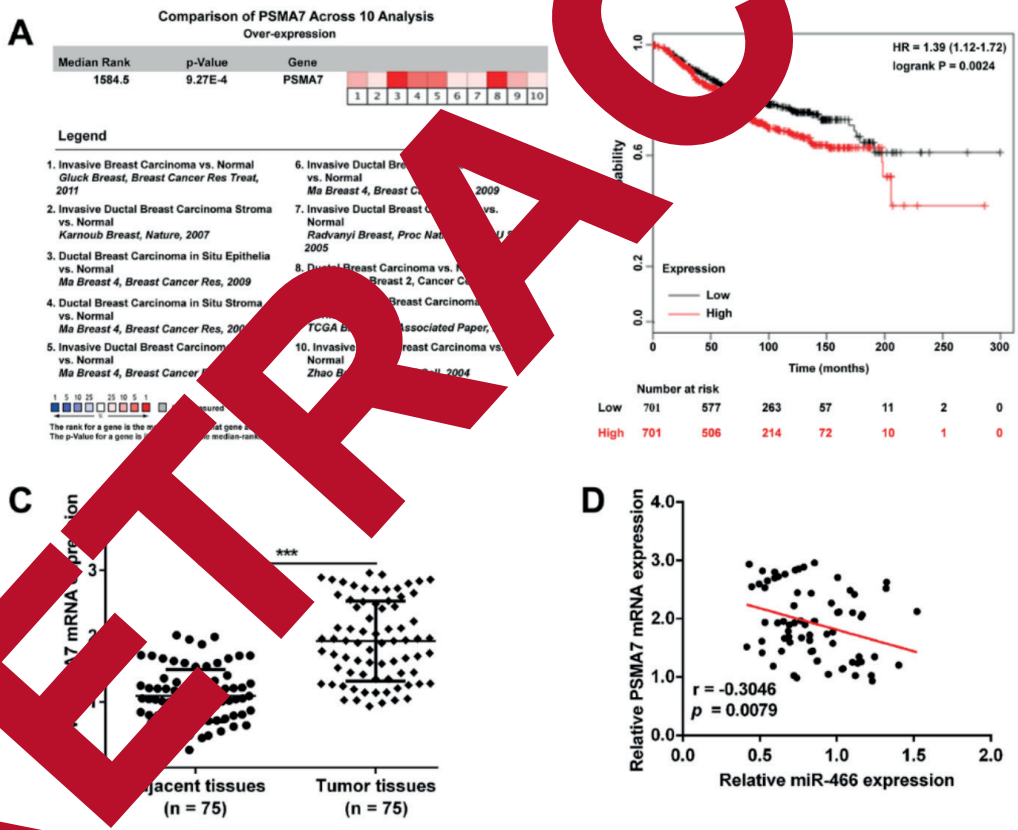


Figure 4. [illegible]gulation of PSMA7 was associated with poor prognosis of BC patients. **A**, Seven microarray datasets regard[illegible] PSMA7 mRNA expression in BC *vs.* normal tissues were included in our meta-analysis. Data are shown as the median [illegible] of PSMA7 through each dataset analysis. *p*-value for PSMA7 was presented using the median ranked analysis about BC [illegible]al tissues. **B**, Kaplan-Meier plots showing the effects of PSMA7 on five-year overall survival in BC. In red: patients wit[illegible]xpression above the median and in black, patients with expressions below the median. **C**, Relative expression of PSMA7 in normal adjacent tissues and tumors from BC patients were detected by RT-qPCR (n = 75); ***$p < 0.001$, compared with adjacent tissues; (**D**) The correlation between miR-466 and PSMA7 expression in BC tissues (n = 75) was analyzed using Spearman's correlation coefficient.

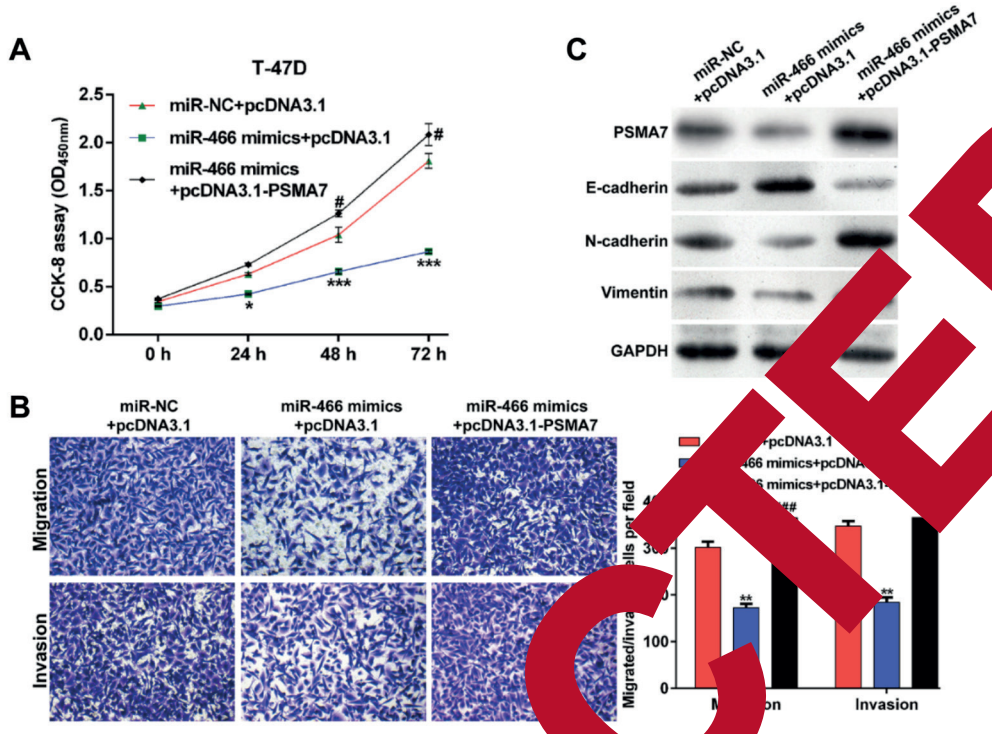


Figure 5. Restoration of PSMA7 counteracted the c iferation, migra nvasion inhibition caused by miR-466 in BC cells. T-47D cells were co-transfected with mi r miR-NC together with pcDNA3.1 or pcDNA3.1-PSMA7. **A**, Cell proliferation rate was determined by CCK- ay d T-47D cells. **B**, Cell migration and invasion were evaluated using transwell assay in transfected T-47D (mag 00). Data were expressed as mean±SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p <$ pared with miR-NC + pcDNA3.1; #$p < 0.05$, ###$p < 0.001$, compared with miR-466 mimics + pcDNA3.1. **C**, The p xpression of PSMA7, E-cadherin, N-cadherin and Vimentin was measured using western blot anal nsfected T cells.

cantly decreased in WT MA7 l porter plasmid when tra of miR- o mimics rather than miR-N nsfection in both T-47D (Fig 3B) and ZR 0 (Figure 3C) cells. Mo qPCR (Fig 3D) and Western blot alysis re 3E) consistently demonstr that PSMA ession levels were remark reduced in T-47D and ZR-75-30 cells after R-466 imics transfection compared wi R-NC nsfection. The above results suggested R-466 nregulated the expression of P 7 by ctly binding to its 3'UTR in cells.

Up-regulation of PSMA7 was Associated with Poor Prognosis of BC Patients

To have a good knowledge of PSMA7 expression levels in BC tissues, we performed meta-analysis of PSMA7 gene expression using public microarray datasets from Oncomine database. As shown in Figure 4A, a total of seven online microarray datasets, including Gluck Breast, Karnoub Breast, Ma Breast 4, Radvanyi Breast, Richardson Breast 2, TCGA Breast and Zhao Breast datasets were included in our study, which consistently indicated that the mRNA expression of PSMA7 was significantly overexpressed in BC tissues compared with normal tissues (gene median rank: 1584.5, $p = 9.27E-4$). Using Kaplan-Meier Plotter database, we evaluated the prognostic value of PSMA7 expression in BC patients and found that higher PSMA7 expression was related to shorter overall survival in BC patients (Figure 4B). Additionally, RT-qPCR analysis further confirmed that PSMA7 mRNA expression levels were notably upregulated in 75 paired tumor tissues compared with matched adjacent tissues derived from BC patients (Figure 4C). Spearman's correlation coefficient analysis demonstrated that miR-466 expression was inversely correlated with PSMA7 expression in the same BC tumor tissues (Figure 4D). Collectively, these data demonstrated that PSMA7 was overexpressed in BC and predicted poor survival prognosis.

Restoration of PSMA7 Reversed the Suppression of Cell Proliferation, Migration and Invasion BC Cells with the MiR-466 Mimics

To further investigate whether PSMA7 participated in the functional regulation of miR-466 in BC cell proliferation, migration and invasion, rescue experiments were performed in T-47D cells after co-transfection with miR-466 mimics and pcDNA3.1-PSMA7. The results from CCK-8 assay indicated that PSMA7 overexpression significantly abolished the suppressive effects of miR-466 overexpression on cell proliferation rate in T-47D cells (Figure 5A). Similarly, transwell assay demonstrated that significantly decreased number of migratory and invasive cells were observed in miR-466 mimics plus pcDNA3.1 transfection compared with miR-NC plus pcDNA3.1-transfection, which was reversed by co-transfection with miR-466 mimics plus pcDNA3.1-PSMA7 (Figure 5B). Western blotting indicated that the overexpression of PSMA7 reversed the decreased PSMA7 protein expression induced by miR-466 overexpression (Figure 5C). Furthermore, we found PSMA7 overexpression attenuated the suppressive effects of miR-466 overexpression on EMT markers (increased E-cadherin, decreased N-cadherin and Vimentin) in T-47D cells (Figure 5C). These data supported that miR-466 suppressed BC cell proliferation, migration and invasion by repressing PSMA7.

Discussion

In the present study, we found that the expression of miR-466 was remarkably downregulated in BC tissues and cell lines compared with corresponding controls. Clinical statistical analysis showed that decreased miR-466 expression was associated with poor prognosis in BC patients. In agreement with our data, Tong et al[11] previously demonstrated that low expression of miR-466 was significantly associated with tumor size, tumor node metastasis stage, lymph node metastasis, distant metastasis and poor prognosis in colorectal cancer patients. Cao et al[12] also reported that miR-466 expression levels were downregulated in osteosarcoma tissues and negatively correlated with metastasis and TNM stage and poor prognosis in patients with osteosarcoma.

Through functional experiments, we found that miR-466 overexpression significantly inhibited the proliferation, migration and invasion of BC cells. Consistent with our *in vitro* data, the suppressive role of miR-466 on tumor growth and metastasis was revealed in prostate cancer[10], colorectal cancer[11], epithelial ovarian cancer[23] and esophageal squamous cell carcinoma. These data indicated that miR-466 might be a tumor suppressor in the progression and development of BC.

Up to now, it has been observed that several target genes, including PUMILIO homolog 1 (Prox1)[25], RUNX2[10,22], CCND1 and PTEN have been validated as the target genes of miR-466 and participated in various diseases. Here, we performed bioinformatics analysis to obtain insight into the molecular mechanism of miR-466 and selected PSMA7 as a potential target of miR-466. As demonstrated by previous studies, PSMA7 serves as a key regulator in the development of cancers[26], including lung cancer[13], colorectal cancer[14] and hepatocellular carcinoma[17]. Here, we further found PSMA7 was overexpressed in BC tissues and negatively correlated with miR-466 expression. Kaplan-Meier Plotter database analysis showed that higher PSMA7 expression was related to shorter overall survival in BC patients. Consistent with our analysis, Romanuik et al[17], Scotto et al[18] and Hu et al[27] consistently found the overexpression of PSMA7 in castration-recurrent prostate cancer, liver cancer, cervical cancer and colorectal cancer, respectively. In addition, high expression of PSMA7 is significantly correlated with liver metastasis in colorectal cancer[27]. Furthermore, rescue experiment showed that the overexpression of PSMA7 reversed the suppressive effects of miR-466 on cell migration, invasion and EMT transcription factors (E-cadherin, N-cadherin, and vimentin). These facts further supported that miR-466 suppressed the migration, invasion and EMT in BC cells might partially through targeting PSMA7.

Conclusions

The present results demonstrated that overexpression of miR-466 suppressed the cell proliferation, migration, invasion and EMT by targeting PSMA7 in BC cells. The novelty of this work is identification of miR-466/PSMA7 axis as a promising therapeutic target for BC treatment. However, these are some limitations to this work as follows: lacking *in vivo* experiments and determination of apoptotic proteins and deeper molecular exploration.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Helsinki Declaration and approved by the Research Ethics Committee of the People's Liberation Army Medical College.

Authors' Contributions

HY designed this research. XY, ZSJ and YX carried out most experiments in this work and drafted this manuscript. WC and LQW helped with the western bolt experiments and helped perform statistical analysis. DHX helped to draft the manuscript. All authors read and approved the final manuscript.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Consent for Publication

We have obtained consents to publish this paper from all the participants of this study.

Conflict of Interest

The authors declare that they have no competing i

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