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, were performed to analyze cell proliferation, migration and invasion ability. The association between miR-466 and proteasome subunit α7 (PSMA7) was confirmed by Luciferase reporter assay.

RESULTS: Here, we first observed that the expression of miR-466 was significantly downregulated in BC tissues and cell lines. The decreased miR-466 expression was significantly associated with tumor size (p = 0.003), lymph node metastasis (p = 0.008), TNM stage (p = 0.032) and poor survival rate. In addition, miR-466 was identified as an independent prognostic factor for BC patients. We further found that the overexpression of miR-466 significantly inhibited cell proliferation, migration and invasion. Mechanistically, PSMA7 was a potential target gene of miR-466 and negatively regulated miR-466 in BC cells. Oncomine database and Kaplan-Meier overall survival analysis indicated that upregulation of PSMA7 was associated with poor prognosis of BC patients. The rescue experiments demonstrated that PSMA7 overexpression reversed the effects of miR-466 on cell proliferation, migration, invasion and EMT transcription factors (E-cadherin, N-cadherin, and vimentin).

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

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

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quently amplified in tumor. 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. Most studies have reported the overexpressed expression of PSMA7 and its oncogenic role in different tumor cells. For instance, Romanuik et al observed the higher or increased PSMA7 expression in castration-recurrent prostate cancer. Shi et al, Scotto et al and Hu et al 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. The shRNA-mediating silencing of PSMA7 decreased cell proliferation, induced cell cycle G0/G1 phase arrest and apoptosis in cervical cancer cells. Xia et al also manifested that PSMA7 knockdown suppresses the proliferation, migration, invasion and subcutaneous tumorigenesis of gastric cancer cells in nude mice. Interestingly, Richardson et al revealed that PSMA7 expression was overexpressed in testicular and BC. Based on these facts, we thus speculated that PSMA7 might promote the malignant cellular behaviors in BC cells by functioning as an oncogene.

In our study, we investigated the expression levels of miR-466 and PSMA7, as well as their prognostic values in BC patients using available tumor tissue samples or online bioinformatic analysis. Through functional experiments, we evaluated the regulatory effects of miR-466 on cell proliferation, migration and invasion. We further confirmed the association between miR-466 and PSMA7 using Luciferase reporter assay. Importantly, whether PSMA7 is downstream of or involved in miR-466-modulating cell functions was additionally demonstrated by performing rescue experiments. These findings might help identify another promising therapeutic target for BC.

Patients and Methods

Clinical Tissue Specimens

A total of 75 paired tumor tissues and matched adjacent tissues were collected from BC patients who underwent 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 specimens were stored in liquid nitrogen for further analysis. After clinical diagnosis, the basic patient information, including age, tumor size and lymph node metastasis are summarized in Table I. Before surgery, all patients signed written informed consents and were confirmed not to receive any anti-tumor treatments, including chemotherapy or immunotherapy. After surgery, each patient was performed five-year follow-up and the corresponding survival data was obtained by telephone communication. 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.

Cell Culture Conditions

Four BC cell lines (MDA-MB-231, MCF-7, T-47D and ZR-75-30) and a normal human breast epithelial cell line MCF-10A were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in humidified incubator containing 5% CO₂.

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 1×10⁵ 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
MiR-466 targets PSMA7 in breast cancer

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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases (n = 75)</th>
<th>miR-466 expression</th>
<th>p-value</th>
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<tbody>
<tr>
<td></td>
<td>Low (n = 48)</td>
<td>High (n = 27)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
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<tr>
<td>&lt; 50</td>
<td>30</td>
<td>20</td>
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</tr>
<tr>
<td>≥ 50</td>
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<td>28</td>
<td>0.003</td>
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<td>Tumor size (cm)</td>
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<tr>
<td>&lt; 3</td>
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<td>32</td>
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<tr>
<td>≥ 3</td>
<td>33</td>
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<td>Lymph node metastasis</td>
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<tr>
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<td>Estrogen receptor status</td>
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<td>Positive</td>
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<td>Epidermal growth factor receptor 2 status</td>
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<td>20</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
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</table>

miR, microRNA; TNM, tumor-node-metastasis classification system.

synthesized using miScript II RT kit (TaKaRa, Dalian, China). Using the miScript II RT kit, we synthesized miRNA assay kit (Applied Biosystems, Carlsbad, CA, USA). For the expression of miR-466, we quantified miR-466 with U6 as the internal control. For PSMA7 quantification, reverse transcription was performed using M-MLV cDNA synthesis kit (Promega Corporation, Madison, WI, USA). Gene expression levels were examined with SYBR Premix Ex Taq II (TaKaRa Bio, Tokyo, Japan) with GAPDH as the internal control. The thermocycling conditions for RT-qPCR were as follows: 95°C for 30 s, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The primer sequences used in this study were as follows: miR-466 forward, 5′-CTACCACGTGGGTCCCCTC-3′; reverse, 5′-GACGGCCGCATCTTTCTTGTT-3′; U6 forward, 3′-GCTTCGGCAGCACATATACTAAT-5′; reverse, 3′-CGCTTCAGATTTCGCTGTCAT-5′; PSMA7 forward, 5′-GACGGCCGCATCTTTCTTGTT-3′; and GAPDH forward, 5′-GACGGCCGCATCTTTCTTGTT-3′; and reverse, 3′-CACACCGACCTTACATTTT-5′. Relative gene expression levels were calculated using the 2-ΔΔCt 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-
seeded 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 in five randomly selected fields under a light microscope at a magnification of ×200. All experiments were repeated three times.

**Luciferase Reporter Assay**

PSMA7 was predicted as a potential target of miR-466 through TargetScan 7.1 (http://www.targetscan.org/vert_71/), which was further validated by performing Luciferase reporter assay. Briefly, the predicted wild-type 3’-UTR of PSMA7 and a mutated sequence within the predicted target site were subcloned into the pGL3 reporter Luciferase vector (Promega Corporation, Madison, WI, USA) to generate WT and MUT PSMA7 plasmids, respectively. Subsequently, BC cells were co-transfected with 50 nM of miR-466 mimics or miR-NC with 0.1 µg of the WT or MUT PSMA7 plasmid and lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) for 48 h. Next, Luciferase activity was determined using Dual-Luciferase reporter assay system (Promega Corporation, Madison, WI, USA) the ratio of Renilla and firefly Luciferase as the relative Luciferase activity. All experiments 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 corresponding concentration was determined by Bicinchoninic Acid (BCA) assay (Beyotime Institute of Biotechnology). Equal amounts of protein samples (30 µg) were separated using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyethylene difluoride (PVDF) membranes. After blocking in non-fat powdered milk for 1 h at room temperature, the membranes were incubated with primary antibodies against PSMA7, E-cadherin, N-cadherin, Vimentin and GAPDH overnight 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.oncomine.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-

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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>Age</td>
<td>0.654 (0.512-1.789)</td>
<td>0.601</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>1.212 (0.785-2.012)</td>
<td>0.032</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>1.065 (0.684-1.892)</td>
<td>0.013</td>
</tr>
<tr>
<td>TNM stage</td>
<td>1.885 (1.237-3.625)</td>
<td>0.027</td>
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<tr>
<td>Estrogen receptor status</td>
<td>0.843 (0.660-2.901)</td>
<td>0.435</td>
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<tr>
<td>Progesterone receptor status</td>
<td>1.193 (0.898-1.597)</td>
<td>0.381</td>
</tr>
<tr>
<td>Epidermal growth factor receptor 2 status</td>
<td>1.489 (0.998-3.021)</td>
<td>0.751</td>
</tr>
<tr>
<td>miR-466 expression</td>
<td>1.453 (0.585-1.978)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

HR: hazard ratio; CI: confidence interval; TNM, tumor-node-metastasis classification system; NA, not analyzed.
MiR-466 targets PSMA7 in breast cancer

Statistical Analysis

All experiments were performed three times and data were expressed as mean ± SD. Statistical analysis was carried out using SPSS version 22.0 software (IBM, Armonk, NY, USA) or GraphPad Prism 6.0 software (GraphPad Software Inc.). The correlation between miR-466 expression and the clinicopathological characteristics of BC was analyzed using the Chi-square test. The overall survival rate was assessed using Kaplan-Meier and log-rank tests. The prognostic significance of miR-466 expression was evaluated using univariate and multivariate Cox regression analyses. The correlation between miR-466 and PSMA7 expression in BC tissues was analyzed using Spearman’s correlation coefficient. Statistical differences between groups were assessed using Student’s t-tests or
Results

Decreased MiR-466 Expression Predicted Poor Prognosis in BC Patients

To evaluate the clinical significance of miR-466 in BC patients, we first determined the expression pattern of miR-466 using RT-qPCR. As shown in Figure 1A, the expression of miR-466 was significantly downregulated in 75 paired tumor tissues compared with matched adjacent tissues derived from BC patients. Next, all patients were divided into high-expression group and low-expression group based on the median level of miR-466 expression 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 Target Gene of MiR-466 in BC Cells

To better understand the mechanisms underlying the suppressive effects of miR-466 on cell proliferation and metastasis, the target genes of miR-466 were predicted online, of which PSMA7 was selected as a candidate target of miR-466 and the predicted interaction between them was illustrated in Figure 3A. Subsequently, Luciferase reporter assay was applied to examine whether miR-466 directly targeted the predicted binding sites in the PSMA7 3’UTR. The results showed that the Luciferase activity was signifi-
Significantly decreased in WT PSMA7 luciferase reporter plasmid when co-transfection of miR-466 mimics rather than the miR-NC transfection in both T-47D (Figure 3B) and ZR-75-30 (Figure 3C) cells. Moreover, RT-qPCR (Figure 3D) and Western blot analysis (Figure 3E) consistently demonstrated that PSMA7 expression levels were remarkably reduced in T-47D and ZR-75-30 cells after miR-466 mimics transfection compared with normal transfection. The above results suggested that miR-466 could repress the expression of PSMA7 by directly binding to its 3’UTR in BC cells.

Upregulation 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, \( p = 9.27 \times 10^{-4} \)). 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.

Figure 5. Restoration of PSMA7 counteracted the cell proliferation, migration and invasion inhibition caused by miR-466 in BC cells. T-47D cells were co-transfected with miR-466 mimics + pcDNA3.1 or pcDNA3.1-PSMA7. A, Cell proliferation rate was determined by CCK-8 assay in transfected T-47D cells. B, Cell migration and invasion were evaluated using transwell assay in transfected T-47D cells (magnification ×200). C, The protein expression of PSMA7, E-cadherin, N-cadherin and Vimentin was measured using western blot analysis in transfected T-47D cells.

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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. previously demonstrated that low expression of miR-466 was significantly correlated with tumor size, tumor metastasis, lymph node metastasis, distant metastasis and poor prognosis in colorectal cancer patients. Cao et al. 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, colorectal cancer, epithelial ovarian cancer 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 proved that several target genes, including PROSPERO homeobox 1 (Prox1), RUNX2, CCND1 and PTEN, have been validated as target genes of miR-466 and participated in different diseases. Here, we performed bioinformatics analysis to obtain insight into the molecular mechanisms 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 tumors, including lung cancer, colorectal cancer and hepatocellular carcinoma. Here, we further found PSMA7 was overexpressed in BC tissues and negatively correlated with miR-466 expression in Kaplan-Meier Plotter database analysis. We found that higher PSMA7 expression was related to shorter overall survival in BC patients. Consistent with our analysis, Romanuik et al., Scotto et al. and Hu et al. 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. 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 blot 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 interests.

References


