LncRNA PSMA3-AS1 activates the progression of triple-negative breast cancer cells by blocking miR-186-5p-mediated PSME3 inhibition

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ABSTRACT

Triple-negative breast cancer (TNBC) is an aggressive malignant tumor with a high death rate in the whole world. This cancer mainly occurs in young women group possessed with poor prognoses. Long noncoding RNAs (lncRNAs) are known for regulating human diseases and cancers. Even though growing researches have illuminated that lncRNAs have a close relation with TNBC progression, the function of lncRNAPSMA3 antisense RNA 1 (PSMA3-AS1) in TNBC has not been discussed and exposed yet. In the present research, the expression pattern and functional role of PSMA3-AS1 were analyzed and unveiled with the help of RT-qPCR and functional assays. The findings demonstrated that PSMA3-AS1 was notably upregulated in TNBC cells. Silencing of PSMA3-AS1 had suppressing effects on TNBC cell growth and migration. Mechanistically, PS-MA3-AS1 induced upregulation of proteasome activator subunit 3 (PSME3) by functioning as a miR-186-5p sponge. Furthermore, rescue assays certified that overexpression of PSME3 or inhibition of miR-186-5p could abrogate the inhibiting role of silenced PSMA3-AS1 on TNBC cell functions. To summarize, PSMA3-AS1 abolishes miR-186-5p-mediated suppression on PSME3 to accelerate TNBC progression.

Introduction

Triple-negative breast cancer (TNBC) is a highly aggressive malignant tumor with a high death rate in the whole world (1,2). At present, cytotoxic chemotherapy is systemic chemotherapy aimed at the early stage of TNBC, but other adjuvant therapies have been emerging such as targeted agents, chemotherapy, platinum-based drugs, etc. (3,4). The patients with TNBC have the worst overall survival, indicating poor prognosis (5,6). To optimize and improve treatment approaches, long noncoding RNAs (lncRNAs) have obtained more attention because of their regulation mechanisms in multiple diseases and tumors.

LncRNAs are a group of RNA molecules with 200 nt in length and they are almost incapable of coding proteins (7). With the continuous improvement of cognition of lncRNA, lncRNA is no longer just a useless transcription noise in people's eyes, but a major regulator that can participate in the process of cell biology (8). Importantly, an increasing number of researches have supported that lncRNAs contribute to the progression of many human cancers due to their dysregulation. Wang et al. (9) have discovered that certain lncRNAs regulated TNBC and this finding offers value clues for the diagnosis and treatments of TNBC. Besides, lncRNA SNHG12 attends to the oncogenic potential of TNBC by interplaying with c-MYC as a transcription factor (10). DANCR expression is upregulated in TNBC and its silence depresses the TNBC progress (11). However, there are some lncRNAs to play a tumor-suppressing role in different cancers. For example,

overexpression of GAS5 can partially damage the tumor promotion to suppress TNBC progression (12). Collectively, important knowledge was acquired by us that lncRNAs exerts different function in cancers when they are expressed at different levels.

PSMA3 antisense RNA 1 (PSMA3-AS1) has been identified in several cancers as an oncogenic lncRNA. For example, PSMA3-AS1 highly expressed in esophageal cancer cells contributed to cancer cell growth through the regulation of the miR-101/EZH2 axis (13). Also, PS-MA3-AS1 was confirmed to facilitate cell activities of lung cancer (14). Recently, it was reported that PSMA3-AS1 could expedite glioma cell processes via modulating miR-302a-3p-inhibited RAB22A (15). However, whether PSMA3-AS1 is a biological participant to affects TNBC is still unknown.

In recent years, evidence has supported that lncRNA could mediate cancer progression by competing endogenous RNA (ceRNA) mechanisms (16). CeRNA network is a regulatory mechanism between RNAs interaction, and it means that lncRNA could sequester microRNAs (miRNAs), so as to release messenger RNAs (mRNAs) from the inhibition of miRNAs (17,18). For example, GAS6-AS2 accelerated cell proliferation of bladder cancer through sponging miR-298 to regulate CDK9 (19).

In the present study, we concentrated on exposing the participation of PSMA3-AS1 in the cell processes of TNBC. Furthermore, the regulatory mechanism and interaction among PSMA3-AS1 and other RNAs were also investigated and discussed.

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Materials and Methods

Cell lines

Human normal breast epithelial cell line MCF-10A and human TNBC cell lines (MDA-MB-468, MDA-MB-231, HCC-1937) were procured from ATCC for cellular experiments. MCF-10A cells were kept in MEBM with an additional MEGM Single Quot Kit and cholera toxin. HCC-1937 cells were maintained in RPMI-1640 medium with an additional 10% FBS. MDA-MB-468 and MDA-MB-231 cells were cultured in Leibovitz's L-15 medium with an additional 10% FBS. All were kept in a 5% CO_2 incubator at 37 °C.

RT-qPCR

Total RNA extraction was achieved with TRIzol Reagent, and then cDNA synthesis was completed using the PrimeScript Reverse Transcriptase Kit. qPCR assay was undertaken with SYBR Green PCR Kit (Takara) on Step-One Plus Real-Time PCR System. The calculation of relative gene expression was made by using the $2^{-\Delta\Delta Ct}$ method. U6 or GAPDH was taken as the internal control gene for standardization.

Plasmid transfection

MDA-MB-468 and HCC-1937 were seeded into 6-well plates for transfection with shRNAs targeting PSMA3-AS1 or PSME3 using Lipofectamine 3000 (Invitrogen). Besides, the full PSME3 sequence was inserted into the pcDNA3.1 vector (Invitrogen) for PSME3 overexpression. The miR-186-5p inhibitors, miR-186-5p mimics and their NCs were procured from Ribobio.

Colony formation

The transfected cells (800) were reaped and planted into 6-well plates. After being incubated for 2 weeks, methanol was added to fix colonies, and then 0.5% crystal violet solution was added to count colonies manually. This assay contained 3 individual repeats.

EdU assay

Cell proliferation detection assay was performed in 96-well plates by use of BeyoClick™ EdU Cell Proliferation Kit, in light of direction. The transfected cells (1 × 104) were fixed and treated with EdU kit for 2 h, followed by DAPI staining at room temperature. Later, stained cells were analyzed by observing under a fluorescence microscope (Olympus, Tokyo, Japan). This assay contained 3 individual repeats.

JC-1 assay

JC-1 assay was implemented in 6-well plates to detect the change of mitochondrial membrane potential (Δψm). 1 mL of cell suspension was prepared and mixed with 2.5 μg/ml of JC-1 dye (Beyotime) for 30 min. Finally, the stained cells were observed by fluorescence microscope (Olympus).

TUNEL assay

The analysis of apoptotic cells was made by using the One-Step TUNEL Apoptosis Assay Kit. Briefly, a total of 1 × 104cells collected after transfection were put into 96-well plates, followed by fixation and permeabilization. Af-ter nuclei staining with DAPI, the results were monitored using an Olympus fluorescence microscope.

Wound healing

To monitor migrating cells, cells were seeded in 6-well plates for wound healing detection. 1 × 106 transfected cells were cultured until reached 100% cell confluence. And then, using a 200-μL pipette tip for making wounds. The wound healing ability was determined and analyzed at two time points of incubation (0 and 24 h). This assay contained 3 individual repeats.

Subcellular fraction assay

RNA abundance in two cellular parts was determined using PARIS™ Kit to conduct subcellular fraction assay in MDA-MB-468 and HCC-1937. GAPDH and U6 were used as controls for RNAs in cytoplasmic and nuclear parts. This assay contained 3 individual repeats.

FISH

Cell samples of MDA-MB-468 and HCC-1937 were processed with a PSMA3-AS1-FISH probe in the hybridization buffer, as per the direction. After nuclei staining with DAPI, the results were monitored using an Olympus fluorescence microscope.

RNA pull-down

Using RIPA lysis buffer to obtain cell lysates, and then proteins were isolated and collected for mixing with PSMA3-AS1-specific biotin probe or control probe. The RNA-protein mixture was captured by adding magnetic beads, followed by digestion and elution. After RNA extraction, RT-qPCR was conducted. This assay contained 3 individual repeats.

RNA immunoprecipitation (RIP)

Using RIPA lysis buffer to obtain cell lysates, and then incubating it in RIP buffer with Ago2 antibody or control IgG antibody. Next, the immunoprecipitates were obtained using the magnetic beads. RNA enrichment was analyzed by RT-qPCR. This assay contained 3 individual replications.

Luciferase reporter assay

The full length of PSMA3-AS1 or PSME3 3'UTR containing the complementary sites with miR-186-5p was used to generate wild-type reporter vector, while the full length of them with the mutant complementary sites was utilized for generating mutant-type vectors. The prepared vectors were co-transfected with miR-186-5p mimics or NC into indicated cells for 48 h. Using a dual-luciferase reporter assay system obtained from Promega to assess the luciferase activity.

Statistical analyses

Data were compared and analyzed using statistical analysis software (SPSS v22.0) by using the Student's t-test or one-way ANOVA method. All experimental data were exhibited in bar plots as the means ± SD from 3 individual replications. Significant data were defined as $p < 0.05$.

Results

### PSMA3-AS1 induces the growth and migration of TNBC cells

To unveil the PSMA3-AS1 function, we first detected its expression in several different TNBC cells and normal cell lines (control) using RT-qPCR. The results help us identify the high expression of PSMA3-AS1 in TNBC cells (Figure 1A). Subsequently, shRNAs targeting PSMA3-AS1 were used for silencing PSMA3-AS1 expression in MDA-MB-468 and HCC-1937 cells. As we expected, PSMA3-AS1 dramatically declined by interfering with it compared with sh-NC groups (Figure 1B). Under this situation, we observed that proliferating cell numbers were decreased by sh-PSMA3-AS1 transfection, suggesting cell proliferation ability was significantly cut down due to knockdown of PSMA3-AS1 (Figure 1C-1D). However, the cell apoptosis rate shown in JC-1 and TUNEL results was remarkably elevated by PSMA3-AS1 depletion (Figure 1E-1F). Finally, the wound healing assay revealed that knocking down PSMA3-AS1 effectively suppressed the quantity of migrated cells (Figure 1G). From this researches, we could get a conclusion that PSMA3-AS1 promoted TNBC cell malignancies.

PSMA3-AS1 is miR-186-5p sponge

To confirm whether PSMA3-AS1 was able to sponge miRNA, we first probed its cellular distribution through subcellular fraction and FISH assays. The results uncovered that it was distributed in the cytoplasm, indicating that PSMA3-AS1 regulated genes level at the posttranscriptional level (Figure 2A-2B). To certify that PSMA3-AS1 could competitively bind to miRNAs, we predicted and found seven possible miRNAs interacting with PSMA3-AS1 from ENCORI (http://starbase.sysu.edu.cn/) with the specific condition (CLIP-Data >= 3; pan-Cancer >= 8). For screening, we tested the underlying miRNA that could combine with PSMA3-AS1 utilizing an RNA pull-down assay. As displayed in Figure 2C, miR-186-5p was abundant and enriched in the biotinylated PSMA3-AS1 probe compared to the control probe, while others had no obvious changes. Besides, ENCORI also helped us to forecast out the matched sites of PSMA3-AS1 in miR-186-5p sequence (Figure 2D). According to the data of the RIP assay, we clarified the enriched PSMA3-AS1 and miR-186-5p in the Anti-Ago2 group (Figure 2E). Afterward, miR-186-5p was effectively overexpressed in both TNBC cells after the transfection of miR-186-5p mimics (Figure 2F). For verifying the binding sites, we performed the luciferase reporter assays and we discovered that miR-186-5p mimics co-transfection overtly restrained the luciferase activity of PSMA3-AS1-WT in both cells, rather than PSMA3-AS1-Mut (Figure 2G). All assays showed that PSMA3-AS1 could bind to miR-186-5p.

### PSME3 is a target of miR-186-5p and is positively affected by PSMA3-AS1

Next, it was naturally performed to explore mRNA, as the downstream of miRNA out of the consideration of the ceRNA regulatory mechanism. Through utilizing the PITA, miRmap, microT, PicTar and TargetScan databases in ENCORI with the specific condition (CLIP-Data >= 5; Degradome-Data >= 1), we screened out 12 mutual target genes (Figure 3A). Then we detected their expression in
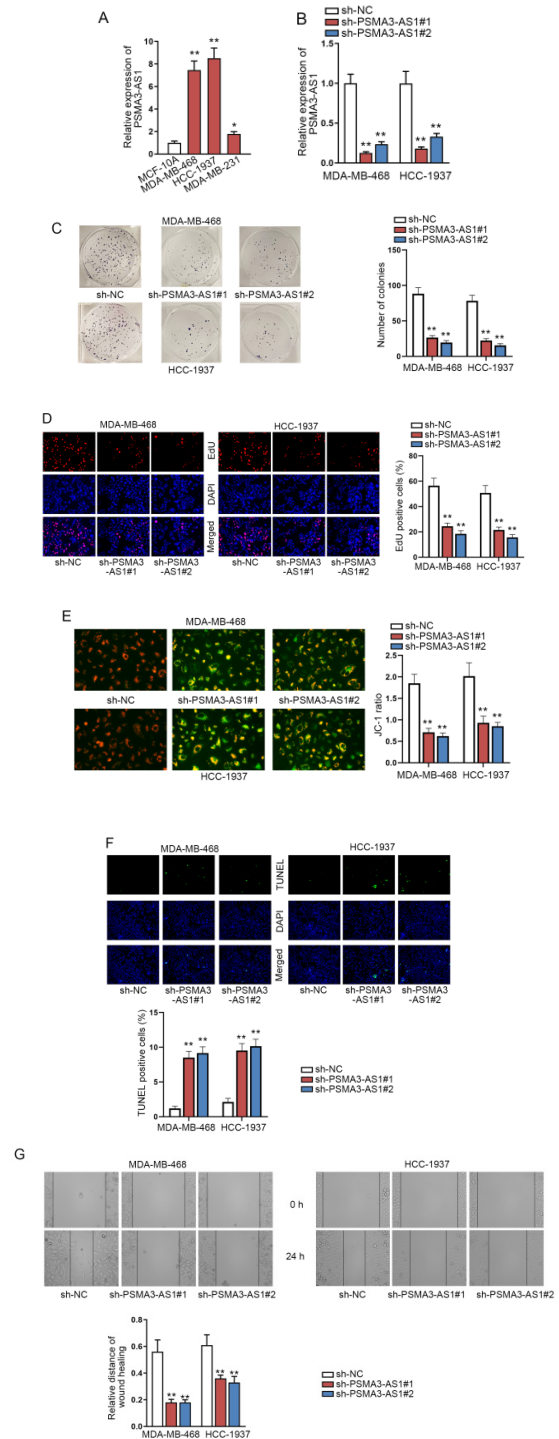
Figure 1. PSMA3-AS1 promotes TNBC cell proliferation and migration, but hampers cell apoptosis. (A) PSMA3-AS1 expression was detected by RT-qPCR in MDA-MB-468, HCC-1937 and MDA-MB-231 and human normal breast cells MCF-10A. (B) The interference efficiency of PSMA3-AS1 was assessed through RT-qPCR. (C-D) The ability of proliferation of MDA-MB-468 and HCC-1937 cells transfected sh-PSMA3-AS1 was measured by colony formation of EDU assay. (E-F) MDA-MB-468 and HCC-1937 cell apoptosis change was evaluated via JC-1 assay and TUNEL assay. (G) TNBC cell migration ability was tested via wound healing assay. *P<0.05, **P<0.01.

miR-186-5p mimics-transfected cells and we found that only two mRNAs (PSME3 and FNDC3A) expression was lowered (Figure 3B). Then we revealed that PSME3 rather than FNDC3A was notably highly expressed mRNA in TNBC cells (Figure 3C). We used ENCORI again to acquire the sequences of PSME3 and miR-186-5p contai-
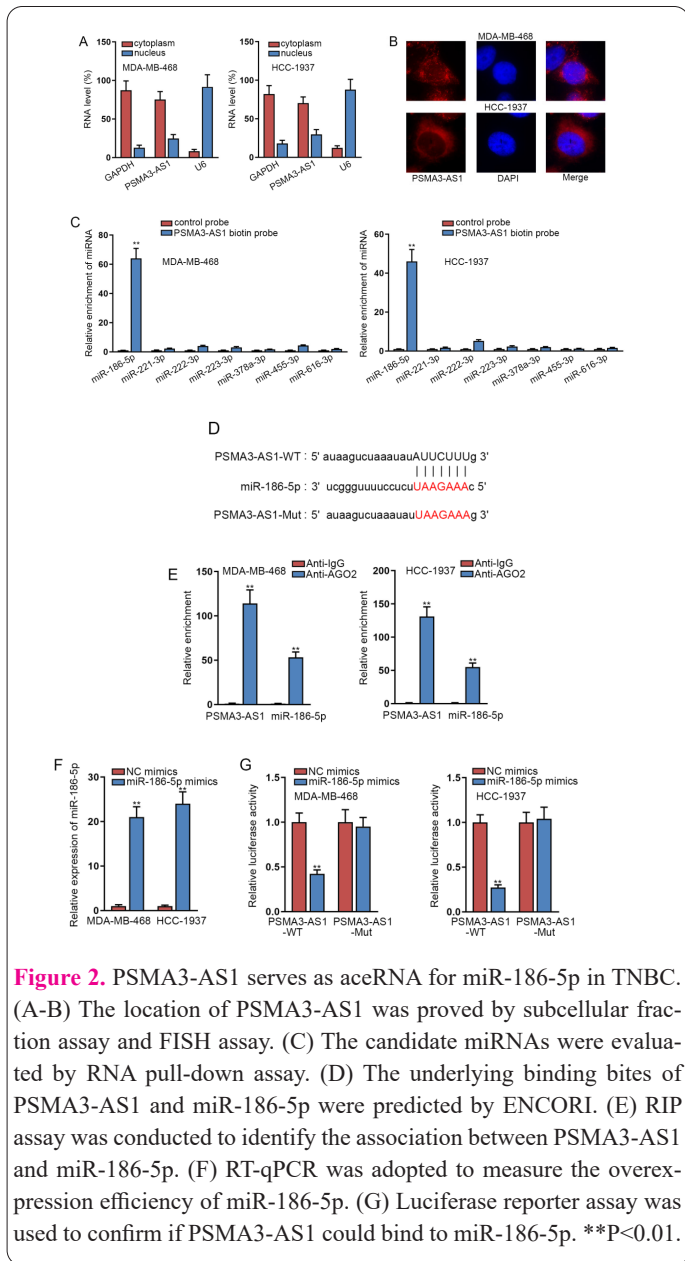
Figure 2. PSMA3-AS1 serves as aceRNA for miR-186-5p in TNBC. (A-B) The location of PSMA3-AS1 was proved by subcellular fraction assay and FISH assay. (C) The candidate miRNAs were evaluated by RNA pull-down assay. (D) The underlying binding bites of PSMA3-AS1 and miR-186-5p were predicted by ENCORI. (E) RIP assay was conducted to identify the association between PSMA3-AS1 and miR-186-5p. (F) RT-qPCR was adopted to measure the overexpression efficiency of miR-186-5p. (G) Luciferase reporter assay was used to confirm if PSMA3-AS1 could bind to miR-186-5p. **P<0.01.

ning complementary sites with each other (Figure 3D). Thus, PSME3 was chosen to be the underlying target gene for follow-up assays. The RIP and luciferase reporter assay also further confirm the combination among three RNAs in TNBC cells. As exposed in Figure 3E, PSMA3-AS1/miR-186-5p/PSME3 was eminently abundant in Anti-Ago2 complex instead of Anti-IgG groups. Also, the luciferase activity of the vector inserted with wild-type PSME3 sequence was visibly cut down due to miR-186-5p upregulation, whereas PSME3-Mut was not affected with the same condition (Figure 3F). In order to illuminate the regulatory network, the rescue assay was carried out. Firstly, miR-186-5p was effectively inhibited in cells with miR-186-5p inhibitor transfection (Figure 3G). Next, we detected the alteration of PSME3 expression. The consequence revealed that PSME3 expression was silenced by sh-PSMA3-AS1#1, but fully recovered by miR-186-5p knockdown (Figure 3H). Taken together, PSME3 was targeted by miR-186-5p and positively associated with PSMA3-AS1.

PSME3 acts as an oncogene in TNBC cells

We further conducted loss-of-functional assays. First-

ly, PSME3 was signally depressed by sh-PSME3#1 or sh-PSME3#2 in comparison with sh-NC in both cells (Figure 4A). Subsequently, we demonstrated that silencing PSME3 prevented TNBC cells from proliferating (Figure 4B-4C). Inversely, the JC-1 assay and TUNEL assay suggested that declining PSME3 was able to accelerate cell apoptosis (Figure 4D-4E). The wound healing assay showed that PSME3 down-regulation also hampered the TNBC cell migration (Figure 4F). Thus, PSME3 acted as an oncogene in TNBC and accelerated TNBC progression.

PSMA3-AS1 promotes TNBC cell malignancies via modulating miR-186-5p/PSME3 axis

In the final step, we designed a train of recuse assay to expound the role of the whole ceRNA network in the progression of TNBC. Firstly, both cells were transfected with PSME3 overexpression vector to enhance the expression of PSME3 (Figure 5A). Following this, the inhibition
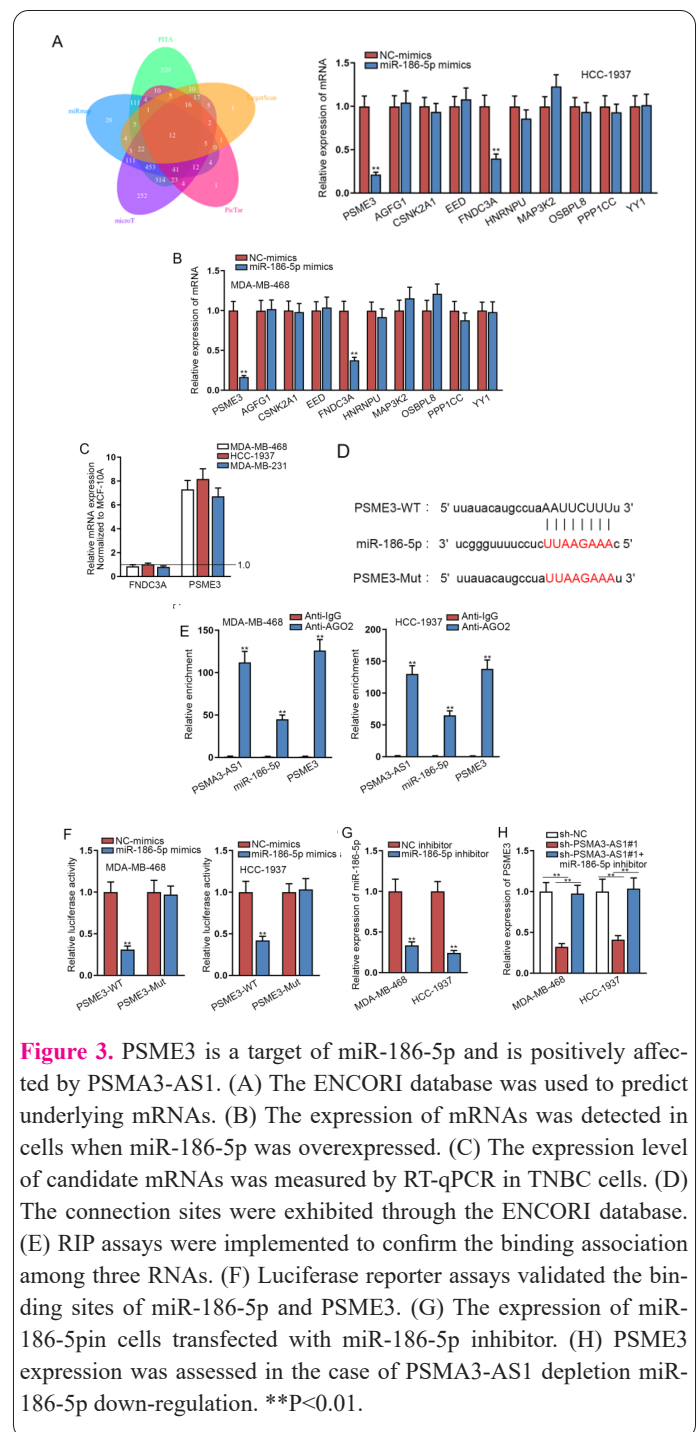
Figure 3. PSME3 is a target of miR-186-5p and is positively affected by PSMA3-AS1. (A) The ENCORI database was used to predict underlying mRNAs. (B) The expression of mRNAs was detected in cells when miR-186-5p was overexpressed. (C) The expression level of candidate mRNAs was measured by RT-qPCR in TNBC cells. (D) The connection sites were exhibited through the ENCORI database. (E) RIP assays were implemented to confirm the binding association among three RNAs. (F) Luciferase reporter assays validated the binding sites of miR-186-5p and PSME3. (G) The expression of miR-186-5pin cells transfected with miR-186-5p inhibitor. (H) PSME3 expression was assessed in the case of PSMA3-AS1 depletion miR-186-5p down-regulation. **P<0.01.
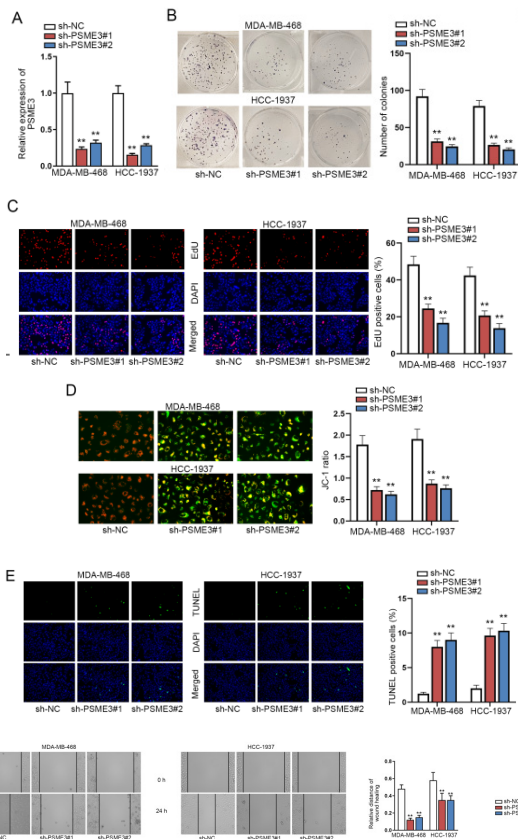
Figure 4. PSME3 acts as an oncogene in TNBC. (A) The interference efficiency of PSME3 was tested with the help of RT-qPCR. (B-C) The cell proliferation capacities were measured by colony formation assay and EdU assay in the case of silencing PSME3. (D-E) JC-1 assay and TUNEL assay were performed to assess the abilities of cell apoptosis. (F) The wound healing assay was used to observe the change of migration in transfected cells with sh-PSME3. **P<0.01.

of TNBC cell proliferation, caused by silencing PSMA3-AS1, was totally reversed with the absence of miR-186-5p or the augment of PSME3 (Figure 5B-5C). Also, inhibiting PSMA3-AS1 expression could promote cell apoptotic capability, while this result was changed by inhibiting miR-186-5p or boosting PSME3 (Figure 5D-5E). In the end, the wound healing assays told us that the cell migration suppression resulting from PSMA3-AS1 down-regulation was completely recovered by miR-186-5p inhibitor or pcDNA3.1-PSME3 (Figure 5F). On the whole, PSMA3-AS1 serves as a facilitator in the progression of PSMA3-AS1 via regulating miR-186-5p/PSME3 axis.

Discussion

TNBC is a severe and aggressive malignant tumor with a high death rate in the whole world. There is no very effective treatment for TNBC, so it is necessary to study its pathogenesis. After lncRNAs were found to have biological functions, a link between lncRNAs and cancers/diseases has been widely reported in recent years (20,21). For instance, the patients with colorectal cancer, who had strong AFAP1-AS1 expression possess the worst poor prognosis (22). LncRNA-AK001085 insufficient is closely associated with ankylosing spondylitis (AS) (23). AFAP1-AS1 is taken for a key tumor-associated lncRNA and its aberrant expression has been found in malignancies until now such as gastric cancer, cholangiocarcinoma, gallbladder cancer,

etc. (24). In this study, we mainly investigated PSMA3-AS1 in TNBC, which has not been studied so far. In previous researches, a high level of PSMA3-AS1 was found in cancer cells, such as esophageal cancer (13), lung cancer (14) and glioma (15), and PSMA3-AS1 exerted a carcinogenic effect in them. Similarly, we detected and discovered as expected that PSMA3-AS1 was highly expressed in TNBC cells. Importantly, we also proved the suppressed TNBC cell growth and migration when PSMA3-AS1 was knocked down. Thus, we confirmed that PSMA3-AS1 had oncogenic lncRNA potential in TNBC.

MiRNAs are known as post-transcriptional regulators through repressing mRNA translation (25). In the ceRNA hypothesis, miRNAs have been considered to be sponged by lncRNAs to regulate the progression of human cancers (26). For example, lncRNA SNHG5/miR-32 axis was confirmed to modulate gastric cancer cell growth by targeting KLF4 (27). SNHG15 could facilitate cell proliferation of osteosarcoma via sponging miR-141 (28). Thus, in our research, we also suspected the miRNA sponge role of PSMA3-AS1 in TNBC cells. Through the bioinformatics prediction and a series of mechanism assays, miR-186-5p was proved to be sponged by PSMA3-AS1. The downregulation of miR-186-5p was able to erase the silenced PSMA3-AS1-induced inhibition of cell proliferation, and migration. It was reported by previous research that miR-186-5p could act as a repressor for cell metastasis of colorectal cancer by regulating ZEB1 (29). Importantly, a recent study has indicated the inhibiting role of miR-186-5p in the cell activities of breast cancer (30).

PSME3 has been proven to involve dissimilar tumors
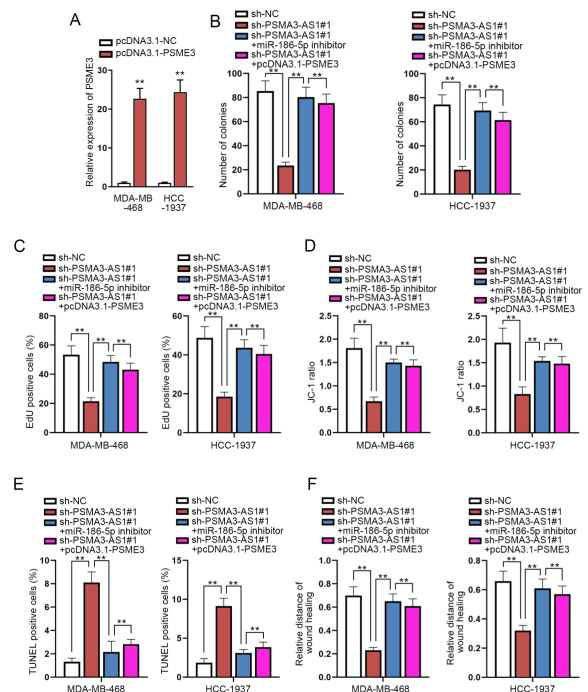
Figure 5. PSMA3-AS1 promotes the progression of TNBC via modulating miR-186-5p/PSME3 axis. (A) The overexpression efficiency of PSME3 was assessed by RT-qPCR. (B-C) The capacity of TNBC cell proliferation was evaluated by colony formation assay and EdU assay in different groups. (D-E) The cell apoptosis ability was measured via JC-1 assay and TUNEL assay in different groups. (F) The cell migration ability was evaluated by wound healing assay in different groups. **P<0.01.

by accumulating researches. For example, PSME3 could be targeted by miR-585-3p and accelerate cell growth of colon cancer (31). PSME3 could also facilitate cell proliferation and invasion of pancreatic cancer through the c-Myc-glycolysis signaling axis (32). Furthermore, PSME3 induced the EMT process of breast cancer cells (33-36). Here, we also proved that PSME3 acted as a target gene of miR-186-5pin TNBC and it played the oncogene effect on TNBC cells. Knocking down PSME3 had the same effect as silencing PSMA3-AS1 with anti-proliferation and anti-migration and pro-apoptosis, reflecting a role of tumor-pushing in TNBC.

In the present assay, we also conducted the rescues assays, whose results demonstrated that PSME3 expression declined with the absence of PSMA3-AS1, and was upregulated with the absence of miR-186-5p. Overexpression of PSME3 could reverse silenced PSMA3-AS1-induced inhibition on TNBC progression.

Conclusion

To make a long story short, PSMA3-AS1 promotes the progression of TNBC via modulating the miR-186-5p/PSME3 axis. Our findings highlight a crucial role for PSMA3-AS1 in the regulation of TNBC and might open new avenues for future TNBC therapies.

Disclosure statement
There are no competing interests in this study.

Data availability statement
Not applicable.

Acknowledgement

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