Long non-coding RNA PSMA3-AS1 enhances cell proliferation, migration and invasion by regulating miR-302a-3p/RAB22A in glioma

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Abstract
Glioma is the most prevalent solid tumor in the central nervous system. Recently, it has been indicated that lncRNAs substantially adjust the development of a variety of human cancers. In this study, it was found and verified via microarray analysis that lncRNA PSMA3-AS1 exhibited a high expression in glioma tissues and cell lines. Then CCK-8, EdU staining, plate clone assay, Transwell assay, Western blotting and nude mouse model were adopted to verify PSMA3-AS1’s effects on glioma. Knockdown of PSMA3-AS1 inhibited the migration, proliferation and invasion of glioma cells in vivo and in vitro. Besides, PSMA3-AS1 bound to miR-302a-3p directly and reduced the expression of miR-302a-3p, thus functioning as an endogenous sponge confirmed by luciferase reporter assay and bioinformatics analysis. PSMA3-AS1 knockdown remarkably enhanced the role of miR-302a-3p overexpression in cell behaviors in glioma. Moreover, these assays also confirmed that RAB22A was a target of miR-302a-3p. In this research, therefore, the PSMA3-AS1/miR-302a-3p/RAB22A pathway regulatory axis may be revealed in the pathogenesis of glioma, and PSMA3-AS1 can be used as an underlying target for the treatment and prognosis of glioma.

Keywords: glioma; PSMA3-AS1; miR-302a-3p; RAB22A

1. Introduction
As the most common solid tumor, Glioma is the most common solid tumor in the central nervous system (CNS)[1, 2]. Glioma in the CNS has been classified into the low and high grade by the World Health Organization (WHO), based on which low-grade glioma is further sub-divided into Grade I and II, while high-grade glioma into Grade III and IV[3]. Generally, cases of Grade IV glioma (glioblastomas; GBM) die within two years, and cases of Grade III anaplastic glioma and Grade II glioma have 2-5-year and 2-year prognosis, respectively[4]. Hence, investigating the potential molecular mechanisms of glioma development and progression should be the top priority for enhancing the diagnosis and treatment of the tumor.

Long non-coding RNAs (lncRNAs) are a type of RNAs with length over 200 nucleotides that have no coding ability[5]. Besides, multiple biological processes, including cell apoptosis, proliferation and metastasis in cancers are modulated by lncRNAs[6-8]. There are many lncRNAs related to glioma that have been reported. For example, Xia L et al. confirmed that FER1L4 adjusts the cycle and proliferation of glioma cells[9], Ni W. et al proposed that FoxD2-AS1 adjusts the PI3K/AKT signaling pathway as well as the miR-185-5p/HMG2A2 axis, so as to accelerate the progression of glioma[10], and Yang C et al. found that lncRNA HERC2P2 is a tumor suppressor in glioma[11]. However, there are still many gaps in our current understanding of lncRNA function. PSMA3-AS1 (ENSG00000257621) is an lncRNA located in Chromosome 14q23.1, and no reports examine the role of PSMA3-AS1 in glioma.

In recent years, it has been widely investigated that lncRNAs interact with miRNAs,
becoming a mechanism associated with the tumor biology mediated by lncRNAs. MiRNAs are relatively conservative with 18-22 nt in length, which are different from lncRNAs[12]. MiRNAs boost the degradation of RNAs in mammals and bind to the 3'-untranslated region (3'UTR) of target mRNAs, thus modulating negative genes[13]. Different biological processes contained and are controlled by a variety of miRNAs.

By reference to the definition of competing endogenous RNAs (ceRNAs), RNAs compete for shared miRNAs to interact with each other, which implies another method of post-transcriptional regulation[14]. Then the miR-302a-3p was selected based on the StarBase database through bioinformatic prediction. Moreover, miR-302a-3p dampens the initiation and development of cancers, such as pancreatic cancer and hepatocellular carcinoma by interaction with various downstream targets[15, 16]. However, the potential mechanism in which miR-302a-3p functions is still rarely reported. As such, it was speculated that PSMA3-AS1 contributed to malignant glioma behaviors by adjusting miR-302a-3p/RAB22A. To validate this, the expression pattern of PSMA3-AS1 in glioma tissues and cell lines was firstly studied, followed by investigation of its biological effects and clinical value in glioma. Ultimately, the PSMA3-AS1/miR-302a-3p/RAB22A pathway was validated to be related to the growth, invasion and migration of glioma, so it was considered as an underlying target in the diagnosis and treatment of glioma.

2. Materials and methods
2.1 Ethical compliance
The research was approved by the Ethics Committee of Heze NO.3 People's Hospital (Shandong, China). All Population-related study was carried out in accordance with the World Medical Association Declaration of Helsinki, and that all subjects written informed consent.

2.2 Clinical tissue samples
Twenty pairs of glioma tissue and adjacent normal tissue samples were extracted from cases who underwent routine surgery with glioma confirmed by pathology in Heze NO.3 People's Hospital from January 2017 to December 2018. All the cases underwent no radiotherapy or chemotherapy prior to surgery, and they signed the informed consent. Immediately after extraction, all tissue samples were frozen in liquid nitrogen for later assays. Detailed information is presented in Table 1. This research was approved by the Ethics Committee of Heze NO.3 People's Hospital.

2.3 Cell lines and cell culture
A172, LN229, U87 and U251, glioma cell lines, and NHA, a human normal astrocyte cell line, were provided by the Cell Bank of CAS (Shanghai, China), purchased from Thermo Fisher Scientific and maintained in DMEM (Gigliomao, Carlsbad, CA, USA) supplemented with 10% FBS (Gigliomao) under the condition of 5% CO₂ at 37°C.
2.4 Quantitative real-time PCR (qRT-PCR)
TRIzol (Life Technologies, Carlsbad, USA) was utilized to separate total RNAs from tissues and cells, and RNAs were synthesized into cDNAs using PrimeScript RT Reagent Kit, or into miRNAs using the PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, Tokyo, Japan) by reference to the manufacturer's schemes. SYBR® Premix Ex TaqTM reagent (TaKaRa) was used for qPCR on the ABI PRISM 7500 RT-PCR system (Applied Biosystems, Foster City, CA, USA). Gene expression was monitored with GAPDH or U6 expression as an internal reference. Designed by RiboBio (Guangzhou, China), primer sequences are shown below: PSMA3-AS1: F: 5'-GUCGUCAGGUUGUGUCUA-3' \text{ and } R: 5'-GCUGUGAAAGUGCCUGUGAA-3'; miR-302a-3p: F: 5'-ACACUCCAGCUGGAGUGGUUUGUACCUC-3' \text{ and } R: 5'-CUCAACUGGUGUCGUGAGUGCACAUUCAGUGUGGAAU-3'; RAB22A: F: 5'-GUGUGUCUGCUCCGGAUCAC-3' \text{ and } R: 5'-GCCCUAAUUGUUGGUGAUU-3'; GAPDH: F: 5'-GTCAACGGATTTGGTCTGTATT-3' \text{ and } R: 5'-AGTCTTCTGGTGCGACTGTTA-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3' and R: 5'-AAGCCTTCAGATTTGCGT-3'. 2^{-\Delta\Delta Ct} \text{ method was adopted to calculate the relative expression level.}

2.5 Subcellular fractionation
The nuclear and cytosolic fractions of LN229 and U251 cells were separated using the PARIS Kit (Invitrogen, USA) according to the manufacturer’s instructions. RNA was extracted from both fractions. qRT-PCR was then performed using GAPDH as the cytosolic control, and U6 as the nuclear control.

2.6 Cell transfection
Small interfering RNAs (siRNAs) specifically targeting PSMA3-AS1, negative control siRNA (si-NC), miR-302a-3p mimics and miR-NC were provided by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequence of PSMA3-AS1 siRNA, miR-302a-3p mimics and miR-NC was as below: si-PSMA3-AS1-1: 5'-UCUCGAAAACCCGAAAGAGA-3’; si-PSMA3-AS1-2: 5'-UUCUAAGAACCACUUCUCC-3’; miR-NC: 5'-UCACAACCUCUAGAAAGAGU-3'; miR-302a-3p mimics: 5'-UAAGUGCUUCAUGUUUGGUGA-3'; miR-302a-3p inhibitor: 5'-UAAGUGCUUCAUGUUUGGUGA-3'. By reference to the manufacturer's protocol, Lipofectamine 2000 kit (Invitrogen, CA, USA) was used to transfect LN229 and U251.

2.7 CCK-8 assay
Transfected glioma cells were cultured for 48h and collected in the logarithmic phase. The cells were generated and seeded in96-well plates at a density of 5000 cells per well and cells were cultured at 37°C for 24h. Then 10 μL CCK-8 solution (Solarbio, Beijing, China) was added into each well for 2 h and the absorbance (450 nm) was

Reference:
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measured by means of a microplate reader (BioTek Instruments, USA). Finally, the cell growth curve was plotted in accordance with the absorbance at each time point.

2.8 5-Ethynyl-2′-deoxyuridine (EdU) staining

Cell-Light EdU DNA Cell Proliferation Kit (RiboBio Co., Ltd, Guangzhou, China) was applied to assess cell proliferation based on the manufacturer's scheme. Specifically, cells were inoculated in 96-well plates at a density of 5,000 cells/well after transfection, and each well were in triplicate. Following culture for 12 h, 4% formaldehyde was added to fix the cells and 100 μl of 1× Apollo® reaction buffer for 30 min of cell incubation. After that, Hoechst 33342 was utilized to stain the cell nuclei for 30 min. The images of cells stained with EdU and Hoechst were observed under an optical microscope (Nikon, Tokyo, Japan), and EdU-positive cells were counted using ImageJ software.

2.9 Colony formation assays

In the first place, cell cultures were trypsinized to prepare single-cell suspensions, followed by inoculation into 6-well plates at 150 cells/well and incubation at 37°C for 14 days. Following formation, methanol was added to fix visible colonies and 0.5% crystal violet was used to stain them, followed by cell count in each colony. Colonies formed with over 50 cells were calculated and recorded for statistical analysis.

2.10 Cell migration and invasion assays

The 24-well Transwell chamber with polycarbonate membrane (pore size: 8 μm) (EMD Millipore, Billerica, MA, USA) was utilized to evaluate the invasion and migration abilities of glioma cells in vitro. In invasion assay, the cells were added with trypsin after transfection and with medium for suspension, and 20 μL Matrigel (BD Biosciences, San Jose, CA, USA) was used to pre-coat the upper surface of the filter. In migration assay, the cells received the same treatment as the invasion assay except that the upper chamber was added with 2×10⁴ cells in medium free of serum, while the lower chamber was added with medium containing 10% FBS. Subsequently, the cells were incubated for 24 h, after which those migrated to the lower chamber were subjected to fixation by 4% paraformaldehyde and dying with 0.05% crystal violet. At least 3 fields of view were selected, in which the stained cells were photographed and calculated.

2.11 Western blotting

RIPA lysis buffer (Beyotime, Beijing, China) was applied for extraction of the total proteins and the Bradford method for protein quantification. SDS-PAGE (10% gel) was carried out to isolate 30 μg protein lysates, and the proteins were then transferred to PVDF membranes (Millipore, Billerica, MA, USA). Thereafter these membranes were sealed with 5% skim milk for 1.5 h, followed by incubation with the following primary antibodies: rabbit anti-human IgG antibodies and antibodies against Vimentin (1: 500, ab45939), β-actin (1: 500, ab8227), Snail 1 (1: 500, ab82846), ZO-1(1: 500, ab96587), TSG101 (1: 1000, ab125011), E-cadherin (1: 500, ab11512) and Twist (1:
Data analysis was conducted using ImageJ software (NIH, Washington, DC, USA), and each assay was repeated for three times, respectively.

2.12 Luciferase activity assay
Some DNA sequences of PSMA3-ASI and RAB22A 3’-UTRs containing the binding sites for wild-type (WT) or mutant (MUT) miR-302a-3p were subjected to PCR amplification and cloned into pmirGLO luciferase vectors (Promega, Madison, USA) to produce RAB220A WT, RAB22A MUT, PSMA3-ASI WT and PSMA3-ASI MUT reporter plasmids, respectively, so as to elucidate whether PSMA3-ASI and RAB22A 3’UTRs were directly targeted by miR-302a-3p. During the luciferase assay, the constructed reporter plasmids and miR-302a-3p or miR-NC were applied to independently co-transfect 293T cells by means of Lipofectamine 2000 (Invitrogen, CA, USA), and the Dual-Luciferase® Reporter Assay kit (Promega) was utilized to examine the luciferase activity at 48 h after transfection based on the manufacturer’s scheme.

2.13 Tumor formation experiment in nude mice
Nude mice at the age of 6 weeks were bought from Cancer Institute of the Chinese Academy of Medical Science. Both sides of the flank area of mice (n=3) in each group were injected with about 1x10⁶ U251 that stably transfected by sh-NC, sh-PSMA3-ASI or sh-PSMA3-ASI+miR-302a-3p inhibitor lentivirus in 0.1 mL PBS. The tumor volume was calculated using a caliper from the 7th day by reference to the formula V = (LxW²)/2 (L: length, W: width of the tumor) every three days. Based on the average tumor volume, the growth curve was drawn in each group. 31 days later, mice were intraperitoneally injected with 3% pentobarbital sodium and were killed by excessive anesthesia with a dose of 90 ml/kg, and the tumors were removed for follow-up study. Animal experiments took place in SPF Animal Laboratory at Taishan Medical University. Subsequently, qRT-PCR was carried out to detect the expression of RAB22A or miR-302a-3p. All assays obtained the approval from the Animal Care and Use committee of the Heze NO.3 People’s Hospital and Taishan Medical University with the approval number of 201710012-1. And all in vivo experiments were performed in Taishan Medical University.

2.14 Statistical analysis
SPSS 22.0 (IBM, SPSS, Chicago, IL, USA) was adopted for statistical processing. The paired-samples t-test was conducted to assess the differences in the expression between glioma tissues and paired adjacent normal tissues, and features of glioma tissues and the expression of PSMA3-ASI were studied via the χ² test. In three independent experiments, data were expressed as mean ± SD. Besides, the Student’s t-test and one-way ANOVA were adopted for assessment of intergroup or intragroup differences, respectively. p<0.05 represented a different that was statistically significant.
3. Results

3.1 Characteristic and expression of PSMA3-ASI in glioma

Through analyzing the GSE103227 microarray, PSMA3-ASI was found to be highly expressed in glioma. Differentially expressed IncRNAs were identified according to the criteria of \( p<0.05 \) and fold change >2. The differently expressed IncRNAs including PSMA3-ASI with most significance was identified in glioma. To ascertain these results, PSMA3-ASI expression was assessed in 20 pairs of glioma tissues and adjacent normal tissues via qRT-PCR. In comparison with paired adjacent tissues, PSMA3-ASI exhibited a notably raised expression in glioma tissues (Figure 1A). The PSMA3-AS1 was elevated in patients with advanced stages of gliomas (III + IV vs I + II, \( P < 0.05 \)). Detailed information is presented in Table 1. Identically, NHA cells had a lower PSMA3-ASI expression than human glioma cells (Figure 1B). Notably, LN229 and U251 had high expressions compared with the other glioma cell lines, so the two cell lines were chosen for the loss-of-function assays. We performed the nuclear and cytoplasmatic separation experiment and found that PSMA3-AS1 mainly located in the cytoplasm (Figure 1C). This result revealed that PSMA3-AS1 will mainly take part in the post-transcriptional regulation of genes related to glioma.

3.2. PSMA3-ASI down-regulation impeded the migration, proliferation and invasion of glioma cells

Since PSMA3-ASI in glioma cell lines was raised, whose biological function was further unfolded in vitro. LN229 and U251 were transfected with si-PSMA3-ASI-1, si-PSMA3-ASI-2 or si-PSMA3-ASI3, respectively. It was found in qRT-PCR that PSMA3-ASI expressions in LN229 and U251 were reduced after transfection, among which the sharpest decrease in PSMA3-ASI expression was caused by si-PSMA3-ASI-2 transfection (Figure 2A). According to CCK-8 assay, the growth of LN229 and U251 was remarkably blocked by si-PSMA3-ASI-2 transfection instead of si-NC transfection (Figure 2B). Subsequently, EdU-positive cells were confirmed to be down-regulated in LN229 and U251 by EdU assay (Figure 2C). The results of the plate clone experiment were consistent with those of the previous experiments, and the colony formation of glioma cells after si-PSMA3-ASI-2 transfection was significantly inhibited (Figure 2D).

Functions of PSMA3-ASI in cell migration and invasion were then examined via Transwell assay. It was found that knockdown of PSMA3-ASI decreased the number of migrated cells in LN229 and U251 (Figure 3A). Similarly, PSMA3-ASI down-regulation suppressed the invasion of LN229 and U251 (Figure 3B). As epithelial-mesenchymal transition (EMT) exerts a pivotal effect on glioma cell invasion and migration, the role of PSMA3-ASI in epithelial characteristics was further investigated. The expressions of ZO-1 (an epithelial marker), Vimentin (a mesenchymal marker) and Snail1 and Twist (EMT-related transcription factors) were measured. As shown in Figure 3C, si-PSMA3-ASI-2 promoted ZO-1 at the mRNA and protein levels, while inhibiting Vimentin, Snail1 and Twist. It can be seen that reduction of PSMA3-ASI evidently suppresses the migration, proliferation and
invasion of glioma cells.

3.3 MiR-302a-3p adjusted the proliferation, migration and invasion of glioma cells by targeting PSMA3-AS1’s 3’UTR

There has been more and more evidence that lncRNAs are able to adjust miRNAs’ effects by sponging them. StarBase revealed that miR-302a-3p and 3’-UTR of PSMA3-AS1 were complementary as they shared binding sites (Figure 4A), which was then validated via the dual luciferase reporter assay. According to the results, it was not the luciferase activity of PSMA3-AS1 MUT but that of PSMA3-AS1 WT that was markedly weakened by miR-302a-3p (Figure 4B). The miR-302a-3p mRNA level was decreased in glioma tissues and was negatively correlated with PSMA3-AS1 (Figure 4C and D). At the same time, the content of miR-302a-3p in glioma cell lines was measured by qRT-PCR, which was lower than that of NHA (Figure 4E). Furthermore, miR-302a-3p in LN229 and U251 exhibited a higher expression in PSMA3-AS1 knockdown group than si-NC group (Figure 4F). Subsequently, the roles of PSMA3-AS1/miR-302a-3p in the migration, proliferation and invasion of glioma cells were continued to be discussed. It could be discovered that miR-302a-3p overexpression prominently blocked cell proliferation (Figure 4G) and decreased the cells suffering from metastasis and invasion (Figure 4H), and PSMA3-AS1 knockdown in U251 cells could enhance these effects.

3.4 MiR-302a-3p targets RAB22A

Bioinformatics analysis was carried out and revealed that miR-302a-3p and 3’-UTR of RAB22A mRNA were complementary as they shared binding sites (Figure 5A). Luciferase reporter assay ascertained that there was a molecular binding within RAB22A and miR-302a-3p (Figure 5B). QRT-PCR (Figure 5C) manifested that RAB22A in glioma cells was markedly higher than that in NHA. The TargetScan and miRDB were used to predict the target gene of miR-302a-3p, and CROT, EDNRB, LATS2, OXR1, ZNF367, RAB22A and NR2C2 were selected. Then the U251 and LN229 cell lines were transfected to detected these genes. Only RAB22A was decreased by miR-302a-3p (Figure 5D-E), and the other genes didn’t change after miR-302a-3p mimics transfection. Meanwhile, the decreased miR-302a-3p could rescue the inhibition effect of si-RAB22A on RAB22A protein expression (Figure 5F). Meanwhile, the decreased cell proliferation, migration and invasion induced by si-RAB22A were recovered by miR-302a-3p inhibitor (Figure 5G-H). Briefly, it can be concluded that miR-302a-3p targets RAB22A, and RAB22A expression may be adjusted by PSMA3-AS1 in a positive manner via sponging miR-302a-3p, evidencing the role of the PSMA3-AS1/miR-302a-3p/RAB22A pathway.

3.5 PSMA3-AS1 partially negatively adjusted miR-302a-3p in vivo to cause tumor formation

Findings in vitro were then ascertained by the establishment of a nude mouse model of xenograft. Substantial differences were observed in tumor formation in the sh-NC, sh-PSMA3-AS1 or sh-PSMA3-AS1+miR-302a-3p inhibitor groups (Figure 6A).
Additionally, it was demonstrated in the time-dependent analysis that sh-PSMA3-AS1 group had smaller tumor volumes than sh-NC group, and sh-PSMA3-AS1+miR-302a-3p inhibitor could rescue the effect of sh-PSMA3-AS1 as a tumor suppressor. (Figure 6B). Identically, tumor weight in all groups had the same results as the tumor volume (Figure 6C). MiR-302a-3p (Figure 6D) and RAB22A (Figure 6E-F) expressions in xenografts were monitored via qRT-PCR and western blot, the results of which illustrated that PSMA3-AS1 knockdown evidently lowered the expression level of RAB22A but elevated that of miR-302a-3p, which was rescued by simultaneous miR-302a-3p inhibition, in consistent with findings in vivo.

4. Discussion
In recent years, an enormous body of research has revealed that lncRNAs are not inhibited in malignant tumors and become targets for diagnosing and treating cancers, including glioma[17-19]. However, there is almost no research related to PSMA3-AS1. Interestingly, it was found in this study that PSMA3-AS1 might be associated with glioma by microarray analysis, which was then further verified and studied in population and cells. Moreover, PSMA3-AS1 was discovered to be remarkably raised in glioma samples and cells, and the reduced PSMA3-AS1 greatly inhibited glioma cell proliferation, migration and invasion, which was verified by assays in vitro and in vivo. These results suggest that PSMA3-AS1 might be a new idea for glioma treatment.

There is a close correlation between lncRNA's biological function and miRNAs and their interactions substantially adjust the progression of numerous cancers[20-22]. In this research, PSMA3-AS1 was ascertained to directly bind to miR-302a-3p and reduce the expression of the latter in glioma cells. Identical to the results of PSMA3-AS1 knockdown, the migration, proliferation and invasion of glioma cells were notably impeded by miR-302a-3p overexpression, but the function of PSMA3-AS1 knockdown in the glioma cell behaviors was remarkably stimulated by it. Actually, miR-302a-3p suppresses multiple tumors, such as hepatoblastoma[16] and gastric cancer[23], so it was hypothesized that PSMA3-AS1, a host gene of snoRNAs, targets miR-302a-3p to accelerate glioma progression.

It has been found that RAB22A, a member of RAS oncogene family, is markedly raised in numerous human cancers[24]. In recent years, epigenetical mechanism has been discovered to adjust RAB22A expression in human cancers, and multiple miRNAs directly target RAB22A to inhibit tumors[25-27]. Moreover, Yin Y et al. demonstrated that miR-204-5p decreases RAB22A so as to strengthen the chemotherapeutic sensitivity[28]. Similarly, it was found that RAB22A was upregulated in glioma. Moreover, RAB22A was regarded as miR-302a-3p's target in glioma examined by bioinformatics analysis. In vivo assays were carried out, which further ascertained that PSMA3-AS1 knockdown was capable of down-regulating RAB22A expression by raising miR-302a-3p, thus slowing down glioma progression.
To sum up, the results of assays in vitro and in vivo unfold that PSMA3-ASI functions as an oncogene to accelerate glioma progression. The findings indicate that tumor formation and the growth, migration and invasion of glioma cells are impeded by PSMA3-ASI knockdown. This study firstly reveals the underlying mechanism that PSMA3-ASI influences the cell proliferation, migration and invasion via miR-302a-3p/RAB22A pathway in the tumorigenesis of glioma, providing new ideas for glioma treatment.

**Competing interests**
The authors declare that they have no competing interests.

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**Author contributions**
Mei-fen Sun made substantial contributions to conception and design. Li-li Zhou made acquisition of data, performed the experiments. Li-li Zhou, Meng Zhang and Yan-zhen Zhang wrote the draft manuscript. All authors contributed to the reviewing of the manuscript, and approved the final manuscript for submission.

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Figure legends

Figure 1: Relative PSMA3-AS1 expressions in glioma tissues and cell lines. (A) The relative expressions of PSMA3-AS1 in glioma tissues and adjacent normal tissues (n=20) detected via qRT-PCR. (B) Relative PSMA3-AS1 expressions in one normal cell line and glioma cell lines. (C) Nuclear and cytoplasmic separation experiment of U251 and LN299. Three independent experiments are conducted. Error bars represent mean ± SD of at least three experiments. *p<0.05; **p<0.01; ***p<0.001.

Figure 2: Regulation of PSMA3-AS1 on the growth and proliferation of LN229 and U251. (A) Assessment of the expressions of PSMA3-AS1 in LN229 and U251
treated with different si-PSMA3-AS1 sequences by qRT-PCR. (B) Measurement of cell viabilities of LN229 and U251 that are stably transfected with si-NC or si-PSMA3-AS1-2, respectively by CCK-8 assay. (C) Verification of the functions of PSMA3-AS1 in the proliferation of LN229 and U251 by EdU assays. EdU-positive cells are counted and recorded. (D) Verification of the functions of PSMA3-AS1 in the proliferation of LN229 and U251 via clone assay. Three independent experiments are conducted. Error bars represent mean ± SD of at least three experiments. *p<0.05; **p<0.01; ***p<0.001.

Figure 3: Regulation of PSMA3-AS1 on the migration and invasion of LN229 and U251. (A-B) The percentage of cell suffering from migration and invasion number in LN229 and U251 after si-PSMA3-AS1-2 transfection is detected. (C) The protein and mRNA levels of ZO-1, Vimentin, Snail1 and Twist in LN229 and U251 upon down-expression of PSMA3-AS1. Three independent experiments are conducted. Error bars represent mean ± SD of at least triplicate experiments. *p<0.05; **p<0.01; ***p<0.001.

Figure 4: MiR-302a-3p is a direct target of PSMA3-AS1. (A) The target miRNA regulated by PSMA3-AS1 is predicted using StarBase. Binding sites between miR-302a-3p and PSMA3-AS1 are shown. (B) 293T cells are co-transfected with the luciferase reporter constructs containing PSMA3-AS1 WT or PSMA3-AS1 MUT sequences and miR-302a-3p or miR-NC, and then the relative luciferase activity is examined. *p<0.01 vs. miR-NC. (C) Relative miR-302a-3p expression in normal and glioma tissues. (D) The correlation of miR-302a-3p and PSMA3-AS1 of glioma tissues(P=0.0243). (E) Relative miR-302a-3p expression in a normal cell line NHA and glioma cell lines. *p<0.05; **p<0.01; ***p<0.001 vs. NHA. (F) LN229 and U251 are transfected with si-NC or si-PSMA3-AS1-2. 48 h later, miR-302a-3p expression is measured via qRT-PCR. ***p<0.001 vs. si-NC. (G-H) The proliferation is determined via CCK-8 assay and migration and invasion via Transwell assay in U251 transfected with miR-NC, miR-302a-3p or miR-302a-3p + si-PSMA3-AS1-2. Three independent experiments are carried out. Error bars represent mean ± SD of at least three experiments. *p<0.05; **p<0.01; ***p<0.001 vs. miR-NC; #p<0.05; ##p<0.01 vs. miR-302a-3p.

Figure 5: PSMA3-AS1 positively regulates RAB22A expression via miR-302a-3p. (A) Predication of interaction between miR-302a-3p and RAB22A via bioinformatics analysis. (B) Luciferase vitality in the combination within miR-302a-3p/miR-NC and RAB22A WT/MUT shown in luciferase reporter assay. ***p<0.001 vs. miR-NC. (C) Relative RAB22A expression in a normal cell line NHA and glioma cell lines. **p<0.01; ***p<0.001 vs. NHA. (D-E) Expression of predicted miR-302a-3p target genes (CROT, EDNRB, LATS2, OXR1, ZNF367, RAB22A and NR2C2) in U251 and LN229 cell lines transfected by miR-302a-3p. (F) RAB22A expressions in LN229 and U251 detected by Western blotting. (G-H) The proliferation is determined via CCK-8 assay and migration and invasion via Transwell assay in U251 transfected
with control, si-RAB22A or si-RAN22A+ miR-302a-3p inhibitor. Three independent experiments are conducted. Error bars represent mean ± SD of at least three experiments. **p < 0.01 vs. miR-NC.

**Figure 6:** *PSMA3-AS1* oncogenic activity is in part through negative regulation of miR-302a-3p in vivo. (A) Distinctive images of tumor formation of xenograft in nude mice from sh-NC, sh-*PSMA3-AS1* or sh-*PSMA3-AS1*+miR-302a-3p inhibitor groups, respectively (n=3 in each group). (B) Tumor volume of mice measured in every three days in sh-NC, sh-*PSMA3-AS1* or sh-*PSMA3-AS1*+miR-302a-3p inhibitor groups, respectively. (C) Changes in the tumor weight in nude mice from sh-NC, sh-*PSMA3-AS1* or sh-*PSMA3-AS1*+miR-302a-3p inhibitor groups, respectively (n=3 in each group). (D) Determination of miR-302a-3p expression in xenografts via qRT-PCR. (E-F) The protein and mRNA levels of RAB22A in xenografts. Three independent experiments are carried out. Error bars represent mean ± SD of at least three experiments. *p<0.05; **p<0.01; ***p<0.001 vs. sh-NC; *p<0.05; **p<0.01 vs. sh-*PSMA3-AS1*. 
**A**

**U251 cell line**

si-NC | si-PSMA3-AS1-2
---|---

**LN229 cell line**

si-NC | si-PSMA3-AS1-2
---|---

**B**

**U251 cell line**

si-NC | si-PSMA3-AS1-2
---|---

**LN229 cell line**

si-NC | si-PSMA3-AS1-2
---|---

**C**

**U251 cell line**

ZO-1 | Vimentin | Snail1 | Twist | β-actin
---|---|---|---|---

si-NC | si-PSMA3-AS1-2
---|---

**LN229 cell line**

ZO-1 | Vimentin | Snail1 | Twist | β-actin
---|---|---|---|---

si-NC | si-PSMA3-AS1-2
---|---

*Note: Images depict experimental results involving cell lines and protein expression levels.*
A

PSMA3-AS1
5' GAGAAGAUCUCCAAAGCACAU A 3'
mir-302a-3p
3'AGUGGUUUGUACCUUUGCAGAU 5'

B

Relative luciferase activity

|          | PSMA3-AS1 WT | PSMA3-AS1 MUT |
|----------|--------------|--------------|
| miR-NC   | 1.0          | 1.0          |
| mir-302a-3p mimics | 0.85         | 0.75         |

C

Relative miRNA level of mir-302a-3p

|          | Normal | glioma |
|----------|--------|--------|
| miR-NC   | 1.0    | 1.0    |
| mir-302a-3p mimics | 0.85   | 0.65   |

D

R^2 = 0.1265
P = 0.0243

E

Relative miRNA level of mir-302a-3p

|          | NHA | U87 | U251 | LN229 | A172 |
|----------|-----|-----|------|-------|------|
| miR-NC   | 1.0 | 1.2 | 1.4  | 1.3   | 1.2  |
| mir-302a-3p | 0.85 | 0.65 | 0.7   | 0.8   | 0.75 |

F

Relative miRNA level of mir-302a-3p

|          | U251 | LN229 |
|----------|------|-------|
| si-NC    | 1.0  | 1.0   |
| si-PSMA3-AS1-2 | 0.85 | 0.55  |

G

H

U251 cell line

50% absorption

|          | miR-NC | mir-302a-3p | mir-302a-3p + PSMA3-AS1-2 |
|----------|--------|-------------|---------------------------|
|          | 1.0    | 0.85        | 0.75                      |

migration

invasion

U251 cell line

Migration of cell number

|          | miR-NC | mir-302a-3p | mir-302a-3p + PSMA3-AS1-2 |
|----------|--------|-------------|---------------------------|
|          | 1.0    | 0.85        | 0.75                      |

Invasion of cell number

|          | miR-NC | mir-302a-3p | mir-302a-3p + PSMA3-AS1-2 |
|----------|--------|-------------|---------------------------|
|          | 1.0    | 0.85        | 0.75                      |
Table 1: Clinical information of glioma patients

| Parameters                              | Group | Cases | PSMA3-AS1 expression | P-value |
|-----------------------------------------|-------|-------|----------------------|---------|
|                                         |       |       | Low  | %    | High | %    |         |
| Gender                                  | Male  | 13    | 7    | 53.8 | 6    | 46.2 | 0.819   |
|                                         | Female| 7     | 4    | 57.1 | 3    | 42.9 |         |
| Age at surgery                          | < 30  | 6     | 4    | 66.7 | 2    | 33.3 | 0.755   |
|                                         | > 30  | 14    | 7    | 50   | 7    | 50   |         |
| Pathological stage                      | WTO:II| 5     | 5    | 100  | 0    | 0    | 0.035   |
|                                         | WTO:III~IV| 15 | 6 | 40 | 9 | 60 | |
| Tumor size (maximum diameter in MRI)   | > = 30 mm | 10 | 6 | 60 | 4 | 40 | 0.613 |
|                                         | < 30 mm | 10 | 5 | 50 | 5 | 50 | |
| Tumor necrosis                         | Yes   | 7     | 3    | 42.9 | 4    | 57.1 | 0.256   |
|                                         | No    | 13    | 8    | 61.5 | 5    | 38.5 |         |
| Total                                   |       | 20    | 10   | 50   | 10   | 50   |         |