Silencing LINC00657 Inhibited the Malignant Biological Behaviors of Glioma by Interaction with PUM2 to Facilitate NANOS3 mRNA Degradation

Lin Wang (ahhwanglin@163.com)
the First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China
https://orcid.org/0000-0002-8819-1686

CuiZhu Mei
The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China

Fang He
The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China

Hao Xu
The Affiliated Hospital of Guizhou Medical University

Primary research

Keywords: Glioma, LINC00657, mRNA degradation, PUM2, NANOS3

DOI: https://doi.org/10.21203/rs.3.rs-105347/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Background: Molecular-targeted therapy exerts a crucial influence on the combined treatment of glioma. LncRNA (Long noncoding RNA) exerts an influence on regulating glioma progression. This current study aiming at unveiling the feasible roles and molecular mechanism of LINC00657 in glioma.

Methods: Cell Counting Kit-8 (CCK-8), Transwell, flow cytometry, RNA pull-down assays, and RNA Immunoprecipitation (RIP) were utilized to elucidate the interactions betwixt LINC00657 and PUM2 (Pumilio RNA binding family member 2).

Results: Silenced LINC00657 attenuated the migration and proliferation of glioma cells as well as promoted cell apoptosis. In the meanwhile, LINC00657 downregulation diminished its binding to PUM2, also intensified the binding of NANOS3 mRNA and PUM2, consequently facilitating the degradation of NANOS3 mRNA. In nude mice, the xenograft tumor growth could be remarkably attenuated owing to the silencing of LINC00657 together with the overexpression of PUM2.

Conclusions: LINC00657/PUM2/ NANOS3 pathway exerts a crucial influence on modulating the biological behavior of glioma cells.

Background

Glioma, one of the extremely usual malignant tumors of the central nervous system, responsible for approximately 20%-60% of the total intracranial tumors. The World Health Organization (WHO) grades them for their level of malignancy into four levels: grade I-II, the so-called low-grade glioma, which is less aggressive; Grade III was an anaplastic glioma; Grade IV was a glioblastoma. III-IV gliomas are highly malignant gliomas[1]. The III-IV gliomas not only have strong proliferation potential, but also are easy to form large masses. Moreover, glioma has the characteristics of super aggressive growth, which can continuously invade the normal brain tissue. Surgery often fails to completely remove the tumor tissue. It is often used as a clinical assistant for radiotherapy and chemotherapy, but patients receiving treatment show poor prognosis[2, 3]. Glioma is characterized by invasive growth, unclear boundary with surrounding brain tissue, and easy to relapse in a short period, resulting in a shorter survival time[4]. The high malignancy of glioma is mainly due to the fast growth of glioma. However, recurrence of glioma is mainly caused by incomplete surgical resection and continuous malignant proliferation of residual tumor cells[5]. Therefore, in the research and treatment of glioma one of the most important directions is the proliferation and migration of glioma.

LncRNAs (Long non-coding RNAs), as RNA transcripts > 200nt, are crucial in modulating the occurrence and development of various tumors[6]. The effects of IncRNA on epigenetics and gene enhancers can both affect gene transcription, IncRNA can also modulate the activity of tumor suppressor genes, and IncRNA can also exert a regulatory influence on the processing and translation of mRNA[7]. Therefore, IncRNA can modulate occurrence and development of tumor transcriptionally, posttranscriptionally, and epigenetically[8]. Previous studies indicated that LINC00657 may work as an oncogene in colorectal
cancer[9], breast cancer cancer[10] and gastric cancer[11]. Nonetheless, the function of LINC00657 in glioma is still unknown.

At the post-transcriptional level, gene expression is prone to be modulated by RNA binding proteins (RBPs), that are supposed to exert a crucial influence on the development and occurrence of tumors. A notable member of PUF family of RBPs is Pumilio RNA binding family member 2 (PUM2), which in humans can combine with 750 unique mRNA targets, thus exert a crucial influence on the maintenance of stem cells together with brain development[12]. Recent researches have demonstrated that PUM2 exerted an influential regulatory influence on diverse solid tumors[13]. Nevertheless, the function of PUM2 in glioma is short of report. In modulating the expression of downstream genes LncRNAs perform as “molecular sponges” or “molecular scaffolds” for RBP[14]. PTBP3 protein can promote hepatocellular carcinoma through recruiting plentiful Inc-nuclear enriched abundant transcript 1 (NEAT1) splicing variants[15]. We adopted bioinformatics software to predict that LINC00657 might bind to PUM2, indicating that LINC00657 may perform its biological duty via binding with PUM2.

Nanos gene, which has been extensively learned, is currently entirely known to modulate diverse biological processes, including PGC (primordial germ cell) migration, maintenance of germline stem cell self-renewal, the differentiation of the anterior–posterior body axis, together with suppression of somatic cell fate in the course of germline development[16]. Nonetheless, in testicular carcinoma, Nanos3 and Nanos2 were dysregulated in situ and emerged as meiosis regulators, in many oncological studies. Upregulation of NANOS3 can promote the oncogenic growth in p-Rb-deficient cells, which suggests in cancer cell proliferation Nanos plays a dynamic role[17]. As reported before, Nanos3 has been proved to promote cell proliferation, migration, chemoresistance, together with invasion in glioblastoma[18]. However, the underlying mechanism remained unknown.

We elucidated the endogenous expression of LINC00657, PUM2, and NANOS3 in glioma cells and tissues in this study. Furthermore, we performed further investigation to probe into the relationship among molecules above, together with their impacts on the biological behaviors of glioma cells, with the intention of unveiling the new mechanism for the progress and morbidity of glioma, and provide novel therapy for glioma.

**Materials And Methods**

**Sample collection**

Tissue specimens of glioma: From January 2010 to June 2019, a total of 60 pathologically confirmed primary glioma specimens were collected from the First Affiliated Hospital of USTC according to the WHO (2000) gliomas grading standard, Ⅰ/Ⅱ/Ⅲ/Ⅳ grade glioma, 15 patients with craniocerebral trauma afterbrain swelling induced by cerebral herniation after cranial decompression surgery for 12 cases of normal brain tissue specimens, as normal controls. All specimens were instantly frozen after collection in liquid nitrogen, then transferred to the -80°C refrigerator for storage. Prior to collection, the above specimens
have gained approval from the ethics Committee of the First Affiliated Hospital of USTC and written consent of the patients and their families has been obtained.

**Cell culture**

Normal brain tissue cells HEB and glioma cells HS683, T98MG, U373, A172, SHG-44, U87 and U251 were all obtained in the cell bank of The Chinese Academy of Sciences, then were cultured in DMEM high glucose medium of 10% fetal bovine serum, at 37°C and 5% CO₂ in an incubator.

**Quantitative real-time PCR (qRT-PCR)**

We extracted total RNA from glioma tissues and cells with Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA). We adopted a Nanodrop spectrophotometer (ND-100) to detect the concentration of RNA and at the 260/280 nm ratio detect its quality. Via a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) we generated the complementary DNA (cDNA). We adopted Two-Step SYBR PrimeScript RT-PCR Kit (Takara Bio, Inc, Japan) to perform qRT-PCR (quantitative real-time PCR) for the assays of LINC00657, PUM2 and NANOS3. RT-qPCR, on a Roche Light Cycler480 system (Roche Diagnositcs, Inc.), in conformity with the manufacturer's instructions. We normalized expressions to endogenous controls (GAPDH), also we used the relative quantification (2^{- ΔΔCt}) method to perform fold change calculation. The sequences of the primers were as follows:

LINC00657 F, 5’-CAGAGGAGGTATGCAGGGAG-3’ and R, 5’-GGATGTCTAGCTCCAAGGGG-3’;
NANOS3 F, 5’-CATTTATTGAGGGCTGACTGGAT-3’ and R, 5’-CGGAACTCCTGTGCTTTGTCT-3’;
PUM2 F, 5’-TCGGGGAATGGGAGAGCTTT-3’ and R, 5’-GCTGGGACATTGAATGGTGAGA-3’;
GAPDH F, 5’-TCAAGATCATCAGCAATGCC-3’ and R, 5’-CGATACAAAGTTGCTATGGA-3’;

**Lentiviral Vector Construction and Infection**

Respectively, we ligated short hairpin RNAs directed against LINC00657 and NANOS3 into the pLV-u6-gfp-Puro lentiviral vector (Genechem, Shanghai, China). And we ligated short hairpin RNA directed against PUM2 into the pLV-u6-red-bsd lentiviral vector (Genechem, Shanghai, China). We also ligated the NANOS3 coding sequence (CDS) into the pLV-cmv-red-bsd lentiviral vector (Genechem, Shanghai, China). We also ligated the PUM2 coding sequence (CDS) into the pLV-cmv-gfp-Puro lentiviral vector (Genechem, Shanghai, China). We harvested lentivirus 48 h after that the packaging vectors co-transfected with the lentiviral vectors or the empty lentiviral vectors (negative control, NC) into U87 and U251 cells. We consequently infected cells with the lentivirus, so as to obtain the sh-LINC00657, sh-PUM2 and sh-NANOS3 cells, or PUM2 and NANOS3 overexpressing cells.

**Cell Proliferation Assay**
To analyze the proliferation of U251 and U87 cells, we performed Cell Counting Kit-8 assays (CCK-8, Dojin, Japan). In 96-well plates we seeded cells in a density of 2,000 cells per well after the transfection. We added 10 µl of CCK-8 solution into every well after 72 h, then at 37°C we incubated the cells for 2 h. With the SpectraMax M5 microplate reader, at 450 nm we measured the absorbance.

**Quantization of Apoptosis by Flow Cytometry**

We quantified cell apoptosis via the Annexin 7AAD/PE staining (Southern Biotech, Birmingham the AL, USA). The cells, which were rinsed with PBS for twice and centrifuged, suffered being stained with Annexin 7AAD/PE and being resuspended in Annexin-V-7AAD/PE binding buffer, in obedience to the manufacturer's instructions. To get the apoptotic fractions, we then used flow cytometry (FACScan, BD Biosciences) to analyze the cells.

**Cell Migration Assay**

To test the invasion and migration of U251 and U87 we adopted Chambers (24-well) with 8-µm pore size (Costar, Corning, NY, USA). Processes were performed in a manner that the cells suffered being resuspended at a density of 10⁵/ml in 100 µl serum-free medium, also being seeded in the upper chamber [or chambers precoated with 500 ng/ml Matrigel solution (BD, Franklin Lakes, NJ)]. In the lower chamber, we placed six hundred microliters medium of 10% FBS for 48 h; with a cotton swab we then wiped the cells on the upper membrane surface out physically. With methanol and being stained with 10% Giemsa, cells which were migrated to the lower side of the membrane were finally fixed. Then, under a microscope we chose five random fields for counting the cells, and took the photographs.

**Western Blot Assay**

We extracted the total protein from frozen cells with RIPA (radioimmunoprecipitation assay) buffer containing 50mM HEPES, 1mM EDTA (ethylenediaminetetraacetic acid) (pH 8.0) on ice. We centrifuged the samples were at 17,000 rpm and 4°C for 40min, and by BCA (bicinchoninic acid) protein assay kit (Beyotime, Shanghai, China) we obtained the protein concentration of the supernatant extracts. After that, they were subjected to SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene fluoride) membranes electrophoretically. We incubated such membranes in Tris-buffered saline which contained 5% nonfat milk at 25°C for 2 h and then incubated them with primary antibodies as follows: PUM2 (1:2,000, ab92390, Abcam, Cambridge, MA, USA), NANOS3 (1:2,000, ab70001, Abcam, Cambridge, MA, USA) and GAPDH (1:1,000, ab8245, Abcam, Cambridge, MA, USA) for 18 h. As secondary antibodies, we used Horseradish peroxidase (HRP)-linked antimouse immunoglobulin G (IgG) and HRP-linked antirabbit IgG antibodies. were Via FluorChem 2.0 software (Alpha Innotech, San Leandro, CA, USA) we quantified the signals.

**RNA Immunoprecipitation Assay**
Via a Magna RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) we performed RNA immunoprecipitation (RIP). We incubated whole-cell lysate with RIP buffer containing magnetic beads, which were conjugated with negative control normal rabbit IgG or with human anti-PUM2 antibody. We incubated samples with Proteinase K and isolated the immunoprecipitated RNA. By a spectrophotometer (NanoDrop, Thermo, Scientific, Waltham, MA, USA) we measured the RNA concentration, and via a bioanalyzer (Agilent, Santa Clara, CA, USA) we assessed the RNA quality. Besides, we extracted purified RNAs and analyzed them to elucidate the presence of the binding targets.

RNA Pull-Down Assay

Via Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher) we examined the interaction between LINC00657 and PUM2. We coincubated biotin-labeled LINC00657 with magnetic beads and protein extract of U251 or U87 cells. For generating the bead–RNA–protein complex we used low speed centrifugation. Washed with Handee spin columns, in SDS buffer the bead compound was boiled, and via the GAPDH control we detected the retrieved protein.

Tumor Xenografts in Nude Mice

We purchased female nude mice from GemPharmatech (Nanjing, China) at 6-8 weeks. This experimental study meets the requirements of animal ethics, minimizing the number of animals used, and reducing the pain of animals under anaesthesia before operation. During the experiment, the room temperature was controlled at about 22°C and the relative humidity was about 60%. During the study we gave autoclaved food and water to the animals received. Into 3 groups the nude mice were divided: NC group, sh-LINC00657 group and sh-LINC00657+PUM2 group. We used the stable-expression U87 cells for in vivo study. We injected 100l cells into the right side of the mouse near armpit. We measured the tumor size with vernier caliper every 4 days after the formation of the transplanted tumor and recorded. We calculated the tumor volume with this formula: volume (mm$^3$) = length × width$^2$/2.

Statistical Analysis

We presented data as mean ± SD. By SPSS 18.0 statistical software we evaluated all statistical analyses, via the Student’s t test or one-way analysis of variance (ANOVA). When P < 0.05 differences are recognized to be influential.

Results

Knockdown of LINC00657 inhibited the malignant biological behaviors of glioma cells

Via QRT-PCR we analyzed LINC00657 expression in glioma samples, together with glioma cell lines of different grades. The results manifested that in comparison with the normal group, LINC00657 expression exhibited an intensifying trend with the increase of glioma grade (Fig. 1A). In glioma cell lines, LINC00657
expression was remarkably increased in HS683, T98MG, U373, SHG44.A172, U87 and U251 compared with normal HEB cells (Fig. 1B). We established stable transfected sh-LINC00657 cells to further explore the function of LINC00657. The viability of sh-LINC00657 cells were remarkably diminished compared with NC cells (Fig. 1C), however, the apoptosis was remarkably intensified (Fig. 1D). When LINC00657 was knocked down, the U251 and U87 cells migration was also inhibited (Fig. 1E).

**Overexpression of PUM2 inhibited the malignant biological behaviors of glioma cells**

Via the bioinformatics software Starbase 2.0 we made a prediction that PUM2 could bind to LINC00657, which indicates LINC00657 may need PUM2 to function. Hence, we detected PUM2 expression in glioma cells and tissues first. In comparison with the normal group, the expression level of PUM2 tends to decrease with the increase of glioma level, as shown in Fig. 2A. In comparison with normal HEB cells, PUM2 expression in HS683, T98MG, U373, SHG44.A172, U87 and U251 was remarkably diminished (Fig. 2B) in glioma cell lines. In U251 and U87 cells, the protein expression levels of PUM2 were remarkably lower, compared with HEB cells (Fig. 2C). Then we established stable transfected PUM2 cells and detected their changes in the biological behaviors. We observed remarkable decrease in the viability and migration of U251 and U87 cells in PUM2 groups as well as increased apoptosis, compared with NC group (Fig. 2D-F).

**Pum2 Bound To Linc00657**

We performed RNA pull-down assay and RIP assay to validate the interaction betwixt LINC00657 and PUM2. LINC00657 was increased in the PUM2 immunoprecipitate of U251 and U87 cells in comparison with the IgG immunoprecipitate, whereas such increasement in PUM2 immunoprecipitate was remarkably diminished after LINC00657 knockdown (Fig. 3A). We then conducted the RNA pull-down assay for determining the binding betwixt LINC00657 and PUM2. The LINC00657 overexpression group has enriched more PUM2 in comparison with the control group (Fig. 3B).

**Knockdown of NANOS3 inhibited the malignant biological behaviors of glioma cells**

It was demonstrated in previous studies that PUM2 was able to degrade target mRNA through binding gene, therefore inhibiting the gene expression. With the bioinformatics software Starbase 2.0 we found NANOS3 was the potential target gene of PUM2. As a result, we then detected the role of NANOS3 in glioma. The expression of NANOS3 was remarkably intensified in WHO III glioma cells and tissues, which was exhibited in Fig. 4A and B. The protein expression level of NANOS3 was remarkably intensified in U251 and U87 cells (Fig. 4C), in comparison with HEB cells. We established the stable transfected cells of sh-NANOS3, and detected their changes in the biological behaviors. The viability of both cell lines in the sh-NANOS3 group was remarkably inhibited in comparison with NC group, however, the apoptosis rate was remarkably diminished (Fig. 4D and E). The cell migration was also attenuated when NANOS3 was knocked down (Fig. 4F).
Pum2 Overexpression And Linc00657 Knockdown Inhibited The Nanos3 Expression

So as to identify the function of PUM2 in modulating NANOS3 expression, we detected the changes of NANOS3 in the expression levels of mRNA and protein after knockdown or overexpression of PUM2. In U251 and U87 cells, the mRNA and protein expression of NANOS3 were remarkably diminished by the overexpression of PUM2, while the silencing of PUM2 could remarkably increase them (Fig. 5A and B). Hence, to evaluate the binding of NANOS3 mRNA to PUM2 we performed RIP. In the PUM2 immunoprecipitate of U251 and U87 cells, NANOS3 mRNA was intensified in comparison with the IgG immunoprecipitate. While this enrichment was remarkably intensified after the knockdown of LINC00657 (Fig. 5C and D). Moreover, we transfected U87 and U251 cells with both sh-LINC00657 and sh-PUM2 for determining the modulating effects of LINC00657 and PUM2 on NANOS3 expression. Results exhibited that the protein and mRNA expression of NANOS3 was diminished by LINC00657 silencing alone, and in LINC00657 silencing combined with PUM2 overexpression group the expression of NANOS3 was lower than in LINC00657 silencing alone. Additionally, PUM2 silencing could rescue the decreased expression which was induced by LINC00657 silencing (Fig. 5E and F). We revealed from these results that PUM2 attenuated NANOS3 expression via binding to its 3′-UTR, and by the knockdown of LINC00657 this inhibitory effect could be intensified.

NANOS3 reverses the effect of PUM2 on the biological behavior of glioma cells

For confirming whether PUM2 modulates the behavior of glioma cells via regulating NANOS3, with PUM2 and NANOS3 we co-transfected U251 and U87 cells. In the PUM2 group, the viability and migration of U251 and U87 cells was diminished in comparison to the NC group, while the apoptosis rate was intensified; in NANOS3 group we found the contrary results. NANOS3 overexpression could reverse the inhibitory influences on the malignant biological behaviors of breast cancer cells of PUM2 overexpression (Fig. 6A–C). We demonstrated from these results that NANOS3 engaged in the modulation of PUM2 on glioma cells on their biological behaviors.

In vivo tumor growth was inhibited by the silencing of LINC00657 in combination with the overexpression of PUM2

In order to further demonstrate the function of LINC00657 and PUM2 in glioma in vivo, on the nude mice we performed the xenograft tumor experiment. In comparison with NC group, the size, weight, and volume of xenograft tumors were all remarkably diminished in the sh-LINC00657, and sh-LINC00657 + PUM2 groups, and smaller tumors emerged in the sh-LINC00657 + PUM2 group (Fig. 7A-C). Consequently, in nude mice, the growth of xenograft tumors was remarkably attenuated by the silencing of LINC00657, together with the overexpression of PUM2.

Discussion
In the samples and glioma cells, LINC00657 was highly expressed; and its silencing attenuated the migration and viability of glioma cells, also promote the apoptosis. In a targeted manner PUM2 was bound with LINC00657. Silencing LINC00657 attenuated the binding betwixt PUM2 and LINC00657. Besides, the binding betwixt PUM2 and NANOS3 mRNA was promoted, while NANOS3 mRNA was degraded. Hence, the expression level of NANOS3 was diminished. With these results we provide a novel experimental basis to probe into the impacts of LINC00657 in glioma.

Nowadays, numerous attentions were focused on the abnormal expression as well as influential regulatory effects of IncRNAs in glioma. For example, long non-coding RNA PSMA3-AS1 in glioma facilitates cell invasion, proliferation, and migration by modulating miR-302a-3p/RAB22A[19]. Besides, LEF1-AS1 in glioma accelerates tumorigenesis through sponging miR-489-3p to facilitate HIGD1A[20]. LINC00657 expression was found markedly upregulated in glioma cells and tissues in this study. And we observed silencing LINC00657 attenuated the migration and proliferation of glioma, whereas apoptosis was facilitated. It was suggested that in glioma LINC00657 might serve as a carcinogenic factor. Reports on the effects of LINC00657 in glioma are lack recently. Through serving as “molecular sponges” of miRNAs(microRNAs) together with enhancers and epigenetic regulators, LncRNAs modulate gene expression. The interaction betwixt RBPs and IncRNAs exerts a crucial influence on the occurrence of malignant tumors development. RBPs can modulate the tumor cells on their biological behaviors by binding IncRNAs, therefore modulating their stability and controlling the tumor cells on their biological behaviors. LncRNA NORAD was able to upregulate the transcripts of Pumilio proteins, also exerts an influence on genomic stability[21]. In liver cancer (HULC), the stability of the IncRNA, which was highly upregulated, was diminished by RBPs via the recruitment of CCR4-NOT complex[22]. Additionally, IncRNAs can affect RBPs and modulate the downstream target gene expression. In the study by Zhang et al[23], IncRNA MEG3, which serves as the molecular scaffold of PTBP1, could recruit Shp mRNA to promote its degradation.

In a targeted manner, PUM2 was bound to LINC00657, while silencing LINC00657 decreased this binding process. In a similar way, TDP-43 was bound with the IncRNA GADD7, diminishing the binding of the mRNA 3′-UTR of target genes with TDP-43, thus decreasing the inhibition on the translation process of these genes[24]. In glioma tissues, expression of PUM2 was remarkably diminished in this study, and in the glioma cells it was expressed at a remarkably low level. PUM2 overexpression can attenuate the migration, proliferation, together with promoting the apoptosis of glioma cells. It was showed in glioma PUM2 functions as a tumor suppresser.

PUM2 sustained the morphology and function of synapses in the mammalian neurons[25]. As reported before, in myeloid leukemia cells PUM2 is highly expressed also it was corelated with modulating the growth of leukemia cells and hematopoietic stem cells[13]. Besides, in a targeted way, mRNA 3′-UTR of downstream molecules can be bound with PUM2, and their degradation is facilitated, with gene expression being attenuated[26, 27]. It was demonstrated by this study that PUM2 was able to bind with NANOS3 mRNA and inhibit NANOS3 expression. PUM2 is able to bind with SCCRO3 mRNA, while overexpression of PUM2 decreased the mRNA expression of SCCRO3, consequently affecting the tumor-
suppression effects of SCCRO3, which is similarl to our results[28]. The inhibition on the protein and mRNA expression of NANOS3 was intensified by LINC00657 silencing in combination with the overexpression of PUM2, in our study, it was indicated that the inhibition of PUM2 on NANOS3 mRNA is intensified when the silencing of LINC00657 happens. In the glioma tissues and cells NANOS3 was highly expressed in our study. Silencing NANOS3 remarkably attenuated the migration and proliferation of glioma cells whereas facilitating the apoptosis, which supposes that in glioma NANOS3 serves as a carcinogenic factor.

As whole, we first demonstrate that in the tissues and cells of glioma LINC00657 is highly expressed. Silencing LINC00657 can reduce its binding to PUM2, consequently promoting the binding of PUM2 to NANOS3 mRNA, thus attenuating the malignant biological behaviors of glioma cells. As a result, we prove the influential function and mechanism of LINC00657 in modulating the glioma cells on their biological behaviors, additionally offer a novel target and a brand novel mechanism for the molecular targeted treatment of glioma.

**Abbreviations**

LncRNA: Long noncoding RNA; CCK-8: Cell Counting Kit-8; RIP: RNA Immunoprecipitation; PUM2: Pumilio RNA binding family member 2; RBPs: RNA binding proteins; qRT-PCR: Quantitative real-time PCR.

**Declarations**

**Acknowledgements**

Not applicable.

**Authors’ contributions**

LW conceived and designed and performed the experiments, and wrote the paper. CM analyzed the data. FH and HX contributed reagents/materials/analysis tools. All authors read and approved the final version of the manuscript and agree to take responsibility for the published article.

**Funding**

None

**Availability of data and materials**

The datasets generated/analyzed during the current study are available.

**Ethics approval and consent to participate**

All study protocols were in accordance with the Declaration of Helsinki, and approved by the Medical and Clinical Research Ethics Committee of the First Affiliated Hospital of USTC. Informed written consent was
obtained from each participant prior to the study. Animal experimental procedures were in line with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Extensive efforts were made to ensure minimal suffering of the included animals.

**Consent for publication**

All authors consent for publication.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol. 2007;114(2):97–109.

2. Li L, Gu M, You B, Shi S, Shan Y, Bao L, You Y. Long non-coding RNA ROR promotes proliferation, migration and chemoresistance of nasopharyngeal carcinoma. Cancer Sci. 2016;107(9):1215–22.

3. Hwang JH, Smith CA, Salhia B, Rutka JT. The role of fascin in the migration and invasiveness of malignant glioma cells. Neoplasia. 2008;10(2):149–59.

4. Ceglie G, Vinci M, Carai A, Rossi S, Colafati GS, Cacchione A, Tornesello A, Miele E, Locatelli F, Mastronuzzi A. *Infantile/Congenital High-Grade Gliomas: Molecular Features and Therapeutic Perspectives*. Diagnostics (Basel) 2020, 10(9).

5. Stummer W, Kamp MA. The importance of surgical resection in malignant glioma. Curr Opin Neurol. 2009;22(6):645–9.

6. Wilusz JE, Sunwoo H, Spector DL. Long noncoding RNAs: functional surprises from the RNA world. Genes Dev. 2009;23(13):1494–504.

7. Hezroni H, Koppstein D, Schwartz MG, Avrutin A, Bartel DP, Ulitsky I. Principles of long noncoding RNA evolution derived from direct comparison of transcriptomes in 17 species. Cell Rep. 2015;11(7):1110–22.

8. Wang Q, Cheng N, Li X, Pan H, Li C, Ren S, Su C, Cai W, Zhao C, Zhang L, et al. Correlation of long non-coding RNA H19 expression with cisplatin-resistance and clinical outcome in lung adenocarcinoma. Oncotarget. 2017;8(2):2558–67.

9. Zhao L, Liu C, Yan S, Hu G, Xiang K, Xiang H, Yu H. LINC00657 promotes colorectal cancer stem-like cell invasion by functioning as a miR-203a sponge. Biochem Biophys Res Commun. 2020;529(2):500–6.

10. Shan Q, Qu F, Yang W, Chen N. Effect of LINC00657 on Apoptosis of Breast Cancer Cells by Regulating miR-590-3p. Cancer Manag Res. 2020;12:4561–71.
11. Yu SY, Peng H, Zhu Q, Wu YX, Wu F, Han CR, Yan B, Li Q, Xiang HG. Silencing the long noncoding RNA NORAD inhibits gastric cancer cell proliferation and invasion by the RhoA/ROCK1 pathway. Eur Rev Med Pharmacol Sci. 2019;23(9):3760–70.

12. Zamore PD, Williamson JR, Lehmann R. The Pumilio protein binds RNA through a conserved domain that defines a new class of RNA-binding proteins. RNA. 1997;3(12):1421–33.

13. Naudin C, Hattabi A, Michelet F, Miri-Nezhad A, Benyoucef A, Pflumio F, Guillonneau F, Fichelson S, Vigon I, Dusanter-Fourt I, et al. PUMILIO/FOXP1 signaling drives expansion of hematopoietic stem/progenitor and leukemia cells. Blood. 2017;129(18):2493–506.

14. Kim J, Abdelmohsen K, Yang X, De S, Grammatikakis I, Noh JH, Gorospe M. LncRNA OIP5-AS1/cyano sponges RNA-binding protein HuR. Nucleic Acids Res. 2016;44(5):2378–92.

15. Yang X, Qu S, Wang L, Zhang H, Yang Z, Wang J, Dai B, Tao K, Shang R, Liu Z, et al. PTBP3 splicing factor promotes hepatocellular carcinoma by destroying the splicing balance of NEAT1 and pre-miR-612. Oncogene. 2018;37(50):6399–413.

16. Grelet S, Andries V, Polette M, Gilles C, Staes K, Martin AP, Kileztky C, Terryn C, Dalstein V, Cheng CW, et al. The human NANOS3 gene contributes to lung tumor invasion by inducing epithelial-mesenchymal transition. J Pathol. 2015;237(1):25–37.

17. Wang L, Du L, Duan W, Yan S, Xie Y, Wang C. Overexpression of long noncoding RNA NORAD in colorectal cancer associates with tumor progression. Onco Targets Ther. 2018;11:6757–66.

18. Zhang F, Liu R, Liu C, Zhang H, Lu Y. Nanos3, a cancer-germline gene, promotes cell proliferation, migration, chemoresistance, and invasion of human glioblastoma. Cancer Cell Int. 2020;20:197.

19. Zhou LL, Zhang M, Zhang YZ, Sun MF. Long non-coding RNA PSMA3-AS1 enhances cell proliferation, migration and invasion by regulating miR-302a-3p/RAB22A in glioma. Biosci Rep 2020, 40(9).

20. Cheng Z, Wang G, Zhu W, Luo C, Guo Z. LEF1-AS1 accelerates tumorigenesis in glioma by sponging miR-489-3p to enhance HIGD1A. Cell Death Dis. 2020;11(8):690.

21. Lee S, Kopp F, Chang TC, Sataluri A, Chen B, Sivakumar S, Yu H, Xie Y, Mendell JT. Noncoding RNA NORAD Regulates Genomic Stability by Sequestering PUMILIO Proteins. Cell. 2016;164(1–2):69–80.

22. Hammerle M, Gutschner T, Uckelmann H, Ozgur S, Fiskin E, Gross M, Skawran B, Geffers R, Longerich T, Breuhahn K, et al: Posttranscriptional destabilization of the liver-specific long noncoding RNA HULC by the IGF2 mRNA-binding protein 1 (IGF2BP1). Hepatology 2013, 58(5):1703–1712.

23. Zhang L, Yang Z, Trottier J, Barbier O, Wang L. Long noncoding RNA MEG3 induces cholestatic liver injury by interaction with PTBP1 to facilitate shp mRNA decay. Hepatology. 2017;65(2):604–15.

24. Liu X, Li D, Zhang W, Guo M, Zhan Q. Long non-coding RNA gadd7 interacts with TDP-43 and regulates Cdk6 mRNA decay. EMBO J. 2012;31(23):4415–27.

25. Zhong J, Zhang T, Bloch LM. Dendritic mRNAs encode diversified functionalities in hippocampal pyramidal neurons. BMC Neurosci. 2006;7:17.
26. Spassov DS, Jurecic R. Cloning and comparative sequence analysis of PUM1 and PUM2 genes, human members of the Pumilio family of RNA-binding proteins. Gene. 2002;299(1–2):195–204.

27. Breslin MB, Zhu M, Lan MS. NeuroD1/E47 regulates the E-box element of a novel zinc finger transcription factor, IA-1, in developing nervous system. J Biol Chem. 2003;278(40):38991–7.

28. Huang G, Stock C, Bommelje CC, Weeda VB, Shah K, Bains S, Buss E, Shaha M, Rechler W, Ramanathan SY, et al. SCCRO3 (DCUN1D3) antagonizes the neddylation and oncogenic activity of SCCRO (DCUN1D1). J Biol Chem. 2014;289(50):34728–42.

**Figures**

**Figure 1**

Knockdown of LINC00657 inhibited the malignant biological behaviors of glioma cells. (A) In comparison with the control group, the WHO Ⅰ, WHO Ⅱ, WHO Ⅲ and WHO Ⅳ LINC00657 gene expression was remarkably increased in gliomas. (B) HS683 (equivalent to the WHO Ⅰ), T98MG (equivalent to the WHO Ⅱ), U373
(equivalent to the WHO ♂), SHG44, A172, U251 and U87 (four glioblastoma, equivalent to the WHO ♀), LINC00657 expression increased remarkably, compared with normal HEB cells. After knockdown the expression of LINC00657, the proliferation ability of U251 and U87 cells was remarkably decreased (C), apoptosis was remarkably increased (D), and migration was remarkably decreased (E).* p < 0.05.

**Figure 1**

Knockdown of LINC00657 inhibited the malignant biological behaviors of glioma cells. (A) In comparison with the control group, the WHO ♂, WHO ♀, WHO ♂ and WHO ♀ LINC00657 gene expression was remarkably increased in gliomas. (B) HS683 (equivalent to the WHO ♂), T98MG (equivalent to the WHO ♀), U373 (equivalent to the WHO ♂), SHG44, A172, U251 and U87 (four glioblastoma, equivalent to the WHO ♀), LINC00657 expression increased remarkably, compared with normal HEB cells. After knockdown the expression of LINC00657, the proliferation ability of U251 and U87 cells was remarkably decreased (C), apoptosis was remarkably increased (D), and migration was remarkably decreased (E).* p < 0.05.
Overexpression of PUM2 inhibited the malignant biological behaviors of glioma cells. (A) In comparison with the control group, the WHO I, WHO II, WHO III and WHO IV level PUM2 gene expression was remarkably reduced in glioma. (B) In comparison with normal HEB cells HS683 (equivalent to the WHO I), T98MG (equivalent to the WHO II), U373 (equivalent to the WHO III), SHG44, A172, U251 and U87 (four glioblastoma, equivalent to the WHO IV), PUM2 expression decreased remarkably. (C) The protein expression level of PUM2 in U251 and U87 cells was remarkably lower compared with HEB cells. The proliferation capacity of U87 and U251 cells was remarkably decreased (D), migration was remarkably decreased (E), and apoptosis was remarkably increased (F), after upregulating the expression of PUM2. * p < 0.05.
Overexpression of PUM2 inhibited the malignant biological behaviors of glioma cells. (A) In comparison with the control group, the WHO I, WHO II, WHO III and WHO IV level PUM2 gene expression was remarkably reduced in glioma. (B) In comparison with normal HEB cells HS683 (equivalent to the WHO I), T98MG (equivalent to the WHO II), U373 (equivalent to the WHO III), SHG44, A172, U251 and U87 (four glioblastoma, equivalent to the WHO IV), PUM2 expression decreased remarkably. (C) The protein expression level of PUM2 in U251 and U87 cells was remarkably lower compared with HEB cells. The proliferation capacity of U87 and U251 cells was remarkably decreased (D), migration was remarkably decreased (E), and apoptosis was remarkably increased (F), after upregulating the expression of PUM2. * p < 0.05.
Figure 3

PUM2 bound to LINC00657 (A) We used cellular lysates from U251 and U87 cells for RNA immunoprecipitation with antibody against PUM2; we detected LINC00657 expression levels via qRT-PCR (quantitative real-time PCR). (B) Detection of PUM2 with Western blot analysis in the sample pulled down by LINC00657 from U251 and U87 cells. *p<0.05.
Figure 4

Knockdown of NANOS3 inhibited the malignant biological behaviors of glioma cells (A) In comparison with the control group, the WHO I, WHO II, WHO III and WHO IV NANOS3 gene expression was correlated with a significant increase in the level glioma. (B) Compared with normal HEB cells, NANOS3 expression in glioma cell lines was remarkably increased. (C) The protein expression level of NANOS3 in U251 or U87 cells was remarkably increased in comparison with HEB cells. After down-regulating the expression of NANOS3, the proliferation ability of U87 and U251 cells was remarkably reduced (D), apoptosis was remarkably increased (E), and migration was remarkably reduced (F). * p < 0.05.
Knockdown of NANOS3 inhibited the malignant biological behaviors of glioma cells (A) In comparison with the control group, the WHO ⁴, WHO ⁵, WHO ⁶ and WHO ⁷ NANOS3 gene expression was coredlated with a significant increase in the level glioma. (B) Compared with normal HEB cells, NANOS3 expression in glioma cell lines was remarkably increased. (C) The protein expression level of NANOS3 in U251 or U87 cells was remarkably increased in comparison with HEB cells. After down-regulating the expression of NANOS3, the proliferation ability of U87 and U251 cells was remarkably reduced (D), apoptosis was remarkably increased (E), and migration was remarkably reduced (F). * p < 0.05.

Figure 5

PUM2 overexpression and LINC00657 knockdown inhibited the NANOS3 expression (A) The mRNA expression amount of NANOS3 modulated by PUM2 knockdown and overexpression. (B) The protein expression amount of NANOS3 modulated by PUM2 knockdown and overexpression. (C and D) NANOS3 mRNA was intensied in the PUM2 immunoprecipitate of U87 and U251 cells. This enrichment was remarkably increased after LINC00657 knockdown. The (E) mRNA and (F) protein expression levels of NANOS3 was diminished by sh-LINC00657 and lower when combined with PUM2, however, the inhibitor effect of sh-LINC00657 could be reversed by sh-PUM2. *p<0.05.
Figure 5

PUM2 overexpression and LINC00657 knockdown inhibited the NANOS3 expression (A) The mRNA expression amount of NANOS3 modulated by PUM2 knockdown and overexpression. (B) The protein expression amount of NANOS3 modulated by PUM2 knockdown and overexpression. (C and D) NANOS3 mRNA was intensified in the PUM2 immunoprecipitate of U87 and U251 cells. This enrichment was remarkably increased after LINC00657 knockdown. The (E) mRNA and (F) protein expression levels of NANOS3 was diminished by sh-LINC00657 and lower when combined with PUM2, however, the inhibitor effect of sh-LINC00657 could be reversed by sh-PUM2. *p<0.05.

Figure 6
NANOS3 reverses the effect of PUM2 on the biological behavior of glioma cells (A) We adopted CCK-8 assay to assess the effect of PUM2 and NANOS3 on the proliferation of glioma cells. (B) With the expression of PUM2 and NANOS3 flow cytometry analysis of glioma cells changed. (C) With the expression of PUM2 and NANOS3 quantification of migration cells changed. We presented representative images and accompanying statistical plots (scale bar = 100μm). *p<0.05.

Figure 6

NANOS3 reverses the effect of PUM2 on the biological behavior of glioma cells (A) We adopted CCK-8 assay to assess the effect of PUM2 and NANOS3 on the proliferation of glioma cells. (B) With the expression of PUM2 and NANOS3 flow cytometry analysis of glioma cells changed. (C) With the expression of PUM2 and NANOS3 quantification of migration cells changed. We presented representative images and accompanying statistical plots (scale bar = 100μm). *p<0.05.
Figure 7

In vivo tumor growth was inhibited by the silencing of LINC00657 in combination with the overexpression of PUM2 (A) We showed the sample tumor from respective group. (B) We excised and weighed the tumor. (C) After injection with transfected U87 cells, we calculated tumor volume every 4 days. *p<0.05.

Figure 7

In vivo tumor growth was inhibited by the silencing of LINC00657 in combination with the overexpression of PUM2 (A) We showed the sample tumor from respective group. (B) We excised and weighed the tumor. (C) After injection with transfected U87 cells, we calculated tumor volume every 4 days. *p<0.05.