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ORIGINAL RESEARCH

LncRNA RHPN1-AS1 Targeting miR-625/REG3A Promotes Cell Proliferation And Invasion Of Glioma Cells

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Introduction: Glioma arises from the proliferation of neuroglial cells differentiated from the ectoderm. Evidence has confirmed that differentially expressed long non-coding RNAs (lncRNAs) may be involved in the development and progression of various tumors. The present study aimed to explore the biological function of lncRNA RHPN1-AS1 in glioma.

Materials and methods: The expressions of RHPN1-AS1 in glioma tissues and cells were examined using RT-PCR. Colony formation assay, MTT assay, wound healing assay and transwell assay were performed to detect cell cloning efficiency, proliferation, migration and invasion of glioma cells, respectively. Western blot was applied to assess the expression levels of migration-related and invasion-related proteins. Online bioinformatic tools and luciferase reporter assay were, respectively, employed to predict and verify the downstream target microRNA/gene of RHPN1-AS1.

Results: RHPN1-AS1 was up-regulated in glioma tissues and cells. The cell proliferation, migration and invasion of glioma were inhibited when the expression of RHPN1-AS1 was down-regulated in glioma cells. The expressions of migration-related and invasion-related proteins were also suppressed in siRHPN1-AS1 groups. Furthermore, we predicted and verified that RHPN1-AS1 was directly targeted to miR-625-5p/REG3A. Our study demonstrated that the knockdown of RHPN1-AS1 inhibited the proliferation, migration and invasion activity of glioma cells via regulating miR-625-5p/REG3A expression.

Conclusion: The results revealed that the lncRNA RHPN1-AS1 may be a molecular target in glioma therapy.

Keywords: glioma, LncRNA, RHPN1-AS1, proliferation, migration, invasion

Introduction

Gliomas, also known as neuroectodermal or neuroepithelial tumors, occur in the neuroectoderm.[1,2] Most glioma tumors originate from different types of neuroglia. However, based on histogenesis and biological characteristics, a similar occurrence of neuroectodermal tumors in a variety of complex tumors is generally referred to as "glioma".[3,4] The incidence of gliomas ranges from 3 to 8 per 100,000 people in China while the global morbidity ranges from 4.67 to 5.73 per 100,000 people.[5,6]

At present, the standard treatment of glioma is concurrent chemotherapy with temozolomide (TMZ) after surgical excision combined with radiotherapy. However, the overall effect is not satisfactory.[7,8] Due to the infiltration of glioma cells to surrounding brain tissue and poor permeability of the blood–brain barrier to chemotherapy drugs,[9] it is still difficult to completely remove the tumor even

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using the current advanced microsurgical techniques.[10,11] Thereby leading to high resistance and tolerance of glioma cells to treatment.[12,13] Therefore, the challenge in the field of nerve tumor therapy is to find new treatment methods, which can effectively inhibit the malignant biological characteristics of glioma, thus prolonging the survival time of patients and improving their quality of life. Further studies to elucidate the molecular pathogenesis of gliomas and search for new therapeutic pathways and gene therapy targets are ongoing.

Long non-coding RNA (lncRNA), a class of RNA, does not encode proteins and has a transcript length of more than 200 nucleotides.[14,15] Previous reports had proved that lncRNA is closely associated with the pathogenesis of different human malignant tumors.[16,17] Some lncRNAs which may be involved in the occurrence and development of tumors are differentially expressed in tumors and normal tissues.[18] LncRNA, as a by-product of RNA polymerase II transcription, was initially considered to have no biological function.[19] However, recent studies have confirmed that lncRNA has many biological functions, including chromatin modification,[20] chromosome silencing,[21] transcriptional regulation and other biological processes,[22] affecting protein function[23] and the content of microRNA.[24] LncRNA RHPN1-AS1 had found to be overexpressed in several cancers in previous studies, including uveal Melanoma[25] and non-small cell lung cancer.[26] However, the effect of lncRNA RHPN1-AS1 on glioma is unclear.

In the current study, we found that lncRNA RHPN1-AS1 was over-expressed in glioma tissues and cell lines. Moreover, several in vitro assays showed that RHPN1-AS1 knockdown suppressed the proliferation, migration and invasion of glioma cells. In addition, we predicted and verified lncRNA RHPN1-AS1 effected on glioma via targeting miR-625-5p/REG3A. These results provide a novel insight of glioma tumorigenesis and therapy.

Materials And Methods

Patients And Tissues

Glioma tissues and peritumoral brain edema (PTBE) tissues were collected from 37 pairs of glioma patients who underwent surgery between Oct 2009 and Dec 2011 at Taian Center Hospital. All specimens were frozen in liquid nitrogen immediately after surgical operation and then stored at −80°C. The research protocol was approved by the Taian Center Hospital and adhered to the ethical guidelines of the 1975 Declaration of Helsinki. All patients enrolled in the study gave written informed consents.

Cell Culture

Human glioma cell lines H4, A172, U251 and LN229 and a normal human astrocytes cell line NHA were purchased from Shanghai Cell Bank, Shanghai Institutes for Biological Sciences (Shanghai, China). Experimental cells were subsequently cultured in DMEM (HyClone, Logan, UT, USA) that consisted of 100 units/mL penicillin (Invitrogen, Shanghai, China), 100 μg/mL streptomycin (Invitrogen) and 10% fetal bovine serum (FBS) (Invitrogen), and then they were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell Transfection And Reagents

Small interfering RNAs (siRNA: 5′-ACAGCTATATCA GCCAACCAGAGT-3′), small interfering negative control (siNC: 5′-GTTTACAACACGCTTCCTCTGA-3′) RNAs targeting RHPN1-AS1, miR-625-5p mimic (5′-AGGGGGAA AGUUCUAUAGUCC-3′)/miR-NC (5′-AGGUCTAAGUUC UAUGCACC-3′), and miR-625-5p inhibitor (5′-GGACTATA GAACTTTCCCCCT-3′) were designed and obtained from GenePharma (Shanghai, China). Glioma cells (U251 and LN229) were cultured to 60–70% confluency. Cells were subsequently transfected with Lipofectamine 2000 Reagent (Invitrogen) complied with manufacturer's instruction. After plasmids transfected, the expression of RHPN1-AS1 was validated by quantitative real-time PCR (qRT-PCR).

Western Blot

Proteins were extracted using a 500-μL Radioimmunoprecipitation assay (RIPA) buffer with 1-mM phenylmethane sulfonyl fluoride (Sigma, St. Louis, MO, USA) to extract the protein lysates. Tris-buffered saline, containing 0.1% Tween-20 (TBS-T) with 5% nonfat dry milk, was used to block the cell membranes for 30 mins at 37°C. TBS-T was applied to wash the membranes for 4 times, and primary antibodies were employed to incubate them overnight at 4°C. Primary antibodies were all provided by Abcam (Cambridge, MA, USA) and the dilutions used in the current study were as follows: anti-GAPDH (1:500; ab8245), anti-N-cadherin (N-cad; 1:1000; ab18203), anti-snail (1:1000; ab53519), anti-twist (1:1000; ab50581) and REG3A (1:500; ab95316). Following extensive washing, the secondary antibody at a dilution

of 1:2000 was used to incubate membranes for 1 hr at room temperature. GAPDH served as the negative control.

RNA Extraction And qRT-PCR

Trizol (Invitrogen) was used to extract miRNAs and total RNA from glioma tissues and cell lines according to the manufacturer's protocol. DNase I (Roche, Indianapolis, IN, USA) was used to remove residual DNA. QRT-PCR was performed to detect the expressions of RHPN1-AS1, miR-625-5p and REG3A. A cDNA synthesis kit (TaKaRa Biotechnology Co., Ltd, Dalian, China) and oligo (dT) primers were applied to transcribe RNA (2 μg) to cDNA, and SYBR Green qPCR Master Mixes (TaKaRa) was then used for following qRT-PCR according to the manufacturer's instruction. GAPDH was used for normalization. The oligonucleotides, used as PCR primers, were as follows: lncRNA RHPN1-AS1 5′-CT AGCCAGGAGGTTTCGC-3′ and 5′-TCCGCAACAAGCA CACA-3′, GAPDH 5′-CACCCACTCCTCCACCTTTG-3′ and 5′- CCACCACCCTGTTGCTGTAG-3′, miR-625-5p 125′-AGCGCGACTATAGAACTTTCC-3′ and 5′-CTCCTA CTCCCTCCCTCATC-3′, REG3A 5′-GCCTGTGTTTGGTG TGC-3′ and 5′-ATGAGGTGGTCAGGTTGG-3′; the ABI 7300 system (Applied Biosystem, Foster City, CA, USA) was performed to initially incubating the samples at 95°C for 10 mins, and then at 95°C for 10 mins, followed by 40 cycles of incubation at 95°C for 15 s and 60°C for 45 s. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the fold changes. All data represent the average of three replicates.

MTT Assay

The MTT Cell Proliferation and Cytotoxicity Assay Kit (Dojindo Laboratories, Tokyo, Japan) were used to assess the proliferation activity of human glioma cell lines (LN229 and U251) according to the manufacturer's instructions. Cell lines were all culture at 96-well plates with a density of 1×10^4 cells/well and then transfected with siRHPN1-AS1, miR-625-5p mimics, or miR-625-5p inhibitor. The cells were incubated for 12, 24, 48 and 72 hrs. After incubation, the MTT solution was removed and replaced with dimethyl sulfoxide (DMSO; 150 μL, 4%; Sigma). A microplate reader (Bio-Tek, Instruments, Neufahrn, Germany) was used to measure the absorbance at 490 nm.

Colony Formation Assay

The cloning ability of glioma cells was detected using a colony formation assay. Briefly, cells in each treatment group were cultured in 6-well culture plates for 2 weeks to form colonies. Colonies were stained with crystal violet (2%) and counted under inverted microscope (Olympus, Tokyo, Japan). The experiments were performed 3 times.

Wound Healing Assay

Experimental cells in this study were implanted into 6-well culture plates with the density of 1.0×10^6 cells/well. After the cells had fused, a scratch was scraped with a pipette tip on the glioma cell monolayer, and PBS (Beyotime, Nanjing, China) was subsequently applied to wash cells for 3 times, and FBS-free medium was used to seed cells. At 0 and 48 hrs incubation, the glioma cell lines were photographed and the scratch area was assessed using Image J software (National Institutes of Health, Bethesda, MD, USA). The migration rate was calculated as follows: (area of original wound-area of wound at different times)/area of original wound×100%.

Cell Invasion Assay

To determine the invasion ability of glioma cells, a transwell invasion assay was applied. The top chambers of Matrigel-coated Transwell inserts (BD Bioscience, San Jose, CA, USA) were implanted with 5×10^4 cells resuspended in serum-free medium. Complete medium, acted as a chemoattractant, was added into the bottom compartment of the chamber. After incubated for 24 hrs, the surface membrane of the top cells of the remaining cells was slightly dried with cotton swabs, and PBS was used to wash the cells that was on the bottom surface of membrane, fixed in formaldehyde was used to fixed cells, crystal violet was used to stain them and then 5 representative microscopic fields were selected to count cells under an Olympus fluorescence microscope (Tokyo, Japan) to measure the rate of invasion. Experiments were conducted 3 times.

Luciferase Reporter Assay

Wt-RHPN1-AS1, mut-RHPN1-AS1 were, respectively, inserted into pmirGLO reporter vectors (Promega, Madison, WI, USA). U251 and LN229 cell lines were transfected with miR-625-5p mimics and wt-RHPN1-AS1 or mut-RHPN1-AS1 via Lipofectamine 2000. A dual-luciferase reporter assay system (Promega) was used to evaluate the relative luciferase activity at 48 hrs post-transfection. Data were exhibited as the ratio of Renilla luciferase activity to firefly luciferase activity. Luciferase reporter assays to verify the direct binding of miR-625-5p to REG3A 3′ UTR were performed as described above.

Statistical Analysis

Data were presented as mean±standard deviation (SD). Statistical analysis was proceeded using SPSS 19.0 software (SPSS, Chicago, IL, USA). Correlation between RHPN1-AS1 expression level and miR-625-5p or REG3A expression levels in glioma tissues was evaluated through Pearson's correlation analysis. Student's *t*-test was performed to analyze the differences between 2 groups. ANOVA followed by Dunnett's multiple comparison was applied to assess the differences among 3 independent groups. $P<0.01$ was considered statistically significant.

Results

LncRNA RHPN1-AS1 is significantly upregulated in glioma tissues and cell lines.

In the beginning of the study, the RHPN1-AS1 expressions in 37 glioma tissue samples and paired adjacent non-tumor tissues were detected via qRT-PCR. Figure 1A shows that the expression level of RHPN1-AS1 in glioma tissues was obviously higher than that in the PTBE tissues ($P<0.001$). In addition, we measured the expression of lncRNA RHPN1-AS1 in glioma cell lines (H4, A172, U251 and LN229) and normal cells (NHA) were also measured using qRT-PCR. The result showed that the expression level of RHPN1-AS1 was remarkably upregulated in H4, A172, U251 and LN229 cells compared with NHA cells ($P<0.01$, Figure 1B).

LncRNA RHPN1-AS1 knockdown inhibits the proliferation of glioma cells in vitro.

The expression levels of RHPN1-AS1 in glioma tissues and cell lines were markedly higher than that in PTBE tissues and NHA cells. In U251 and LN229 cells transfected with siRHPN1-AS1, the expression of RHPN1-AS1 was significantly decreased when compared to the siNC group (Figure 2A). To verify the effect of RHPN1-AS1 silence on glioma cell proliferation ability, MTT assay and colony formation assays were applied. The results of MTT and colony formation assays demonstrated that lncRNA RHPN1-AS1 knockout suppressed the proliferation ability of glioma cells (Figure 2B–E).

LncRNA RHPN1-AS1 knockdown inhibits the migration and invasion activity of glioma cells in vitro.

To explore the role of RHPN1-AS1 acted in the glioma cells migration and invasion, wound healing assay and transwell invasion test were performed. Figure 3A and B exhibited that the relative wounding width of siRHPN1-AS1 group was significantly greater than siNC group after 24 hrs culture in both U251 and LN229 cell lines ($P<0.001$). The result of transwell invasion test was shown in Figure 3C and D; the invasion cell number of siRHPN1-AS1 group was obviously less than siNC group ($P<0.001$). These results revealed that lncRNA RHPN1-AS1 knockdown effectively reduced the migration and invasion viability of glioma cells ($P<0.001$). To better understand the molecular mechanism of the effects of RHPN1-AS1 on migration and invasion of glioma cells, the expression levels of N-cadherin, snail, and twist, proteins associated with cell migration and invasion, were examined by Western blot. Figure 3E–G demonstrated that the expression levels of N-cad, snail and twist were all significantly inhibited in siRHPN1-AS1 group when compared with the siNC group ($P<0.001$). These results revealed that RHPN1-AS1 knockdown inhibited the migration and invasion of glioma cells.

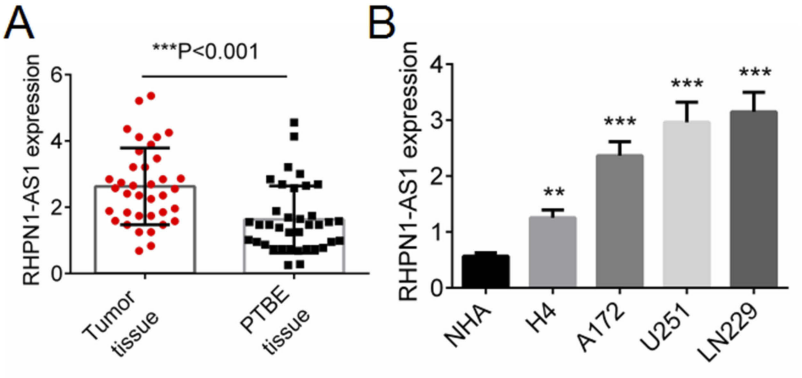

Figure 1 LncRNA RHPN1-AS1 is upregulated in glioma tissues and cells. (**A**) Relative expression levels of lncRNA RHPN1-AS1 in 37 pairs of glioma tissues and PTBE tissues were identified using RT-PCR. (**B**) Relative expression levels of lncRNA RHPN1-AS1 in normal human astrocytes cell line NHA and 4 glioma cell lines (H4, A172, U251 and LN229) were identified using RT-PCR. Data were shown as mean±SD. **$P<0.01$, ***$P<0.001$ compared with PTBE tissues or NHA cell line.

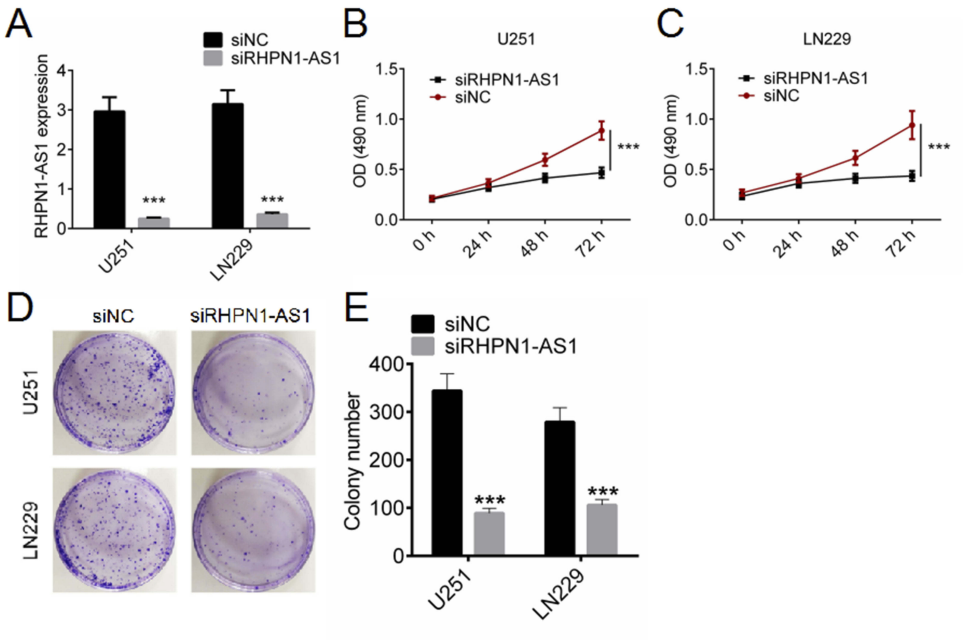

Figure 2 LncRNA RHPN1-AS1 promotes glioma cell proliferation and colony formation. (**A**) LncRNA RHPN1-AS1 expression levels were detected using RT-PCR after transfection with siRHPN1-AS1 or siNC in glioma cell lines U251 and LN229. (**B**) Cell proliferation of U251 cells was identified by MTT assay. (**C**) Cell proliferation of LN229 was identified by MTT assay. (**D**) Clonogenic abilities of cell line U251 and LN229 were assessed via colony formation assay after transfection with siRHPN1-AS1 or siNC. (**E**) Colony numbers of U251 and LN229 cells were counted in colony formation assay after transfection with siRHPN1-AS1. Data were shown as mean±SD. ***P<0.001 compared with siNC group.

Bioinformatic methods predict the interaction between lncRNA RHPN1-AS1 and miR-625-5p.

We predicted that miR-625-5p was the target microRNA of lncRNA RHPN1-AS1 by miRDB. To verify the prediction, RHPN1-AS1-wt and RHPN1-AS1-mut luciferase reporter plasmids were conducted (Figure 4A). As shown, miR-625-5p mimic suppressed the relative luciferase activity after being transfected with RHPN1-AS1-wt (Figure 4B). When the binding site of miR-625-5p in RHPN1-AS1 was mutated, miR-625-5p mimic had no effect on the relative luciferase activity (Figure 4B). To further confirm the interaction between RHPN1-AS1 and miR-625-5p, U251 and LN226 cells were transfected with siRHPN1-AS1, and siRHPN1-AS1 transfection was found to promote miR-625-5p expression (Figure 4C).

MicroRNAs can regulate gene expression via targeting mRNA for degradation.[27] To further predict the downstream target mRNAs of miR-625-5p, TargetScan was used. Among the potential target genes, REG3A got the highest score. We cloned the REG3A-wt and REG3A-mut luciferase reporter plasmids (Figure 4D). We transfected miR-625-5p mimic or miR-NC into glioma cells for luciferase assay. Figure 4E shows that transfecting miR-625-5p mimic obviously reduced the relative luciferase activity. In addition, the expression of REG3A was detected via RT-PCR and Western blot, as shown in Figure 4F–H, overexpression of miR-625-5p decreased the expression of REG3A expression in U251 and LN229 cells. In summary, lncRNA RHPN1-AS1 promoted proliferation and invasion of glioma cells via targeting miR-625-5p/REG3A.

Rescue experiment verified lncRNA RHPN1-AS1 accelerate proliferation, migration and invasion of glioma cells via targeting miR-625-5p.

To verify the molecular mechanism of lncRNA RHPN1-AS1, a rescue experiment was performed by transfecting siRHPN1-AS1 or miR-625-5p inhibitors into U251 cells. The results of the MTT assay and cell colony experiments showed that the addition of miR-625-5p inhibitor recovered cell proliferation ability after siRHPN1-AS1 transfection (Figure 5A–C). Similarly, the wound healing assay and transwell invasion test confirmed that miR-625-5p inhibitor enhanced the migration and invasion activities of U251 cells when the cell lines were transfected with siRHPN1-AS1 (Figure 5D–G). The expression of N-cad, snail and twist were also seen to be remarkably increased with the addition of miR-625-5p inhibitor, via Western blotting (Figure 5H and I). Overall, lncRNA RHPN1-AS1 promoted the proliferation, migration and invasion of glioma cells by targeting miR-625-5p.

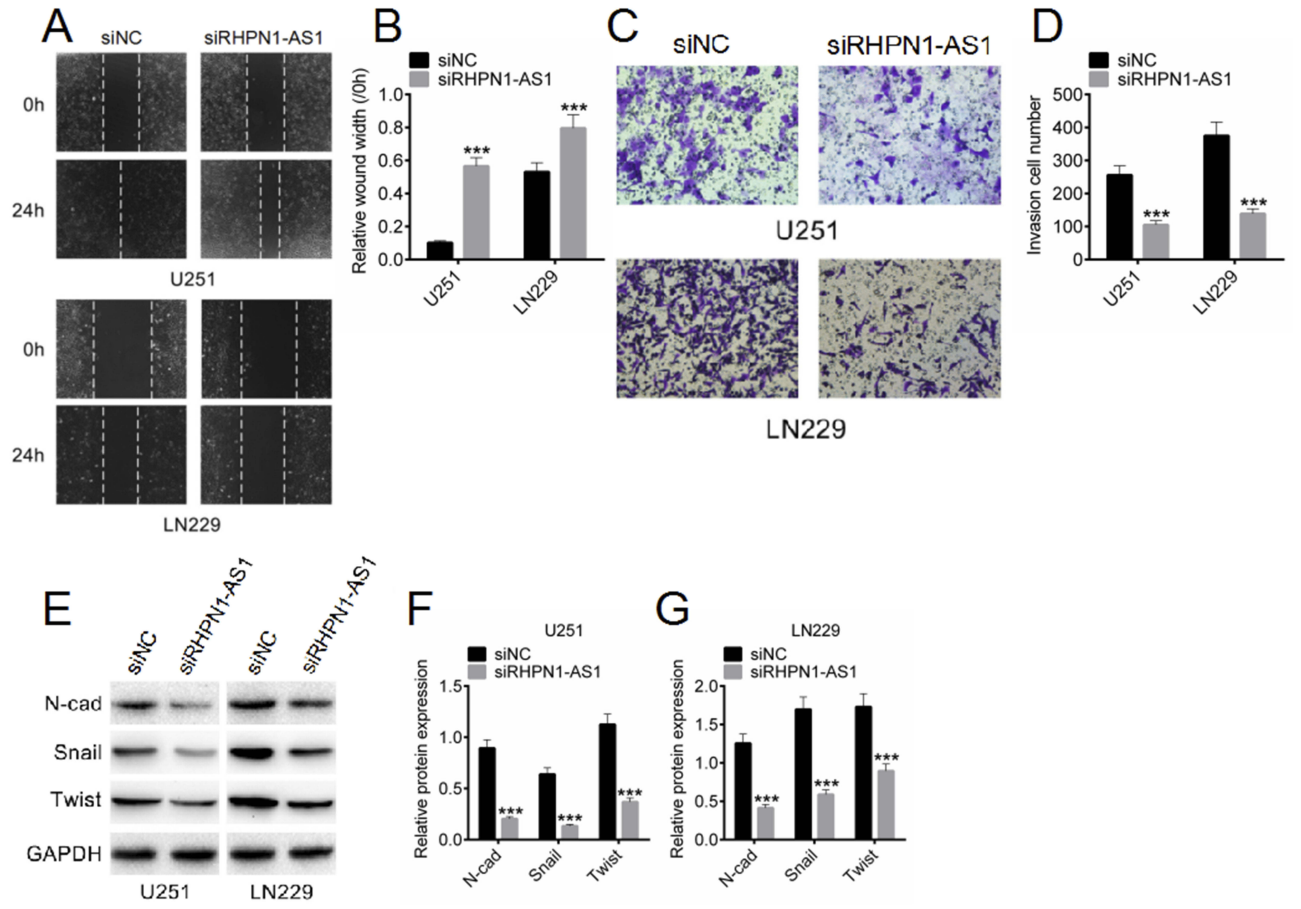

Figure 3 LncRNA RHPN1-AS1 promotes glioma cell migration and invasion. (**A, B**) Cell migration was evaluated by wound healing assays (magnification: 200×). (**C, D**) Cell invasion was assessed by transwell invasion assays (magnification: 400×). (**E–G**) The expression levels of proteins (N-cad, snail and twist) that correlated with cell migration and invasion were detected via Western blot. Data were shown as mean±SD. ***P<0.001 compared with siNC group.

MiR-625-5p overexpression inhibits the proliferation, migration and invasion of glioma cells.

To define the function of miR-625-5p in glioma cells, miR-625-5p mimic was constructed and then transfected into glioma cell lines U251 and LN229. Data from RT-PCR displayed that miR-625-5p mimic transfection obviously up-regulated the expression level of miR-625-5p in U251 and LN229 cell lines (P<0.001, Figure 6A). The cell proliferation was subsequently detected using MTT assay, the OD value of miR-625-5p mimic group was significantly lower than that of miR-NC group both in U251 and LN229 cell lines (P<0.001, Figure 6B). As shown in Figure 6C, colony cell number in miR-625-5p groups of the two cell lines were also dramatically less than that in miR-NC groups (P<0.001). Wound healing assay was applied to measure the migration activity of cells and the results were shown in Figure 6D. Apparently, the relative wound width of miR-625-5p groups was less than that of miR-NC groups (Figure 6E). The results from transwell assay showed that miR-625-5p mimic transfection notably declined the invasion cell number in glioma cell lines compared to that in miR-NC group (P<0.001, Figure 6F). Figure 6 displays that the protein expressions of N-cadherin, snail and twist were remarkably repressed in miR-625-5p groups when compared with that in miR-NC groups (P<0.01). These results demonstrated that the overexpression of miR-625-5p could suppress the cell proliferation, colony formation, migration and invasion in human glioma cell lines.

Discussion

Various lncRNAs show irregular expression and play functional roles in human tumours.[28,29] Liu et al[30] reported that lncRNA loc285194 inhibited p53-regulated tumor suppression by suppressing miR-211. Cui et al[31] reported that lncRNA SNHG1 overexpression promoted the activity of non-small cell lung cancer by inhibiting miR-101-3p and regulating the Wnt/β-catenin signaling pathway. Jin et al[32]

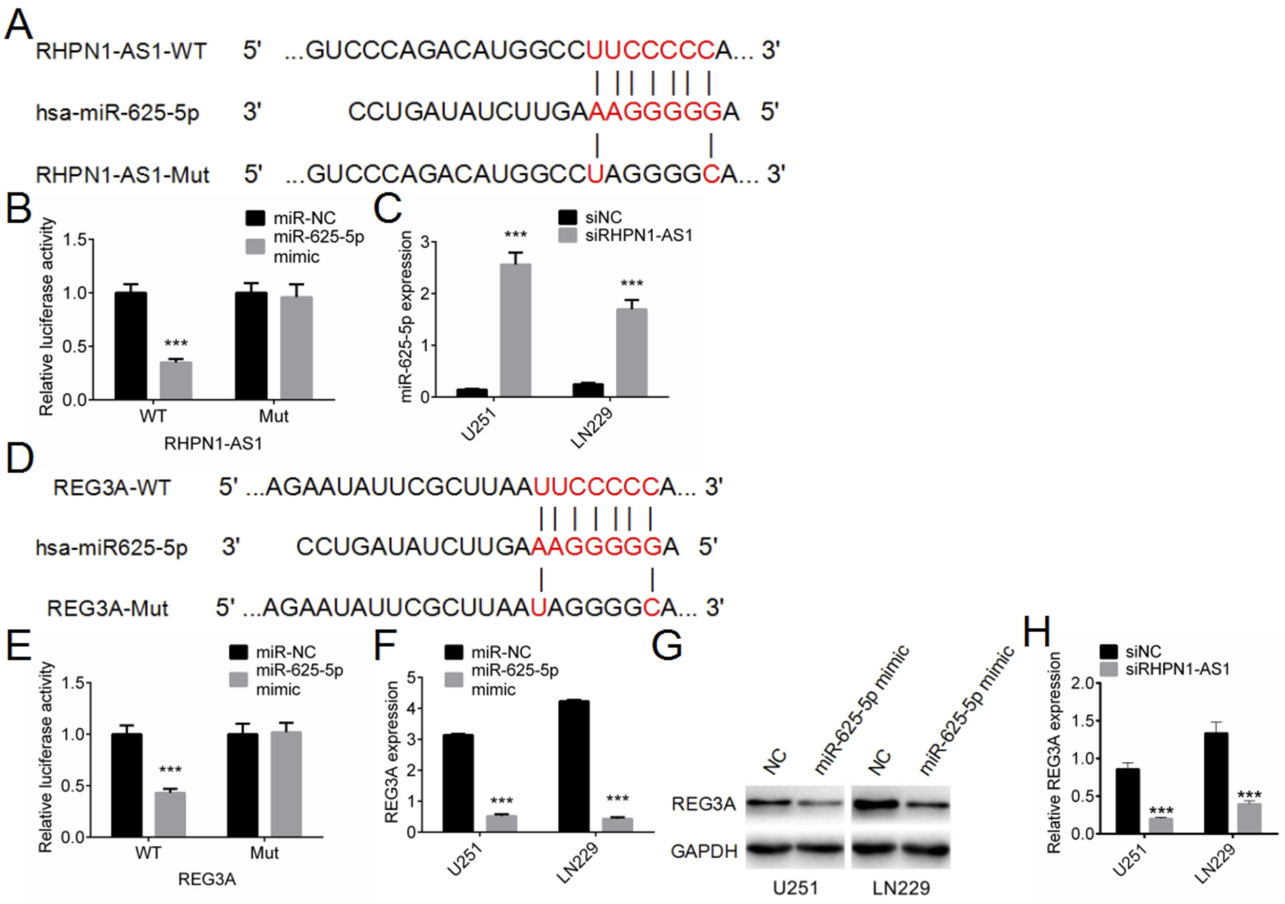

Figure 4 LncRNA RHPN1-AS1 interacts with miR-625-5p/REG3A in glioma cells. (**A**) A binding site of miR-625-5p in RHPN1-AS1 was predicted by the miRDB. (**B**) Relative luciferase activity was evaluated after co-transfection with miR-625-5p mimics and wt-RHPN1-AS1 or mut-RHPN1-AS1. (**C**) The expression levels of miR-625-5p in U251 and LN229 cells were detected by RT-PCR after transfection with siRHPN1-AS1. (**D**) A binding site of REG3A in miR-625-5p was predicted by the TargetScan. (**E**) Relative luciferase activity was evaluated after co-transfection with miR-625-5p mimics and wt-REG3A or mut-REG3A. (**F**) The expression levels of REG3A in U251 and LN229 cells were detected by RT-PCR after transfection with miR-625-5p mimics. (**G, H**) The expression levels of REG3A in U251 and LN229 cells were detected by Western blot after transfection with miR-625-5p mimics. Data were shown as mean±SD. ***P<0.001 compared with miR-NC or siNC group.

found that the upregulation of PlncRNA-1 accelerated cell proliferation and tumor metastasis of prostate cancer. Li et al[33] reported that lncRNA TUG1 inhibited glioma by promoting cell apoptosis. RHPN1-AS1 was reported to be highly expressed in a variety of tumors and was considered as an oncogene.[26] However, the role and pathogenic mechanism of lncRNA RHPN1-AS1 in human gliomas has not been completely understood.

In the present study, we first identified that the expression of lncRNA RHPN1-AS1 was enhanced in human glioma tissues and cell lines compared with PTBE tissues and NHA cell lines. As expected, RHPN1-AS1 knockdown remarkably suppressed the proliferation of U251 and LN229 cells. In addition, the migration and invasion of U251 and LN229 cells were inhibited after transfecting siRHPN1-AS1. Furthermore, we detected the expression level of several proteins (N-cadherin, snail and twist), which were correlated with cell migration and invasion,[34,35] and we found that these proteins were downregulated after lncRNA RHPN1-AS1 knockdown. These results revealed that lncRNA RHPN1-AS1 might play a carcinogenic role in glioma oncogenesis and development.

Previous studies had confirmed that lncRNA modulates tumorigenesis and development by targeting microRNAs.[36,37] As reported in multiple myeloma, lncRNA OIP5-AS1 knockdown-induced microRNA-410 overexpression and regulated cell proliferation and apoptosis via targeting KLF10 and modulating PTEN/PI3K/AKT signal pathway.[38] LncRNA SNHG12 was found to regulate the proliferation and viability of human gastric cancer cells (BGC-823) via targeting microRNA-199a/b-5p.[39] In this study, we predicted that miR-625-5p acted as the target microRNA of lncRNA RHPN1-AS1 via miRDB, and then we found that the expression of miR-625-5p negatively correlated with the expression

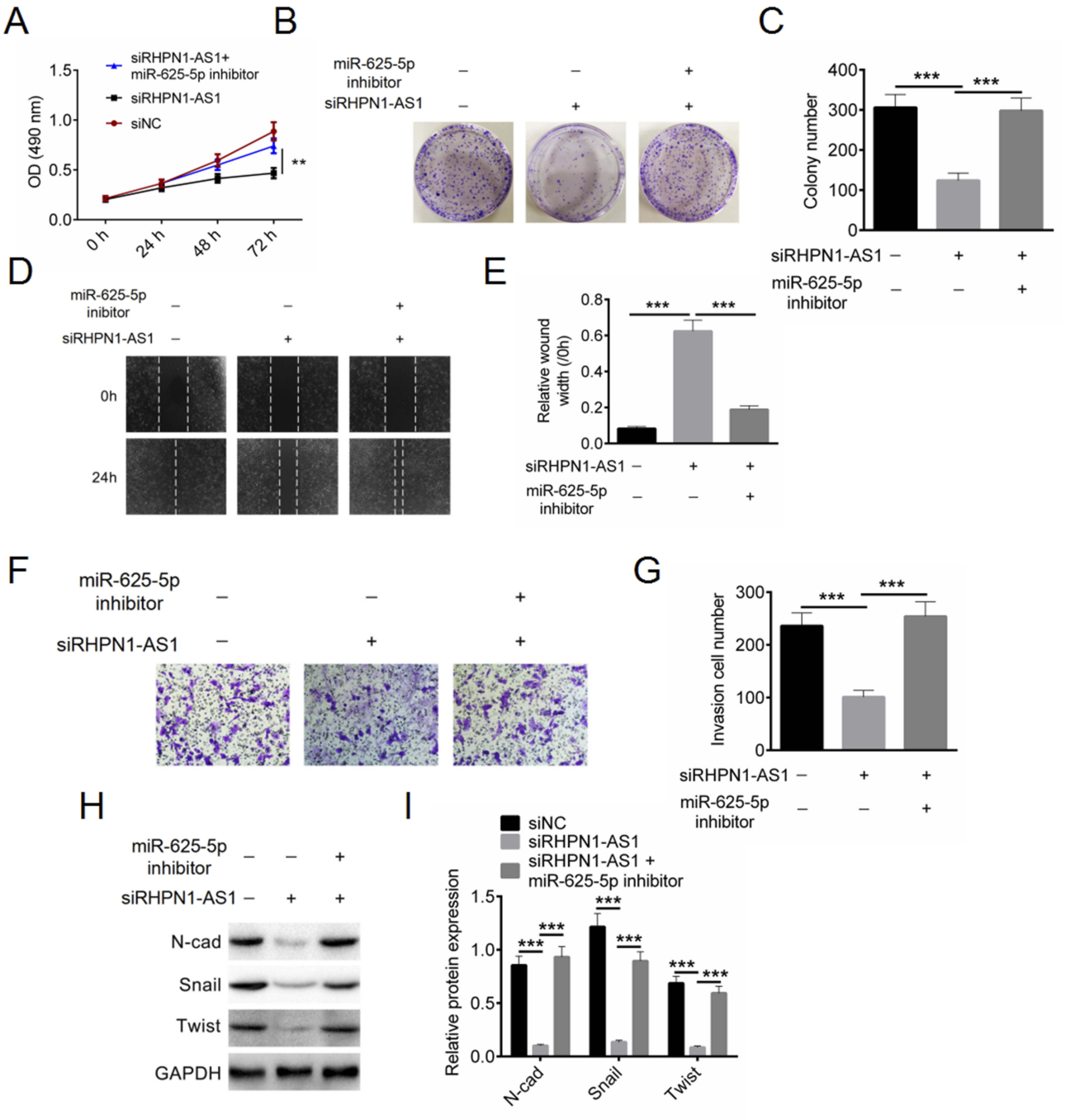

Figure 5 LncRNA RHPN1-AS1 affects glioma cell proliferation, migration, and invasion by targeting miR-625-5p. (**A**) Proliferation of U251 cells was measured through the MTT assay after rescuing the expression of miR-625-5p. (**B, C**) Clonogenic ability of U251 cells was evaluated through the colony formation assay after rescuing the expression of miR-625-5p. (**D E**) Cell migration of U251 cells was measured through the wound healing assay after rescuing the expression of miR-625-5p (magnification: 200×). (**F, G**) Cell invasion of U251 cells was detected through the transwell invasion assay after rescuing the expression of miR-625-5p (magnification: 400×). (**H, I**) The expression levels of proteins (N-cad, snail, and twist) that correlated with cell migration and invasion were detected via Western blot after rescuing the expression of miR-625-5p. Data were shown as mean±SD. $^{**}P<0.01$, $^{***}P<0.001$ compared with control group.

of RHPN1-AS1 in glioma cell lines (U251 and LN229 cells) by luciferase reporter assays. Previous studies have reported that miR-625-5p may be a modulator in several tumors including gastric cancer,[40] hepatocellular carcinoma[41] and esophageal cancer,[42] RHPN1-AS1 might act on glioma via regulating miR-625-5p.

Also, REG3A was predicted as the target gene of lncRNA RHPN1-AS1/miR-625-5p in our study. It has

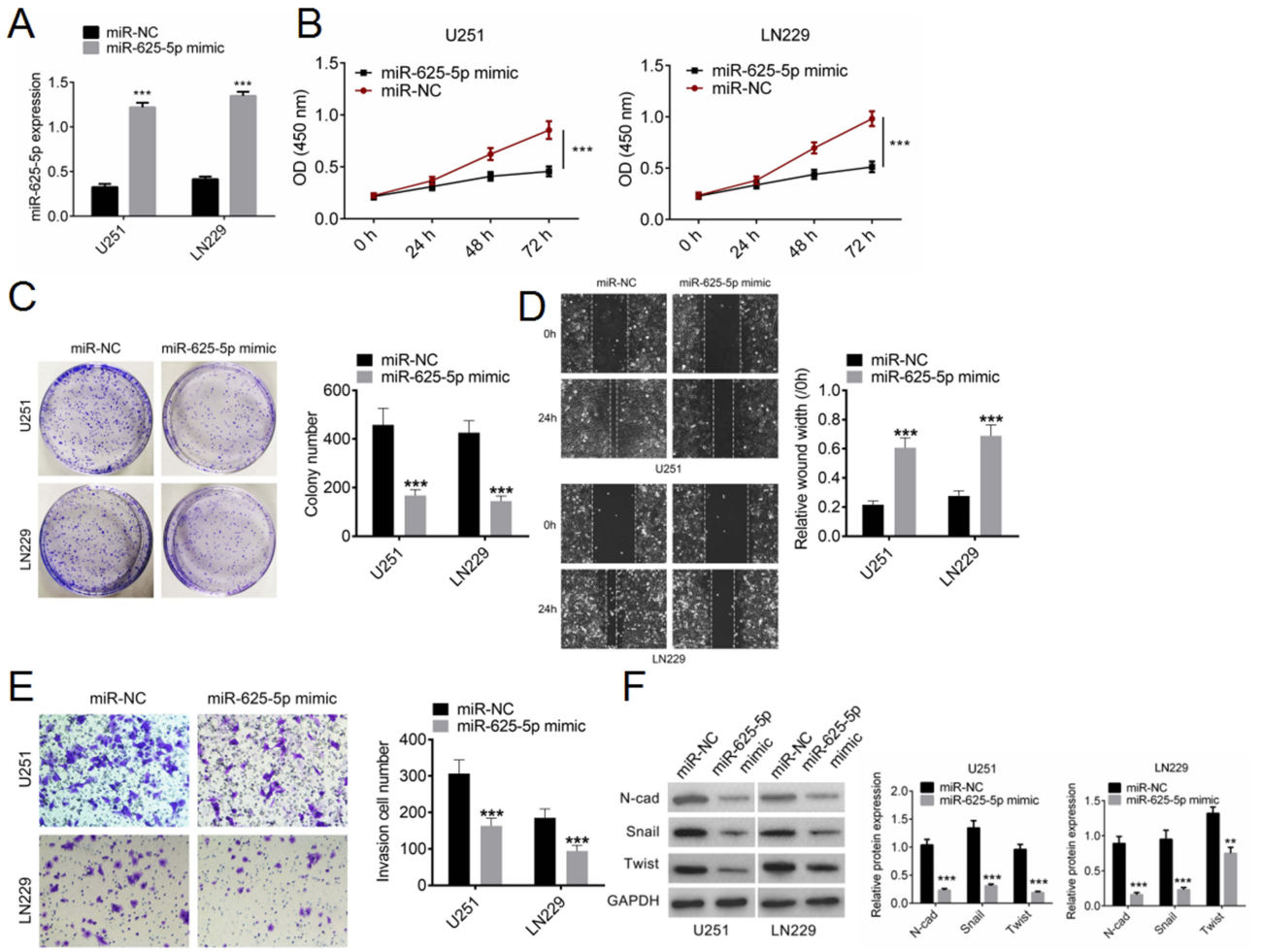

Figure 6 miR-625-5p mimic inhibits glioma cell proliferation, colony formation, migration and invasion. (**A**) The expression level of miR-625-5p in glioma cell lines with miR-625-5p mimic/miR-NC transfection was detected using RT-PCR. (**B**) The cell proliferation was detected using MTT assay. (**C**) The cell colony number was measured using colony formation assay. (**D**) The cell migration was assessed using wound healing assay (magnification: 200×). (**E**) The cell invasion was examined using transwell assay (magnification: 400×). (**F**) The protein expressions of N-cad, snail and twist were determined using Western blot. Data were shown as mean ± SD. ***P*<0.01, ****P*<0.001 compared with miR-NC group.

been reported that REG3A was widely over-expressed in various cancers such as pancreatic cancer,[43] colorectal cancer[44] and gastric cancer.[45] In the current study, we elucidated that the expression of REG3A was significantly decreased when the expression of miR-625-5p was upregulated in U251 and LN229 cells. In addition, the knockdown of RHPN1-AS1 repressed the expression of REG3A. These results confirmed that lncRNA RHPN1-AS1 might be correlated with the expression of miR-625-5p/REG3A.

To further verify the above prediction, an miR-625-5p inhibitor was used in a rescue experiment. We found that the miR-625-5p inhibitor somewhat restored the proliferation of glioma cells with siRHPN1-AS1 transfection. In addition, the migration and invasion of glioma cells with siRHPN1-AS1 were also recovered after adding the miR-625-5p inhibitor. Furthermore, the expression levels of N-cad, snail, and twist after adding miR-625-5p inhibitor were detected, the results showed that the expression levels of these proteins were increased with miR-625-5p inhibitor. In addition, we demonstrated that the overexpression of miR-625-5p inhibited the cell proliferation, colony formation, migration and invasion viability in glioma cells. These results elucidated that RHPN1-AS1 knockdown suppressed the proliferation, migration, and invasion via targeting miR-625-5p.

In conclusion, the current data indicated that lncRNA RHPN1-AS1 played a stimulative role in the pathogenesis of human glioma, and downregulation of RHPN1-AS1 decreased the proliferation, migration, and invasion viability of glioma cell lines via targeting miR-625-5p/REG3A. The current results revealed that lncRNA RHPN1-AS1 may be a molecular therapeutic target in glioma.

Ethics approval and consent to participate

The project protocol was approved by the Taian Center Hospital.

Availability of data and materials

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

Author contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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