

ORIGINAL ARTICLE

LncRNA MALAT1 promotes glioma cell growth through sponge miR-613

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Summary

Purpose: To explore the effect of LncRNA MALAT1 on the growth of glioma cells and its related mechanism.

Methods: The expression of LncRNA MALAT1 and its target gene miR-613 in glioma cells was detected by RT-PCR, and the proliferation, invasion and apoptosis rates and the expression of related proteins were detected by CCK-8, invasion and apoptosis rate tests.

Results: The results of qRT-PCR showed that LncRNA MALAT1 was up-regulated and miR-613 was down-regulated in glioma tissues and cells, and LncRNA MALAT1 was negatively correlated with miR-613. Cell tests confirmed that LncRNA MALAT1 played a role in promoting oncogene, including promoting proliferation and invasion of glioma cells and promoting apoptosis. Bioinformatics prediction

and subsequent experiments proved that miR-613 was the direct target of LncRNA MALAT1. Furthermore, the proliferation and invasion ability of glioma cells were evidently inhibited, the apoptosis rate was evidently enhanced, and the expressions of pro-apoptosis related proteins bax and Cle-Caspase-3 were up-regulated while the expression of anti-apoptosis related protein bcl-2 was down-regulated.

Conclusion: LncRNA MALAT1 can promote the proliferation and invasion of glioma cells and inhibit the apoptosis of glioma cells by sponging miR-613. LncRNA MALAT1 can be applied as a new potential target for glioma treatment.

Key words: apoptosis, glioma, invasion, LncRNA MALAT1, miR-613, proliferation

Introduction

Glioma, as a central nervous system disease, is also the most aggressive malignant neoplasm, with high mortality [1]. Despite the development of medical technology, the great progress made in cancer immunotherapy, and new radiotherapy and chemotherapy approaches, its mortality has not improved due to the high metastasis and recurrence rate of glioma [2,3]. Accumulating evidence shows that molecular heterogeneity is the main obstacle to improve the outcome of glioma [4]. So it is urgent to clarify the molecular mechanism of glioma proliferation and invasion, to find new treatment targets and schedules, and to improve prognosis. Long non-coding RNA (LncRNA) is a set of non-

protein coding transcripts with more than 200 nucleotides, which acts in many malignant neoplasms including glioma [5]. The current research [6-8] shows that LncRNA not only actively participates in gene expression regulation, alternative splicing modification, histone modification and gene chromatin rearrangement, but also acts as a competitive endogenous RNAs (CeRNAs) to adjust microRNA (miR) targets, thus affecting cell function. Metastasis-associated lung adenocarcinoma transcript -1 (MALAT1) is LncRNA with about 8000-nucleotides, which is highly-conserved [9]. Previous studies show that MALAT1 is usually over-expressed in neoplasms, and acts as a carcino-

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gen in breast carcinoma [10], colon carcinoma [11] and cervical carcinoma [12]. In addition, MALAT1, as one of the LncRNA, was highly expressed in glioma in the past, and it has high value for predicting the poor prognosis of glioma [13]. Although some studies [14] have explored its mechanism in glioma, MALAT1 may have multiple targets as ceRNA, so its possible mechanism in glioma still needs further exploration.

In this study, glioma from patients and various glioma cells were applied to test the expression, clinical significance, function and potential mechanism of MALAT1.

Methods

Clinical specimens

From March 2016 to March 2018, 95 patients who came to our hospital for glioma resection were selected as the research participants. The average age of all patients was 59.42 ± 4.15 years. With the consent of patients, 95 cases of glioma tissues were obtained as the research group (RG) during the operation, and 52 cases of normal brain tissues were taken as the control group (CG) with the consent of patients with traumatic brain injury from accidents. The samples were placed in a liquid nitrogen tank. All patients have signed the informed consent form.

Cell culture and transfection

U251, T98MG and U87 and normal astrocyte cell line NHAS were taken from American ATCC Company and cultured in Dulbecco Modified Eagle's Medium (DMEM), which includes 10% fetal bovine serum (FBS) and 100U/ml penicillin or streptomycin 100µg/mL with 5%CO₂. SI-MALAT1 and its negative control (SI-NC), miR-613 mimics, miR-613 inhibitor and miR-NC were taken. MALAT1 sequence was inserted into pcDNA3.1 to establish an over-expression plasmid (pcDNA3.1-MALAT1). The cells were inoculated in 96-well plates and transfected for 48 h by Lipofectamine 2000, and the transfected cells were used for further experiments.

qPCR-RT

Total RNA was taken using TRIzol reagent. The purity, concentration and integrity were tested by ultraviolet spectrophotometer. The specific steps of reverse transcription using TaqMan reverse transcription reagents kit were strictly carried out according to the instructions

of the kit. SYBR Premix ExTaq II was applied for amplification, and ABI 7500PCR was applied for amplification. The amplification procedure was as follows: 10µL of SYBR Premix Ex Taq II (2x), 2µL of cDNA, 0.8µL of upstream and downstream primers, and 20µL of sterile purified water. The amplification conditions were as follows: 95°C for 30s, 95°C for 5s, and 60°C for 30s, for a total of 40 cycles. Each sample was placed in 3 wells, and the experiment was conducted 3 times. U6 was applied as the internal reference of miR-613, GAPDH for MALAT1, and 2^{-ΔΔCt} was applied to test the data (Table 1).

Proliferation

The proliferation was tested by CCK-8. Cells were taken 48 h after transfection, adjusted to 3×10^4 cells/ml, and placed into 96-well plates. Each well was cultured with 100 µl of cells, and 10 µL of CCK8 solution was put at 0 h, 24 h, 48 h and 72 h after adherent growth. After placing the reagent, it was cultured at 37°C with 5% CO₂ for 2 h, and then optical density (OD) value was evaluated at 450 nm to get the proliferation. This procedure was performed 3 times.

Invasion (Transwell assay)

Cells transfected for 24 h were collected, adjusted to 3×10^5 , cultured on 6-well plates, washed with phosphate buffered saline (PBS) and moved into the upper chamber, added with 200 µL DMEM culture solution into the upper chamber, added with 600 mL DMEM including 10% FBS, and cultured at 37°C for 48 h. The matrix and cells that have not passed through the membrane surface were moved and washed, then the cells were fixed with paraformaldehyde for 10 min, and washed with double distilled water for 3 times, dyed with 0.5% crystal violet and the invasion was assessed.

Apoptosis

Cells were treated with 0.25% trypsin, rinsed, put with 100 µL of binding buffer, adjusted into 1×10^6 , added with AnnexinV-FITC and propidium iodide (PI), cultured for 5 min, and tested with FC500MCL flow cytometer system for a total of 3 times.

Western blot

The cultured cells were lysed with RIPA at 4°C for 30 min, then the total protein was extracted, separated by 12% SDS-PAGE, moved to PVDF, dyed with Ponceau S, rinsed in phosphate buffer solution containing tween 20 (PBST) for 5 min, AND THEN MEMBRANES WERE BLOCKED with 5% skim milk powder for 2 h, and added with Bax (1: 500), Bcl-2 (1: 500) and sealed overnight at

Table 1. Primer sequence

Gene	Upstream primer 5'-3'	Downstream primer 5'-3'
MALAT1	ATGCGAGTTGTTCTCCGTCT	TATCTGCGGTTTCCTCAAGC
GAPDH	CAACTACATGGTTTACATGTT	GCCAGTGGAATCCACGAC
miR-613	GGGGTACCGTATTATAAGTAATCTG	CCAAGCTTTCACCGAGAAGAGATCAACCAATA
U6	GCACCTTAGGCTGAACA	AGCTTATGCCGAGCTCTTGT

4°C. The membrane was rinsed to discard the primary antibody, added with horseradish peroxidase labeled goat anti-rabbit, cultured at 37°C for 1 h, washed with PBS for 3 times, 5 min each time. The excess liquid on the dry film was discarded with filter paper, and the expressions of the proteins were observed with electrochemiluminescence immunoassay.

Detection of double fluorescein reporter enzyme

Candidate miRNA that can bind MALAT1 was searched using bioinformatics database (Starbase v2.0). The oligonucleotide containing MALAT1 target sequence was amplified and cloned into pmirGLO plasmid, and the wild type (Wt) of pmirGLO-MALAT1-3'UTR and the mutant (mut) of pmirGLO-MALAT1-3'UTR were established respectively. The constructed plasmids were sequenced and identified by transferring them to the downstream of luciferase reporter gene. The luciferase reporter plasmid and miR-613- mimic or miR-NC were moved into glioma cells with Lipofectamine 2000. After 48 h, the luciferase activity was evaluated.

RNA pull-down assay

With magnetic ribonucleic acid protein pull-down kit, 1 µg biotin-labeled MALAT1 was put into Eppendorf (EP) test tube, then 500 µL structure buffer was placed at 95°C for 2 min, followed by ice bath for 3 min. Fifty µL fully resuspended beads were cultured in EP tube overnight at 4°C. Beads were centrifuged at 1500xg for 3 min, and the supernatant was discarded, then the beads were washed three times with 500 µL RNA binding protein immunoprecipitation (RIP) washing solution. After washing, 10 µL of cell lysate was cultured at room tempera-

ture for 1 h, then the cultured bead-RNA-protein mixture was centrifuged at low speed, and the supernatant was washed three times with 500 µL RIP washing buffer. A 10 µL supernatant of cell lysate was processed with input protein, and the protein was tested by Western blot.

RIP analysis

Firstly, glioma cells were obtained by centrifugation, and RIP lysis buffer was used for the extraction of total proteins. Then, the suspension was centrifuged and the supernatant was extracted. One part of cell extract was applied for Input, and the other part was cultivated with antibody for coprecipitation. The magnetic bead-antibody complex was rinsed and suspended in 900 µL RIP washing buffer, and then cultivated with 100 µL cell extract at 4°C for a night. Then, the sample was cultivated on a magnetic substrate to get the magnetic globin complex. At last, sample digestion and RNA extraction were conducted with proteinase k, and qRT-PCR was conducted to test the enrichment of MALAT1 and miR-613. The antibody AGO2 (1:2000, abcam, USA) was applied for RIP assay, and immunoglobulin G (IgG) (1:1000, abcam, USA) was applied as negative control.

Statistics

SPSS19.0 was applied to test the data, GraphPad 7 to visualize the figures. Independent t test was applied for comparison between groups, single factor analysis of variance was applied for multiple groups, LSD-t test was applied for comparison after the event, repeated measurement of variance was applied for multi-time analysis, and Bonferroni was applied for back testing. When $P < 0.05$, there were statistical differences.

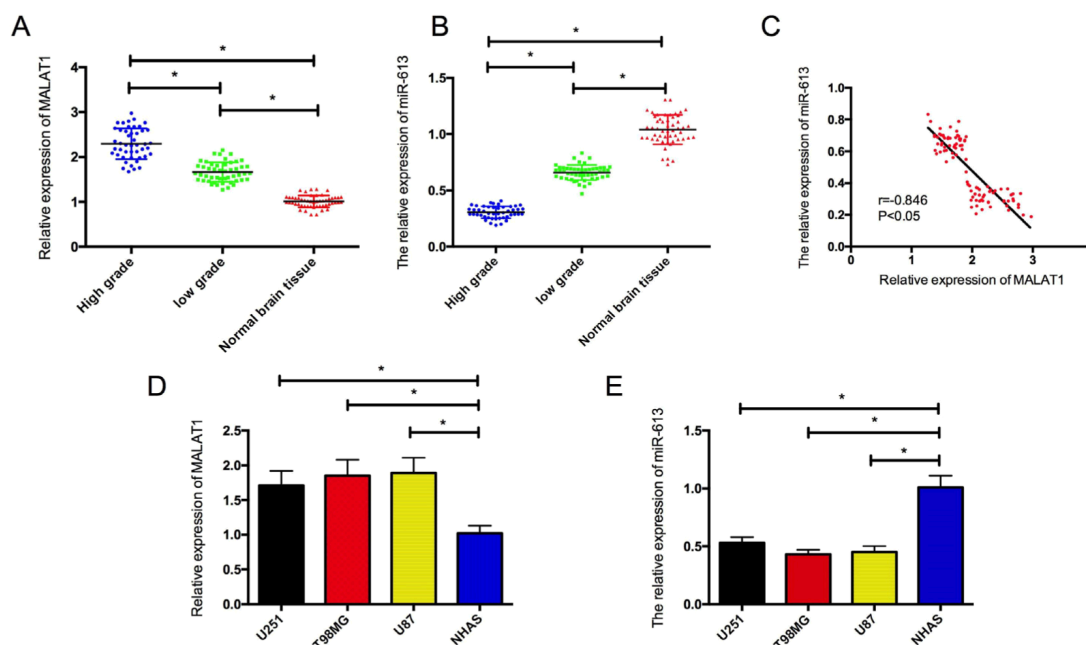


Figure 1. Expression of MALAT1 and miR-613 in glioma. **A,B:** The expression of MALAT1 and miR-613 in glioma tissues of different grades was evaluated by qRT-PCR; **C:** Correlation between MALAT1 and miR-613 expression in glioma tissue was evaluated by linear regression; **D,E:** The expression of MALAT1 and miR-613 in glioma cells of different grades was evaluated by qRT-PCR. * $p < 0.05$.

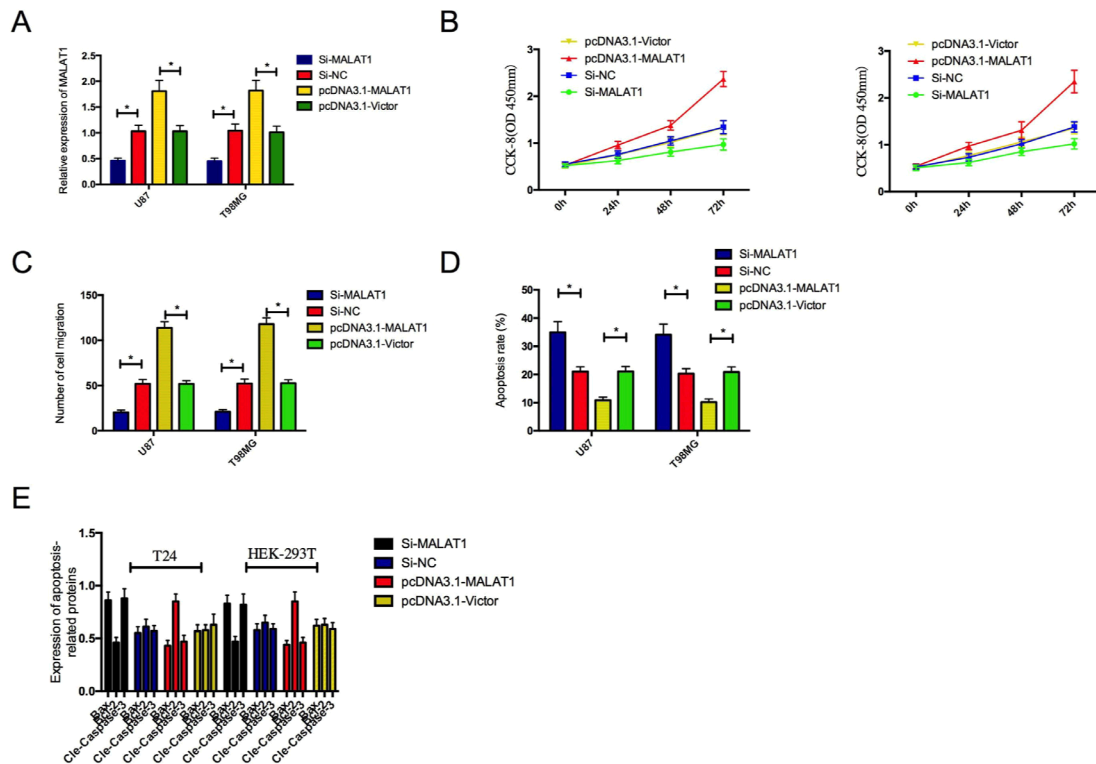


Figure 2. Effect of MALAT1 on proliferation, invasion and apoptosis of glioma cells. **A:** Expression of MALAT1 in glioma cells after transfection. **B:** Effect of MALAT1 on proliferation of glioma cells. **C:** Effect of MALAT1 on the invasion ability of glioma cells. **D:** Effect of MALAT1 on apoptosis of glioma cells. **E:** Effect of MALAT1 on apoptosis-related cells in glioma cells. * $p < 0.05$.

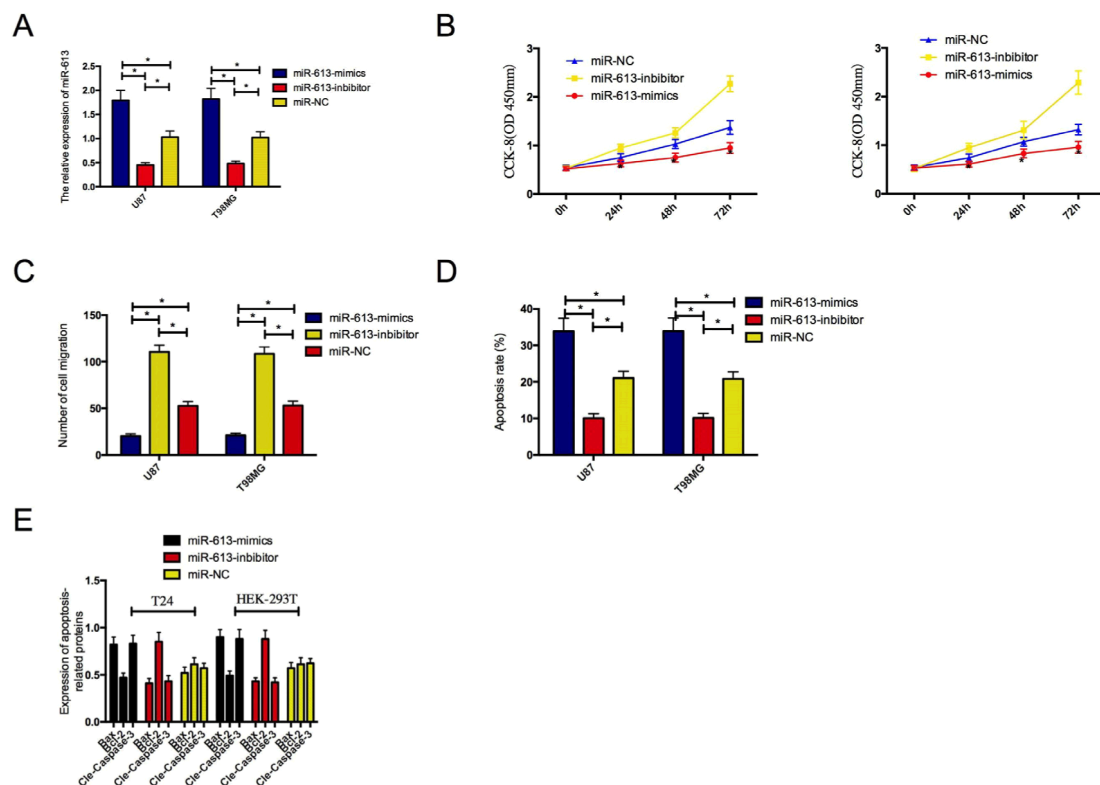


Figure 3. Effects of miR-613 on proliferation, invasion and apoptosis of glioma cells. **A:** Expression of miR-613 in glioma cells after transfection. **B:** Effect of miR-613 on proliferation of glioma cells. **C:** Effect of miR-613 on the invasion ability of glioma cells. **D:** Effect of miR-613 on apoptosis of glioma cells. **E:** Effect of miR-613 on apoptosis-related cells in glioma cells. * $p < 0.05$.

Results

Increase of MALAT1 and decrease of miR-613 in glioma tissues

MALAT1 and miR-613 in glioma tissues were tested by qRT-PCR. Compared with normal brain nerve tissue, MALAT1 in glioma tissues with different pathological grades enhanced evidently, and MALAT1 in glioma cells was also evidently higher than that in normal glial cells. Compared with normal brain nerve tissue, miR-613 in glioma tissue was evidently decreased, and the expression level was closely related to neoplasm grade. miR-613 in glioma cells was also evidently reduced than that in normal glial cells. In addition, MALAT1 had a negative correlation with miR-613 (Figure 1).

Effects of MALAT1 on biological function of glioma cells

U87 and T98MG were transfected with Si-MALAT1, pcDNA3.1-MALAT1, Si-NC and pcDNA3.1-Vector. It was found that MALAT1 in U87 and T98MG transfected with Si-MALAT1 was evidently reduced compared with that in Si-NC cells.

Compared with Si-NC, MALAT1 in U87 and T98MG transfected with Si-MALAT1 was obvi-

ously reduced ($p < 0.05$), and MALAT1 expression in pcDNA3.1-MALAT1 cells was obviously enhanced ($p < 0.05$) compared with pcDNA3.1-Vector cells. In addition, CCK-8 assay, invasion and flow apoptosis were performed. The result showed that down-regulation of MALAT1 evidently hindered the proliferation and invasion of U87 and T98MG ($p < 0.05$), and promoted the apoptosis of cells.

Furthermore, WB revealed that bax and Cle-Caspase-3 enhanced, while bcl-2 reduced ($p < 0.05$). pcDNA3.1-MALAT1 transfected cells evidently enhanced proliferation and invasion ability, and evidently reduced apoptosis rate ($p < 0.05$). Moreover, bax and Cle-Caspase-3 decreased, while bcl-2 enhanced ($p < 0.05$) (Figure 2).

Role of miR-613 on proliferation, invasion and apoptosis

U87 and T98MG were transfected with miR-613-mimics, miR-613-inhibitor and miR-NC. It was found that miR-613 in U87, T98MG cells transfected with miR-613-mimics was evidently enhanced than that in miR-NC cells. The proliferation and invasion of transfected miR-613-mimics cells were evidently inhibited, and the apoptosis was evidently enhanced ($p < 0.05$). miR-613 in cells

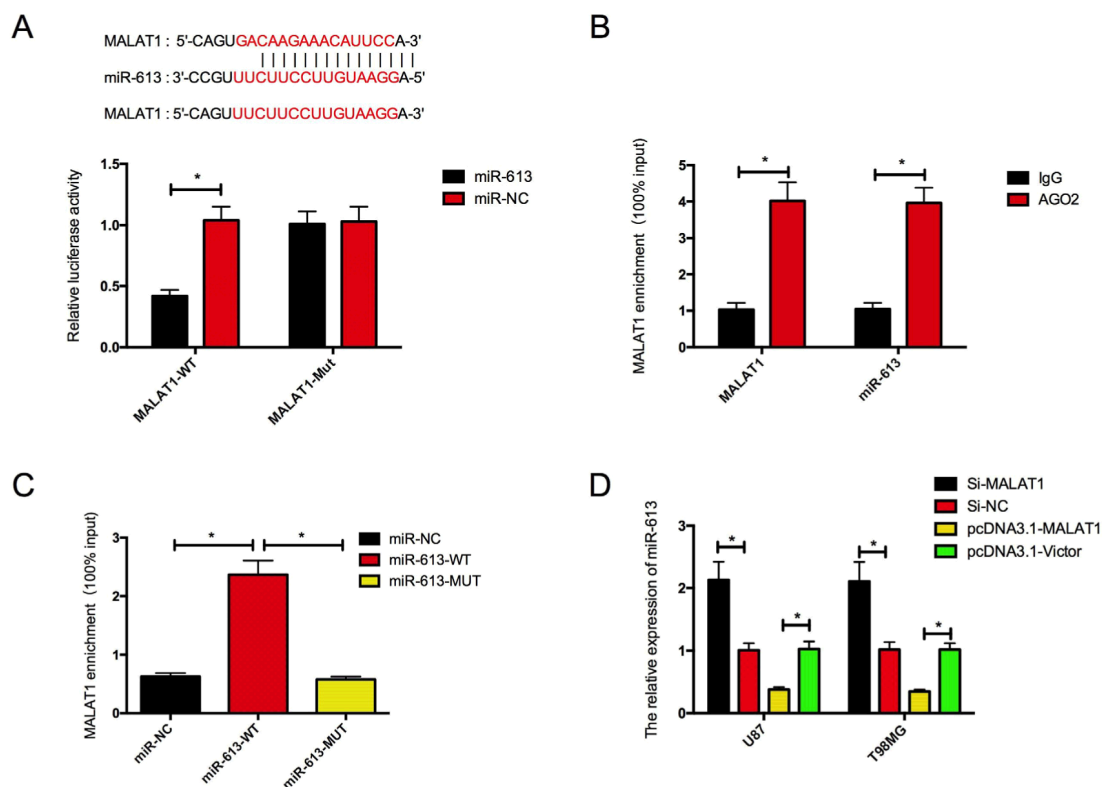


Figure 4. Relationship between MALAT1 and miR-613. **A:** Double luciferase report confirmed the binding relationship between MALAT1 and miR-613. **B:** Assessment of MALAT1, miR-613 and AGO2 enrichment by RIP assay. **C:** Determination of the enrichment ability of MALAT1 and miR-613 by RNA pull-down. **D:** Effect of MALAT1 on miR-613 expression in glioma cells. * $p < 0.05$.

transfected with miR-613-inhibitor was evidently down-regulated. The proliferation and invasion of cells transfected with miR-613-inhibitor were evidently enhanced, and the apoptosis was evidently inhibited ($p < 0.05$) (Figure 3).

MALAT1 acts as a sponge for miR-613

Through online prediction of potential miR of MALAT1 by starbase, we concluded that there is a potential binding target between MALAT1 and miR-613, so we further tested it by double luciferase report, RIP experiment and pull-down experiment. The fluorescence activity of MALAT1-WT was obviously inhibited by miR-613-mimics. RIP experiment showed that MALAT1 and miR-

613 levels precipitated by Ago2 antibody were evidently higher than IgG. However, knocking down MALAT1 evidently enhanced miR-613 in U87 and T98MG cells. Over-expression of MALAT1 evidently inhibited miR-613 in U87 and T98MG cells (Figure 4).

Restoration of miR-613 expression saved MALAT1 from promoting the growth of glioma cells

To further find out whether MALAT1 can act in targeting miR-613 in glioma cells, U87MG were co-transfected with pcDNA3.1-MALAT1 and miR-613-mimics, and then a series of functional analyses were carried out. Western blot showed that co-transfection with miR-613 over-expression vector

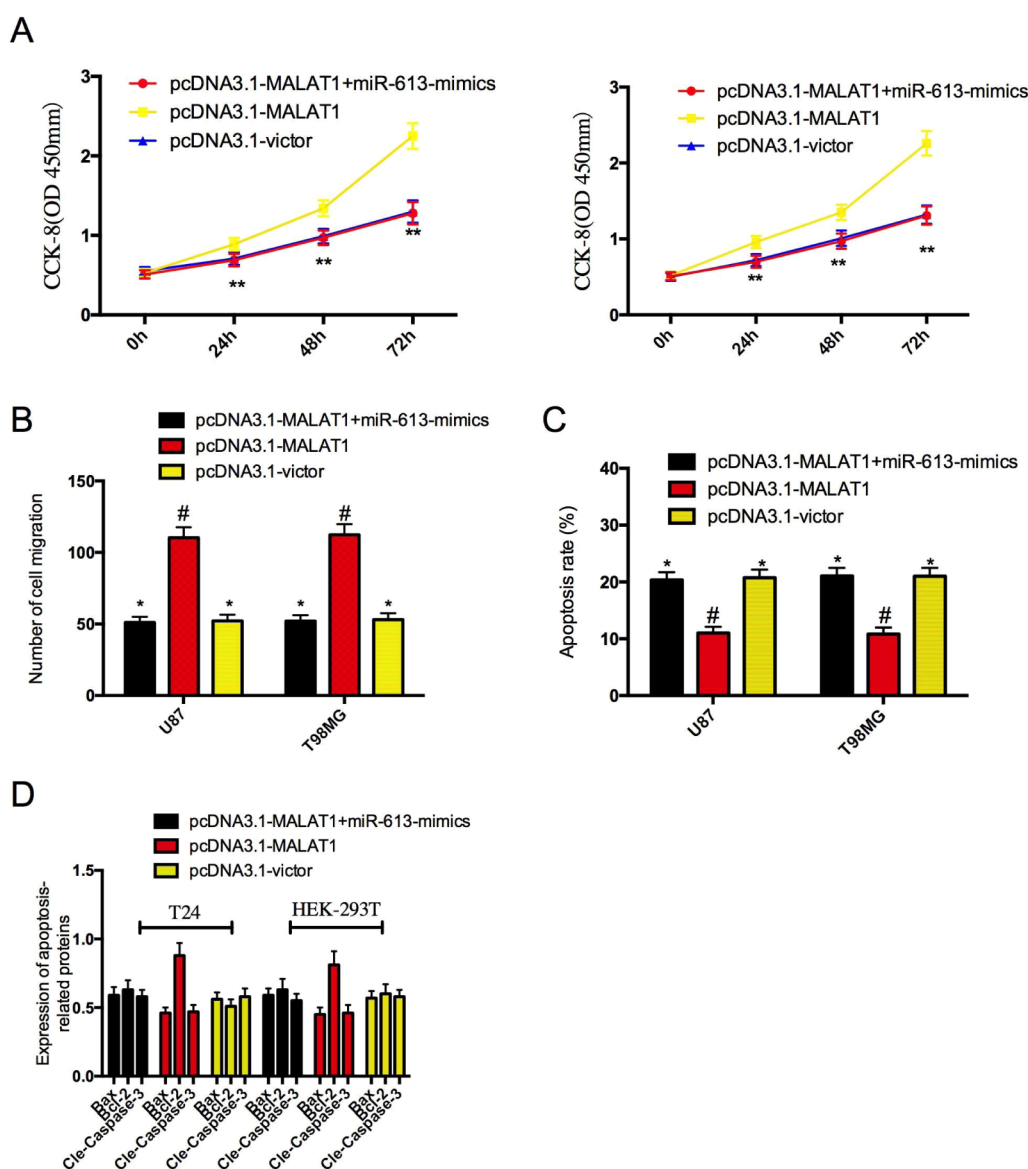


Figure 5. Rescue experiment. **A:** Effect of pcDNA3.1-MALAT1+miR-613-mimics on the proliferation of glioma cells. **B:** Effect of pcDNA3.1-MALAT1+miR-613-mimics on the invasion ability of glioma cells. **C:** Effect of pcDNA3.1-MALAT1+miR-613-mimics on the apoptosis rate of glioma cells. **D:** Effect of pcDNA3.1-MALAT1+miR-613-mimics on apoptosis-related proteins in glioma cells. *means compared with #, $p < 0.05$; ** $p < 0.05$.

successfully reduced the restriction of miR-613 expression induced by pcDNA3.1-MALAT1. CCK-8 analysis showed that MALAT1 over-expression accelerated the proliferation and invasion of U87MG, which could be reversed by introducing MALAT1. Meanwhile, the inhibitory effect of pcDNA3.1-MALAT1 on apoptosis was reversed by miR-613-mimics. These data showed that MALAT1 can act as an oncogene in glioma by adjusting miR-613 (Figure 5).

Discussion

Glioma is an invasive malignant neoplasm, and the imbalance of LncRNA has become one of the main characteristics of glioma [15]. Therefore, it is important to find new LncRNA in glioma to obtain more attractive therapeutic targets. Past reports have indicated that MALAT1 acts as an oncogene in many neoplasms. For example, some researchers [16] found that MALAT1 activated the proliferation of colorectal carcinoma cells by inhibiting miR-101, but inhibited the apoptosis. Other authors [17] found that MALAT1 could accelerate the occurrence and growth of cervical carcinoma by acting as the sponge of miR-429.

In our study, we also found that MALAT1 in glioma enhanced abnormally, and MALAT1 expression enhanced with pathological grade. Previous studies [18] also pointed out that MALAT1 was abnormal in glioma, which was consistent with our observation. Then we regulated MALAT1 in glioma cells to test its role. The results showed that up-regulating MALAT1 would further accelerate the proliferation and invasion, but silencing MALAT1 could hinder the proliferation and invasion. In addition, MALAT1 could also hinder the apoptosis of glioma cells by inhibiting Bax and Cle-Caspase-3 and promoting Bcl-2. Previous studies [19] indicated that MALAT1 was up-regulated in SHG139S, and MALAT1 knockout accelerated the proliferation of SHG139S. MALAT1 was enhanced in primary glioma tissues and glioma cell lines. After MALAT1 gene knockout, the cell survival rate decreased evidently, while the apoptosis rate enhanced significantly [20]. This is similar with our results. It is suggested that MALAT1 can be applied as oncogene to promote the occurrence and development of glioma, and LncRNA MALAT1 is expected to be a treatment biomarker and target of glioma.

In human diseases, LncRNAs may affect cell function through various mechanisms. For example, LncRNAs can also combine with miRNAs as miRNA sponge, thus regulating the expression of miRNA target genes [21]. For example, some au-

thors [22] found that LncRNA HOXA11-AS can be applied as miRNA sponge, targeting miR-613 to promote the development of glioma. Other authors [23] concluded that LncRNA PCA3 accelerated the progression of prostate carcinoma by sponging miR-218-5p. In this research, we proceeded in further tests and showed that LncRNA, as CERN, was involved in the post-transcriptional adjustment of mRNA in the nosogenesis of glioma. We found that miR-613 could bind to MALAT1 through biological analysis, and confirmed their direct targeting relationship by double fluorescein reporter enzyme, RIP and pull-down experiments. In the past, miR-613, as a neoplasm suppressor gene, was found to be down-regulated in many carcinomas. For example, some authors [24] found that miR-613 hindered the migration and invasion of triple negative breast carcinoma cells by targeting Daam1/RhoA signaling pathway. miR-613 in glioma tissues and cells was evidently down-regulated. When we overexpressed miR-613, we found that the proliferation and invasion were evidently hindered, and the apoptosis was significantly enhanced. In the past, some researchers [25] found that miR-613 acted as an anti-carcinoma in glioma cells by directly targeting SOX9, which is consistent with our observation. All these data proved that miR-613 acted as a neoplasm suppressor gene in glioma, but our research firstly proved that MALAT1 can act as a neoplasm suppressor gene in glioma by sponging miR-613. In order to further determine whether MALAT1 can promote the growth of glioma cells by adjusting miR-613, we also carried out rescue experiments and the results showed that Si-MALAT1 could eliminate the promotion of miR-613 on the proliferation and invasion and the inhibition of apoptosis. The above results revealed that MALAT1 could inhibit miR-613 in glioma cells to act in the proliferation, invasion and apoptosis of glioma cells.

Conclusion

In conclusion, MALAT1 is enhanced in glioma, indicating poor prognosis of glioma patients. MALAT1 can accelerate the proliferation and invasion and hinder the apoptosis by regulating miR-613. However, we also found some shortcomings. For example, we have not conducted *in vivo* experiments. Secondly, we have not further explored the possible mechanism of miR-613 in glioma. In the future, we will conduct more basic tests to improve our experimental data.

Conflict of interests

The authors declare no conflict of interests.

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