Long non-coding RNA LINC00473 promotes glioma cells proliferation and invasion by impairing miR-637/CDK6 axis

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Introduction

Glioma is the most frequent intracranial malignant brain tumors in adults, characterized by rapid growth, early metastasis, and high lethality [1,2]. Despite accumulating achievements in treatments of glioma, the prognosis is still unsatisfactory [3]. Although many studies have been performed to detect the molecules associated with gliomas, only a few have been revealed [4,5]. Therefore, it is urgent to discover novel molecular targets for therapy of glioma patients.

Long non-coding RNAs (lncRNAs) consist of a group of RNA molecules that are longer than 200 nucleotides in length with limited protein-coding potential [6]. Increasing evidence showed that lncRNAs might play important roles in the regulation of gene transcription, translation and widespread regulators [7–9]. lncRNA dysregulation contributed to the progression of multiple cancers. For example, Zhang et al. showed that high MALAT1 expression was correlated with advanced tumor progression and poor prognosis in clear cell renal cell carcinoma [10]. Liu et al. found that lncRNA GHET1 promoted esophageal squamous cell carcinoma cells proliferation and invasion via EMT processes [11]. Zhang et al. reported that lncRNA HNFT1-AS1 promoted lung cancer cell proliferation and invasion via regulating miR-17-5p expression [12].

LINC00473 is an intergenic long noncoding RNA which has been reported to be aberrantly expressed in several malignancies. For example, Chen et al. showed that LINC00473 could act as a therapeutic target for LKB1-inactivated lung cancer [13]. Zhu et al. found that LINC00473 acted as an oncogene to promote Wilms tumour growth via miR-195/IKKx axis [14]. Shi et al. suggested that LINC00473 promoted cervical cancer progression by the miR-34a/ILF2pathway [15]. However, the expression and biological functions of LINC00473 in glioma remain poorly understood.

In the present study, LINC00473 expression was significantly increased in glioma tissues and cell lines. High LINC00473 expression was associated with advanced clinical features and poor prognosis. In mechanism, we found that LINC00473 promoted glioma cells proliferation, invasion and EMT processes by acting as a ceRNA of miR-637 to regulate CDK6 expression. Taken together, we suggested that LINC00473/miR-637/CDK6 axis might act as a potential therapeutic target for glioma treatment.

Materials and methods

Tissue samples

A total of 31 glioma specimens and 14 normal brain tissues (NBTs) were obtained from The First Affiliated Hospital of Zhengzhou University and The First Affiliated Hospital of Zhengzhou University Hospital.
College of Clinical Medicine of Henan University of Science and Technology. Diagnosis was based on pathological evidence, and collected tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until used. All patients were not received preoperative treatments before surgery. Written informed consents were obtained from all participants. The study was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University and The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology.

Cell culture

Human glioma cell lines (LN229, LN18, LN308, U87, U251 and SNB19) and normal human astrocytes (NHA) were purchased from Chinese Academy of Medical Sciences (Beijing, China). All cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) medium with 10% fetal bovine serum (Gibco, NY, USA) supplemented with 100 units/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C.

RNA extraction and qRT-PCR analyses

Total RNA was extracted from tissue samples or cultured cells with TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols. RNA was reversely transcribed into cDNAs by using a Reverse Transcription Kit of the PrimeScript™ one step (Takara, Dalian, China). qRT-PCR was performed using the ABI7500 System (Applied Biosystems, CA, USA) and the SYBR Green PCR Master Mix kit (TaKaRa). After normalizing to the endogenous control GAPDH or U6, the relative expression was calculated utilizing the 2^{-ΔΔCt} method. Each experiment was repeated in triplicate at least.

Western blot

Total protein from cells was extracted using RIPA assay and loaded for electrophoresis (Beyotime, Shanghai, China). After transferring on a PVDF membrane at 300 mA for 100 min, the membrane was blocked in 5% skim milk for 2 h, incubated with primary antibodies at 4°C overnight. The membranes were then treated with horseradish peroxidase-conjugated secondary antibodies for 2 h. The bands were detected using an ECL Detection Systems (Thermo Scientific, Beijing, China).

MTT assay

MTT assay was used to detect cell proliferation. Briefly, transfected cells were seeded in 96-well plate (2000 cells per well) and culture for 24 h, 48 h, 72 or 96 h. Then transfected cells were incubated with 20 μL (10 mg/mL) MTT solution was added into each well and incubated at 37 °C for another 4 h. The absorbance was measured at a wavelength of 490 nm.

Colony formation assay

For colony formation assay, transfected cells were plated into 6-well plates and then incubated at 37 °C for 2 weeks. The colonies were subsequently washed with PBS, fixed with methanol and then stained with crystal violet. A coulter counter was then utilized to count the cell colonies.
Transwell invasion assay

Transwell invasion assay was processed according to manufacturer's instructions and previous study [10].

Luciferase reporter assay

The wild-type (WT) and mutated-type (MUT) 3'UTR with LINC00473 were synthesized by site mutation method, amplified by PCR and then were inserted into pmiRGlO (Promega, Madison, Wisconsin, USA). Afterwards the Wt-LINC00473 or Mut-LINC00473 constructs were transfected into cells with the company of miR-637 mimics or miR-NC, the process was implemented inside a 24-well plates. After 48 h, luciferase activity was detected using the Dual-Luciferase Reporter Assay system (Promega). Renilla luciferase activities were used for normalization.

RIP assay

RIP assay was conducted on cells by using EZ-Magna RIP Kit (Millipore, Billerica, MA, USA) following the manufacturer’s instructions. The cells were lysed in complete RNA lysis buffer, then incubated using RIP buffer that contains magnetic beads conjugated with human antibody (Millipore) or negative control mouse IgG. The protein was digested using Proteinase K, and immunoprecipitated RNA was isolated. Purified RNA was subjected to qRT-PCR analysis.

In vivo tumorigenesis assays

Nude athymic BALB/c mice (6-week-old) were purchased from the Laboratory Animal Center of Southern Medical University, and all procedures were approved by the Ethics Committee of the Fifth Affiliated Hospital of Zhengzhou University. For
tumorigenesis assay, the U251 cells transfected with sh-NC or sh-LINC00473 were injected subcutaneously into the flank of nude mice, followed by measurements of the tumor volume at 7-day intervals using the formula: volume = 0.5 × length × width². At 7 weeks after injection, the mice were sacrificed, and tumors were harvested for the determination of tumor mass as well as the IHC analysis of the expression levels of Ki-67.

Statistical analyses
The whole data analysis employed SPSS 17.0 software. The format of mean ± standard deviation was used to present the results. Two-tailed Student’s t-test, or One-way analysis of variance was applied for statistical analysis. \( p < .05 \) was recognized to have statistical significant difference.

Results

LINC00473 is upregulated in glioma
In the present study, qRT-PCR was used to detect LINC00473 expression in 31 paired glioma tissues and normal brain tissues (NBTs). qRT-PCR showed that LINC00473 expression was increased in glioma tissues (Figure 1(A)). High LINC00473 expression was associated with advanced WHO grade, and

\[ \text{volume} = 0.5 \times \text{length} \times \text{width}^2 \]
low KPS score (Figure 1(B,C)). Patients displaying a high expression of LINC00473 contributed to poor overall survival (OS) and disease free survival (DFS) (Figure 1(D,E)). Moreover, LINC00473 expression was significantly upregulated in glioma cells (LN229, LN18, LN308, U87, U251 and SNB19) compared to NHA cells (Figure 1(F)). These data suggested that LINC00473 might play critical roles in glioma progression.

**LINC00473 promotes glioma proliferation and invasion**

To investigate the roles of LINC00473 on tumor progression, glioma cells were transfected with si-LINC00473 or si-NC. qRT-PCR showed that si-LINC00473 significantly reduced LINC00473 expression in LN18 and U251 cells, especially in si-LINC0073-1 (Figure 2(A)). MTT and colony formation assays showed that LINC00473 suppression significantly decreased LN18 and U251 cells viability (Figure 2(B,C)). Transwell assay showed that LINC00473 inhibition reduced the invasion capacity of LN18 and U251 cells (Figure 2(D)). To further determine the underlying mechanism, qRT-PCR and Western blot assays were used to determine MMP9 (invasion marker), E-cadherin (the epithelial marker), and N-cadherin (the mesenchymal markers) expression in glioma cells. Results showed that LINC00473 suppression significantly reduced MMP9, N-cadherin expression, while increased E-cadherin expression both in mRNA and protein levels (Figure 2E-H).

**LINC00473 negatively regulates miR-637 in glioma cells**

Increasing evidence showed that cytoplasmic IncRNAs could function as sinks for pools of active miRNAs, functionally liberating mRNAs to mediate several biological processes [16]. Thus, we firstly explored the distribution of LINC00473 by IncLocator. Results showed that the vast majority of LINC00473 was located in the cytoplasm (Figure 3(A)), and the result was further confirmed by qRT-PCR (Figure 3(B)). Subsequently, DIANA was used to predict the target of LINC00473, and result represented that LINC00473 could target miR-637 (Figure 3(C)). Luciferase reporter assay revealed that miR-637 overexpression significantly decreased the luciferase activity of Wt-LINC00473 rather than Mut-LINC00473 (Figure 3(D)). RIP assay revealed that LINC00473 and miR-637 were both enriched in the immunoprecipitation (Figure 3(E)). qRT-PCR showed that si-LINC00473 significantly increased miR-637 expression in LN18 and U251 compared to si-NC group (Figure 3(F)).

Next, the expression level of miR-637 was also measured in glioma tissues, results showed that miR-637 expression was decreased in glioma tissues (Figure 3(G)). Low miR-637 expression was associated with advanced WHO grade, and low KPS score (Figure 3(H,I)). Moreover, our results showed that the expression of miR-637 was negatively correlated with LINC00473 expression in glioma tissues (Figure 3(J)).

**CDK6 is targeted by miR-637**

It has been shown that miRNAs could regulate gene expression by directly binding with the 3’UTR of their target genes [17]. TargetScan showed that CDK6 have a potential binding sites of miR-637 (Figure 4(A)). Next, we explored CDK6 expression in glioma, results showed that CDK6 was significantly increased in glioma (Figure 4(B,C)), which was consistent with TCGA database results (Figure 4(D)). Kaplan Meier analysis showed that high CDK6 expression was associated with poor OS and DFS of patients with glioma (Figure 4(E,F)). Luciferase reporter assay
suggested that miR-637 mimics reduced the luciferase activity of Wt-CDK6 rather than Mut-CDK6 group (Figure 4(G)). Ectopic expression of miR-637 reduced CDK6 expression both in mRNA and protein levels in LN18 and U251 cells (Figure 4(H,I)). Moreover, CDK6 expression was negatively correlated with miR-637 expression in glioma tissues (Figure 4(J)).

**LINC00473/miR-637/CDK6 axis promotes glioma progression**

Next, we explored whether LINC00473 could act as a ceRNA in regulating CDK6 by competitively binding to miR-637 in glioma. QRT-PCR and Western blot showed that CDK6 expression was reduced by LINC00473 inhibition in LN18 cells, and the reduction could be reversed by miR-637 inhibitors (Figure 5(A,B)). Correlation analysis showed that LINC00473 expression was positively correlated with CDK6 expression in glioma tissues (Figure 5(C)). Moreover, rescue assays showed that the effects of LINC00473 inhibition on glioma cells proliferation and invasion could be abolished by miR-637 inhibitors (Figure 5(D,E)). Similarly, qRT-PCR and Western blot results showed that miR-637 inhibition could reverse the effects of LINC00473 inhibition on N-cadherin, MMP9, and E-cadherin expression in LN18 cells (Figure 5(F,G)). These data indicated that LINC00473 could promote glioma cells progression by regulating the miR-637/CDK6 axis.

**LINC00473 promotes tumor growth in vivo**

To explore the roles of LINC00473 in vivo, a xenograft mouse model was used. Results showed that tumor growth and weights were significantly suppressed in sh-LINC00473 group compared to sh-NC group (Figure 6(A–C)). Ki67 assay indicated that LINC00473 inhibition reduced tumor cell proliferation (Figure 6(D)). Moreover, we explored LINC00473/miR-637/CDK6 axis expression in xenograft tissues, results showed that LINC00473 expression and CDK6 expression were significantly decreased, while miR-637 expression was upregulated in sh-LINC0047 (Figure 6(E,F)). Thus, we suggested that LINC00473 could promote glioma progression by impairing the miR-637/CDK6 axis (Figure 6(G)).

**Discussion**

Glioma is challenging for the neurosurgeon due to its rapid proliferation and infiltrative growth, leading to invasion into normal brain tissue, and resulting in incomplete resection...
Recently, increasing evidence showed that lncRNAs contributed to glioma tumorigenesis and progression. For example, Gong et al. found that lncRNA CASC7 suppressed glioma proliferation and invasion through regulating Wnt/β-catenin pathway [19]. Xu et al. suggested that lncRNA MEG3 reduced glioma cells proliferation and migration via regulation of Sirt7 and PI3K/AKT/mTOR pathway [20]. Shen et al. found that lncRNA FOXD2-AS1 promoted glioma tumorigenesis via targeting the miR-185-5p/CCND2 axis [21].

In the present study, we determined the roles of LINC00473 in glioma. QRT-PCR showed that LINC00473 expression was upregulated in glioma tissues and cell lines. High LINC00473 expression was correlated with advanced WHO grade, and low KPS score. Patients displaying a high expression of LINC00473 contributed to poor OS and DFS. LINC00473 inhibition suppressed glioma cells proliferation, invasion and EMT processes in vitro and reduced tumor growth in vivo. These data demonstrated that LINC00473 could act as an oncogenic lncRNA in glioma tumorigenesis.

Increasing evidence showed that cytoplasmic lncRNAs mainly function as ceRNAs or molecular sponges in carcinogenesis, including glioma [22]. For example, Liu et al. showed that IncRNA HOTAIR promoted glioma progression by acting as a molecular sponge of miR-126-5p [23]. Chen et al. found that HOXD-A51 promoted metastasis in glioma via miR-130/E2F8 axis [24]. Therefore, we speculated that LINC00473 might act as a ceRNA in glioma progression. In the current study, we showed that the majority of LINC00473 is located in cytoplasm, and bioinformatics analysis indicated that miR-637 could bind to LINC00473. The correlation between LINC00473 and miR-637 was further confirmed by Dual-luciferase reporter and RIP assays. Rescue assays showed that the effects of LINC00473 inhibition on glioma cells progression could be abolished by miR-637 inhibitors. Moreover, miR-637 expression was negatively associated with LINC00473 expression in glioma tissues. Thus, we suggested that LINC00473 exerted its oncogenic roles in glioma progression at least partly by regulating miR-637 expression.

CDK6 is a kinase catalytic subunit of a protein kinase complex that participates in G1 progression and G1/S transition [25]. Recent studies showed that CDK6 could play critical roles in glioma progression. Our data showed that LINC00473 inhibition suppressed glioma cells proliferation, invasion and EMT processes in vitro and reduced tumor growth in vivo. These data demonstrated the potential of LINC00473 as a therapeutic target in glioma.
roles in tumor progression. For example, Deng et al. found that miR-218 suppressed gastric cancer cell cycle progression through the CDK6/Cyclin D1/E2F1 axis [26]. Lu et al. found that IncRNA NNT-AS1 promoted metastasis in hepatocellular carcinoma by the miR-363/CDK6 axis [27]. In the present study, we showed that CDK6 expression was significantly increased in glioma and associated with poor OS and DFS. Subsequently, CDK6 was confirmed to be a target gene of miR-637 in glioma cells. Furthermore, we showed that LINC00473 suppression reduced CDK6 expression, and the effects could be partially abolished by miR-637 inhibitors. In addition, LINC00473 expression was positively correlated with CDK6 expression in glioma tissues. Thus, we suggested that LINC00473 promoted glioma proliferation and invasion by sponging miR-637 and upregulating CDK6.

Conclusions

Our study revealed that high LINC00473 expression promoted the proliferation and invasion of glioma cells by regulating miR-637/CDK6 axis, which provided a potential therapeutic target for glioma treatment.

Disclosures

No potential conflict of interest was reported by the authors.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article.

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