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ORIGINAL ARTICLE

EZH2-mediated long-chain non-coding RNA LINC00963 promotes proliferation and invasion of glioma cells through inhibiting p21 expression

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Summary

Purpose: *In the early stage, bioinformatics analysis revealed that the expression of long-chain non-coding RNA LINC00963 in glioma tissues was remarkably increased, but its biological effects on glioma and the potential molecular mechanisms have not been reported. This study aimed to conduct a preliminary discussion on the impact of LINC00963 on glioma, so as to provide new ideas for the treatment of this cancer.*

Methods: *GEPIA database was consulted to determine the expression level of LINC00963 in gliomas. In addition, the interplay between LINC00963 expression and the prognosis of glioma patients was analyzed by Kaplan-Meier method. Effects of LINC00963 on the proliferation and migration of glioma cells were determined using Cell Counting Kit (CCK-8) and Transwell assay. The subcellular localization of LINC00963 was determined by nuclear separation experiments. At the same time, the regulation of LINC00963 on p21 expression was verified through qRT-PCR and Western blot experiments.*

Results: *By analyzing the GEPIA database, we found that LINC00963 was highly expressed in glioma tissues. Meanwhile, qRT-PCR results revealed that LINC00963 level in glioma tissues and cell lines was remarkably higher than that in the normal control group. Kaplan-Meier and log-rank test revealed that there was no statistically significant association between the expression level of LINC00963 and the prognosis of patients with glioma. In addition, in vitro cell assay results indicated that downregulation of LINC00963 markedly suppressed the proliferation and invasiveness of glioma cells. Finally, the related mechanism analysis revealed that LINC00963 may inhibit p21 expression through modulation of EZH2.*

Conclusions: *LINC00963 can inhibit p21 expression through EZH2 and thus enhance the proliferative and invasive capacities of glioma cells. Consequently, LINC00963 may be a potential therapeutic target for gliomas.*

Key words: *glioma, LINC00963, p21, cell proliferation, cell invasion*

Introduction

Central nervous system (CNS) tumor is one of the most common tumors in the world, with an increasing incidence year by year [1]. Glioma is the most common malignant primary tumor among all kinds of brain tumors [2,3]. Among them, malignant glioma accounts for about 60%, and surgical treatment combined with radiotherapy and chemotherapy is the main means of brain glioma treatment [4]. Given to the difficulty in the early diagnosis and treatment of glioma,

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this disease has become a threat to human health which is characterized by high incidence, high recurrence rate, high mortality and low cure rate. Generally, glioma is divided into grade I ~ IV. The higher the grade, the worse the prognosis of patients, especially glioblastoma, with a median survival of only 14 months [5]. Despite remarkable progress been made in the diagnosis and treatment of glioma in recent years with the progress of imaging diagnosis and the application of microneurosurgical techniques, the overall prognosis of glioma is still poor. Therefore, it is of great significance to study the occurrence and development of glioma and its molecular mechanism for its treatment.

LncRNAs are a group of RNAs that are more than 200 nt in length and do not have the ability to encode proteins. These lncRNAs transcripts were once regarded as simple transcription "noise" or "cloning artifacts" and were not valued by scientists [6]. LincRNA functions through a variety of mechanisms, such as regulation of target gene transcription factors [7], regulation of translation and shearing, as a miRNA precursor [8], and ceRNA [9]. In recent years, with the completion of human genome project and the rapid development of molecular genetics technology of lncRNAs in cancer biology, the role of lncRNAs in tumor biology (such as tumor cell proliferation, angiogenesis, apoptosis, migration, and invasion) is receiving more and more attention. These lncRNAs can exert the effects of oncogenes or tumor suppressor genes by activating or inhibiting specific oncogenes [10,11]. Accumulating evidence shows that abnormal lncRNA changes are relevant to the occurrence, development and metastasis of tumor [12]. For example, previous studies have shown that LINC00963 is involved in the transition and metastasis of prostate cancer through the epidermal growth factor receptor (EGFR) signaling pathway [13]. Increased expression of LINC00963 in human non-small cell lung cancer (NSCLC) is associated with prognosis, and can promote invasion of NSCLC cells *in vitro* [13]. Therefore, finding lncRNAs with specific expression and understanding its molecular mechanism in the pathological process of glioma will be helpful to enrich the treatment methods of glioma, so as to advance the survival rate of patients.

In our research, we found that LINC00963 was remarkably highly expressed in glioma tissues. Therefore, we conducted follow-up studies on the potential biological effects and underlying molecular mechanisms of LINC00963, so as to provide a new vision for the diagnosis and treatment of glioma.

Methods

GEPIA database

GEPIA database (http://GEPIA.cancer-pku.cn/index.html) was used to assess the expression level of LINC00963 and survival prognosis of glioma patients. The database can be used to analyze differential genes in tumors, including gene co-expression analysis, gene overall survival (OS) and disease-free survival (DFS) curve analysis, and gene and clinical stage correlation analysis.

Sample collection

All tissue samples in this study were collected from patients undergoing neurosurgery at the Affiliated Yantai Yuhuangding HospitaI of Qingdao University Medical College. Before the operation, all patients signed the informed content, and the sample was quickly stored in liquid nitrogen tank until use. All samples were pathologically confirmed as glioma tissues or normal tissues. The experiment was approved by the ethical committee of the Affiliated Yantai Yuhuangding HospitaI of Qingdao University Medical College and the study followed the rules of the Helsinki Declaration.

Cell culture

Human normal astrocyte (NHA) and four glioma cell lines (LN299, A172, U87, U251) were purchased from ATCC (ATCC, Rockville, MD, USA). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, NY, USA) and antibiotics (100 U/mL penicillin/streptomycin) at 37°C with 5% CO_2.

Cell transfection

U87 and U251 cells with good growth status were selected for transfection and subsequent experiments. si-Linc00963, p21-siRNA and their respective negative control sequences were purchased from Riobobio (Guangzhou, China). Transfection was performed according to the manufacturer's instructions using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The transfection efficiency was detected by qRT-PCR 24-48 h later.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues and cultured glioma cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was subjected to reverse transcription using a TAKARA reverse transcription kit (Takara, Dalian, China) according to the manufacturer's instructions, and then the cDNA obtained by the reaction was used for further qRT-PCR detection. QRT-PCR was performed using SYBR GREEN real-time quantitative PCR kit (Toyobo, Osaka, Japan), and the procedure was carried out according to the product manual. The quantification results of Linc00963 and P21 were calculated by relative quantification method, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used to standardize the results as internal parameters. The primer sequences

were as follows: Linc00963 Forward: 5'-GGTAAATCGAGGCCCAGAGAT-3', Reverse: 5'-ACGTGGATGACAGCGTGTGA-3'; p21 Forward: TGTCCGTCAGAACCCATGC, Reverse: AAAGTCGAAGTTCCATCGCTC; GAPDH Forward: 5'-AGTCCACTGGCGTCTTCA-3', Reverse: 5'-GAGTCCTTCCACGATACCAA-3' ; U6 Forward: 5'-ATTGGAACGATACAGAGAAGATT-3', Reverse: 5'- GGAACGCTTCACGAATTTG-3'; p15 Forward: 5'-CTTCCAGCGAGTTCCAAATCA-3', Reverse: 5'-CAGATGACAACAAGGACCGTG-3'; P16 Forward :5'-GATCCAGGTGGGTAGAAGGTC-3', Reverse: 5'-CCCCTGCAAACTTCGTCCT-3';p27 Forward: 5'-AACGTGCGAGTGTCTAACGG-3' Reverse: 5'-CCCTCTAGGGGTTTGTGATTCT-3'; P57 Forward: 5'-GCGGCGATCAAGAAGCTGT-3' Reverse: 5'-GCTTGGCGAAGAAATCGGAGA-3'

Cell Counting Kit (CCK-8) assay

Twenty four h after transfection, U87 and U251 cells were digested with trypsin, centrifuged, suspended in complete medium, and plated into 96-well plates at 2000 cells per well. After culture for 8-12 h, the cells were completely adhered, and 10 μL of CCK8 reagent+100 μL of culture medium was added in 96-well plates per well and incubate cells in an incubator for 2 h. After that, the optical density (OD) value of each well was measured with a microplate reader at 450 nm absorption wavelength.

Transwell assay

Artificial Matrigel (Cambridge, MA, USA) was evenly spread on a flat surface of a polycarbonate membrane (microporous membrane pore size of 8 μm) in a Transwell chamber (Costar Cambridge, UK) at 40 μL per well, and air-dried at 4°C. Each group of cells was taken, starved for 24 h, and then digested. After the digestion was terminated, the culture solution was discarded after centrifugation, and cells were washed twice with phosphate buffered saline (PBS) and resuspended in serum-free Roswell Park Memorial Institute 1640 (RPMI-1640) medium to adjust the cell density to 10^6/mL. 100 μL of the cell suspension was added to the Transwell upper chamber and 500 μL of 10% serum medium was added to the lower chamber and cells were cultured for 24 h. The cells in the upper chamber were wiped with a cotton swab, washed three times with PBS, fixed with 95% ethanol for 15 min, stained with HE, and photographed under a microscope at 200x magnification. Cells that migrated to the underlying layer were counted using 8 random fields of view. All samples were tested three times and the invasiveness of the tumor cells was expressed by the number of cells passing through the Matrigel gel.

Nuclear separation experiment

The cytoplasmic and nuclear RNA of glioma cells were extracted according to the instructions of a nuclear isolation kit (NE-PER). When the number of cells in the medium increased to 1×10^6, the cells were homogenized with 200 μL lysis buffer J. Buffer SK and ethanol were added to the liquid of cytoplasmic RNA and nuclear RNA, respectively. Cytoplasmic RNA and nuclear RNA were separated by centrifugation through a separation column.

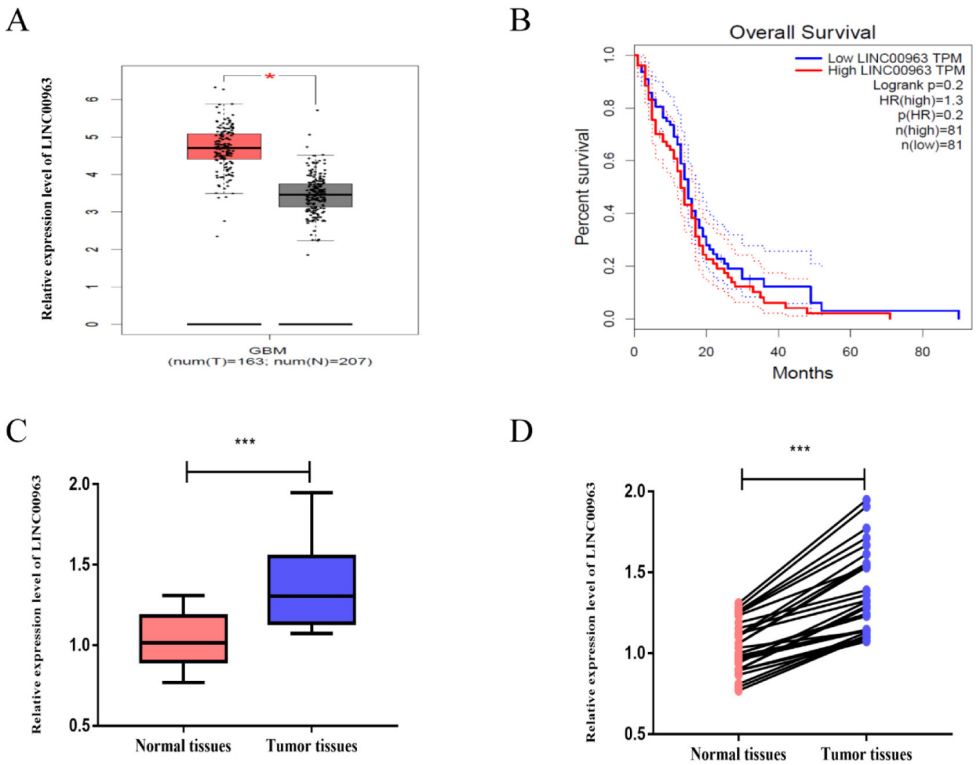

Figure 1. LINC00963 was upregulated in glioma. **A:** LINC00963 levels in glioma tissues analyzed by GEPIA database. **B:** Correlation between LINC00963 level with the prognosis of glioma patients analyzed by GEPIA database. **C:** LINC00963 levels in glioma tissues and adjacent normal ones detected by qRT-PCR. **D:** Differential expression of LINC00963 in glioma tissues and adjacent normal ones. *p<0.05, ***p<0.001.

RNA co-immunoprecipitation experiment (RIP)

RIP was performed using the Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore, Boston, MA, USA) according to the manufacturer's instructions. RIP (anti-EZH2) antibody was purchased from Abcam (Cambridge, MA, USA).

Chromatin immunoprecipitation experiment (CHIP)

Glioma cells (2×10^6) were cultured in plates and their DNAs were dissociated to a length of 300-1000 bp, and then the isolates were treated with 1% formaldehyde to crosslink the DNA with the protein. After crosslinking of the protein-DNA complex, an equal amount of chromatin supernatant was incubated on a shaker overnight at 4°C, including anti-H3K27me (Abeam, Cambridge, MA, USA) or 1 μg of anti-IgG antibody (Abeam, Cambridge, MA, USA). After immunoprecipitation, the relative levels were determined by real-time PCR.

Statistics

Measurement data were expressed as mean ± standard deviation (SD) of three independent replicates, and statistical analyses were performed using SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA). Independent t-test sample and analysis of variance (ANOVA) were used to evaluate qRT-PCR, cell proliferation and invasion test results. GEPIA survival analysis was performed using Kaplan-Meier analysis and log-rank test was used to estimate significant differences. The difference was statistically significant at $p<0.05$.

Results

LINC00963 expression was remarkably increased in glioma

First, we found that LINC00963 level in glioma tissues was remarkably higher than that in the normal control group (Figure 1A) by searching the GEPIA database. At the same time, the high expression of LINC00963 in the GEPIA database indicated a poor prognosis of glioma patients, but the results were not statistically different (Figure 1B). Subsequently, qRT-PCR results indicated that LINC00963 was markedly highly expressed in glioma tissues compared with the normal ones (Figure 1C). Mean-

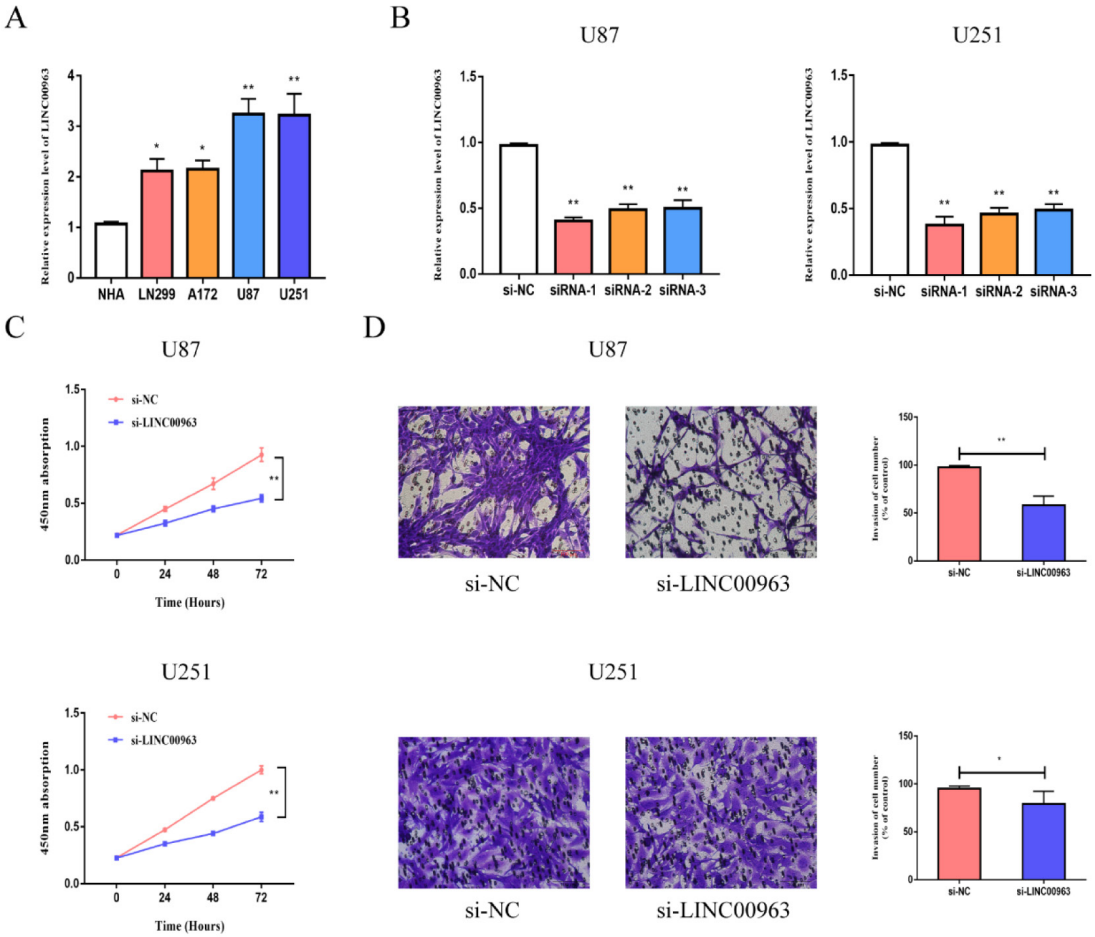

Figure 2. Knockdown of LINC00963 inhibited proliferative and invasive abilities of glioma cells. **A:** LINC00963 levels in glioma cell lines detected by qRT-PCR. **B:** Transfection efficacy of LINC00963 siRNAs (siRNA-1, siRNA-2 and siRNA-3). C: CCK-8 assay showed proliferation in U87 and U251 cells regulated by LINC00963. D: Transwell assay showed invasiveness in U87 and U251 cells regulated by LINC00963. *p<0.05, **p<0.01.

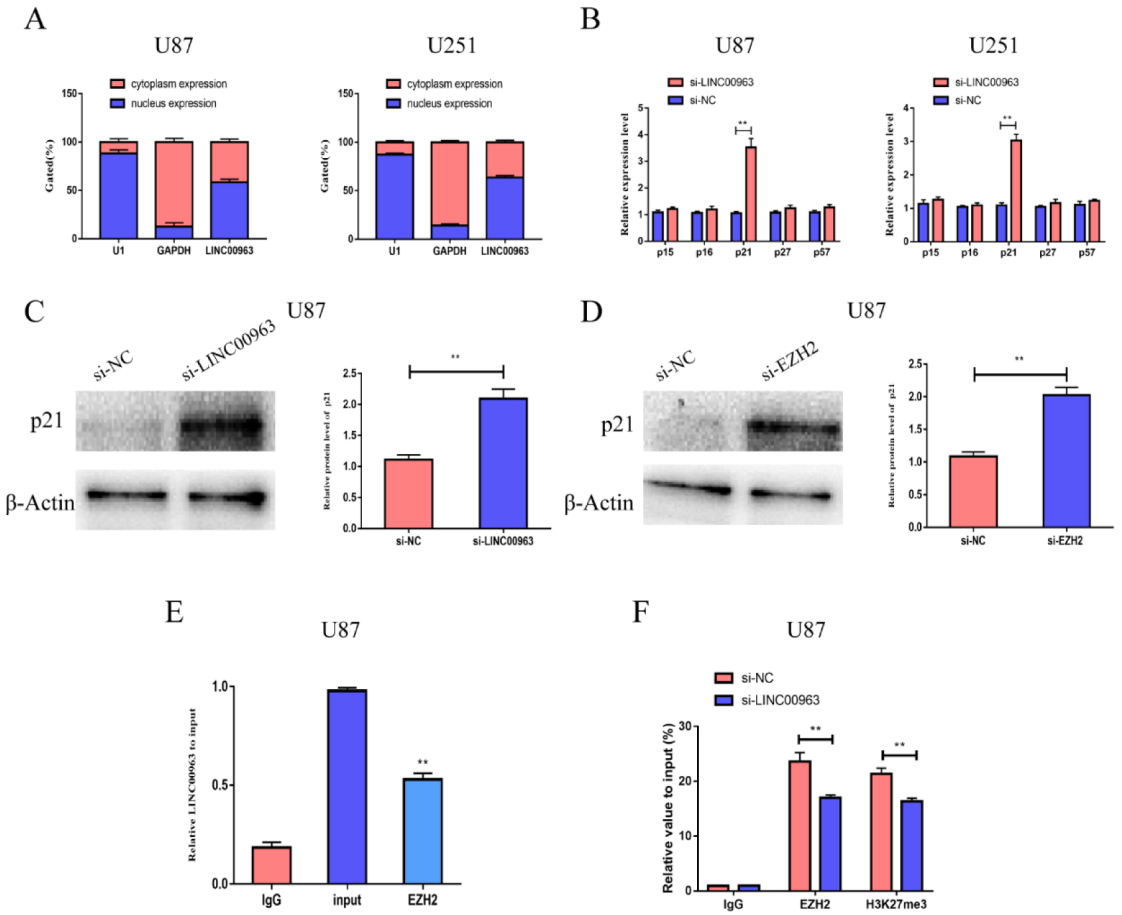

Figure 3. LINC00963 inhibited p21 level through EZH2. **A:** Subcellular distribution of LINC00963. **B:** Relative levels of cell cycle genes (p15, p16, p21, p27 and p57) in U87 and U251 cells with LINC00963 knockdown detected by qRT-PCR. **C:** Protein level of p21 in U87 cells with LINC00963 knockdown detected by Western blot. **D:** Protein level of p21 in U87 cells with EZH2 knockdown detected by Western blot. **E:** Interaction between LINC00963 and EZH2 detected by RIP assay. **F:** Binding relationship in the promoter regions of EZH2 and p21 detected by ChIP assay. **p<0.01.

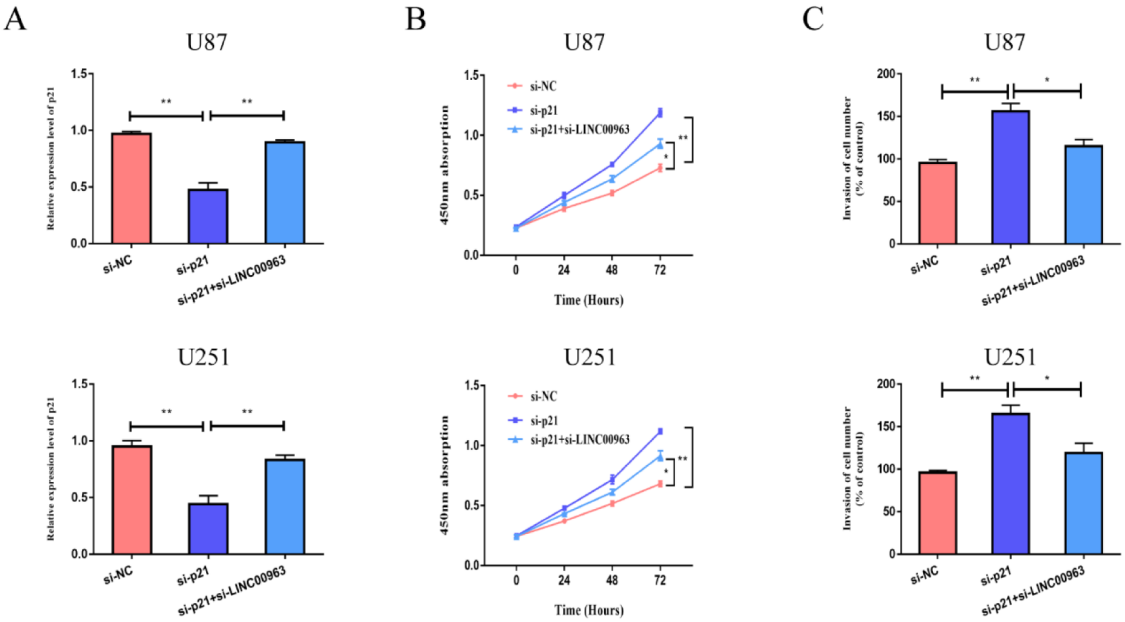

Figure 4. P21 partially abolished the oncogenic role of LINC00963 in glioma. **A:** P21 level in U87 and U251 cells with co-silenced p21 and LINC00963. **B:** CCK-8 assay showed proliferation in co-transfected glioma cells. **C:** Transwell assay showed invasiveness in co-transfected glioma cells. *p<0.05, **p<0.01.

while, paired t-test revealed that LINC00963 level in glioma tissues was also remarkably higher than that of adjacent paracancer ones (Figure 1D). The above results suggested that LINC00963 may play a pivotal role in the progression of glioma.

Down-regulation of LINC00963 inhibited proliferation and invasion of glioma cells

Subsequently, we examined LINC00963 level in glioma cell lines. As shown in Figure 2A, LINC00963 level in glioma cell lines (LN299, A172, U87, and U251) was markedly higher than in control cells. Subsequently, si-LINC00963 was transfected into glioma cells (U87 and U251) and qRT-PCR was conducted to verify the transfection efficiency. The results demonstrated that si-LINC00963 remarkably suppressed LINC00963 level (Figure 2B), and siRNA-1 with high interference efficiency was used for subsequent experiments. Subsequently, CCK8 assay showed that the proliferation ability of U87 and U251 cells was significantly attenuated after down-regulating of LINC00963 (Figure 2C). Besides, we examined the impact of LINC00963 on cell invasion through performing Transwell assay. As shown in Figure 2D, down-regulation of LINC00963 remarkably inhibited the invasiveness of U87 and U251 cells. These results indicated that LINC00963 may contribute to the progression of disease by affecting the proliferative and invasive abilities of glioma cells.

LINC00963 mediated inhibition of p21 expression via EZH2

It is well known that the subcellular localization of LncRNA can determine its biological effects, so nuclear separation experiment was conducted to examine the subcellular localization of LINC00963, and the results indicated that LINC00963 was mostly located in the nucleus (Figure 3A), which meant it may play a role at the transcriptional level. We subsequently inhibited LINC00963 level in glioma cells and detected the expression of cell cycle-related genes by qRT-PCR. As shown in Figure 3B, p21 expression was found remarkably elevated, suggesting that LINC00963 may affect p21 expression in gliomas. In addition, we downregulated LINC00963 and EZH2 expression respectively and then detected the protein expression level of p21 by Western blot. The results confirmed that either inhibition of LINC00963 or EZH2 could enhance the protein expression of p21 in U87 cells (Figure 3C,3D). As the subunit core of PRC2, EZH2 can be recruited by lncRNA to further regulate the expression of transcription factors. It was found by RIP that LINC00963 was able to bind to EZH2 (Figure 3E). We thus hypothesized that LINC00963 may recruit EZH2 to bind to the promoter region of p21 and therefore inhibit p21 expression, and CHIP assay was performed to confirm that EZH2 indeed binds to the promoter region of p21 (Figure 3F). The above results indicated that LINC00963 may exert a biological effect by inhibiting p21 expression via binding to EZH2.

p21 could reverse the cancer promoting function of LINC00963

To further demonstrate whether LINC00963 could inhibit p21 expression by binding to EZH2 to affect cell functions, we performed reverse experiments in U87 and U251 cells. First, we downregulated p21 expression and LINC00963 in the above two cell lines and detected p21 level by qRT-PCR. As shown in Figure 4A, si-LINC00963 partially reversed the inhibitory effect of si-p21 on p21 expression. Subsequently, we co-transfected si-p21 and si-LINC00963 in U87 and U251 cells, and examined the proliferation and migration abilities of glioma cells through *in vitro* cell experiments. CCK8 and Transwell assays revealed that simultaneous transfection of LINC00963 siRNA relatively attenuated the enhanced proliferation and invasiveness of cells caused by p21 knocking down (Figures 4B, 4C). The above results indicated that LINC00963 can suppress the proliferative ability and invasiveness of cells via downregulating p21 expression by binding to EZH2.

Discussion

As the most common primary tumor of the central nervous system, glioma can be divided into astrocytoma, oligodendroglioma and ependymoma. Astrocytoma and glioblastoma account for about 70% of all gliomas, and the mortality and recurrence rates are very high [14,15]. Glioblastoma multiforme (GBM) originates from glial cells of the brain, and about 80% of the adult brain cancer are infiltrating astrocytoma, which has the highest grade of malignancy in all glioma pathological grades, and the WHO defines it as a grade IV malignant tumor [16]. As GBM grows invasively and has no obvious boundary with normal brain tissue, it is difficult to be completely removed. Therefore, the prognosis of glioma patients is not remarkably improved by traditional surgical treatment. Therefore, the search for new molecular targeted drugs for the treatment of glioma has become a research hotspot, and is one of the feasible plans to achieve the clinical cure target in the future.

In recent years, with the development of sequencing technology, more and more non-coding RNAs are considered to participate in a variety of

physiological and pathological processes [17,18]. Many lncRNAs are considered as promising diagnostic biomarkers and therapeutic targets [19]. Studies have confirmed that lncRNAs play a vital role in the occurrence of glioma. For example, lncRNA TUG1 is reported to play a crucial role in maintaining the stem cell characteristics of glioma. LncRNA -TUG1 can promote the apoptosis of glioma cells by inhibiting the expression of anti-apoptotic gene bcl-2 [20]. LncRNA ATB activates astrocytes and promotes the migration and invasion of glioma cells by inhibiting the expression of microRNA 2043p [21]. Moreover, lncRNA HOXD-AS2 promotes glioma progression by regulating cell cycle, which may be a potential diagnostic biomarker and therapeutic target for glioma [22].

EZH2 (Enhancer of Zeste Homolog 2) is the core catalytic component of PRC2 (polycomb repressive complex 2), which can also affect the progression of cancer by affecting the trimethylation and silent transcription of histone H3K27 [23]. Many lincRNAs regulate gene expression through interaction with EZH2 [24,25]. For example, HOTAIR interacts with EZH2 to inhibit tumor development [26]. The p21 gene is a cyclin-dependent kinase inhibitor located downstream of the p53 gene. It is an important cell cycle regulatory protein that inhibits tumor growth and is an important tumor suppressor gene. Abnormal expression of P21 will affect the regulation of cell proliferation and differentiation, leading to the occurrence of malignant tumors [27].

In this study, LINC00963 was found to be abnormally expressed in glioma through the detection database. Subsequently, it was found by qRT-PCR that LINC00963 was up-regulated in both glioma tissues and cells. *In vitro* experiments revealed that inhibiting LINC00963 expression in glioma cells remarkably attenuated the cell proliferation capacity as well as invasiveness. Subsequently, RIP and CHIP assays revealed that LINC00963 may inhibit p21 expression by binding EZH2.Through *in vitro* recovery test, we found that downregulation of LINC00963 in glioma cells could partially reverse the promotion effect of si-p21 on cell proliferation and invasion. These results suggest that LINC00963 is involved in glioma progression by inhibiting p21 expression in combination with EZH2.

Conclusions

This study preliminarily demonstrated that LINC00963 level was remarkably up-regulated in glioma tissues and cells, and the low-expression LINC00963 could inhibit the proliferation and invasion ability of glioma cells, which may be involved in glioma progression through inhibiting p21 expression in combination with EZH2.

Conflict of interests

The authors declare no conflict of interests.

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