European Review for Medical and Pharmacological Sciences 2020; 24: 766-772

LINC00467 promotes proliferation and invasion in glioma *via* interacting with miRNA-485-5p

X.-H. JIANG[1], Y.-Y. LIU[2]

[1]Department of Neurosurgery, Sichuan Academy of Medical Sciences, Sichuan Provincial People's Hospital, Chengdu, Sichuan Province, China
[2]Department of Dermatology, Sichuan Academy of Medical Sciences, Sichuan Provincial People's Hospital, Chengdu, Sichuan Province, China

Abstract. – OBJECTIVE: Long non-coding RNA (lncRNA) LINC00467 was found to be upregulated in glioma tissues by analyzing The Cancer Genome Atlas (TCGA) database. This study aims to uncover the biological role of LINC00467 in influencing the progression of glioma and to provide novel directions for clinical treatment of glioma.

PATIENTS AND METHODS: The expression levels of LINC00467 in glioma tissues were analyzed in the downloaded Gene Expression Profiling Interactive Analysis (GEPIA) dataset. Meanwhile, LINC00467 levels in glioma tissues collected in our hospital and glioma cell lines were determined as well. Proliferative, apoptotic, and invasive changes in U87 and U251 cells transfected with si-LINC00457 or si-NC were assessed. The binding between LINC00467 and microRNA-385-5p (miR-385-5p) was predicted through online bioinformatics and verified by the Dual-Luciferase reporter gene assay. The interaction between LINC00467 and miR-385-5p involved in the progression of glioma was finally verified through rescue experiments.

RESULTS: LINC00467 was upregulated in glioma. The knockdown of LINC00467 attenuated proliferative and invasive abilities, and induced apoptosis in U87 and U251 cells. LINC00467 could bind miRNA-485-5p and negatively regulate its level. Moreover, miRNA-485-5p was responsible for the development of glioma influenced by LINC00467.

CONCLUSIONS: LINC00467 aggravates the progression of glioma by negatively regulating miRNA-485-5p, which may be a potential therapeutic target for glioma.

Key Words:
Glioma, LINC00467, MiR-385-5p, Proliferation, Invasion.

Introduction

Glioma is the most common intracranial malignancy, originating from the neuroepithelial ectoderm, which accounts for about 40% of brain tumors. It is highly heterogeneous and invasive, characterized by uncontrolled proliferation, diffuse infiltration, anti-apoptosis, and necrosis induction[1]. The incidence of glioma is on the rise and presents a younger onset. Pleomorphic glioma is the most prevalent subtype of glioma, manifested as rapid progression and diverse symptoms. Increased intracranial pressure (including symptoms of headache, vomiting, papilledema, and disturbance of consciousness) and focal symptoms and signs (i.e., dysmotility, sensory disturbances, and epilepsy) are the major manifestations of glioma[2-4]. The surgery combined with various adjuvant therapies does prolong the survival of glioma. However, life quality and long-term prognosis of glioma patients are extremely poor[5]. High rate of postoperative recurrences is the leading cause of death in glioma patients. Strong invasiveness of glioma results in a vague boundary between normal tissues and glioma tissues, which will enhance therapeutic difficulties. Therefore, in-depth researches on the mechanism underlying invasive glioma are necessary.

Non-coding RNAs (ncRNAs) are functional RNA molecules that cannot be translated into proteins. These functional molecules exert epigenetic, pre-transcriptional, and post-transcriptional regulations. Long non-coding RNAs (LncRNAs) are a type of ncRNAs with over 200 nt long[6]. Critical roles of lncRNAs in the occurrence and progression of glioma have been well concerned[7]. Individualized therapy targeting glioma-associated lncRNAs may be a promising approach for improving clinical outcomes of glioma patients. With the progression achieved in the high throughput sequencing and microarray analyses, a plenty of lncRNAs associated with glioma development have been dis-

Corresponding Author: Yangying Liu, MM; e-mail: 1450064113@qq.com

covered[8]. For example, LINC00052 suppresses glioma cells to proliferate and metastasize by upregulating KLF6[9,10]. LncRNA BRCAR4 stimulates proliferative ability in glioma *via* activating the EGFR/PI3K/AKT pathway[10]. By targeting the nuclear factor-κB (NF-κB) and P38/MAPK pathway, lncRNA-ATB accelerates the transforming growth factor-β (TGF-β)-induced invasiveness of glioma[11]. LINC00515/miR-16/PRMT5 regulatory loop contributes to accelerate proliferation and suppress apoptosis in glioma[12].

In this paper, we investigated the role of LINC00467 in aggravating the malignant phenotypes of glioma and the underlying mechanism. Our findings could provide new ideas for glioma treatment.

Patients and Methods

GEPIA Data Analysis

Differential expressions and prognostic potentials of LINC00467 in glioma tissues were assessed by analyzing Gene Expression Profiling Interactive Analysis (GEPIA) database (http://GEPIA.cancer-pku.cn/index.html), an online database containing gene sequencing data. Here, GEPIA database containing 163 glioma tissues and 207 normal tissues were downloaded for analyses.

Sample Collection

Paired glioma tissues and normal brain tissues were harvested from 30 glioma patients undergoing surgery in the Sichuan Provincial People's Hospital. All samples were pathologically confirmed and preserved in liquid nitrogen. Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of Sichuan Provincial People's Hospital following the Declaration of Helsinki.

Cell Culture

Human astrocytes NHA and glioma cells LN299, A172, U87, and U251 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C. The medium was regularly replaced.

Transfection

The cells were inoculated in a 6-well plate with 1×10^6 cells per well and cultured at 80% confluence. The cells were then incubated in the serum-free medium for 2 h and transfected using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, the cells were collected for functional experiments.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The cells were lysed to harvest RNAs using TRIzol (Invitrogen, Carlsbad, CA, USA), and RNA concentration was detected using a spectrometer. The extracted RNAs were subjected to reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. The relative level was calculated using $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: LINC00467, F: 5'-GCAGGGACACCATTACAGC-3', R: 5'-GCAACATGTACCGCGGCAC-3'; miRNA-485-5p, F: 5'-GGTTACTAAAGTCCGTCGGACGTG-3', R: 5'-GATTACGCTCATGATCGAAC-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell Counting Kit (CCK-8) Assay

The cells were seeded into 96-well plates with 5×10^4 cells per well. At the appointed time points, 10 μl of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Flow Cytometry

The cells were washed with phosphate-buffered saline (PBS) twice, digested, and suspended in the binding buffer. Subsequently, cells were incubated with 5 μL of AnnexinV-FITC (fluorescein isothiocyanate) and 5 μL of Propidium Iodide (PI) at room temperature in the dark. Apoptotic rate was examined by flow cytometry on EPICS XL-MCL FACScan (Becton-Dickinson, Mountain View, CA, USA).

Transwell

5×10^4 cells were inoculated in the upper side of a transwell chamber (Corning, NY, USA). In the bottom side, 600 μL of medium containing 20% FBS was added. After 24 h cell culture, those invade to the bottom side were fixed in 4% paraformaldehyde for 20 min, stained with crystal violet for 20 min, and counted using a microscope. The number of invasive cells was counted in 5 randomly selected fields per sample.

Dual-Luciferase Reporter Gene Assay

5×10^4 cells per well were inoculated in a 24-well plate. The cells were co-transfected with LINC00467-WT/LINC00467-MUT and miRNA-485-5p mimics/NC for 24 h. The Luciferase activity was finally determined using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY USA) was used for data analysis. GraphPad Prism 7.0 (La Jolla, CA, USA) was utilized for depicting figures. Data were expressed as mean ± standard deviation ($\bar{x}$ ± SD). Spearman's rank correlation analysis was conducted to compare the expression relationship between the two genes. Kaplan-Meier method was introduced for survival analysis. Data between the two groups were compared using the *t*-test. $p<0.05$ considered the difference was statistically significant.

Results

LINC00467 Was Upregulated in Glioma

By analyzing the downloaded GEPIA dataset containing 163 glioma tissues and 207 normal tissues, LINC00467 was found to be upregulated in glioma tissues (Figure 1A). However, dataset analysis did not reveal a significant correlation between the LINC00467 level and the prognosis of glioma patients (Figure 1B). In 30 glioma tissues collected in our hospital, LINC00467 was identically highly expressed than that of normal tissues (Figure 1C). Higher abundance of LINC00467 was found in glioma patients

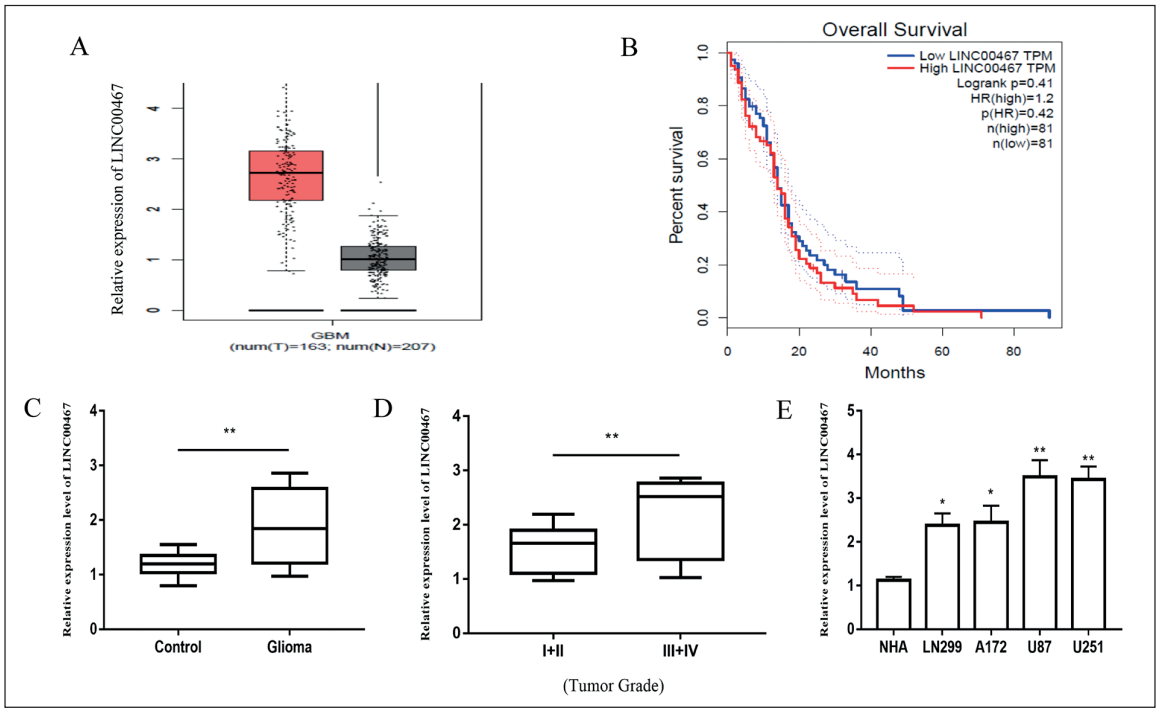

Figure 1. LINC00467 was upregulated in glioma. **A,** LINC00467 levels in glioma tissues (n=163) and normal tissues (n=207) analyzed in the GEPIA dataset. **B,** Overall survival in glioma patients with high or low level of LINC00467 analyzed in the GEPIA dataset. **C,** LINC00467 levels in 30 matched glioma tissues and normal tissues. **D,** LINC00467 levels in glioma patients with grade I+II or grade III+IV. **E,** LINC00467 levels in human astrocytes NHA and glioma cells LN299, A172, U87, and U251. **p*<0.05; ***p*<0.01.

with grade III+IV than those with grade I+II (Figure 1D). Compared with human astrocytes, LINC00467 was highly expressed in glioma cells as well (Figure 1E).

Silence of LINC00467 Suppressed Proliferative and Invasive Abilities, and Induced Apoptosis in Glioma

Transfection of si-LINC00467 remarkably downregulated LINC00467 in U87 and U251 cells, presenting an effective transfection efficacy (Figure 2A). CCK-8 results revealed the decreased viability after the knockdown of LINC00467 in glioma cells (Figure 2B). Apoptotic rate was greatly elevated in glioma cells transfected with si-LINC00467 (Figure 2C). However, invasive cell number was markedly reduced by transfection of si-LINC00467 in glioma cells (Figure 2D). It is suggested that LINC00467 accelerated the proliferative and invasive abilities, as well as attenuated apoptosis in glioma.

LINC00467 Exerted its Biological Function Through Sponging MiRNA-485-5p

By interacting with target miRNAs, lncRNAs are able to regulate diverse biological activities. Here, we predicted that miRNA-485-5p was the target miRNA binding LINC00467 (Figure 3A). Luciferase activity declined in glioma cells co-transfected with LINC00467-WT vector and miRNA-485-5p mimics, confirming their interaction (Figure 3B). MiRNA-485-5p, conversely to that of LINC00467, was downregulated in glioma tissues (Figure 3C). In addition, the Spearman's rank correlation analysis uncovered a negative correlation between the expression levels of miRNA-485-5p and LINC00467 in glioma tissues (Figure 3D). Furthermore, miRNA-485-5p was downregulated in glioma cells overexpressing LINC00467 (Figure 3E). On the contrary, LINC00467 was downregulated in glioma cells overexpressing miRNA-485-5p, further verifying their negative relationship (Figure 3F).

MiRNA-485-5p Reversed the Regulatory Effect of LINC00467 on Glioma

To ascertain the involvement of miRNA-485-5p in the progression of glioma influenced by LINC00467, rescue experiments were conducted. The miRNA-485-5p level was partially enhanced in glioma cells co-transfected with si-LINC00467 and miRNA-485-5p inhibitor com-

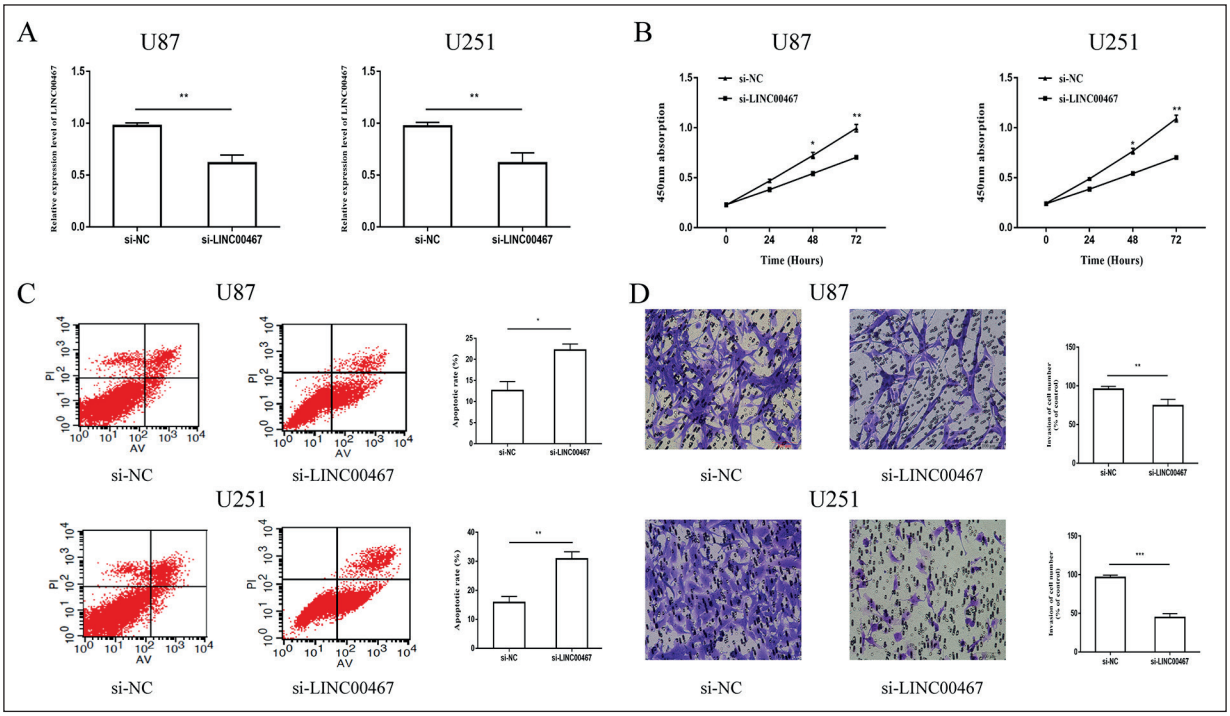

Figure 2. Silence of LINC00467 suppressed proliferative and invasive abilities, and induced apoptosis in glioma. **A,** Transfection efficacy of si-LINC00467 in U87 and U251 cells. **B,** Viability in U87 and U251 cells transfected with si-NC or si-LINC00467. **C,** Apoptotic rate in U87 and U251 cells transfected with si-NC or si-LINC00467. **D,** Invasive cell number in U87 and U251 cells transfected with si-NC or si-LINC00467 (magnification: 20×). *p<0.05; **p<0.01; ***p<0.001.

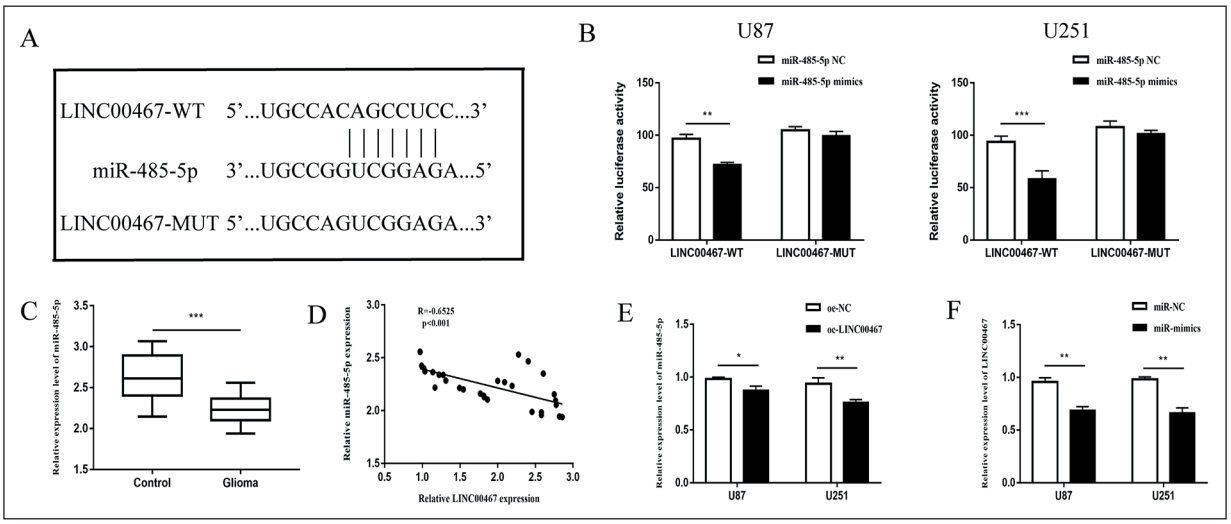

Figure 3. LINC00467 exerted its biological function by sponging miRNA-485-5p. **A,** Binding sequences in the promoter regions of LINC00467 and miRNA-485-5p. **B,** Luciferase activity in U87 and U251 cells co-transfected with LINC00467-WT/LINC00467-MUT and miRNA-485-5p mimics/NC. **C,** MiRNA-485-5p levels in 30 matched glioma tissues and normal tissues. **D,** Spearman's rank correlation analysis on relationship between LINC00467 and miRNA-485-5p in glioma tissues. **E,** MiRNA-485-5p level in U87 and U251 cells transfected with oc-NC or oc-LINC00467. **F,** LINC00467 level in U87 and U251 cells transfected with miRNA-485-5p mimics or NC. *p<0.05; **p<0.01; ***p<0.001.

pared with those transfected with si-LINC00467 (Figure 4A). Notably, decreased viability and invasive cell number in glioma cells with LINC00467 knockdown were partially reversed after the co-silence of LINC00467 and miRNA-485-5p (Figures 4B, 4D). The knockdown of LINC00467 accelerated apoptosis in U87 and U251 cells, which was attenuated by the co-transfection of si-LINC00467 and miRNA-485-5p inhibitor (Figure 4C). Therefore, miRNA-485-5p was responsible for the progression of glioma influenced by LINC00467.

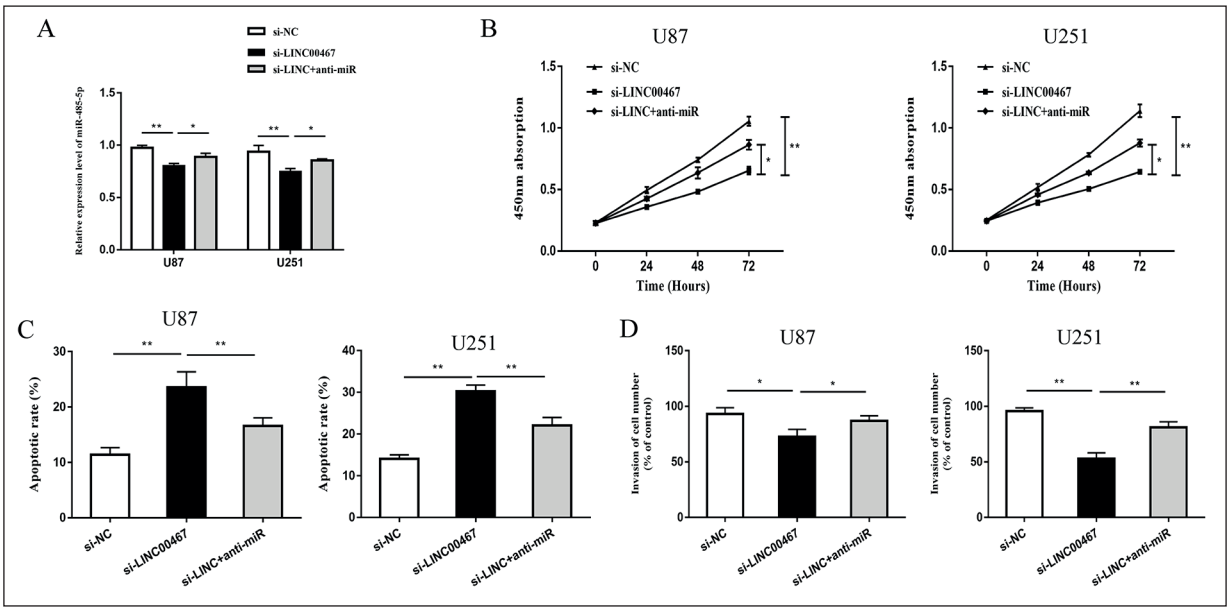

Figure 4. MiRNA-485-5p reversed the regulatory effect of LINC00467 on glioma. U87 and U251 cells were transfected with si-NC, si-LINC00467 or si-LINC00467+miR-487-5p inhibitor. **A,** MiRNA-485-5p level. **B,** Viability. **C,** Apoptotic rate. **D,** Invasive cell number. *p<0.05; **p<0.01.

Discussion

Glioma is the most common malignant tumor of the adult central nervous system, posing a great threat to public health. The incidence of glioma is about 5/100,000, showing a gradual increase each year[13]. Glioma is characterized as high malignancy, poor prognosis, and high mortality. It is classified into four grades, with low-grade glioma in grade I-II, and high-grade one in grade III-IV. The majority of glioma patients seek medical advice because of headache, exercise discomfort, and psychiatric symptoms. Nevertheless, the detective rate of glioma in early stage is relatively low, leading to an extremely poor prognosis. Comprehensive therapies for glioma include surgery and postoperative chemotherapy or radiotherapy. However, the median survival of glioma patients is only 14 months even after active treatment[14]. It is urgent to develop more effective approach for improving the prognosis of glioma.

Relevant protein-encoding genes involving in the occurrence and progression of glioma have been discovered in the past decade[15,16]. LncRNAs, as a type of newly emerged functional RNAs, are important regulators in the progression of glioma. They exert transcriptional and epigenetic regulations on gene expressions. Many reports supported their functions in various genetic activities by interacting with DNAs, RNAs, and proteins. In tumor diseases, differentially expressed lncRNAs are the key mediators influencing tumor cell behaviors[17]. For instance, LINC00673-v4 enhances the invasiveness of lung adenocarcinoma by activating the Wnt/β-catenin pathway[18]. LncRNA HNF1A-AS1 influences the progression of non-small cell lung cancer (NSCLC) by targeting the miR-149-5p/Cdk6 axis[19]. LINC00210 enhances the malignant level of thyroid cancer cells by targeting the miR-195-5p/IGF1R/Akt regulatory loop[20].

This study discovered that LINC00467 was upregulated in glioma. Moreover, the LINC00467 level was positively correlated to tumor grade of glioma. Subsequently, *in vitro* experiments demonstrated that the knockdown of LINC00467 attenuated the proliferative and invasive abilities, and induced apoptosis in U87 and U251 cells. LINC00467 could bind miRNA-485-5p and negatively regulate its level. Moreover, rescue experiments confirmed that miRNA-485-5p was responsible for the progression of glioma influenced by LINC00467. Collectively, the LINC00467/miRNA-485-5p axis aggravated the severity of glioma.

Conclusions

Taken together these results detected that LINC00467 aggravates the progression of glioma by negatively regulating miRNA-485-5p, which may be a potential therapeutic target for glioma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Wood MD, Halfpenny AM, Moore SR. Applications of molecular neuro-oncology - a review of diffuse glioma integrated diagnosis and emerging molecular entities. Diagn Pathol 2019; 14: 29.
2) Sarkisian MR, Semple-Rowland SL. Emerging roles of primary cilia in glioma. Front Cell Neurosci 2019; 13: 55.
3) Guo Y, Hong W, Wang X, Zhang P, Korner H, Tu J, Wei W. MicroRNAs in microglia: how do microRNAs affect activation, inflammation, polarization of microglia and mediate the interaction between microglia and glioma? Front Mol Neurosci 2019; 12: 125.
4) Yan C, Wang J, Yang Y, Ma W, Chen X. Molecular biomarker-guided anti-angiogenic targeted therapy for malignant glioma. J Cell Mol Med 2019; 23: 4876-4882.
5) Liang S, Fan X, Zhao M, Shan X, Li W, Ding P, You G, Hong Z, Yang X, Luan G, Ma W, Yang H, You Y, Yang T, Li L, Liao W, Wang L, Wu X, Yu X, Zhang J, Mao Q, Wang Y, Li W, Wang X, Jiang C, Liu X, Qi S, Liu X, Qu Y, Xu J, Wang W, Song Z, Wu J, Liu Z, Chen L, Lin Y, Zhou J, Liu X, Zhang W, Li S, Jiang T. Clinical practice guidelines for the diagnosis and treatment of adult diffuse glioma-related epilepsy. Cancer Med 2019; 8: 4527-4535.
6) Li J, Liu C. Coding or noncoding, the converging concepts of RNAs. Front Genet 2019; 10: 496.
7) Gugnoni M, Ciarrocchi A. Long noncoding RNA and epithelial mesenchymal transition in cancer. Int J Mol Sci 2019; 20: 209-215.
8) Wang J, Zhou J, Jiang C, Zheng J, Namba H, Chi P, Asakawa T. LNRRIL6, a novel long non-coding RNA, protects colorectal cancer cells by activating the IL-6-STAT3 pathway. Mol Oncol 2019: 13: 2344-2360.
9) Xu CF, Liu P, Tan J, Hu DF. Long noncoding RNA LINC00052 suppressed the proliferation, migration and invasion of glioma cells by upregulating KLF6. Eur Rev Med Pharmacol Sci 2019; 23: 4822-4827.

10) Wei L, Yi Z, Guo K, Long X. Long noncoding RNA BCAR4 promotes glioma cell proliferation via EGFR/PI3K/AKT signaling pathway. J Cell Physiol 2019; 234: 23608-23617.

11) Tang F, Wang H, Chen E, Bian E, Xu Y, Ji X, Yang Z, Hua X, Zhang Y, Zhao B. LncRNA-ATB promotes TGF-β-induced glioma cells invasion through NF-κB and P38/MAPK pathway. J Cell Physiol 2019; 234: 23302-23314.

12) Wu Z, Lin Y. Long noncoding RNA LINC00515 promotes cell proliferation and inhibits apoptosis by sponging miR-16 and activating PRMT5 expression in human glioma. Onco Targets Ther 2019; 12: 2595-2604.

13) Tamtaji OR, Mirzaei H, Shamshirian A, Shamshirian D, Behnam M, Asemi Z. New trends in glioma cancer therapy: targeting Na (+) /H (+) exchangers. J Cell Physiol 2019. doi: 10.1002/jcp.29014. [Epub ahead of print].

14) Hallal S, Ebrahimkhani S, Shivalingam B, Graeber MB, Kaufman KL, Buckland ME. The emerging clinical potential of circulating extracellular vesicles for non-invasive glioma diagnosis and disease monitoring. Brain Tumor Pathol 2019; 36: 29-39.

15) Kopp F. Molecular functions and biological roles of long non-coding RNAs in human physiology and disease. J Gene Med 2019; 21: e3104.

16) Rynkeviciene R, Simiene J, Strainiene E, Stankevicius V, Usinskiene J, Miseikyte KE, Meskinyte I, Cicenas J, Suziedelis K. Non-coding RNAs in glioma. Cancers (Basel) 2018; 11. pii: E17.

17) Guo J, Liu Z, Gong R. Long noncoding RNA: an emerging player in diabetes and diabetic kidney disease. Clin Sci (Lond) 2019; 133: 1321-1339.

18) Guan H, Zhu T, Wu S, Liu S, Liu B, Wu J, Cai J, Zhu X, Zhang X, Zeng M, Li J, Song E, Li M. Long noncoding RNA LINC00673-v4 promotes aggressiveness of lung adenocarcinoma via activating WNT/β-catenin signaling. Proc Natl Acad Sci U S A 2019; 116: 14019-14028.

19) Liu L, Chen Y, Li Q, Duan P. LncRNA HNF1A-AS1 modulates non-small cell lung cancer progression by targeting miR-149-5p/Cdk6. J Cell Biochem 2019; 120: 18736-18750.

20) Du P, Liu F, Liu Y, Shao M, Li X, Qin G. Linc00210 enhances the malignancy of thyroid cancer cells by modulating miR-195-5p/IGF1R/Akt axis. J Cell Physiol 2019. doi: 10.1002/jcp.29016. [Epub ahead of print].