IncRNA LINC01494 Promotes Proliferation, Migration And Invasion In Glioma Through miR-122-5p/CCNG1 Axis

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Background: Long noncoding RNAs (IncRNAs) are recognized as key effectors in tumor, including glioma. LINC01494 is an uncharacterized novel IncRNA. In this research, we aimed to investigate the function of LINC01494 in glioma.

Methods: Gene relative expression was analyzed by qRT-PCR method. CCK8, colony formation and Transwell assay was used to determine cell proliferation, migration and invasion. Bioinformatics analyses were used to predict the target of LINC01494 and miR-122-5p. Luciferase reporter assay was utilized to validate the interactions between LINC01494 and miR-122-5p or CCNG1 and miR-122-5p.

Results: LINC01494 was identified as a significantly upregulated IncRNA in glioma through bioinformatics analysis. Furthermore, LINC01494 upregulation indicated poor prognosis. Meanwhile, in vitro investigation indicated that silencing LINC01494 with siRNAs obviously inhibited the proliferation, cell cycle, migration and invasion of glioma cells. Besides, it is found that LINC01494 expression was negatively correlated with miR-122-5p. We demonstrated that LINC01494 inhibited miR-122-5p to upregulate CCNG1 expression through direct interaction. Rescue assay further demonstrated that LINC01494/miR-122-5p/CCNG1 signaling cascade plays a critical role in regulating glioma cell proliferation, migration and invasion.

Conclusion: Taken together, our findings demonstrated the essential function and molecular mechanism of LINC01494 in glioma progression.

Keywords: LINC01494, miR-122-5p, CCNG1, glioma, proliferation

Introduction
Glioma is one of the most aggressive cancers in the nervous system and causes a large number of deaths every year worldwide.1 Currently, surgery combined with adjuvant therapy is the main strategy for glioma treatment.2 Nevertheless, prognosis of glioma patients is quite poor.3,4 The effective biomarkers for glioma diagnosis and prognosis and the therapeutic targets for glioma intervention are still lacking. Therefore, defining molecular mechanisms regulating glioma development is very necessary and urgently needed.

Long noncoding RNAs (IncRNAs) are characterized with over 200 nucleotides in length and little protein-coding potential.5 As a novel member of the noncoding RNA family, IncRNAs have attracted much attention in recent years. Accumulating studies have indicated that IncRNAs play critical roles in regulating multiple biological processes, including division, differentiation and metastasis.6 Aberrant
expression of lncRNAs is associated with tumorigenesis. For example, lncRNA CASC11 is upregulated in osteosarcoma and promotes tumor metastasis. Overexpression of lncRNA DANCR initiates progression of lung cancer. LncRNA miR155HG upregulation in glioblastoma regulates proliferation, migration and invasion by miR-185/ANXA2 signaling. Additionally, lncRNA NEAT1 as an oncogene in breast cancer is significantly upregulated in tumor tissues. Therefore, understanding the function of novel lncRNAs will be benefit for elucidating the mechanism of glioma development.

LINC01494 is a novel lncRNA. To date, the function of LINC01494 in tumor is unknown. In the present research, we found that LINC01494 expression was upregulated in glioma. Overexpression of LINC01494 is associated with a low survival rate. Knockdown assays indicated that LINC01494 silencing suppressed glioma cell proliferation and cell-cycle. Moreover, the potential of tumor cell metastasis were also impaired after LINC01494 knockdown. We identified that LINC01494 sponged miR-122-5p to upregulate expression of oncogene CCNG1. In conclusion, our findings elucidated critical roles of LINC01494 in glioma progression and revealed a novel mechanism.

Materials And Methods

Human Tissue Specimens

All 39 glioma tissues and responding normal controls were obtained from China-Japan Union Hospital of Jilin University. None of them were treated with antitumor treatment prior to surgery. All tissues were stored in liquid nitrogen until use. Our study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University and written informed consent was got from patients. Experiments involving human tissues were conducted in accordance with the Declaration of Helsinki.

Cell Culture

Glioma cell lines, including U87, U251, LN229 and A172 cells, and Human astrocyte cell line (NHA) were from the American Type Culture Collection (ATCC) and cultured using DMEM medium (Gibco, CA) containing 10% FBS (Gibco).

Cell Transfection

All siRNAs were bought from GenePharma (Shanghai, China). MiR-122-5p mimics, miR-122-5p inhibitors, and negative controls were bought from GenePharma. These vectors were transfected into U87 and U251 cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s protocol. 48 h later, the transfection efficiency was confirmed by qRT-PCR.

Real-Time Quantitative PCR

Total RNAs were obtained from tissues and cell lines using Trizol reagent (Invitrogen). RNAs were transcribed into cDNA using Prime-Script RT Reagent Kit (Takara, Dalian, China). qPCR was carried out using SYBR Prime Script RT-PCR kits (Takara) on ABI 7300 Fast Real-Time PCR system m (Applied Biosystems, Foster City, CA) following the manufacturer’s protocols. U6 was as a normalized control for miRNA and GAPDH was used as normalized control for other genes. Relative expression was calculated using the 2^−ΔΔCt method. The primer sequences were as follows: LINC01494 (Forward: 5ʹ-CCCGACCTTAGAGTTCTGCG-3ʹ, reverse: 5ʹ-CCCCCCA GGAGAGGGTAAGAT-3ʹ), CCNG1 (Forward: 5ʹ-GTTACC GCTGAGAGCTGACAGT-3ʹ, reverse: 5ʹ-GCAGCCATC CTGGATGATTCA-3ʹ) and miR-122-5p (Forward: 5ʹ- GTGACAATGGTGGAATGTGG-3ʹ, reverse: 5ʹ-AAGCACA ACGATGCCAAAC-3ʹ). U6 (Forward: 5ʹ-CTCGTTCGG CAGACA-3ʹ, reverse: 5ʹ-AACGTTTCAGGAATTCGGT-3ʹ) and GAPDH (Forward: 5ʹ-AGCCACATCGTCAGACA-3ʹ, reverse: 5ʹTGGACTCCACGACGTACT-3ʹ).

Cell Proliferation Assay

Cellular proliferation was detected using the cell counting kit-8 (CCK8; Beyotime, Beijing) assay and colony formation assay. For CCK8 assay, cells (2000 cells/well) were seeded into 96-well plates and cultured for described days. Then CCK8 solution was added and incubated for 2 h. Absorbance at 450 nm was determined using a microplate reader (ELx800; BioTek Instruments, Inc, Winooski, VT). For colony formation assay, 500 cells were plated in the 6-well plates and cultured for 2 weeks. Then clones were fixed and stained. Colony numbers were counted.

Luciferase Assay

The binding sites between LINC01494 and miR-122-5p were predicted by using miRDB. Binding sites between miR-122-5p and CCNG1 were analyzed by using TargetScan. For luciferase reporter assay, the sequence of LINC01494 or CCNG1 containing the wild-type (WT) or mutant (Mut) binding site for miR-122-5p was constructed into pmirGLO dual-luciferase vector (Promega, Madison, WI). Then reporter vectors were transfected into cells together with miR-122-5p.
mimics or controls. 48 h later, the luciferase activity was determined by using a Dual-luciferase Reporter Assay System (Promega) based on the manufacturer’s instructions.

**Transwell Assay**
For cell migration and invasion analysis, the Transwell assay was performed according to a previous research.\(^{11}\)

**Statistical Analysis**
Graphpad Prism software was used for statistical analysis. Results were expressed as the mean ± SD. All experiments were independently repeated at least three times. The Student’s t-test or ANOVA test was used for analysis of statistical differences. \(P < 0.05\) was considered statistically significant.

**Results**
**The Expression Of LINC01494 In Glioma Tissues**
We analyzed a GEO dataset and identified a novel lncRNA LINC01494 that was upregulated in glioma tissues compared to normal tissues (Figure 1A). To further validate it, we collected 39 pairs of tumor and nontumor tissues. Northern blotting and qRT-PCR results showed LINC01494 expression was higher in glioma samples (Figure 1B and C).

Consistently, LINC01494 level was elevated in tumor cell lines in contrast to NHA cells (Figure 1D). Then glioma tissues were divided into LINC01494 high expression and low expression groups and analyzed whether LINC01494 could be a prognostic biomarker. Notably, LINC01494 upregulation was correlated with a low survival rate in glioma patients (Figure 1E).

**Effects Of LINC01494 Knockdown On Glioma Proliferation, Migration And Invasion**
To analyze the role of LINC01494 in glioma, two independent siRNAs targeting LINC01494 were obtained. As shown, LINC01494 expression was effectively reduced in U87 and U251 cells by siRNAs (Figure 2A). Through CCK8 assay, we found that LINC01494 knockdown suppressed the proliferation of U87 and U251 cells markedly (Figure 2B). We obtained a similar result by using colony formation assay (Figure 2C). Interestingly, LINC01494 knockdown caused increased cells arrested in G0/G1 phase and decreased cells in S and G2/M phase (Figure 2D), suggesting that LINC01494 knockdown suppressed cell cycle. Then Transwell assay was performed. We found that depletion of LINC01494 impaired the migration and invasion of glioma cells (Figure 2E and F).

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**Figure 1** The expression of LINC01494 in glioma tissues. (A) Expression levels of LINC01494 in glioma tissues and normal tissues based on the GEO database (GSE51146). (B) Northern blotting assay was used to detect LINC01494 expression in three pairs of tissues. (C) Relative expression of LINC01494 in 39 glioma tissues and their adjacent normal controls by qRT-PCR. (D) Relative expression of LINC01494 in glioma cell lines. (E) Kaplan-Meier survival curve according to LINC01494 expression in glioma patients. *\(P<0.05\), **\(P<0.01\) and ***\(P<0.001\).
LINC01494 Overexpression Promotes Glioma Progression

To further confirm the roles of LINC01494 on glioma, we overexpressed LINC01494 in U87 and U251 cells (Figure 3A). According to CCK8 and colony formation assays, we found that LINC01494 overexpression promoted the proliferation, migration and invasion of U87 and U251 cells (Figure 3B–D). Thus, LINC01494 promotes glioma progression.

Figure 2 Effects of LINC01494 knockdown on glioma proliferation, migration and invasion. (A) Two independent siRNAs against LINC01494 were transfected into U87 and U251 cells and the efficiency of LINC01494 knockdown was confirmed. (B) CCK8 assay was performed to test cellular proliferation. (C) LINC01494 knockdown led to reduced colony numbers. (D) The effect of LINC01494 knockdown on cell cycle was analyzed by FACS. (E and F) LINC01494 knockdown suppressed the migration and invasion of U87 and U251 cells. *P<0.05, **P<0.01 and ***P<0.001.
LINC01494 Regulates miR-122-5p/CCNG1 Axis In Glioma

To research the molecular mechanism, we analyzed the potential targets of LINC01494 by bioinformatics approach. We identified miR-122-5p which has the highest score. Furthermore, we also analyzed the potential mRNA targets of miR-122-5p and identified CCNG1, which is an important regulator of cell cycle. Interestingly, we observed an inverse expression correlation between LINC01494 and miR-122-5p or between miR-122-5p and CCNG1 in glioma tissues (Figure 4A). Thus, we further investigated whether there were direct interactions among them through luciferase reporter assays. We found that miR-122-5p mimic transfection significantly suppressed the activity of WT-LINC01494 or WT-CCNG1 reporter in U87 and U251 cells (Figure 4B). However, mutation of the predicted binding sites for miR-122-5p in the reporter abrogated this trend (Figure 4B). Thus, LINC01494 directly bound to miR-122-5p while miR-122-5p targeted CCNG1. Furthermore, we found that LINC01494 knockdown significantly increased the expression of miR-122-5p and vice versa (Figure 4C). And miR-122-5p inhibited the expression of CCNG1 (Figure 4D). Importantly, LINC01494 knockdown suppressed expression whereas addition of miR-122-5p inhibitors reversed it (Figure 4E), suggesting that LINC01494 promoted CCNG1 expression by sponging miR-122-5p. We also found that miR-122-5p expression was downregulated in glioma tissues (Figure 4F), whereas CCNG1 levels were remarkably upregulated in glioma tissues (Figure 4G and H), implying potential functions of miR-122-5p and CCNG1 in glioma.

Effects Of The LINC01494/miR-122-5p/CCNG1 Axis On Glioma Cells

To analyze the function of the LINC01494/miR-122-5p/CCNG1 signaling, we performed rescued assays. We confirmed the transfection efficiency by measure CCNG1 signaling and CCK8, colony formation and Transwell assays indicated that addition of miR-122-5p inhibitors abrogated the suppressive functions of LINC01494 knockdown on proliferation, migration and invasion (Figure 5B–E). However, together transfection with CCNG1 siRNAs further suppressed the proliferation,
migration and invasion of glioma cells (Figure 5B–E). In conclusion, these data demonstrated that LINC01494 promotes glioma progression through modulating miR-122-5p/CCNG1 axis.

**Discussion**

As the most malignant tumor in nervous system, glioma displays a poor prognosis and threatens human health. Currently, the mechanism regulating glioma development is still poorly elucidated. In the recent years, lncRNAs have been reported to participate in glioma progression. Therefore, it is important to further explore the correlation between lncRNA and glioma. In this study, we identified a novel lncRNA LINC01494 with high expression level in glioma tissues and cell lines. We found that LINC01494 high expression predicted a poor prognosis. Functional experiments indicated that loss of LINC01494 significantly repressed the proliferation, migration and invasion of glioma cells. Moreover, the cell cycle was arrested after LINC01494 knockdown. We also demonstrated that LINC01494 could interact with miR-122-5p and promote CCNG1 expression. Through upregulating CCNG1, LINC01494 contributes to glioma development.

LncRNA has been acknowledged as competitive endogenous RNA (ceRNA) to regulate expression post-transcriptionally. A lot of lncRNAs have been proven to sponge miRNAs to regulate tumorigenesis. For instance, lncRNA TUG1 increases migration and invasion of osteosarcoma cells through sponging miR-143-5p and modulating HIF-1α expression. LncRNA MALAT1 as ceRNA inhibits miR-140-5p to promote development of tongue squamous cell carcinoma. Additionally, LncRNA SNHG3 modulates miRNA-151a-3p/RAB22A signaling and contributes to osteosarcoma metastasis. In our study, we also identified LINC01494 as a sponge for miR-122-5p. We demonstrated their direct interaction through luciferase reporter assay. Several studies have suggested a suppressive role of miR-122-5p in tumor. For example, miR-122-5p suppresses growth and metastasis of bile duct carcinoma. MiR-122-5p attenuates gastric cancer metastasis in vitro. However, the role of miR-122-5p in glioma remains unclear. Our data indicated that miR-122-5p expression was downregulated in...
glioma tissues, implying miR-122-5p as a tumor suppressor in glioma.

Afterwards, we investigated the targets of miR-122-5p through bioinformatics analysis. We found that CCNG1 may be a target. Luciferase reporter assay demonstrated their association. Moreover, we showed that CCNG1 expression was inhibited by miR-122-5p in glioma. CCNG1 is a pivot regulator of cell cycle. Researches have revealed an important correlation between CCNG1 and tumorigenesis. For example, CCNG1 upregulation promotes esophageal squamous cell carcinoma growth. In pancreatic ductal adenocarcinoma, CCNG1 overexpression also promotes tumor cell proliferation. Additionally, a critical oncogenic role of CCNG1 is still reported in ovarian cancer, gastric cancer and lung carcinoma. Nevertheless, the role of CCNG1 in glioma has not been uncovered. In our study, we also demonstrated that CCNG1 expression was regulated by LINC01494/miR-122-5p axis. Moreover, we found that CCNG1 expression was significantly upregulated in glioma tissues, suggesting its oncogenic roles. Thus, we then performed rescue assays. We demonstrated that CCNG1 is a pivot effector in LINC01494/miR-122-5p/CCNG1 axis and found that silencing of CCNG1 really suppressed proliferation, migration and invasion of glioma cells. However, mouse experiments are required in the further to further demonstrate the important roles of LINC01494/miR-122-5p/CCNG1 axis in glioma.

In summary, our findings demonstrated the novel lncRNA LINC01494 is an oncogenic gene in glioma. LINC01494 promotes glioma proliferation, migration and invasion through modulating miR-122-5p/CCNG1 axis.
Our study expands the knowledge on how lncRNAs regulate tumorigenesis.

Disclosure

The authors report no conflicts of interest in this work.

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