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LINC00887 aggravates the malignant progression of glioma *via* upregulating CCND1

X.-M. SHEN[1], S. HAN[1], N. LIU[1], H.-Q. XU[2], C.-X. YAN[1], C.-J. YU[1]

[1]Department of Neurosurgery, Sanbo Brain Hospital, Capital Medical University, Beijing, China
[2]Department of Neurosurgery, The People's Hospital of Anyang City, Anyang, China

Xueming Shen and Song Han contributed equally to this work

Abstract. – OBJECTIVE: This study aims to explore the impact of LINC00887 on the malignant progression of glioma via upregulating CCND1.

PATIENTS AND METHODS: LINC00887 and CCND1 levels in glioma patients in different tumor grades or metastasis statuses were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Kaplan-Meier curves were depicted for analyzing the prognostic potential of LINC00887 in glioma patients. Meanwhile, Pearson correlation test was conducted to assess the expression correlation between LINC00887 and CCND1 in glioma tissues. After knockdown of LINC00887 in LN229 and U251 cells, proliferative abilities were examined by cell counting kit-8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assays. Subcellular distribution of LINC00887 was determined. Thereafter, RNA Binding Protein Immunoprecipitation (RIP) was performed to uncover the interaction between LINC00887 and CCND1. After α-amanitin induction in glioma cells overexpressing LINC00887, RNA degradation of CCND1 was examined at 0, 6, 12 and 24 h, respectively. Finally, the synergistic regulation of both LINC00887 and CCND1 on glioma proliferation was explored by CCK-8 assay.

RESULTS: It was found that LINC00887 was upregulated in glioma tissues, especially in stage III+IV or metastatic glioma cases. Overall survival was remarkably worse in glioma patients expressing a high level of LINC00887 than those with a low level. CCND1 was upregulated in glioma tissues as well, showing a positive correlation to LINC00887. In addition, LINC00887 was mainly distributed in the cytoplasm and interacted with CCND1, and it shortened the half-life of CCND1. Moreover, the knockdown of LINC00887 inhibited glioma cell proliferation, and this inhibitory effect was abolished by overexpression of CCND1.

CONCLUSIONS: LINC00887 is upregulated in glioma tissues, and it aggravates the malignant progression of glioma by upregulating CCND1.

Key Words:
LINC00887, CCND1, Glioma.

Introduction

Glioma is one of the most lethal tumors, leading to more than 400,000 deaths each year[1]. Its 5-year survival is lower than 15%[2]. Currently, classical glioma biomarkers, including serum squamous cell carcinoma antigen (SCCA), carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), are limited in clinical application owing to their insufficient sensitivities and specificities[3,4]. Therefore, it is critically important to develop novel glioma biomarkers.

Long non-coding RNAs (LncRNAs) cannot encode proteins, but they are capable of regulating tumor progression and metastasis by mediating carcinogenic or tumor-suppressor pathways[5,6]. LncRNAs are abnormally expressed in many types of tumors, which are considered as potential diagnostic or prognostic indicators[7-11]. LncRNA ATB has been identified to be upregulated in glioma cells, and its high level predicts a poor prognosis in glioma patients[12]. CRNDE drives glioma proliferation and invasiveness *via* the mTOR signaling[13]. Tian et al[14] reported the ability of LINC00887 in accelerating the canceration of non-small-cell lung cancer. The potential functions of LINC00887 in glioma remain elusive.

As a cell cycle regulator, overexpression of CCND1 shortens G1 phase and weakens dependence on mitogens. As a result, CCND1 has been confirmed to be a carcinogenic gene[15]. Cyclin D1 level is closely related to the malignant level of tumors, such as glioma, breast cancer and bladder cancer[16-18]. Zhang et al[19] pointed out that GATAD1 triggers glioma progression by targeting CCND1. This study mainly explores the underlying influences of LINC00887 and CCND1 on glioma progression, so as to provide novel ideas for the clinical treatment of glioma.

Corresponding Authors: Changxiang Yan, MD; e-mail: yancx65828@ccmu.edu.cn
Chunjiang Yu, MD; e-mail: yucj@ccmu.edu.cn

Patients and Methods

Collection of Pathological Tissues

A total of 42 glioma tissues were surgically collected from glioma patients who were confirmed by preoperative pathology. Meanwhile, adjacent normal tissues away from 2 cm of tumor lesions were harvested as well. All samples were diagnosed according to the World Health Organization criteria. Inclusion criteria: patients with no other treatment prior to surgery, those whose glioma tissues were confirmed by pathology, and those with complete follow-up data. Exclusion criteria: patients who suffered from other tumors, those receiving preoperative chemoradiotherapy, or those with other fatal diseases cause death. Tissue samples were frozen in the liquid nitrogen for RNA extraction. Recruited patients did not receive neoadjuvant chemotherapy or radiotherapy before operation, and there was no contraindication of operation. Follow-up was conducted for recording survival time. This study was approved by the Ethics Committee of Sanbo Brain Hospital, Capital Medical University and conducted after informed consent was obtained from each subject.

Cell Culture

Human astrocytes (NHA) and glioma cell lines (U87, U251, A172 and LN229) were provided by the Institute of Developmental Biology, Fudan University (Shanghai, China). They were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) and 1% penicillin in a humidified incubator with 5% CO_2 at 37°C. Fresh medium was replaced with an interval of 3 days and passaged using trypsin. For assessing RNA degradation, cells were induced with α-amanitin for 0, 6, 12 and 24 h, respectively.

Cell Transfection

Cells were washed in phosphate-buffered saline (PBS) for three times, digested in trypsin for 2 min and centrifuged. Then, they were implanted in 6-well plates with 4×10^5 cells per well. Until cell confluence reached 70%, they were transfected using Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 6-8 h. At 48 h, medium containing 2 μg/mL puromycin was applied for 72 h of cell culture. Afterwards, cells were implanted into another 6-well plate for 1-2 weeks of cell culture. Cell colonies were selected for extended culture.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The concentration and purity of RNA extracted from cells were determined using a spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was carried out using the commercial kit (Applied Biosystems, Foster City, CA, USA). Following 10 min of pre-denaturation at 95°C, complementary deoxyribose nucleic acids (cDNAs) were amplified at 95°C for 15 s and 60°C for 15 s, for a total of 45 cycles. Fluorescence signal was captured at 60°C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as the internal reference. Primer sequences were as follows: LINC00887: Forward 5'-TGGCCAGTGTTTCACCTGTT-3' and reverse 3'-TGATTTCCTCCAACGTGCCA-5'; CCND1: Forward 5'-GCATGTTCGTGGCCTCTAAG-3' and reverse 5'-CGTGTTTGCGGATGATCTGT-3'; GAPDH: forward 5'-CCACGATAACACCAGCTTCG-3' and reverse 5'-ACTTGAGCATGTAGGCCTGT-3'.

Cell Counting Kit-8 (CCK-8)

A total of 1×10^3 cells were implanted in each well of a 6-well plate and cultured for indicated time points, and 10 μL of CCK-8 solution was added (TaKaRa, Dalian, China). After 1-h culturing in the dark, absorbance at 450 nm was measured using a microplate reader. Blank group was set by adding medium and experimental solution without cells.

5-Ethynyl-2'-Deoxyuridine (EdU)

Cells were inoculated in 48-well plates and cultured to 70-90% confluence. 10 μL of EdU (50 μmol/L) was applied in each well for cell labeling. After 12 hours, cells were incubated with 4% paraformaldehyde, followed by PBS washing and incubation with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for another 20 min. 1 mL of DAPI was applied for nucleus staining in the dark. Finally, cells were washed in PBS and captured.

Chromatin Fractionation

At least 1×10^6 cells were lysed in 200 μL of Lysis Buffer J and centrifuged for obtaining the supernatant, which was the cytoplasmic fraction. The remaining was incubated with Buffer SK and absolute ethanol. Nuclear RNAs were obtained by column centrifugation, and cytoplasmic and nuclear RNA levels were determined by qRT-PCR.

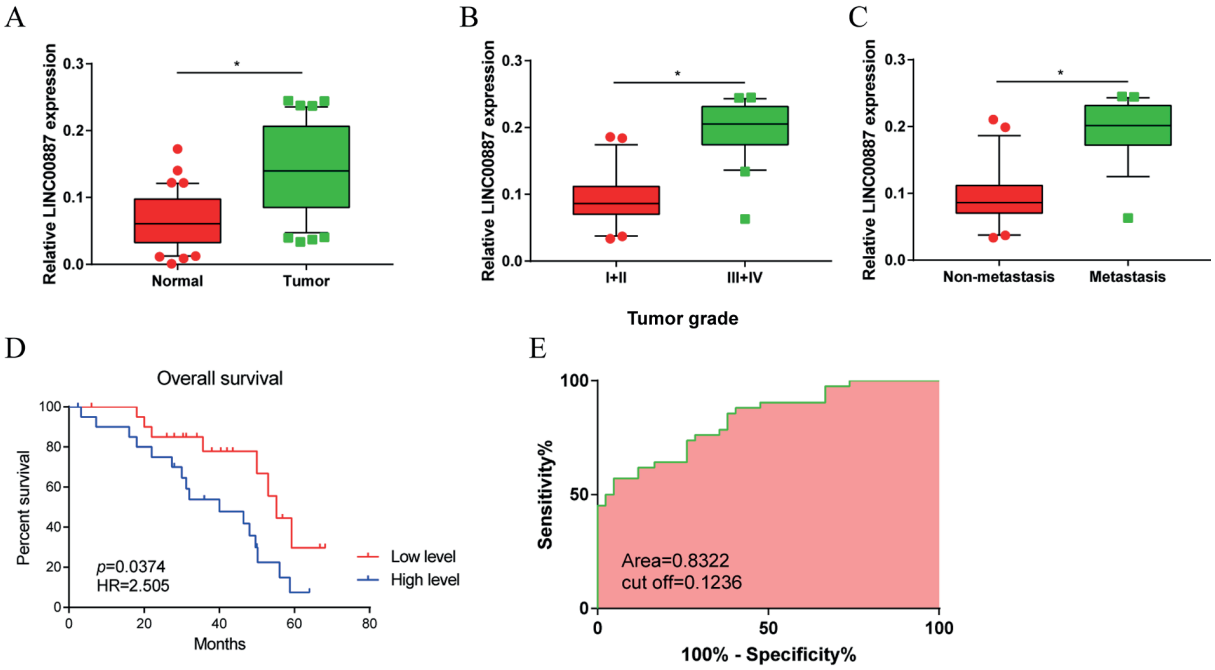

Figure 1. Upregulation of LINC00887 in glioma. **A,** LINC00887 levels in glioma tissues (n=42) and adjacent normal tissues (n=42). **B,** LINC00887 levels in stage I-II and stage III-IV glioma patients; **C,** LINC00887 levels in metastasis and non-metastasis glioma cases. **D,** Overall survival in glioma patients of high-level and low-level LINC00887 group; **E,** ROC curve analysis showed that AUC=0.8322, and cut-off value =0.1236. **p*<0.05.

RNA Binding Protein Immunoprecipitation (RIP)

Anti-Ago2 antibody and the Magna RIP Kit (Millipore Inc, Billerica, MA, USA) were used for exploring the interaction between LINC00887 and CCND1. The monoclonal mouse anti-human IgG was the internal reference. Enrichment of CCND1 was detected by qRT-PCR.

Statistical Analysis

Data processing was conducted using Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA). Diagnostic potentials of LINC00887 was assessed by depicting receiver operating characteristic (ROC). Survival analysis was carried out by depicting Kaplan-Meier curves, followed by Log-rank test for comparing survival differences. Besides, Pearson correlation test was conducted to assess the expression correlation between LINC00887 and CCND1 in glioma tissues. The *t*-test was used to compare the pairwise differences between groups. Significant difference was set at $p<0.05$.

Results

Upregulation of LINC00887 In Glioma

Upregulated LINC00887 was detected in glioma tissues compared with that in adjacent normal tissues (Figure 1A). In particular, stage III+IV glioma patients expressed a higher level of LINC00887 than stage I+II patients (Figure 1B). Moreover, higher abundance of LINC00887 was detected in metastatic glioma patients than in non-metastatic patients (Figure 1C). It is suggested that upregulated LINC00887 in glioma tissues may be involved in glioma progression as an oncogene. To ascertain the impact of LINC00887 on prognosis in glioma patients, recruited glioma patients were divided into high-level LINC00887 group (n=21) and low-level LINC00887 group (n=21), respectively. By analyzing their follow-up data, Kaplan-Meier curves revealed poor prognosis in high-level LINC00887 group (HR=2.505, $p=0.0374$) (Figure 1D). Depicted ROC curves proved the diagnostic potential of LINC00887 in glioma (AUC=0.8322, cut-off value=0.1236, Fig-

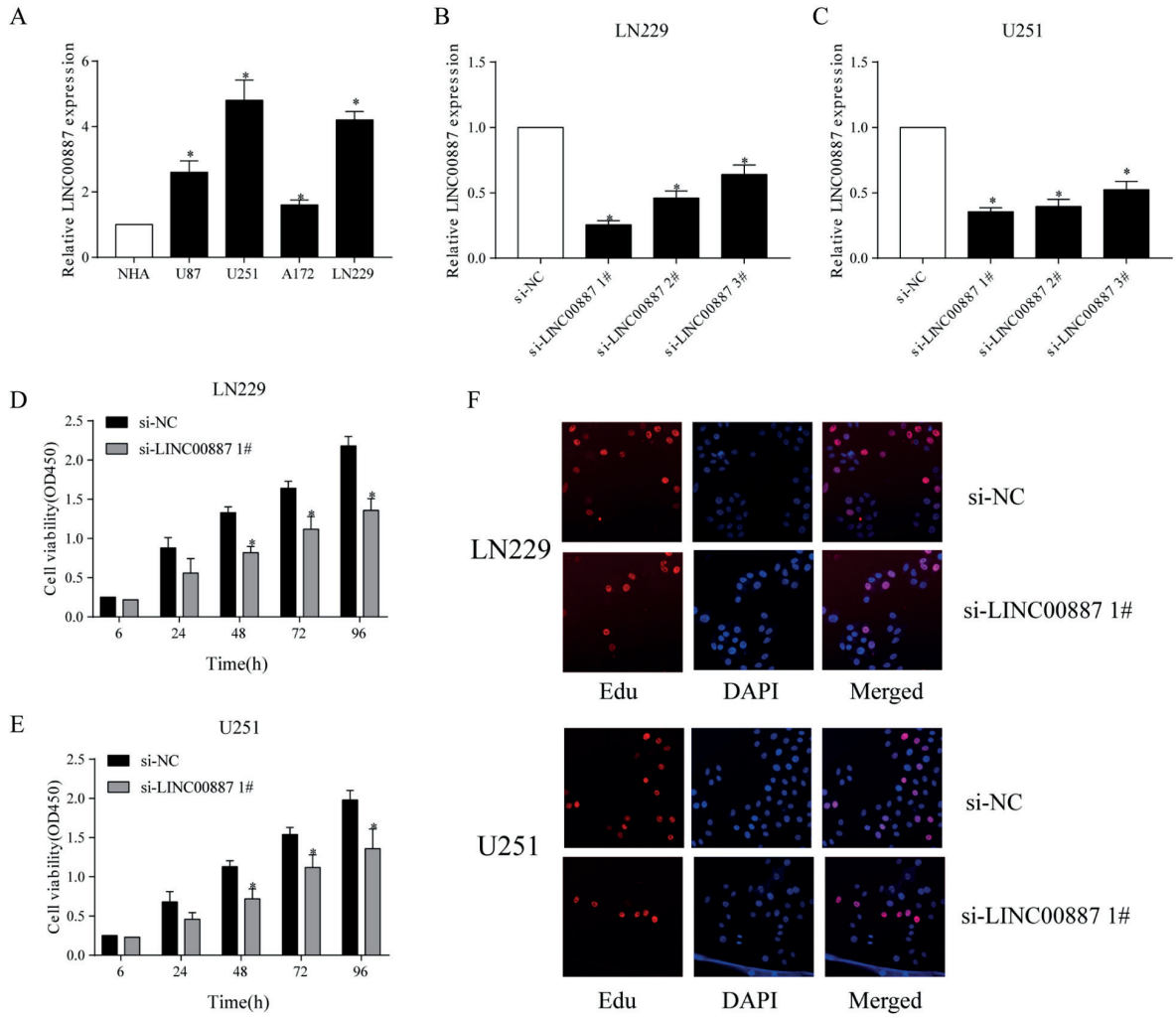

Figure 2. LINC00887 promoted glioma proliferation. **A,** LINC00887 level in glioma cell lines. **B, C,** Transfection efficacy of si-LINC00887 1#, si-LINC00887 2# and si-LINC00887 3# in LN229 (**B**) and U251 cells (**C**). **D, E,** Viability in LN229 (**D**) and U251 cells (**E**) transfected with si-NC or si-LINC00887 1#. **F**, EdU-stained cells in LN229 and U251 cells transfected with si-NC or si-LINC00887 1#. (magnification: 400×) *$p<0.05$.

ure 1E). It could be concluded that LINC00887 was unfavorable to the prognosis in glioma.

LINC00887 Promoted Glioma Proliferation

Compared with human astrocytes, LINC00887 was upregulated in glioma cell lines as well (Figure 2A). Among the four tested glioma cell lines, U251 and LN229 cells expressed the highest level of LINC00887, which were used for the following *in vitro* experiments. Then, three LINC00887 siRNAs were constructed, and their knockdown efficacy was tested. Transfection of either of them effectively downregulated LINC00887 in LN229 and U251 cells (Figure 2B, 2C). Notably, si-LINC00887 1# presented the best efficacy to silence LINC00887 in glioma cells. Knockdown of LINC00887 markedly decreased viability in LN229 and U251 cells at 24, 48, 72 and 96 h (Figure 2D, 2E). Identically, EdU-stained cells were fewer in glioma cells transfected with si-LINC00887 1# than those transfected with si-NC (Figure 2F). Collectively, LINC00887 was capable of promoting proliferative ability in glioma.

LINC00887 Upregulated CCND1

LINC00887 was mainly enriched in the cytoplasmic fraction of U87 and LN229 cells (Figure 3A, 3B). Subsequently, the differential level of CCND1 in glioma and normal tissues were ex-

amined. CCND1 was highly expressed in glioma tissues (Figure 3C), and it presented a positive correlation to LINC00887 level (R^2=0.4113, p<0.0001) (Figure 3D). Moreover, the transfection with si-LINC00887 1# downregulated CCND1 in glioma cells, further supporting their positive relationship (Figure 3E). In addition, transfection with pcDNA3.1-CCND1 effectively upregulated CCND1 in glioma cells, showing a pronounced transfection efficacy (Figure 3F).

Interaction Between LINC00887 and CCND1

In both LN229 and U251 cells, LINC00887 was mainly enriched in anti-CCND1, confirming the interaction between LINC00887 and CCND1 (Figure 4A, 4B). To explore the role of LINC00887 in regulating RNA stability of CCND1, glioma cells overexpressing LINC00887 were induced with α-amanitin for 0, 6, 12 and 24 h, respectively to block RNA synthesis. It was shown that LINC00887 remarkably shortened the half-life of CCND1, indicating that LINC00887 could promote CCND1 synthesis in glioma cells (Figure 4C, 4D). Of note, the overexpression of CCND1 was able to abolish the decreased viability in glioma cells with LINC00887 knockdown (Figure 4E, 4F). It is concluded that LINC00887 and CCND1 synergistically promoted the proliferative potential in glioma.

Discussion

Glioma is a malignant tumor in the central nervous system (CNS), accounting for 31% of CNS tumors and 81% of primary CNS malignant tumors[20]. Based on CNS tumor grade, glioma is subtyped into low-grade (I-II) and high-grade (III-IV) cases. Higher tumor grade predicts worse prognosis in glioma[21]. Surgery is the preferred treatment for glioma, and some patients are required to be treated with postoperative chemotherapy and/or radiotherapy. Nevertheless, the 5-year survival of glioblastoma is lower than 5%[21].

In the past decades, lncRNAs were considered as transcription noises. The biological functions of lncRNAs have been gradually identified owing to the improvement on gene analyses[22]. LncRNAs exert a regulatory role in the process of epigenetic, transcriptional and post transcriptional regulation of cancer[23]. Thousands of lncRNAs expressed in the nucleus or cytoplasm can be encoded by human genomes. LncRNAs specifically distributed in the brain may contribute to the diagnosis and treatment of glioma[24]. Ma et al[25] reported that

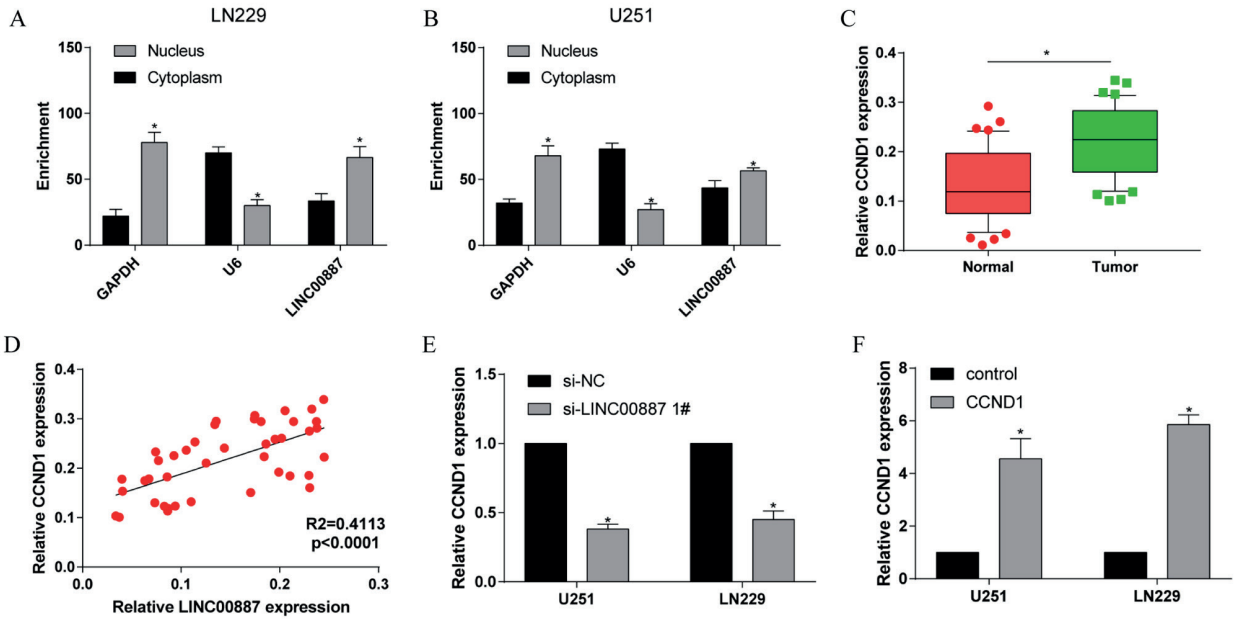

Figure 3. LINC00887 upregulated CCND1. **A, B,** Subcellular distribution of LINC00887 in LN229 (**A**) and U251 cells (**B**). **C,** CCND1 levels in glioma tissues (n=42) and adjacent normal tissues (n=42). **D,** A positive correlation between LINC00887 and CCND1 in glioma tissues. **E,** CCND1 level in U251 and LN229 cells transfected with si-NC or si-LINC00887 1#. **F,** CCND1 level in U251 and LN229 cells transfected with pcDNA3.1-NC or pcDNA3.1-CCND1. *p<0.05.

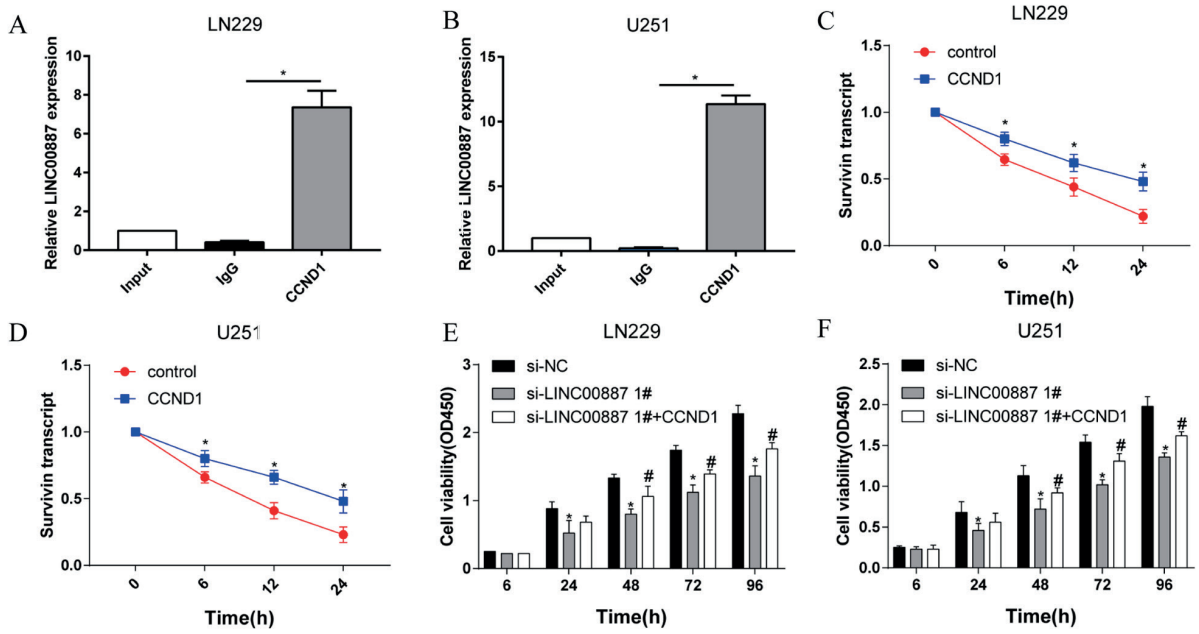

Figure 4. Interaction between LINC00887 and CCND1. **A, B,** Enrichment of LINC00887 in input, anti-IgG and anti-CCND1 in LN229 (**A**) and U251 cells (**B**). **C, D,** RNA degradation of CCND1 in α-amanitin-induced LN229 (**C**) and U251 cells (**D**) overexpressing LINC00887. **E, F,** Viability in LN229 (**E**) and U251 cells (**F**) transfected with si-NC, si-LINC00887 1# or si-LINC00887 1#+pcDNA3.1-CCND1. *p<0.05 *vs.* si-NC group, #p<0.05 *vs.* si-LINC00887 1# group.

MALAT1 is highly expressed in glioma tissues, and its level is positively linked to tumor size and histological grade, but it is inversely proportional to the overall survival of glioma patients, suggesting that MALAT1 may be as a biomarker predicting the prognosis in glioma. In addition, Zhao et al[26] showed that UCA1 was expressed highly in glioma. UCA1 expression level is not associated with patient gender, age, tumor diameter, KPS score, tumor site, but is proven to be related to tumor grade in glioma patients. However, the regulatory mechanisms of glioma-specific lncRNAs in cancer progression should be further validated. This study demonstrated that LINC00887 was upregulated in glioma tissues and predicted a poor prognosis in affected patients. *In vitro* evidence supported the findings that LINC00887 promoted glioma proliferation. Thereafter, it was speculated that LINC00887 may drive the malignant progression of glioma.

Cyclins, extensively distributed in the eukaryotes, are structurally stable and involved in cell cycle progression by binding cyclin-dependent kinases[27]. CCND1, a member of the Cyclins family, is upregulated in many types of tumor cells, serving as an oncogene[28]. Ma et al[29] observed that upregulated CCND1 in glioma is responsible for triggering proliferative potential and accelerating cell cycle progression. CDCA7L stimulates glioma growth by upregulating CCND1[30]. Fortunately, this study found that there seems to be some regulatory relationship between LINC00887 and CCND1. Here, it was identically found that CCND1 was upregulated in glioma samples. CCND1 level was positively linked to LINC00887 in glioma tissues and their interaction was verified by RIP assay. Furthermore, the overexpression of CCND1 could abolish the function of LINC00887 in regulating proliferative potential in glioma cells. Hence, LINC00887 may be a novel glioma biomarker used for diagnosis and treatment. However, the specific regulatory role of LINC00887 and CCND1 in glioma is still unclear, which needs further study in the future.

Conclusions

This study first found that LINC00887 is elevated in glioma tissues, and it aggravates the malignant progression of glioma by upregulating CCND1, opening a new field of vision for the diagnosis and targeted therapy of elevated.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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