Long non-coding RNA (lncRNA) LINC01125 had anti-tumor effects in glioma by vitro and vivo study

Jinmiao Zhang*, Lian Zheng, Guo Chen

Department of Neurosurgery, The Fifth People's Hospital of Chongqing, Chongqing 40062, China

* Corresponding author

E-mail: 14624480@qq.com

Abstract

Aim: The aim of this study was to explore the effects mechanisms of lncRNA LINC01125 in glioma development by vitro and vivo study.

Methods: Evaluating lncRNA LINC01125 expression in glioma cancer and adjacent normal tissues by ISH assay and HE staining. In cell experiment, the lncRNA LINC01125 were transfected in the U251 and U87 cell lines, measuring the cell proliferation by CCK-8 assay and cell apoptosis and cell cycle by flow cytometer; evaluating cell invasion and migration by transwell and wound healing assay. The relative proteins expression by WB assay. In the vivo study, we measured the tumor size and weight.

Results: Compared with adjacent normal, the LINC01125 expression of cancer tissues were significantly down-regulation. With LINC01125 supplement, the cell proliferation rate of lncRNA groups were significantly depressed with cell cycle kept in G1 phase; invasion cell number and wound healing rate of lncRNA groups were significantly suppressed compared with that of Model groups. By WB assay, the PTEN and Bax proteins expression were significantly increased and the PI3K, AKT, Bcl-2, MMP-2 and MMP-9 proteins expressions were significantly increased. In vivo study, the tumor volume and weight were significantly suppressed in nude mice.

Conclusion: LINC 01125 had anti-tumor effects by vitro and vivo study.

Key words: lncRNA; LINC01125; glioma; Biiologica activities

Introduction

Glioma is a common type of tumor that starts in the glial cells of the brain. According to the 2016 WHO CNS classification, gliomas are classified into four grades (grade I to IV), with a higher grade indicating a poorer prognosis, especially in patients with glioblastomas whose survival period is 14 months on average (1, 2). Among those diagnosed with primary grade IV glioma, about 90% had no history of lower-class gliomas (3). So far, surgical intervention, in combination with radiotherapy and chemotherapy, is still the mainstay of treatment for patients with glioma (4, 5). Despite the progress of relevant treatment techniques over time (6), the overall prognosis in glioma patients is far from satisfactory. The dismal sensitivity to chemotherapy and radiotherapy is largely attributed to the heterogeneous nature of glioma cells (7, 8). In order to identify potential therapeutic targets and perfect treatment for glioma, it is of significant importance to probe into the molecular mechanism of glioma development and progression.

Long non-coding RNAs (lncRNAs) are traditionally defined as a type of RNA that generally has over 200 nucleotides and does not encode proteins. LncRNAs engage various mechanisms that regulate transcription factors of target genes (9), control translation and clipping processes, serve as precursors for micro-RNAs (miRNAs) (10) and act as competing endogenous RNAs (ceRNAs) (11). Studies show that lncRNAs play an important role in the development and progression of glioma. To identify a new lncRNA, in situ hybridization (ISH) was performed in this study through which abnormal expression of LINC01125 was observed in gliomas. The U251 and U87 human glioma cell lines were examined in vivo and in vitro to preliminarily explore the effect of LINC01125 on the biological behaviors of glioma cells, aiming to provide a reference for the identification of potential therapeutic targets and prognostic markers in glioma.

Materials and Methods

Clinical specimens

In this study, tumor tissue and normal adjacent tissue (NAT) were resected from 30 patients who were admitted by our hospital for primary glioma between May 2017 and June 2018. All specimens were subject to conventional paraffin processing and HE staining.

ISH assay

The specimens were divided into several groups and fixed in 10% neutral formalin buffer solution to prepare conventional tissue slices after 24 h. The ISH was performed as described in previous studies (12-14). The ISH results were observed with a light microscope (200x) while each group's integrated optical density (IOD) was analyzed using ImageJ.

Transfection

Tumor cells were inoculated into a DMEM containing 10% FBS and placed in an incubator at 37℃, 5% CO2, for culture and passage. The medium was removed 24 h before transfection. The cells were washed three times with PBS and 0.25% trypsin was added for digestion at 37℃ until the cells were retracted and rounded. Then, the digestion was terminated by adding a stop buffer. After that, the cells were centrifuged by applying 100 g centrifugal force for 10 min and the supernatant fluid were removed after centrifugation. The cells were inoculated into a 6-well plate, at the density of (0.5-2) × 10^5 cells per well, after resuspension using a penicillin/streptomycin complete medium. When the cells were 60-80% confluent, 100 μmol lncRNA LINC01125 were diluted in a 250 ml Opti-MEM while 5 μl pcDNA3.1 was diluted in 250 μl Opi-MEM. Then, the solutions were mixed thoroughly and placed at room temperature for 5 min. The pcDNA3.1 group was transfected with blank pcDNA3.1; The lncRNA group was transfected with LINC01125-containing pcDNA3.1.

Cell proliferation assay

After transfection for 12 h, the cells were digested with trypsin and then inoculated into a 96-well plate at the density of 5,000-8,000 cells per well, with each concentration having internal triplicates. When the cells remained adhered to the wall,

10μl CCK8 was added 48 later and the cells were cultured in an incubator at 37℃, 5% CO2 for 2 h. Finally, absorbance at 450 nm in all the wells was measured using an enzyme-labeled instrument.

Cell apoptosis assay

The cells were digested with trypsin 24 h after transfection. Then, the cells were washed twice with pre-cooled PBS and resuspended in a binding buffer (1×) to ensure the final concentration reached about 1×10^6 cells/ml. A flow tube was used for resuspension by adding 100 μl resuspension solution in it. Following that, 5 μl Annexin V-FITC and 5 μl PI were added to the suspension and mixed gently. After reaction in a dark environment at room temperature for 15 min, 400μl binding buffer (1×) was added to the suspension, and then the suspension was injected into the flow cytometer instrument to complete the apoptosis detection within 1 h.

Transwell assay

After digestion with trypsin for 12 h, the cells were inoculated into the Transwell chambers at the concentration of 5×10^5 per well, with the upper chamber filled with 300 μl serum-free DMEM and the lower with 700 μl DMEM containing 20% FBS. The cells were removed from the Transwell chambers 8 h later and washed twice with PBS before fixation at room temperature for 2 h using methanol and acetone (1:1). Then, the cells were stained with 0.5 mg crystal violet for 30 min. The cells in the upper chamber were wiped away carefully and observed in five fields of vision using an upright microscope.

Wound healing assay

Scratches were made evenly using a marker pen before cell culture. Then, 2 ml cell suspension was added in each well (at the density of about 3×10^5 cells/ml) and placed in an incubator at 37℃, 5% CO2, for overnight culture before transfection. After that, the transfected cells were cultured using a serum-free medium and 24 h later, the medium was replaced with DMEM (a complete medium). New scratches were made using a pipette tip

Then, the cells were put back in the incubator. After continuous incubation for 48

h, the 6-well plate was washed twice with D-Hank and the scratches made by the marker pen was gently wiped clean using alcohol. Finally, the remaining scratches were observed and photographed under a microscope.

WB assay

The cells were lysed using chloride-potassium (ACK) lysis buffer (10 mmol/L Tris-HCl, pH = 8.0, 140 mmol/L NaCl, 300 mmol/L KCl, 1 mmol/L EDTA, 0.5% Triton X-100 and 0.5% sodium deoxycholate). After that, 1% protease inhibitor (Roche) was added in the lysis buffer. Protein concentration was measured using the BCA protein assay kit (Bio-Rad). The western blotting procedures were subject to previous reports (15). Signal collection was performed using the Enhanced Chemiluminescence Western Blotting kit (GE Healthcare, UK).

Animal modeling

U87 cells during the exponential phase of growth were collected (2×10^6 cells/ml) and inoculated subcutaneously into 10 nude mice from their right lateral dorsal axillary lines. The mice were kept in a sterile environment for 30 d. When the tumor xenografts reached 1.0 cm in diameter, the tumor-bearing nude mice were killed and the tumors were removed and washed several times with physiological saline. All necrotic tissue was removed and the tumor tissue in good conditions was sliced into 3-4 mm3 using a surgical blade. Then, the tissue slices were xenografted into the rest 60 nude mice via their lateral dorsal axillary lines using a special tissue inoculator. The tumor-bearing nude mice were kept in a sterile environment.

After 14 days of feeding, all nude mice were killed to harvest the tumors from their bodies for tumor weight and volume measurement.

Statistical Treatment

The software SPSS 19.0 was used for data analysis, and the experimental results were expressed in the form of “mean± SD (standard)”. Measurement data were examined using the chi-squared test while intergroup comparison was analyzed through one-way ANOVA. $P < 0.05$ indicated a difference of statistical significance.

Results

The pathological and LINC 01125 expression

Compared with Adjacent tissues, the cell invasion and migration were increased in glioma tissues (Figure 1A). Meanwhile, the LINC 01125 expression of glioma tissues were significantly depressed compared with that of adjacent normal tissues by ISH assay (P＜0.001, Figure 1B).

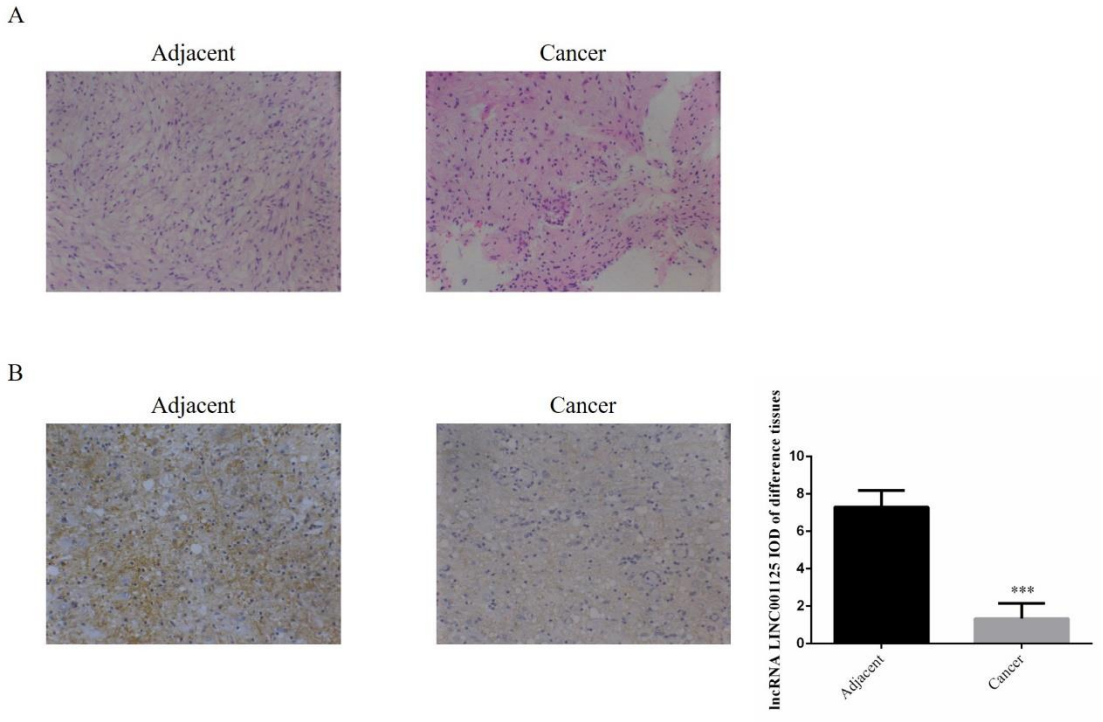

Figure 1. The pathological and LINC 01125 expression (200×)

A. The pathological of adjacent and cancer tissues by HE staining (200×)

B. The lncRNA LINC01125 expression of adjacent and cancer tissues by ISH assay

***: P＜0.001, compared with adjacent normal tissues

LINC 01125 overexpression inhibited cell proliferation by CCK-8 assay

Compared with NC group, the cell proliferation rate of U87 and U251 cell lines were significantly depressed (P＜0.001, Figure 2). The relative data were shown in Figure 2.

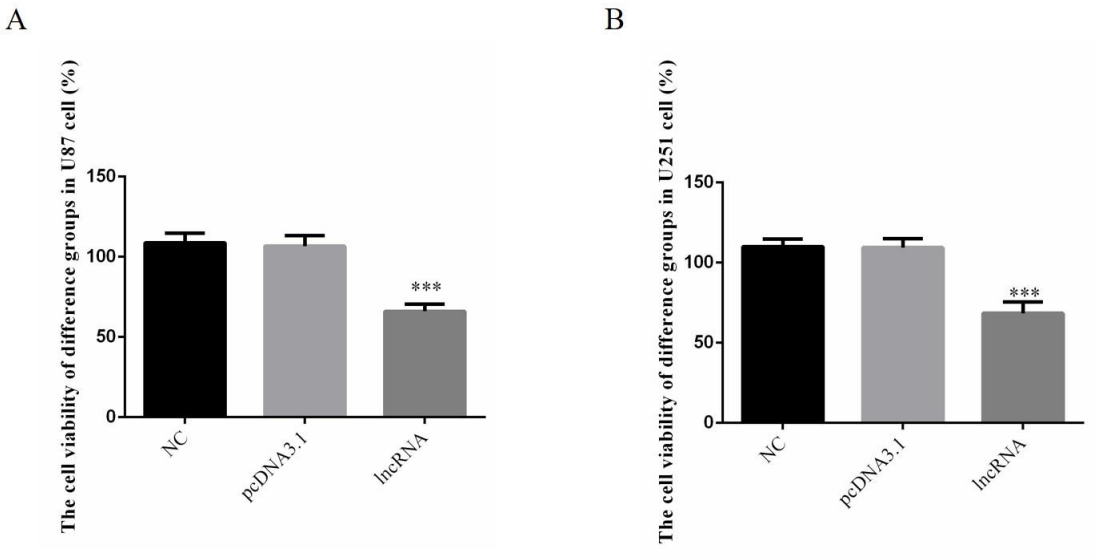

Figure 2. LINC 01125 overexpression inhibited cell viabilities by CCK-8 assay

A. The cell viability of difference groups by CCK-8 in U87 cell

***: P＜0.001, compared with NC group

B. The cell viability of difference groups by CCK-8 in U261 cell

***: P＜0.001, compared with NC group

LINC 01125 overexpression enhanced cell apoptosis rate of difference groups

Compared with NC group, the apoptosis rates of lncRNA group were significantly increased in U87 and U251 cell lines (P＜0.001, respectively, Figure 3A &3B). The relative data were shown in Figure 3.

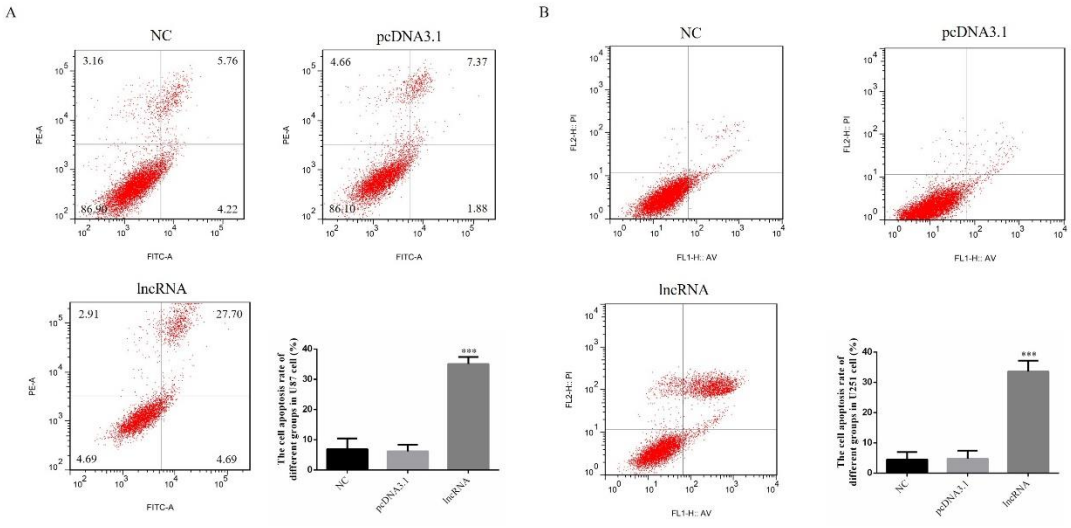

Figure 3. LINC 01125 overexpression improved cell apoptosis rate of difference groups

A. The cell apoptosis rate of difference groups in U87 cell lines

***: P＜0.001, compared with NC group

B. The cell apoptosis rate of difference groups in U251 cell lines

***: P＜0.001, compared with NC group

LINC 01125 overexpression depressed cell invasion number in U87 and U251 cell lines

LINC 01125 overexpression had effects to suppress invasion cell number compared with that of NC groups in U87 and U251 cell lines (P＜0.001, respectively, Figure 4). The relative data were shown in Figure 4.

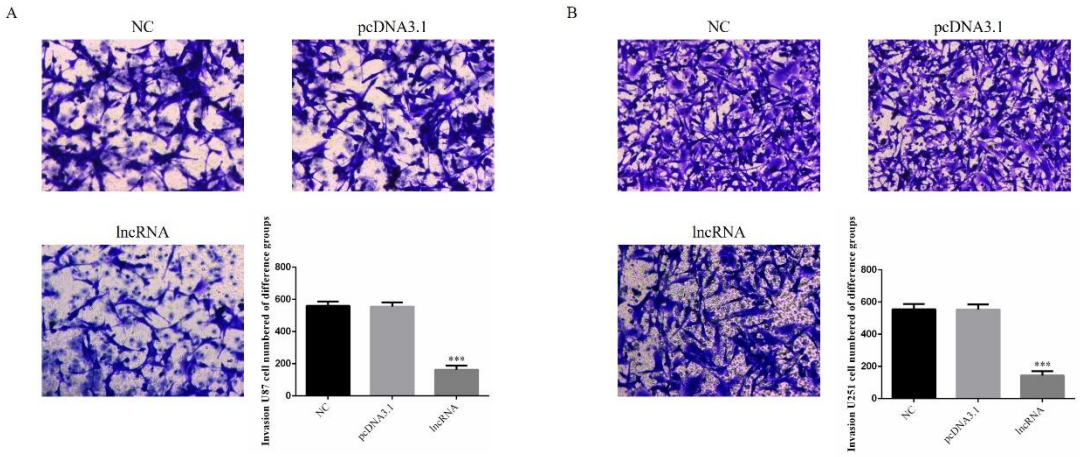

Figure 4. LINC 01125 overexpression depressed cell invasion number in U87 and U251 cell lines (200×)

A. LINC 01125 overexpression suppressed invasion U87 cell number by transwell assay

***: P＜0.001, compared with NC group

B. LINC 01125 overexpression suppressed invasion U251 cell number by transwell assay

***: P＜0.001, compared with NC group

LINC 01125 overexpression suppressed wound healing rate of difference groups in U87 and U251 cell lines

Compared with NC group, the wound healing rate of lncRNA groups were significantly depressed in U87 (Figure 5A) and U251 (Figure 5B) cell lines by wound healing assay (P＜0.001, respectively, Figure 5).

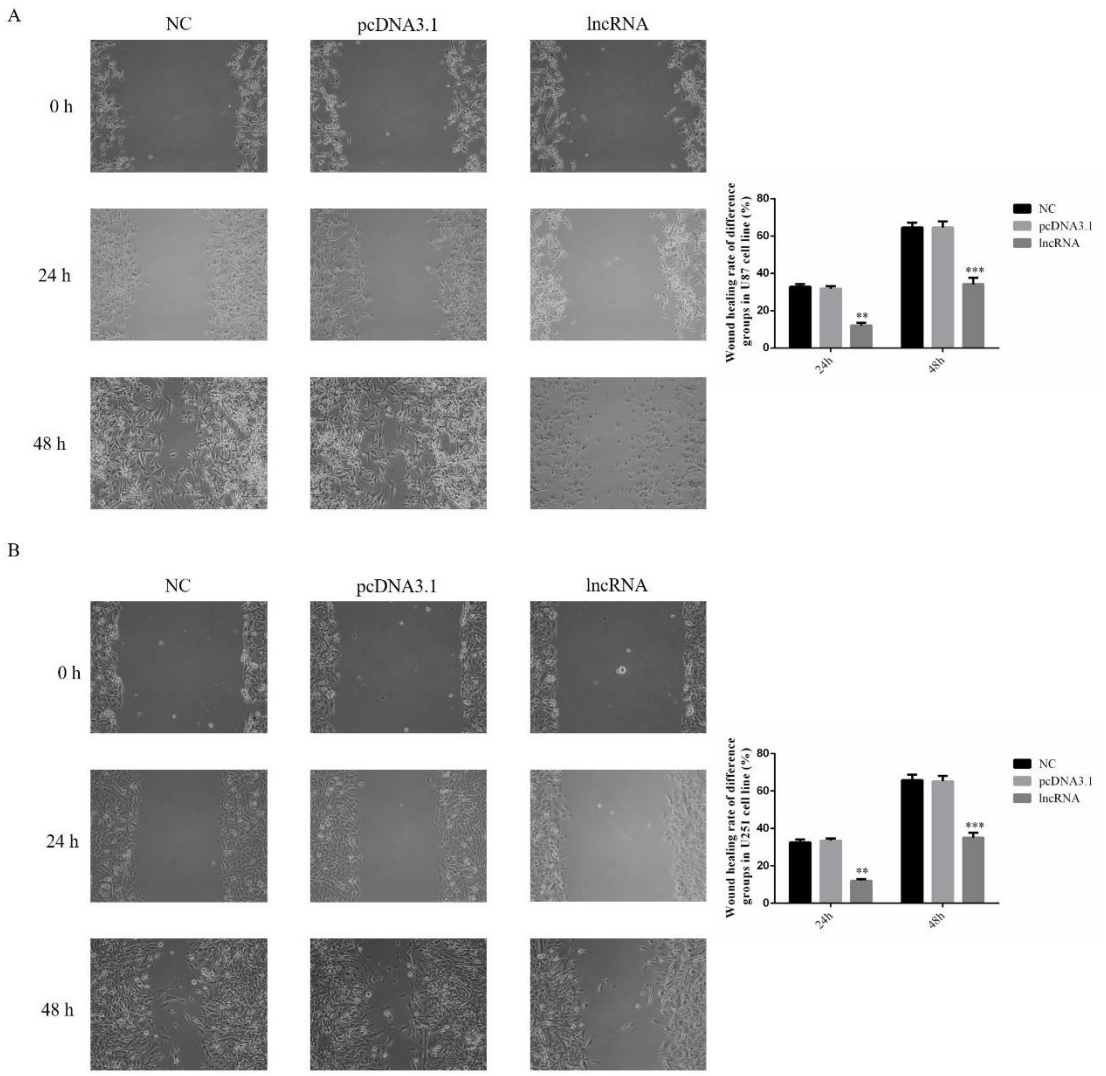

Figure 5. LINC 01125 overexpression suppressed wound healing rate of difference groups in U87 and U251 cell lines (100×)

A. The wound healing rate of U87 cell by wound healing assay (100×)

***: P＜0.001, compared with NC group

B. The wound healing rate of U251 cell by wound healing assay (100×)

***: P＜0.001, compared with NC group

LINC 01125 had effects to relative proteins expression by WB assay

Compared with NC group, PTEN and Bax proteins expression of lncRNA groups were significantly up-regulation in U87 and U251 cell lines by WB assay; PI3K, AKT, Bcl-2, MMP-2 and MMP-9 proteins expression of lncRNA groups were significantly

down-regulation in U87 and U251 cell lines by WB assay (P＜0.001, respectively, Figure 6A＆6B). The relative data were shown in Figure 6.

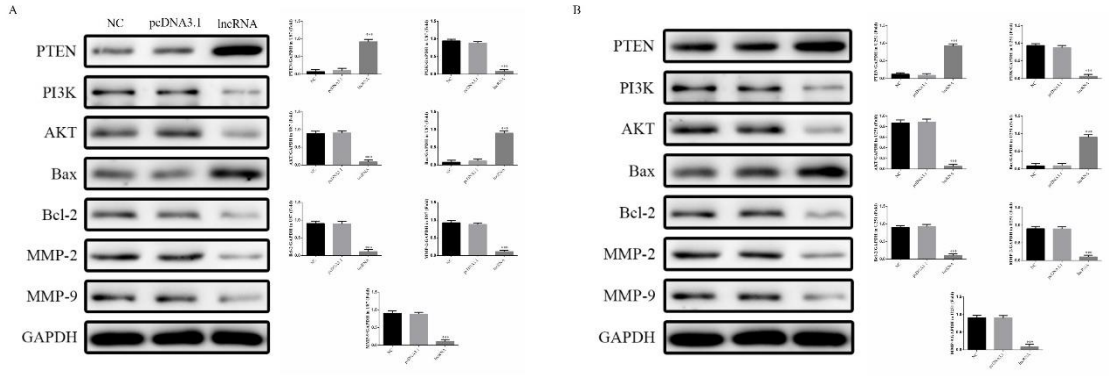

Figure 6. The relative proteins expression by WB assay

A. The relative proteins expression in U87 cell line by WB assay

***: P＜0.001, compared with NC group

B. The relative proteins expression in U251 cell line by WB assay

. ***: P＜0.001, compared with NC group

LINC 01125 had effects to cell proliferation in vivo

The Figure 7A were shown that, the body weight and size of difference groups were no significantly. By vivo study, the results were shown that the tumor volume and weight were significantly depressed compared with those of NC group (P＜0.001, Figure 7B).

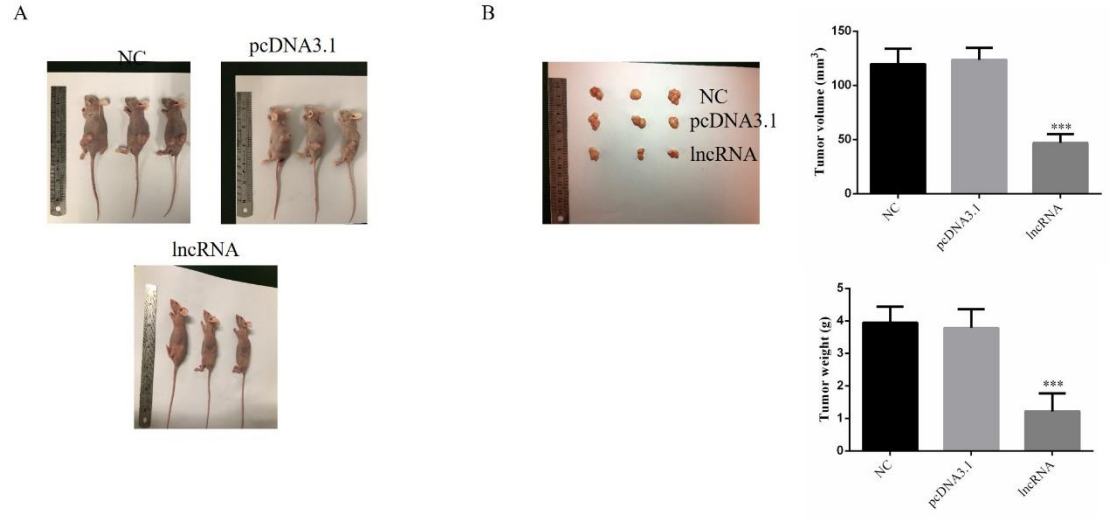

Figure 7. LINC 01125 had effects to cell proliferation in vivo

A. The tumor in body

B. The tumor tissues were taken out and the tumor volume and weight

***:P＜0.001, compared with NC group

Discussion

As the incidence of glioma grows year by year, the disease has become a threat to the world's population. Fortunately, surgical intervention produces favorable outcomes in glioma cases. Yet, grade WIIOIV GBM has a poorer prognosis compared to other gliomas and lacks an effective method to evaluate the risk of recurrence. Against this backdrop, it is of great importance to dive into the molecular mechanism regarding the development and progression of glioma. As we gain a better understanding of the disease, it is found that lncRNAs play a role in the development and progression of atherosclerosis (16), tumors, and diabetes (17), making lncRNAs a popular interest of research. Despite the lncRNA-related reports that keep popping up, we still have little knowledge of the functions of lncRNAs. In this study, we used the ISH to compare glioma tissue and its NAT and found that LINC01125 expression in the tumor tissue was significantly lower than in the NAT. Hence, we infer that LINC01125 overexpression may effectively inhibit the bioactivity of glioma cells.

This study focused on the U87 and U251 cell lines and saw decreased proliferation, invasiveness, and migration of the U87 and U251 cells transfected with LINC01125, which indicated that LINC01125 was possibly a suppressor against glioma.

The PTEN gene is an anti-oncogene closely related to human cancers. A previous study (18) held that PTEN affected the development and progression of pancreatic cancer by means of loss of heterozygosity (LOH), mutation, promoter methylation, and miRNA regulation. Also, it is reported that the development and progression of malignant tumors can be inhibited by controlling the proliferation, invasion, and apoptosis of tumor cells through downregulation of PTEN expression (19-20). Likewise, this study demonstrated that LINC01125 inhibited the proliferation, invasion, and apoptosis of glioma cells by regulating PTEN expression. Further, the

PTEN gene downregulates Akt expression and reduces Akt phosphorylation, thereby activating the Akt pathway and promoting the proliferation and migration of pancreatic cancer cells (21-22). The key of this mechanism probably lies in the dual-specificity protein phosphatase activity of the PTEN gene that dephosphorylates PIP3 and converts it to PIP2 to reduce Akt phosphorylation. Abnormal tumor growth largely occurs when the equilibrium between Bax and Bcl-2 expression is compromised (23, 24). This study showed that LINC01125 overexpression could effectively regulate the PTEN/PI3K/AKT signaling pathways, upregulate Bax expression and inhibit Bcl-2 expression. This is probably the underlying mechanism of how LINC01125 inhibits the proliferation and apoptosis of glioma cells.

The invasion of glioma cells can be described as follows: the glioma cells first adhere to the extracellular matrix, followed by degradation of the extracellular matrix; then, the glioma cells penetrate through the extracellular matrix and enter the adjacent brain tissue (25, 26). The mechanism underlying the invasion of glioma cells is a primary interest of research in the field. Through transwell and wound healing assays, this study noted that the migration and invasiveness of glioma cells were effectively inhibited after transfection with LINC01125. According to the WB test results, LINC01125 could downregulate MMP-2 and MMP-9 expression, indicating that the decreased migration and invasiveness of glioma cells might be associated with the downregulation of the MMP-2 and MMP-9 proteins by LINC01125 overexpression.

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