Long noncoding RNA LINC00152 promotes glioma cell proliferation via Src-YAP signaling pathway

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Research

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Abstract

**Objective** The study is designed to observe the influence of LncRNA LINC00152 on the proliferation of glioma cells and explore the role of Src-YAP signal pathway in the process.

**Methods** U251 and U87 cell were used for in vitro experiments and xenograft studies; transfection method was adopted to build a LINC00152 overexpressing cell strain, and cell viability was determined by MTT assay; cell colony formation ability was measured through colony formation assay, and protein expression was determined by Western blot; YAP protein expression distribution is detected through Immunofluorescence.

**Key findings** LINC00152 overexpressing can enhance the U251 and U87 cell proliferation, the colony formation and the growing ability of subcutaneously transplanted tumor, and it induces YAP nuclear import and down-regulates p-LATS1 and p-YAP protein expression, and up-regulates p-Src protein expression. Src inhibitor 1 can inhibit the changes in protein expression and the cell colony formation of p-LATS1, p-YAP and p-Src, which are induced by overexpression of LINC00152 in U251 and U87 cells.

**Conclusions** LINC00152 promotes cell proliferation of glioma U251 and U87, and the action might be associated with process of activation of Src, inhibition of phosphorylation cascade reaction of LATS1-YAP pathway and final inducing of YAP nuclear import.

Introduction

Glioma is a primary malignant tumor frequently observed, and it occurs at the central nervous system, and it has features such as high recurrence rate and high mortality. Currently, conventional therapies for brain glioma include resection, chemical treatment and radiotherapy. However, its general therapeutic effect isn’t ideal, especially for high-grade gliomas, and as shown by data, its 5-year survival rate is only 9.8%. Therefore, it is quite urgent to find a innovative therapeutic method for clinical purpose. LINC00152 belongs to long noncoding RNA(LncRNA), and it has abnormal expression at malignant tumors such as liver cancer, gastric cancer and colon cancer etc. It has been proved that, LINC00152 is involved in the biological behaviors of malignant tumor cells such as proliferation, migration and cancerometastasis etc. The latest clinical studies have shown that, LINC00152 is associated with poor clinical prognosis of brain glioma, and in the models of nude mouse with subcutaneously transplanted tumor, it is found that it helps inhibit the in vivo growth of brain glioma by down-regulating LINC00152 expression. It suggests that, LINC00152 is associated with malignant biological behaviors of glioma cells, and it has cancer promoting effects. However, the cancer promoting mechanism of LINC00152 remain unclear, which requires further exploration.

Yes-associated protein (YAP) is a transcriptional co-activator, and it is involved in the regulation of cell proliferation and apoptosis in the downstream of hippo pathway. In Mammals, MST1/2, LATS1/2 and
YAP are core constituents of hippo pathway. During interphase of cell, ST1/2 is combined with members of Sav, RAS-related protein family, and LATS1/2 is activated through phosphorylation; the phosphorylated LATS1/2 further phosphorylates YAP, forcing the latter translocate from the nuclei to cytoplasm, which further loses transcriptional activity. As for glioma cells, it has been proved that YAP is involved in the malignant biological behavior such as cell proliferation, apoptosis, autophagy and epithelial-mesenchymal transition etc. However, there is no report on whether YAP is involved in the regulation of brain glioma growth by LINC00152. With glioma U251 and U87 cells as the object of study, we are going to produce a cell strain with over-expression of LINC00152, so as to verify the in vivo and in vitro relationship between LINC00152 and cell proliferation and discuss the role that YAP-related signal pathway plays in the process. The research aims to provide basic theoretical support to the clinic treatment of brain glioma.

**Materials And Methods**

**Cell Culture and Reagents**

Human GBM cell lines, U251 and U87, were obtained from ATTC and stored in the Cell Bank of Shanghai, the Chinese Academy of Sciences (CAS). U251 and U87 cells were maintained in modified RPMI-1640 (HyClone, USA), supplemented with 10% FBS (Gibco, USA) and 1% penicillin/streptomycin (HyClone, USA). All cell lines were grown in a 37 °C incubator with 5% CO₂.

**Transfections**

6-Well plates are inoculated with U251 and U87 cells, and the cells at each well amounted to 6 × 10^5. When the growth of cells reaches a confluence of 90%, the transfection is performed. pcDNA3.1(+) -LINC00152, the overexpression vector of LINC00152, is transfected to overexpress LINC00152, and empty vector pcDNA3.1(+) acts as a control. Both of them carry green fluorescence tag. When transfection is performed, RPMI-1640 medium (doesn't contain serum) serves as medium to dilute the liposome Lipofectamine™ 2000. At the same time, transfection vehicles are prepared and diluted; transfection mixed solution is prepared by mixing these solutions, and the mixed solution is added to each well, and then they are gently shaken. The culture plate is placed in CO₂ incubator at the temperature of 37 °C for 8 hours, and then the medium is replaced with RPMI-1640 containing 10% FBS and then cultured for another 40 hours. Stably expressed cell strain is screened with G418 disulfate (MCE, USA) for multiplication culture. Fluorescent expression of cells is observed under fluorescence microscope, and LINC00152 RNA expression level is detected with RT-qPCR.

**Extraction of total RNA and RT-qPCR**

Cultivate the required groups of U251 and U87 cells to log phase. Total RNA extraction using TRizol (Solarbio, China). The total RNA levels were measured using SYBR Premix Ex Taq™ (TaKaRa, China). Reverse transcription using 2 µg total RNA to obtain cDNA. Quantitative Real-time PCR for Amplification
and Quantification of cDNA. GAPDH is normalized as an internal reference. Relative RNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Primers were synthesized by Invitrogen (Shanghai, China).

**MTT assay**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Solarbio, China) method is used to detect cell viability. Prepare 5 mg/mL MTT solution. At the detection time, 10 µL MTT solution was added to each detection well and incubated for 4 h at 37 °C and 5% CO$_2$. The resulting crystals of formazan were fully dissolved with 100 µL of DMSO for 2 min. The absorbance of the cells was measured at 490 nm using an SpectraMax M2e plate reader (Molecular Devices, California, USA).

**Colony formation assay**

Three hundred cells were seeded into each well of 6-well plates and cultured for 10 days. Pretreat with Src inhibitor 1 (MCE, USA) if necessary. The cultured cells were then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Colonies containing more than 50 cells were counted under an inverted microscope using the formula: colony formation rate (%) = colony number/300 cells × 100%.

**Mouse xenograft model**

Animal experiments were conducted in accordance with the Declaration of Helsinki and the Regulations for Care and Use of Laboratory Animals of the State Food and Drug Administration of China. The experimental protocols were approved by the Animal Ethics Committee of CANGZHOU People's Hospital (Approval number: 20181623). BALB/c-nu mice (male) were obtained from Beijing Vital River Laboratory Animal Technology (Beijing, China) [License # SCXK(jing)2018-0001] and kept on a 12-h day/night cycle with free access to food and water. U251 or U87 cells were divided into two groups: pcDNA3.1(+) group and pcDNA3.1(+)-LINC00152 group. The cells were then washed with PBS for two times and the number of cells was adjusted to $2.5 \times 10^7 \text{cell/mL}$ in RPMI-1640 and inoculated subcutaneously into the right lower limb of the mouse near the back. Tumor sizes were measured three times per week and the volume was calculated following the formula: volume (mm$^3$) = length × width$^2$ × 1/2. The animals were dislocated executed on day 21 after inoculation, and tumors were collected and weighed.

**Western Blot Analysis**

Cultivate the required groups of U251 and U87 cells to log phase. Pretreat with Src inhibitor 1 if necessary. Cells were lysed in RIPA lysis buffer (Solarbio, China), and cell lysates are prepared as protein samples with protein loading buffer. Protein samples were separated on a 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). Then incubated with first antibodies to anti-p-Src (monoclonal, rabbit anti-human, 1:1000), anti-Src (monoclonal, rabbit anti-human, 1:1000), anti-p-LATS1 (monoclonal, rabbit anti-human, 1:1000), anti-LATS1 (polyclonal, rabbit anti-human, 1:2000), anti-p-YAP (monoclonal, rabbit anti-human, 1:1000), anti-p-YAP (monoclonal, rabbit anti-human, 1:1000), anti-p-MST1/2 (monoclonal, rabbit anti-human, 1:1000), anti-MST1 (monoclonal, rabbit anti-human, 1:1000), anti-MST2 (monoclonal, rabbit anti-human, 1:1000), and anti-GAPDH (monoclonal, rabbit anti-human,
(Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. PVDF membranes were washed three times with TBS-T. Afterwards, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies of sheep to mouse and sheep to rabbit (ZSGB-BIO Co., Ltd., China) at 4 °C for 1 h. PVDF membranes were washed three times with TBS-T. Protein bands were visualized by using an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA). Protein bands were detected by GE Al680 system (GE, Chicago, IL, USA). The densitometric analysis of protein bands was performed using ImageJ.

**Statistical analysis**

Statistical analysis was performed using GraphPad_Prism 6.0 and SPSS 19.0 for Windows. All results were shown as mean ± standard deviation (SD). Statistical significance was calculated using one-way analysis of variance (ANOVA) followed by Fisher's multiple comparison test. *P* < 0.05 was considered significant.

**Results**

**Overexpression of LINC00152 promotes the proliferation and colony formation of U251 and U87 cells.**

To observe the biological effects of LINC00152 on glioma cells U251 and U87, first, we need to achieve exogenous overexpression of LINC00152 through transfection. LINC00152 over-expression vector carries green fluorescence tag, and under the fluorescence microscope, it is found that, U251 and U87 cells express green fluorescence in both group empty vector group (pcDNA3.1(+)) and LINC00152 expression group (pcDNA3.1(+)−LINC00152) (Fig. 1a and b). It is indicated that, overexpression vector enters U251 and U87 cells. RT-qPCR result shows that, in the group of pcDNA3.1(+)−LINC00152, both U251 and U87 cells have over-expression of LINC00152 (Fig. 1c).

We have detected viability of cells in both groups, that is, pcDNA3.1(+) and pcDNA3.1(+)−LINC00152, at 12 h, 24 h, 48 h and 72 h through MTT assay. The results show that, compared with pcDNA3.1(+), for U251 and U87 cells in the group of pcDNA3.1(+)-LINC00152, their cell viability is significantly improved (Fig. 1f and 1g).

At the same time, we perform plate clone to observe the colony formation ability of two cells in both groups, that is, pcDNA3.1(+) group and pcDNA3.1(+)−LINC00152 group. The results show that, either for U251 or U87 MG cells, compared with pcDNA3.1(+), the cell colony formation rate is significantly improved in group pcDNA3.1(+)−LINC00152(Fig. 1d and 1e).

**Over-expression of LINC00152 promotes the subcutaneously transplanted tumor growth in U251 and U87 cells.**

In vitro experiment reveals that, LINC00152 over-expression promotes proliferation and colony formation of U251 and U87 cells. For this reason, we have created models of U251 and U87 subcutaneously transplanted tumor to further observe the biological effect caused by LINC00152 over-expression in vivo.
The data shows that, at the end point (21 Day), in the models with U251 and U87 subcutaneously transplanted tumor, compared with group pcDNA3.1(+), the tumor volume and weight are significantly increased in group pcDNA3.1(+) -LINC00152 (Fig. 2).

**Over-expression of LINC00152 promotes YAP nuclear import at U251 and U87 cells.**

YAP is a kind of transcriptional co-activator, and it is located at the cytoplasm during the phosphorylation, and it enters nuclei after dephosphorylation, and takes part in the transcription of target gene, further promoting the cell proliferation. Our research have shown that, there are obvious expression of YAP in cytoplasm and nuclei at U251 and U87 cells in group pcDNA3.1(+); however, in group pcDNA3.1(+) -LINC00152, it is mainly expressed in the nuclei, while the cytoplasm expression isn't obvious. It suggests that LINC00152 promotes YAP nuclear import(Fig. 3).

**LINC00152 promotes U251 and U87 cell proliferation through Src-LATS1-YAP signal pathway, rather than MST1/2-LATS1-YAP signal pathway.**

To describe YAP's upstream signal regulation mechanism, we have observed classical MST1/2-LATS1-YAP pathway. The results show that, compared with cDNA3.1(+), for U251 and U87 cells in group pcDNA3.1(+) -LINC00152, both LATS1 and YAP phosphorylation levels are down-regulated significantly. However, it is quite interesting that, no similar result is observed for MST1/2. It suggests that, the regulation of YAP activity depends on LATS1, rather than MST1/2. At the same time, we also find that, compared with group pcDNA3.1(+), for U251 and U87 cells in group pcDNA3.1(+) -LINC00152, Src phosphorylation is significantly up-regulated ; however, after pretreatment with Src inhibitor 1, LATS1 and YAP phosphorylation level is significantly reversed in group pcDNA3.1(+) -LINC00152. It suggests that, in this study, Src is the upstream regulation target of LATS1-YAP(Fig. 4a ~ 4d).

We have performed plate clone experiment to further observe the role of Src in the promotion of cell proliferation by LINC00152. The results show that, after pretreatment with Src inhibitor 1, the colony formation of U251 and U87 cells is greatly inhibited in group pcDNA3.1(+) -LINC00152(Fig. 4e and 4f). It suggests that, LINC00152 might inhibit LATS1 and YAP phosphorylation by promoting Src phosphorylation, which further promotes U251 and U87 cell proliferation.

**Discussion**

LncRNA refers to a non-coding RNA of base compositions ranging from 200 nt to 100000 nt NA, and it plays an important role in the biological processes such as cell cycle regulation, epigenetic inheritance regulation, transcription regulation and post-transcription regulation etc., and it is involved in a series of diseases in nervous system, cardiovascular system and malignant tumor etc.. [13–16] With the deepening of research, it is found that, more and more LncRNA are closely related to the generation and development of brain glioma. [17, 18] LINC00152 refers to a member of LncRNA family, and it has abnormal expression at malignant tumors such as liver cancer, gastric cancer and colon cancer etc.. [19, 20] The latest clinical studies have shown that, LINC00152 is associated with poor clinical prognosis of
brain glioma. It inhibits the growth of glioma cells in the subcutaneously transplanted tumors in the nude mice by down-regulating LINC00152 expression. It suggests that, LINC00152 might have cancer promotion effect on glioma, and it promotes the in vivo growth of glioma cells.

Through gene transfection, we have up-regulated LINC00152 expression level at U251 and U87 cells in an exogenous way, and cell strains with stably expressed LINC00152 are screened with G418 disulfate. The results show that, after over-expression of LINC00152, for U251 and U87 cells, their cell viability, colony formation ability and growing ability of subcutaneously transplanted tumors in mice are significantly enhanced, which is similar with the report of literature. Further Immunofluorescence experiment shows that, under normal conditions, there are obvious YAP expression in cytoplasm and nuclei at U251 and U87 cells; however, after over-expression of LINC00152, the YAP expression of cytoplasm isn't obvious at two cells. It suggests that, LINC00152 can mediate cytoplasm YAP nuclear translocation at U251 and U87 cells. Generally speaking, YAP phosphorylation is located at the cytoplasm during the phosphorylation, and it enters nuclei after dephosphorylation, and takes part in the transcription of target gene, further promoting the cell proliferation. To further verify YAP's action mechanism, we have observed core elements MST1/2 and LATS1/2 in addition to hippo pathway. The results show that, it can promote down-regulation of LATS1 and YAP phosphorylation level through LINC00152 over-expression; however it is interesting that, phosphorylation level of MST1/2 doesn't change. It suggests that, for LINC00152, the dephosphorylation cascade reaction of LATS1-YAP isn't mediated by MST1/2.

The latest studies have shown that, Src kinase may take part in the regulation of YAP phosphorylation level. Therefore, we have observed Src kinase. The results show that, LINC00152 over-expression can greatly induce Src phosphorylation of U251 and U87 cells; however, by performing pretreatment with Src inhibitor 1, it inhibits LINC00152-induced down-regulation of LATS1 and YAP phosphorylation level. It suggests that, LINC00152 might inhibit LATS1 and YAP phosphorylation by activating Src, rather than MST1/2. However, it remains unclear as to how Src regulates LATS1-YAP signal axis, which requires further studies.

In summary, LINC00152 promotes cell proliferation of glioma U251 and U87, and the action might be associated with process of activation of Src, inhibition of phosphorylation cascade reaction of LATS1-YAP pathway and final inducing of YAP nuclear import.

**Conclusions**

LINC00152 promotes cell proliferation of glioma U251 and U87, and the action might be associated with process of activation of Src, inhibition of phosphorylation cascade reaction of LATS1-YAP pathway and final inducing of YAP nuclear import. Therefore, our research has partly revealed biological effects of LINC00152 in glioma cells.

**Declarations**
Acknowledgements

Not applicable

Conflict of interest

The authors do not have any possible conflicts of interest.

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Patient consent for publication

Not applicable

Authors’ contributions

All listed authors designed the study, performed the experiments and the statistical analysis, and wrote the manuscript. All authors have read the manuscript and approved the final version.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Animal experiments protocols were approved by the Animal Ethics Committee of CANGZHOU People's Hospital (Approval number: 20181623).

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**Figures**
Figure 1
LINC00152 promotes U251 and U87 cell proliferation through Src-LATS1-YAP signal pathway, rather than MST1/2-LATS1-YAP signal pathway. (a) results of p- Src, p- LATS1, p- YAP and p- MST1/2 Western blot of U251 cells in each group. (b) results of p- Src, p- LATS1, p- YAP and p- MST1/2 Western blot of U87 cells in each group. (c, d) Relative protein expression levels of p- Src, p- LATS1, p- YAP, p- MST1/2 at U251 and U87 cells in each group. (e) Influence of pretreatment with Src inhibitor 1 on the plate clone of U251 and U87 cells in group pcDNA3.1(+)-LINC00152. (f) Cloning efficiency in each group. Compared with group pcDNA3.1(+), **P < 0.01; compared with group pcDNA3.1(+)-LINC00152, △△P < 0.01.

Figure 2

Over-expression of LINC00152 promotes YAP nuclear import at U251 and U87 cells.
Figure 3

Over-expression of LINC00152 promotes the subcutaneously transplanted tumor growth in U251 and U87 cells. (a, b) In the models with U251 and U87 subcutaneously transplanted tumor, the changes in tumor volume observed in group pcDNA3.1(+) and group pcDNA3.1(+)-LINC00152. (c, d) In the models with U251 and U87 subcutaneously transplanted tumor, the photo of tumor tissues in group pcDNA3.1(+) and pcDNA3.1(+)-LINC00152. (e, f) In the models with U251 and U87 subcutaneously transplanted tumor, changes in tumor weights observed in group pcDNA3.1(+) and group pcDNA3.1(+)-LINC00152. ** P<0.01.
Overexpression of LINC00152 promotes the proliferation and colony formation of U251 and U87 cells. (a, b) LINC00152 over-expression vectors carrying green fluorescence tags successfully enter U251 and U87 cells.
cells. (b) relative LncRNA expression level of U251 and U87 cells in different groups. (d) cloning efficiency of U251 and U87 cells in different groups. (e) plate clone diagram of U251 and U87 cells in different groups. (f, g) viability of U251 and U87 cells in different groups. Compared with group pcDNA3.1(+), ** P < 0.01.