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Long noncoding RNA LINC00052 suppressed the proliferation, migration and invasion of glioma cells by upregulating KLF6

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Abstract. – OBJECTIVE: Recent studies have discovered a class of dysregulated long noncoding RNAs (lncRNAs) related to carcinogenesis. This study aims to uncover the molecular functions of lncRNA LINC00052 in the tumorigenesis of glioma.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was performed to detect LINC00052 expression in 40 glioma samples and 4 glioma cell lines. Besides, regulatory effects of LINC00052 on the *in vitro* behaviors of glioma cells were evaluated by the proliferation assay, transwell assay and wound healing assay. Furthermore, the interaction between LINC00052 and kruppel-like factor 6 (KLF6) in mediating the progression of glioma was studied by performing qRT-PCR and Western blot.

RESULTS: LINC00052 expression was remarkably downregulated in glioma samples compared with that in adjacent samples. Moreover, cell proliferation, invasion, and migration of glioma were inhibited after overexpression of LINC00052 *in vitro*. Besides, LINC00052 overexpression upregulated mRNA and protein level of KLF6. Besides, the expression of KLF6 in tumor tissues was positively correlated to the expression of LINC00052.

CONCLUSIONS: These results suggested that LINC00052 could repress cell migration, invasion and proliferation in glioma through upregulating KLF6, which may offer a new therapeutic intervention for glioma patients.

Key Words:
Long noncoding RNA, LINC00052, Glioma, KLF6.

Introduction

Glioma is a common primary intracranial tumors in adults worldwide and is a huge threat on public health[1]. Annually, the incidence of glioma is 5/100,000. Although great strides have been made in the therapeutic treatment in the past decades, the mortality rate remains high in glioma patients worldwide. The median survival rate of glioma patients is approximately 15 months, which is the worst five-year survival rate among all cancers[2,3]. Therefore, it is extremely urgent to uncover new mechanisms underlying the development of glioma and find out potential therapeutic targets for glioma. Noncoding RNA accounts for more than 98% of all the sequences. As one subtype of noncoding RNA, long noncoding RNAs (lncRNAs) participate in various cellular processes and pathways during the tumorigenesis and tumor progression[4]. For instance, lncRNA-CCHE1 expression is positively correlated to the malignancy of colorectal carcinoma *via* regulating ERK/COX-2 pathway[5]. Downregulation of lncRNA linc-ITGB1 inhibits cell invasion, migration and epithelial-mesenchymal transition in non-small cell lung cancer through downregulating Snail expression[6]. Activated by ZEB1, lncRNA HCCL5 accelerates cell viability, migration, epithelial-mesenchymal transition and the malignancy of hepatocellular carcinoma[7]. Through the modulation of OIP5 expression, lncRNA OIP5-AS1 promotes cell proliferation and inhibits cell apoptosis in bladder cancer[8]. However, the role of lncRNA LINC00052 in the progression of glioma remains unexplored. In this study, we found out that the expression level of LINC00052 was remarkably downregulated in glioma samples. Subsequent experiments revealed that LINC00052 depressed cell proliferation, invasion and migration in glioma. Furthermore, we discovered that LINC00052 exerted its function in glioma by upregulating kruppel-like factor 6 (KLF6).

Patients and Methods

Patients

Human tissues were obtained from 40 glioma patients who underwent surgery at Liaocheng

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People's Hospital. All tissues were preserved at -80°C. Written informed consent was offered by each glioma patient before the surgery. This study was authorized by the Ethics Committee of Liaocheng People's Hospital.

Cell Culture and Lentiviral Virus Transfectoin

The glioma cell lines U251, U87, T98, SHG44 and U373 were obtained from the Neuroscience Institute of Soochow University (Suzhou, China). The normal human astrocyte 1800 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). For transfection, lentiviral virus targeting LINC00052 was compounded and then cloned to pLenti-EF1a-EGFP-F2A-Puro vector (BiosettiaInc., San Diego, CA, USA). LINC00052 lentiviruses (LINC00052) and empty vector were packaged in 293T cells. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to isolate total RNA from tissues and cells. SYBR green (Roche, Basel, Switzerland) was conducted to determine the relative expression levels between LINC00052 and KLF6 mRNA. Expressions were normalized to the mRNA level of β-actin. QRT-PCR analyses were conducted in triplicate. The primers were used as following: LINC00052, forward, 5'-CCTATCCCTTTCTCTAAGAA-3' and reverse, 5'-ACTTCTGCAAAAACGTGCTG-3'; β-actin, forward, 5'-GATGGAAATCGTCAGAGGCT-3' and reverse, 5'-TGGCACTTAGTTGGAAATGC -3'. The thermal cycle was as follows: 30 s at 95°C, 5 s for 40 cycles at 95°C and 35 s at 60°C. The relative expression was calculated by performing $2^{-\Delta\Delta CT}$ method.

Cell Proliferation Assay

Following the protocol (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), cell proliferation was monitored by cell counting kit-8 (CCK-8) assay every 24 h. Spectrophotometer (Thermo Scientific, Waltham, MA, USA) was utilized to measure the absorbance at 450 nm.

Wound Healing Assay

Cells seeded into the 6-well plates were cultured in DMEM overnight. After scratched with a plastic tip, cells were cultured in serum-free DMEM. 48 h later, wound closure was viewed. Each assay was repeated for three times independently.

Transwell Assay

Cells were seeded on the top of Matrigel-coated transwell chambers (24-well insert, 8-lm pore size; BD Biosciences, Franklin Lakes, NJ, USA) with 200 μL of serum-free DMEM. The bottom chamber was applied with DMEM containing fetal bovine serum (FBS) as a chemoattractant.

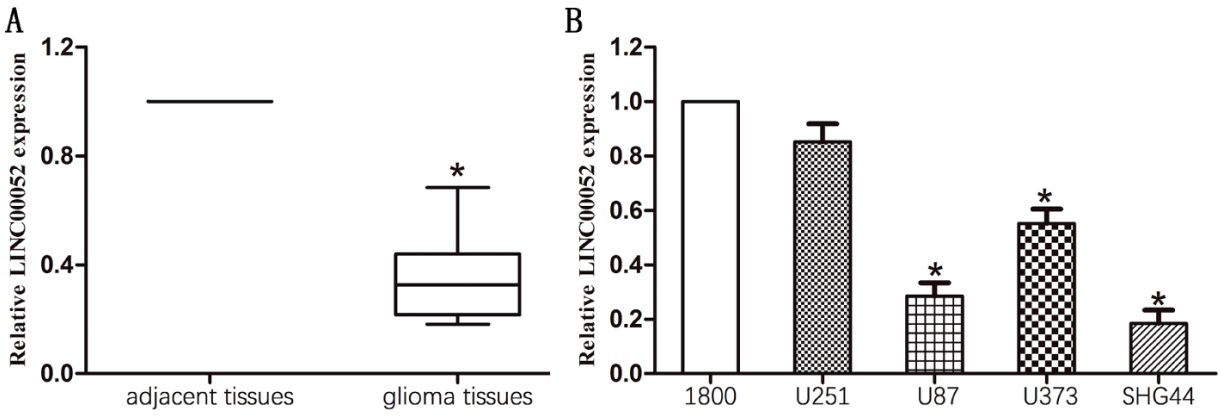

Figure 1. Expression levels of LINC00052 were decreased in glioma tissues and cell lines. ***A,*** LINC00052 expression was significantly decreased in the glioma tissues compared with adjacent tissues. ***B,*** Expression levels of LINC00052 relative to β-actin were determined in the human glioma cell lines and normal human astrocyte 1800 cell line by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

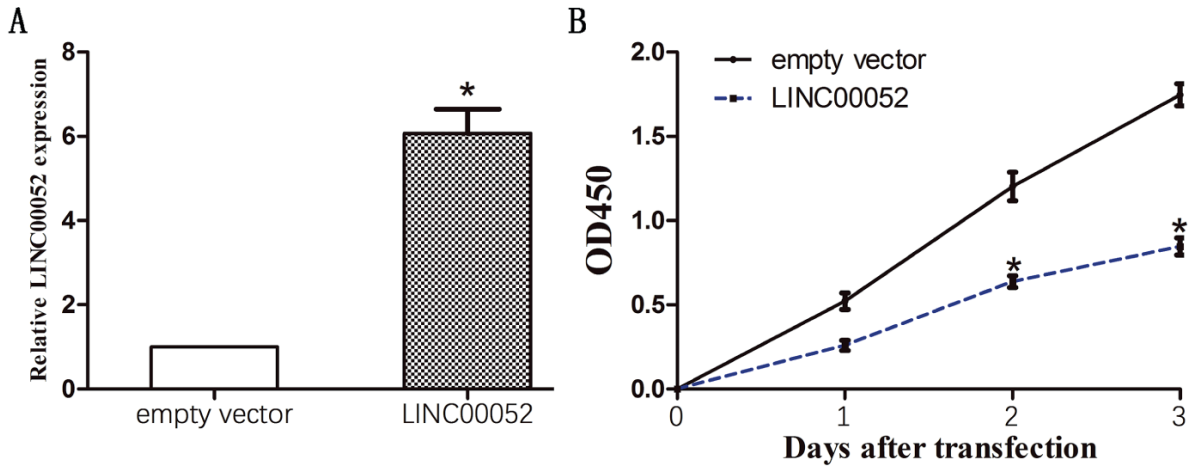

Figure 2. Overexpression of LINC00052 inhibited glioma cell proliferation. ***A,*** LINC00052 expression in glioma cells transduced with empty vector or LINC00052 lentivirus (LINC00052) was detected by RT-qPCR. β-actin was used as an internal control. ***B,*** CCK8 assay showed that overexpression of LINC00052 significantly repressed cell proliferation in SHG44 glioma cells. The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells. *$p<0.05$.

After incubation for 48 h, non-invading cells was removed from the inner part of the insert using a cotton swab. After fixation in 4% formaldehyde, cells on the bottom membrane surface were stained with 0.1% crystal violet. Invading cells were counted in 5 randomly selected fields per sample using a microscope.

Western Blot Analysis

Anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-KLF6 (Abcam, Cambridge, MA, USA) were used as the primary antibodies. After separated with 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein samples were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The primary antibodies were utilized to incubate the membranes at 4°C overnight. After washing with PBS, membranes were incubated with goat anti-rabbit secondary antibody (ProSci, Poway, CA, USA) for 2 h. Enhanced chemiluminescence (ECL) Western blotting detection reagents (Pierce antibodies, Rockford, IL, USA) were used for band exposure. Chemiluminescent film was applied for assessment of protein expression with Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 (SPSS Inc., Chicago, IL, USA) was utilized to conduct the statistical analysis. Two-tailed Student's *t*-test was performed to analyze the significance. When $p<0.05$, the data were considered statistically significant.

Results

Expression Level of LINC00052 in Tissues and Cells of Glioma

First, qRT-PCR was conducted for detecting LINC00052 expression in 40 glioma tissues and 4 glioma cell lines. As a result, LINC00052 was significantly downregulated in tumor tissue samples when compared with that in adjacent tissues (Figure 1A). Besides, LINC00052 level was significantly lower in glioma cells than that in normal human astrocyte 1800 cell line (Figure 1B).

LINC00052 Overexpression Repressed Cell Proliferation in Vitro

According to LINC00052 expression in glioma cells, SHG44 cells were chosen for overexpression of LINC00052. The LINC00052 lentivirus (LINC00052) and the empty vector were synthetized and transfected into SHG44 cells. LINC00052 expression was then determined by qRT-PCR (Figure 2A). Moreover, CCK-8 assay suggested that cell proliferation of glioma cells was repressed after LINC00052 overexpression (Figure 2B).

LINC00052 Overexpression Repressed Cell Migration and Invasion In Vitro

Wound healing assay found that overexpression of LINC00052 inhibited glioma cell migra-

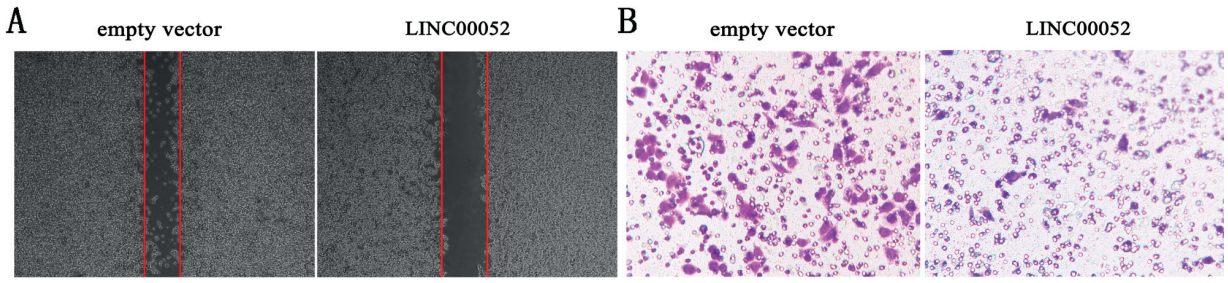

Figure 3. Overexpression of LINC00052 repressed glioma cell migration and invasion. ***A,*** Wound-healing assay showed that the migrated length of cells in LINC00052 lentivirus group was significantly decreased compared with empty control group in SHG44 glioma cells. ***B,*** Transwell assay showed that overexpression of LINC00052 significantly repressed cell invasion in SHG44 glioma cells. The results represent the average of three independent experiments (mean ± standard error of the mean).

tion (Figure 3A). Furthermore, transwell assay indicated that cell invasion of glioma cells was inhibited after LINC00052 overexpression (Figure 3B).

LINC00052 Inhibited Glioma Tumorigenesis via KLF6

QRT-PCR results demonstrated that the mRNA expression of KLF6 was upregulated in glioma cells transfected with LINC00052 lentivirus (Figure 4A). Furthermore, Western blot analysis results revealed that the protein expression of KLF6 was upregulated in glioma cells after transfected with LINC00052 lentivirus (Figure 4B). To explore the interaction between LINC00052 and KLF6, the expression level of KLF6 was detected in tumor tissues. As a result, KLF6 expression was significantly downregulated in glioma tissues when compared with that in adjacent tissues (Figure 4C). Meanwhile, the results of linear correlation analysis showed that in glioma tissues, the expression of KLF6 was positively correlated to LINC00052 expression (Figure 4D).

Discussion

Evidence showed that lncRNAs participate in the regulation of glioma development. For example, lncRNA ATB promotes[9] cell migration and invasion in glioma by activating astrocytes through suppressing the expression of microRNA 2043p. LncRNA HOXD-AS2 facilitates the progression of glioma by regulating cell cycle, which may be utilized as a potential diagnostic biomarker and therapeutic target for glioma[10]. Silence of lncRNA OIP5-AS1 inhibits cell proliferation, migration and promotes cell apoptosis in glioma by blocking the Wnt-7b/beta-catenin pathway and upregulating miR-410[11]. In addition, by regulating Sirt7 and PI3K/AKT/mTOR pathway, lncRNA MEG3 depresses cell proliferation and invasion, and induces autophagy in glioma[12]. Recent researches[13] have indicated that LINC00052 plays an important role in tumorigenesis in several cancers. For instance, LINC00052 depresses cell migration and invasion in hepatocellular carcinoma by upregulating EPB41L3, which is modulated by miR-452-5p. Through activation of Wnt/β-catenin signaling pathway, LINC00052 acts as an oncogene in gastric cancer by promoting cell proliferation and metastasis[14]. In addition, upregulation of LINC00052 enhances the progression of breast cancer by HER3-mediated downstream signaling, which could be used as a potential biomarker for diagnosis and therapy[15]. Our study showed that the expression of LINC00052 decreased in both glioma tissues and cells. Furthermore, overexpression of LINC00052 suppressed the abilities of cell growth, migration and invasion. These data indicated that LINC00052 functioned as a tumor suppressor and inhibits the tumorigenesis of glioma. Known as a tumor suppressor, Krüppel-like factor 6 (KLF6) participates in the regulation of a variety of biological processes in many carcinomas. For example, KLF6 functions as an anti-oncogene in aggressive clear cell renal cell carcinoma through transcriptional depression of KLF6-E2F1 axis[16]. As a target of miR-630, KLF6 accelerates cell proliferation and invasion of epithelial ovarian cancer[17]. Overexpression of KLF6 inhibits cell migration and invasion of oral cancer through attenuating the activity of MMP-9[18]. Moreover, KLF6 constrains the progression of hepatocellular carcinoma dissemination by regulating a VAV3-RAC1 signaling axis[19]. In our

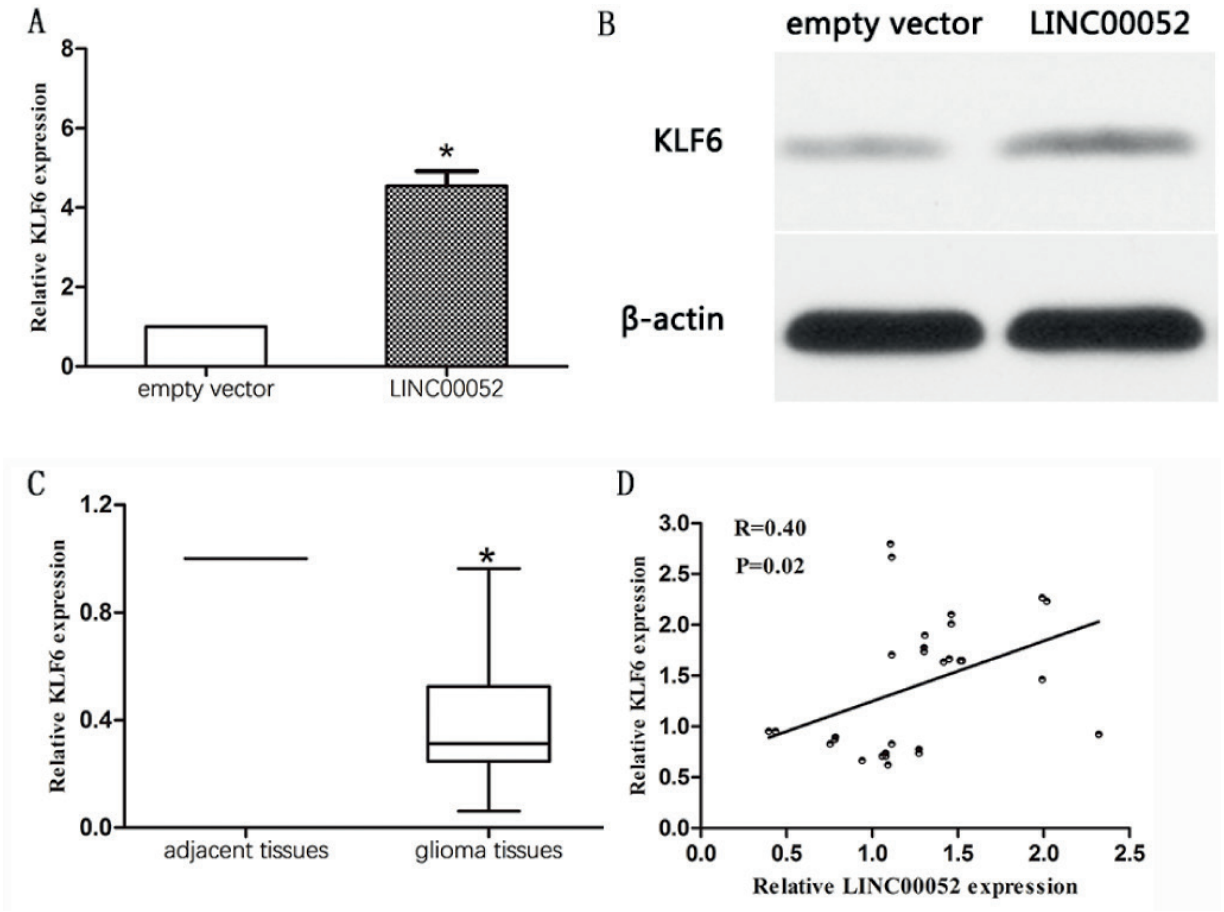

Figure 4. Interaction between KLF6 and LINC00052 in glioma. ***A,*** The RNA expression level of KLF6 in LINC00052 cells was significantly increased compared with empty control cells in SHG44 cells. ***B,*** Protein expression of KLF6 was increased after overexpression of LINC00052 in SHG44 cells. ***C,*** KLF6 was significantly downregulated in glioma tissues compared with adjacent tissues. ***D,*** The linear correlation between the expression level of KLF6 and LINC00052 in glioma tissues. The results represent the average of three independent experiments Data are presented as the mean ± standard error of the mean. *$p<0.05$.

work, qRT-PCR and Western blot indicated that KLF6 was upregulated after LINC00052 overexpression *in vitro*. Moreover, KLF6 expression was remarkably downregulated in glioma samples when compared with that in adjacent tissues. A positive correlation was discovered between KLF6 and LINC00052 expression in glioma tissues. The above results revealed that LINC00052 mediated the progression of glioma *via* regulating KLF6.

Conclusions

We demonstrated that LINC00052 could repress cell migration, invasion and proliferation in glioma through upregulating KLF6. Our research suggests that LINC00052 as a new biomarker in the development of glioma.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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