Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

LncRNA LINC01446 promotes glioblastoma progression by modulating miR-489-3p/TPT1 axis

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ARTICLE INFO

Article history:
Received 12 July 2018
Accepted 12 July 2018
Available online xxx

Keywords:
Long noncoding RNA
LINC01446
miR-489-3p
Glioblastoma
TPT1

ABSTRACT

Accumulating evidence indicates that long noncoding RNA (lncRNA) is implicated in human diseases, including cancers. However, how lncRNA regulates glioblastoma (GBM) progression is poorly understood. Our study revealed a novel lncRNA LINC01446 whose expression was elevated in GBM tissues. Besides, high expression of LINC01446 indicated a poor prognosis in GBM patients. Functionally, LINC01446 knockdown dramatically inhibited GBM cell proliferation, arrested cell-cycle progression and attenuated invasion *in vitro*. Furthermore, the xenograft mouse model showed that LINC01446 silence led to impaired tumor growth *in vivo*. Mechanistically, bioinformatics analysis showed that LINC01446 acted as a sponge for miR-489-3p which targeted TPT1. Though inhibiting miR-489-3p availability, LINC01446 promoted TPT1 expression in GBM cells. Rescue experiments demonstrated that restoration of TPT1 could significantly rescued the effects of LINC01446 silence or miR-489-3p overexpression. Taken together, this study demonstrates a novel singling pathway of LINC01446/miR-489-3p/TPT1 cascade that regulates GBM progression.

1. Introduction

Glioma includes astrocytic tumors, oligodendrogliomas, ependymomas, and mixed gliomas. As one kind of astrocytic tumor, glioblastoma (GBM) accounts for more than 80% of all tumors in brain, thus making it the most common malignant central nervous system tumor [1]. According to WHO (World Health Organization), there are four grade of gliomas based on histopathological criteria. Grade I gliomas has low proliferative rate, while grade IV gliomas are highly aggressive and invasive. GBM belongs to grade IV of gliomas [2] and is a deadly disease with extremely poor prognosis [3]. GBM patients can survive for only 14–15 months after diagnosis [4]. Thus it is critical to understand the disease better. Since now, numerous efforts have been made. Yet, no big progress has been made. But people realize that GBM is driven by signaling networks, so it is critical to understand the mechanism to treat the disease. Here in this paper, we found that LINC01446 plays an important role in GBM progression.

LncRNAs are evolutionarily conserved RNAs transcripts with more that 200 nt, and have no protein-coding potential [5,6]. Researches have shown that lncRNAs are more abundant than protein-coding genes [7], and are more uniquely expressed [8,9]. However, the function of the vast majority of these transcripts remains to be identified. Recently, with the genome-wide approaches, researchers have identified that lncRNAs are differentially transcribed in tumor tissues compared with normal tissues [8,10]. This suggests the potential of discover lncRNA biomarkers for specific cancers. Actually, there have already been several lncRNAs have been used for cancer biomarkers [11], and PCA3 has been approved by FDA prostate cancer diagnosis [11]. In GBM cancers, people have discovered that numerous lncRNAs were differentially expressed [12], but the function of lncRNAs in GBM

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https://doi.org/10.1016/j.bbrc.2018.07.067
0006-291X/

has not been fully revealed.

In this study, we found that LINC01446 was elevated in GBM tissues, and LINC01446 deficiency inhibited GBM cancer progression. Mechanistically, LINC01446 sponged miR-489-3p function, which leaded to TPT1 overexpression. In all, this study demonstrates a critical role of LINC01446/miR-489-3p/TPT1 singling pathway in regulating GBM progression.

2. Materials and methods

2.1. Human samples

31 pairs of GBM samples and adjacent normal tissues were obtained from The First Affiliated Hospital of Kunming Medical University. Patients received no radiotherapy or chemotherapy prior to surgery. All samples were stored in liquid nitrogen before usage. The study was approved by the Ethnic Committee of The First Affiliated Hospital of Kunming Medical University. Written informed consent was obtained from each participant.

2.2. Cell culture and transfection

NHA, A172, U87, U251 and T98G cells were obtained from American Type Culture Collection (ATCC, USA), and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and cultured at 37 °C with 5% CO_2. Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

shRNAs targeting LINC01446 (5′-AGAGCATACGGGAGAGATGAA-3′), scramble shRNA (5′-AATTCTCCGAACGTGTCACGT-3′), miR-489-3p mimic (5′-GUGACAUCACAUAUACGGCAGC-3′) and miR-489-3p inhibitors (5′-GCUGCCGUAUAUGUGAUGUCAC-3′) were bought from GenePharma (Shanghai, China). For LINC01446 stable silence, shRNA was inserted into pLKO.1 vector. For lentiviral packaging, the pLKO.1-shLINC01446 vector or scramble shRNA as well as packaging plasmids was transfected into 293T cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Then, U251 and A172 cells were infected with lentivirus containing the pLKO.1-shLINC01446 vector or scramble shRNA, and were selected with 2 μg/ml puromycin for 10 days. The knockdown efficiency was confirmed by qRT-PCR.

2.3. qRT-PCR

Total RNAs were isolated with Trizol reagent following the manufacturer's protocol as previously described [13]. After that, cDNAs were synthesized with reverse transcriptase (Promega, USA), and qRT-PCR was performed with SYBR Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, USA) with GAPDH or U6 as control.

2.4. Flow cytometry

Cells were fixed with 70% ethanol, and stained with 50 μg/ml propidium iodide (PI), 100 μg/ml RNaseA in 0.2% Triton X-100 PBS buffer for 30 min at 4 °C. After that, the samples were detected with Fortessa (BD Biosciences, USA). Cell cycle distributions of the cells were calculated with ModFit LT software.

2.5. CCK-8 assay

3000 A172 and U251 cells were seeded into 96-well plate, and stimulated with ethanol. Every 24 h, 10 μl CCK-8 was added to the wells and incubated for 2 h, and the absorbance was measured at 450 nm with Multiscan MS spectrophotometer (Labsystems, Sweden), and the experiments were continued for 4 days.

2.6. Xenograft model

In brief, four-week-old female BALB/c nude mice were injected with 4×10^5 cells ($n = 5$), and maintained in SPF condition. The tumor size was regularly checked and measured. 30 days post implantation, the mice were sacrificed and the tumors were further analyzed. The animal protocols were approved by the Ethnic Committee of The First Affiliated Hospital of Kunming Medical University.

2.7. Transwell assays

Before the experiments, the upper chamber of the inserts was coated with 50 mg/l Matrigel. Then, 1×10^4 transfected cells in DMEM without serum were plated into the upper chamber; In the meanwhile, full medium was added to the lower chamber of the insert. 24 h later, the inserts were fixed with 4% formaldehyde and stained with 0.1% crystal violet, 6 fields of view were counted, and the invasion ability of the cells was calculated.

2.8. Luciferase assays

LINC01446 sequence containing the potential miR-489-3p binding sites or mutants of each site was constructed into pMIR-REPORT plasmids (Promega, Madison, WI, USA). Similarly, the predicted miR-489-3p response element (wild-type or mutant) in the 3′-UTR of TPT1 was cloned into a pMIR-REPORT vector. A172 cells were placed in a 24-well plate and were co-transfected with luciferase plasmids and miR-489-3p, control miRNA. After 24 h of transfection, firefly and Renilla luciferase activities were detected with a dual luciferase reporter assay system (Promega) according to the manufacturer's instructions.

2.9. Western blotting

Cells were treated with RIPA buffer, a total of 50 μg lysates were separated with SDS-PAGE. After transfer, the membrane with probed with primary antibodies overnight at 4 °C, followed by a secondary antibodies incubation for 1 h at room temperature. The signals were visualized with ECL.

2.10. Statistical analyses

All data were analyzed according to Student's t-test or one way ANOVA with SPSSS software, and results were shown as mean ± SD. $P < 0.05$ was considered significant.

3. Results

3.1. LINC01446 was overexpressed in GBM tissues

In order to identify lncRNAs involved in GBM progression, we performed lncRNA analysis of GBM and normal brain tissues from a public database GSE51146 and identified 50 up-regulated lncRNAs in GBM tissues compared with normal brain tissues (Fig. 1A and B). Among the 50 robustly up-regulated lncRNAs, LINC01446 was the most up-regulated lncRNA (Fig. 1A). To further confirm LINC01446 overexpressed in GBM tissues, we performed RT-PCR to validate LINC01446 expression in GBM tissues. The results showed that LINC01446 was up-regulated in GBM tissues compared with adjacent normal brain tissues (Fig. 1C). We then divided these 31 samples into two groups (LINC01446 low and LINC01446 high) according to the median expression value of LINC01446, followed

Please cite this article in press as: L. Zhang, et al., LncRNA LINC01446 promotes glioblastoma progression by modulating miR-489-3p/TPT1 axis, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.07.067