Knockdown of IncRNA LSINCT5 suppresses growth and metastasis of human glioma cells via up-regulating miR-451

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ABSTRACT

Background: Glioma is a major cause of brain-cancer relevant death. The present paper designed to reveal the possible role of LSINCT5 in human glioma GL15 cells.

Methods: LSINCT5 and miR-451 expression in glioma tissues was examined using qRT-PCR. The impacts of LSINCT5, miR-451 and Rac1 in GL15 cells were checked by carrying out CCK-8 assay, trans-well assay, and flow cytometric analysis. Further, the target gene of LSINCT5 and miR-451 was explored. Accumulation of PI3K/AKT, Wnt/β-catenin and NF-κB pathway proteins was examined using Western blot.

Results: LSINCT5 was highly expressed while miR-451 low expressed in glioma tissues when compared to normal controls. Down-regulating LSINCT5 effectively declined GL15 cells viability, migration and invasion, but accelerated apoptosis. Nonetheless, the above-mentioned effects of LSINCT5 down-regulation were weakened when miR-451 was silenced. Rac1 was a target of miR-451. The tumour-suppressive effects of miR-451 on GL15 cells were weakened when Rac1 was overexpressed. Further, LSINCT5-miR-451-Rac1 axis could impact the activation of PI3K/AKT, Wnt/β-catenin and NF-kB pathways.

Conclusion: Down-regulation of LSINCT5 represses glioma cells growth and metastasis in vitro likely through targeting miR-451 and thereby inhibiting Rac1-regulated PI3K/AKT, Wnt/β-catenin and NF-κB pathways.

Introduction

Glioma, the prevalent and aggressive brain tumour, is a major reason for brain-cancer relevant death [1]. Although a noticeable advancement has been acquired in the management of glioma during the last several decades, the long-term survival of glioma patients remains unsatisfactory, only with an average survival time of 14 months post-diagnosis [2,3]. Knowing more about the mechanisms responsible for glioma growth and metastasis are still required.

Long noncoding RNAs (lncRNAs) are a newly discovered sort of noncoding RNA transcripts with 200 nucleotides to 100 kilobases. LncRNAs are one of the most recent interests of scientists to study underlying pathophysiology of human cancers [4]. Numerous studies provided a correlation between lncRNAs expression and the pathogenesis of cancers [5,6]. Long stress-induced non-coding transcript 5 (LSINCT5), a member of stress-induced IncRNAs family with 12 members (LSINCT1-12), seems to play a role in cancer development [7,8], LSINCT5 was observed to play significant role in cancer progression and metastasis, and also participate in regulating cellular processes like cell proliferation and apoptosis [8]. It was reported that patients with colorectal or gastric cancer who possess higher levels of LSINCT5 behave poor survival rates [8]. In vitro data illustrated that LSINCT5 silence effectively repressed gastrointestinal cancer cells proliferation [8]. Likewise, ectopic expression of LSINCT5 enhanced breast and ovarian cancer cells proliferation [9]. These studies suggested LSINCT5 as a crucial lncRNA implicated in the onset and development of human cancers.

Recently, scientists hypothesize that lncRNAs function to tumour cells by working as molecular sponges for microRNAs (miRNAs), which further quarantining the targeted mRNAs from degradation by miRNAs [11]. These lncRNAs are termed “competing endogenous RNAs (ceRNAs)”, which compose a considering fraction of gene regulation. The ceRNAs hypothesis protestes the impression that an RNA must be translated into a protein to exert its function [12]. miRNAs are essential regulators in several biological procedures like cell differentiation, proliferation, invasion and apoptosis [13,14]. Recently, the aberrant expression of a number of miRNAs in malignant glioma has been confirmed, such as miR-21, miR-451, miR-181d, miR-23b, miR-10b, miR126, and so forth [15–17]. Among which, miR-451 is a frequently investigated miRNA in glioma. According to several research groups, miR-451 expression is repressed in glioma tissues and cells [18,19]. Besides, miR-451 acted as a tentative switch in mediating glioma cells growth and migration [20–22]. Thus, targeting miR-451 has been regarded as a potential adjuvant therapy for glioma besides...
chemotherapy [23]. In this study, whether miR-451 is related to LSINCT5 in glioma cells is studied. Herein, the specific role of LSINCT5 in human glioma GL15 cells was explored. Interactions among LSINCT5, miR-451 and Rac1 (a pleiotropic regulator of many cellular processes) and their respective regulatory roles were emphatically investigated. In this study, LSINCT5 may represent a target for gene therapy in glioma.

**Methods**

**Cell culture**

GL15 cells (ATCC; Manassas, VA) were cultivated in DMEM (Gibco, Carlsbad, CA) complementing with 10% fetal bovine serum (FBS; Gibco). Cells were cultured at 37 °C in a moistened incubator with 5% CO2.

**Cell transfection**

shRNAs specifically targeting LSINCT5 (sh-LSINCT5) and Rac1 (sh-Rac1), as well as their scrambled respective control (sh-NC), were provided by GenePharma (Shanghai, China). Full-length Rac1 were intercalated into pEX-2 plasmids (GenePharma) for constructing a Rac-1 expression vector (pEX-Rac1). miR-451 mimic, inhibitor and the respective controls (mimic and inhibitor NC) were from Invitrogen (Carlsbad, CA). Transfections were conducted for 48 h by using Lipofectamine 3000 (Invitrogen).

**Clinical specimens**

Glioma tissues were collected from 56 glioma patients who went through tumour resection in Jining No.1 People's Hospital. None of these individuals received any treatment pre-resection. Normal brain tissues from 16 patients with cerebral trauma or epilepsy surgery were used as control. Written informed consent was signed before the usage of clinical specimens and this work was authorized by the ethics committee of Jining No.1 People's Hospital. The tissues were immediately washed twice by cooling PBS and stored at −80 °C before usage.

**qRT-PCR**

Cellular and tissue RNAs were extricated by TRIzol (Invitrogen). LSINCT5 expression was detected using One Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (TaKaRa, Dalian, China). GAPDH served as endogenous control for LSINCT5 expression. For the relative quantification of miR-451 expression, Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and Taqman Universal Master Mix II (Applied Biosystems) were utilized for synthesizing cDNA and conducting PCR. U6 served as endogenous control. For Rac1 detection, RT-PCR was carried using the RNA PCR Kit (AMV) Ver.3.0 (TaKaRa) and GAPDH served as an endogenous control. Relative gene expression was figured out by $2^{-\Delta\Delta Ct}$ method.

**Cell viability**

The transfected cells in 96-well plate with 5000 cells/well were added with 20 μL CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD). Then, the plates were incubated at 37 °C for 1 h and the optical density (OD) was examined at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

**Cell apoptosis**

The transfected cells were gathered into a centrifuge tube and then stained with PI/FITC-Annexin V (Beijing Biosea Biotechnology, Beijing, China) for 1 h at room temperature away from light. Cell apoptosis detection was carried out using a flow cytometry (Beckman Coulter, Fullerton, CA).

**Transwell assay**

Cell migration was examined using the transwell system chambers 8 μM pore. Cell invasion ability was examined using the Chamber matrigel invasion 24-well kit (BD Biosciences, San Jose, CA). Briefly, 5 × 10^4 cells were suspended in serum-free DMEM and placed into the upper side while complete medium was placed to the lower side. 24 h later, the cells in lower chambers were stained with 0.1 mg/mL crystal violet.

**Dual-luciferase reporter assay**

The luciferase assay was carried out using the Dual-luciferase reporter assay system (Promega, Madison, WI). LSINCT5 sequence containing the forecasted miR-451 binding site was inserted into a pmirGlo Vector (Promega) to constitute LSINCT5-wild-type (LSINCT5-wt) reporter plasmid. The primary sequences of LSINCT5 used in these processes were listed as follows. LSINCT5 forward: 5’-CTCGAGTGTAGTCCCCAGCTACTCAG’, containing Xho I restriction site; reverse: 5’-GAGCTCTGGAAGACCATCTTGCTAACA-3’, containing Mlu I restriction site. The sequence of presumed binding site was reconstituted as indicated and was referred as LSINCT5-mutated-type (LSINCT5-mt). Likewise, Rac1-wild-type (Rac1-wt) and Rac1-mutated-type (Rac1-mt) were formed. The primary sequences of Rac1 were as follows: Rac1 forward: 5’-GGGTACCTTTTGAGCCGTCCTGCA-3’, containing Kpn I restriction site; reverse: 5’-GGAATTCGCGTCTTGCACTTGTACGCA-3’, containing EcoR I restriction site. The vectors and miR-451 mimics were transfected into cells using Lipofectamine 2000 (Invitrogen). 48 h later, relative luciferase activity was examined.

**Western blot**

Cell proteins were lysed using RIPA lysis buffer (Thermo Scientific, MA). The primary antibodies against Rac1 (ab97732), Wnt3a (ab28472), Wnt5a (ab72583), β-catenin (ab6302), p-65 (ab86299), p65 (ab16502), IkBα (ab32518), p-IkBα (ab32518), PI3K (ab86714), p-PI3K (ab182651), AKT (ab18785), p-AKT (ab38449) and GAPDH (ab9485, Abcam, Cambridge, MA) were used for protein immunoblotting. Signals were visualized using ECL Substrates (Pierce, Rockford, IL).
Statistics

The data were expressed as the mean ± SD. Statistical analysis was carried out in GraphPad 6.0 statistical software (GraphPad Software Inc, San Diego, CA). p Values were worked out using ANOVA or independent-Samples T-test. p Values less than .05 were considered statistically significant.

Results

LSINCT5 knockdown repressed cell viability, migration, and invasion but accelerated apoptosis of GL15 cells

First, LSINCT5 expression in glioma tissues was examined using qRT-PCR assay. As seen in Figure 1(A), LSINCT5 expression in 56 glioma tissues was dramatically higher than that in normal controls. LSINCT5 knockdown repressed the growth and metastasis of GL15 cells. (A) LSINCT5 expression in 56 glioma tissues and 16 normal controls was detected using qRT-PCR. (B) LSINCT5 expression was analyzed using qRT-PCR after transfection with sh-LSINCT5 #1 and #2. After transfection, (C) cell viability was examined using CCK-8 assay, (D,E) apoptosis was examined using Annexin V-FITC/PI double staining method, and (F) cell migration and invasion were examined using transwell assay. (G) Relative migration and (H) invasion based on the results obtained from transwell assay. *p < .05, **p < .01, ***p < .001.

Figure 1. Knockdown of LSINCT5 repressed the growth and metastasis of GL15 cells. (A) LSINCT5 expression in 56 glioma tissues and 16 normal controls was detected using qRT-PCR. (B) LSINCT5 expression was analyzed using qRT-PCR after transfection with sh-LSINCT5 #1 and #2. After transfection, (C) cell viability was examined using CCK-8 assay, (D,E) apoptosis was examined using Annexin V-FITC/PI double staining method, and (F) cell migration and invasion were examined using transwell assay. (G) Relative migration and (H) invasion based on the results obtained from transwell assay. *p < .05, **p < .01, ***p < .001.
16 normal controls \( (p < .001) \). Next, we explored the growth and metastasis of LSINCT5 in glioma GL15 cells. To this end, shRNA specific for LSINCT5 was utilized to repress LSINCT5 expression. qRT-PCR data displayed that sh-LSINCT5 knocked down LSINCT5 expression in GL15 cells as relative to shNC group. Lower expression of LSINCT5 was made by sh-LSINCT5 #1 \( (p < .01) \) rather than sh-LSINCT5 #2 \( (p < .05, \text{Figure 1(B)}) \). Therefore, sh-LSINCT5 #1 was selected for use in subsequent assays. Further investigation illustrated that LSINCT5 knockdown reduced GL15 cells viability \( (p < .05, \text{Figure 1(C)}) \). In addition, LSINCT5 knockdown dramatically promoted the apoptosis of GL15 cells \( (p < .001, \text{Figure 1(D,E)}) \).

To further explore the impact of LSINCT5 knockdown on GL15 cells metastasis, transwell assay was carried out. The migrating and invasive capacities of GL15 cells transfected with sh-LSINCT5 were significantly decreased as relative to shNC group \( (p < .05 \text{ and } p < .01, \text{Figure 1(F,H)}) \).

**LSINCT5 acted as a molecular sponge for miR-451**

LncRNAs have been found to function as ceRNAs to specific binding with miRNAs \[2\]. Since miR-451 has been frequently described as a tumour suppressive miRNA in glioma \[20–22\], the present work attempted to reveal whether LSINCT5 functioned to GL15 cells via regulating miR-451. qRT-PCR data displayed that LSINCT5 knockdown facilitated miR-451 expression in GL15 cells \( (p < .001, \text{Figure 2(A)}) \). Also, LSINCT5 expression was declined in cells transfected with miR-145 mimic \( (p < .01, \text{Figure 2(B)}) \). We suspected that miR-451 might have relevant binding sites in LSINCT5. Luciferase reporter assay result illustrated that miR-451 overexpression declined luciferase activity of the pMIR luciferase reporter containing LSINCT5-wt \( (p < .05) \). But, none such significant results were found in LSINCT5-mt group \( (p < .001) \).

**LSINCT5 knockdown suppressed GL15 cells growth and metastasis via regulating miR-451**

Next, the role of miR-451 in glioma was studied. As seen in Figure 3A, miR-451 expression was remarkably low in glioma tissues as relative to normal controls \( (p < .001) \). Then, sh-LSINCT5 and miR-451 inhibitor were co-transfected into GL15 cells, to investigate if miR-451 was implicated in the tumour-suppressive effects of LSINCT5 knockdown. As seen in Figure 3B, miR-451 silence obviously enhanced cell viability even if LSINCT5 was suppressed in cells \( (p < .05) \). In addition, miR-451 silence repressed cell apoptosis, as the apoptosis rate was largely decreased \( (p < .01, \text{Figure 3(C,D)}) \). Furthermore, miR-451 inhibition abolished the suppressive effects of LSINCT5 knockdown on migration and invasion \( (p < .05, \text{Figure 3(E–G)}) \).

**Rac1 was the target of miR-451**

Rac1 is a main member of GTPase family that is regarded as a key regulator in glioma cells growth and metastasis \[24,25\]. Interestingly, a previous study demonstrated the role of miR-451 in switching between proliferation and migration in glioma cells partially via mediating Rac1 \[19\]. Thus, this study further verified whether miR-451 functioned to GL15 cells via Rac1. We found that the mRNA level of Rac1 was repressed after miR-451 was overexpressed in GL15 cells \( (p < .05) \) while was dramatically increased when miR-451 was silenced \( (p < .001, \text{Figure 4(A)}) \). The protein level of Rac1 was...
Figure 3. LSINCT5 suppressed GL15 cells via regulation of miR-451. (A) miR-451 expression in 56 glioma tissues and 16 normal controls was detected using qRT-PCR. (B) The cell viability, (C,D) cell apoptosis, (E) cell migration and invasion were determined when GL15 cells were transfected with sh-LSINCT5, miR-451 inhibitor, or their negative controls (shNC and inhibitor NC). (F) Relative migration and (G) invasion based on the results obtained from transwell assay. *p < .05, **p < .01, ***p < .001.
Determined and similar results were displayed in Figure 4(B). Next, the dual-luciferase reporter assay result illustrated that co-transfection of miR-451 mimic and Rac1-wt strongly declined the luciferase activity (p < .01), whereas miR-451 mimic plus Rac1-mt did not change it significantly (Figure 4(C)). Overexpression of miR-451 suppressed GL15 cells growth and metastasis via regulating Rac1

To confirm the effects of Rac1 on GL15 cells, Rac1 expression was altered by transfection with pEX-Rac1 or sh-Rac1. Figure 5(A) displayed that the mRNA level of Rac1 was obviously increased by pEX-Rac1 and repressed by sh-Rac1 (both p < .01). Same trends were observed in Rac1 protein expression (Figure 5(B)). Next, data exhibited miR-451 overexpression significantly inhibited cell viability (p < .05, Figure 5(C)), migration and invasion (both p < .01, Figure 5(F–H)), nevertheless largely accelerated apoptosis (p < .001, Figure 5(D,5E)), which sufficiently revealed its anti-glioma function. However, co-transfection of miR-451 mimic and pEX-Rac1 inhibited the tumour-suppressive effect of miR-451 on GL15 cells.

LSINCT5-miR-451-Rac1 axis affected PI3K/AKT, Wnt/β-catenin and NF-κB pathways

Next, we explored the specific function of Rac1 in GL15 cells. As PI3K/AKT, Wnt/β-catenin and NF-κB pathways are the significant signalling pathways involved in glioma [26–28], we examined whether LSINCT5-miR-451-Rac1 axis could regulate the activation of these three pathways. Western blot was utilized to see the protein accumulation of several well-known proteins of these pathways. As seen in Figure 6(A,B), LSINCT5 silence, miR-451 overexpression, as well as Rac1 silence, was capable of reducing the phosphorylation of PI3K, AKT, p65 and IκBα, and repressing the expression of Wnt3a, Wnt5a and β-catenin. However, Rac1 overexpression led to a contrary impact towards these protein accumulations.

Discussion

LncRNAs are recently-discovered RNA transcripts that are abnormally expressed in various kinds of tumours [5,6]. Numerous researches evidenced that lncRNAs can be used as biomarkers of cancers [4]. LSINCT5 is a stress-regulated lncRNA involved in response to oxidative stress and implicated in cell proliferation and cancer development [29,30]. Here, we designed to see the role of LSINCT5 in glioma cells.

To determine the role of LSINCT5 in GL15 cells, the effect of LSINCT5 knockdown on cell growth and metastasis were firstly analyzed. It was found that LSINCT5 knockdown suppressed cell viability, migration and invasion, and accelerated apoptosis. These data evidenced LSINCT5 as an oncogenic gene in glioma and thereby down-regulating its expression would suppress the development of glioma. LSINCT5 can be dramatically up-regulated in breast and ovarian cancers, and knocking down its expression in cancer-derived cells led to a suppression in cellular proliferation [9]. Likewise, increased LSINCT5 expression was found in gastrointestinal cancer tissues, which was related with the progression of gastric cancer, and knockdown of LSINCT5 significantly inhibited cell metastasis [8]. The findings were in line with our present report, as LSINCT5 expression was also high expressed in glioma tissues. Besides, LSINCT5 exerted as an oncogene and played the tumour-suppressive effect in glioma cells when down-regulating its expression.

In recent years, a novel regulatory mechanism has been widely reported that many lncRNAs can function as ceRNAs to sequester miRNAs, thereby regulating the expression of miRNA targets [11]. In this study, if LSINCT5 can serve as a ceRNA in human glioma was further explored. According to our results, LSINCT5 might regulate miR-451 through directly binding effects, and inhibition of miR-451 abolished the anti-glioma effects, and inhibition of miR-451 abolished the anti-glioma effects.
effect of LSINCT5 silence. Therefore LSINCT5 silence might exert the anti-glioma effect by up-regulating miR-451.

The functions of miR-451 in cancers were widely reported. Increasing the level of miR-451 repressed breast cancer cell proliferation and colony formation but promoted cell apoptosis via repressing HER2, EGFR and MAPK signalling [31]. Besides, miR-451 overexpression inhibited liver cancer cells proliferation through inhibiting IKK-β [32]. As for glioma,
miR-451 could repress glioma by targeting CAB39 and inhibiting PI3K/AKT pathway [15]. The tumour-suppressive effect of miR-451 was observed, but its specific function in GL15 cells should be further studied.

Rac1, a member of the Rho GTPase family, appears to be deregulated in both expression and activity in diverse tumour cells [33]. Some studies reported that Rac1 was up-regulated in glioma and down-regulating Rac1 could greatly inhibit glioma cell growth [34, 35]. In this study, Rac1 was the target of miR-451 which exhibited anticancer effect through negatively regulating Rac1. Thus, Rac1 might be involved in the actions of LSINCT5 and miR-451 in glioma cells. It was reported that Rac1 functioned as a critical mediator of tumour angiogenesis and metastasis through mediating several signal pathways [36]. The PI3K/AKT, Wnt/β-catenin and NF-κB activities regulated by Rac1 were related to many cancer types [37–40]. Since these three signalling pathways are closely related to glioma development [26–28], the effect of LSINCT5-miR-451-Rac1 axis on these pathways in GL15 cells was explored. We found that the pathways were inhibited by LSINCT5 silence, miR-451 overexpression and Rac1 silence. Therefore we speculated that LSINCT5-miR-451-Rac1 axis impacted glioma cells growth and metastasis possibly by indirect mediating PI3K/AKT, Wnt/β-catenin and NF-κB pathways.

In summary, this study illustrated that LSINCT5 knockdown inhibited glioma cells growth and metastasis by up-regulating miR-451. miR-451 overexpression exerted its anti-glioma effects by negative regulating Rac1. LSINCT5 may represent a target for gene therapy in human glioma.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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