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Original Article

MicroRNA-19a promotes glioma cell growth by repressing LRIG1

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Abstract: Growing evidence indicates that deregulation of miRNAs contributes to the development of glioma. In present study, we found that the level of miRNA-19a was significantly elevated in glioma tissues and cell lines. Moreover, down-regulation of miRNA-19a dramatically repressed glioma cell growth *in vitro* and *in vivo*. Meanwhile, the expression of LRIG1, a tumor suppressor in glioma, was increased following miRNA-19a knockdown. Furthermore, luciferase reporter assay confirmed that LRIG1 was a direct target of miRNA-19a. In addition, silencing of LRIG1 could reverse the suppressive effect of miRNA-19a inhibitor. Taken together, our results demonstrated that down-regulation of miRNA-19a could suppress the growth of glioma cells, at least in part, through up-regulating LRIG1.

Keywords: miRNA-19a, LRIG1, glioma, proliferation

Introduction

Malignant gliomas are the most common and incurable brain tumors [1]. Despite the advance in surgery, ionizing radiation and chemotherapy, the prognosis of patients is still poor [2]. Comprehensive understanding of the molecular mechanisms underlying gliomagenesis is fundamental to develop specific therapeutic strategies.

microRNAs (miRNAs) are a class of small non-coding RNAs which negatively regulate gene expression at the post-transcriptional level by directly binding to the 3'untranslated region (3'UTR) of target mRNAs [3, 4]. Accumulating evidence suggests that deregulated miRNAs play critical roles in the initiation and progression of gliomas [5, 6], such as miRNA-21 [7], miRNA-221/222 [8], et al. Previous miRNA profiling data showed that the expression of miRNA-19a was increased during the progression of glioma [6]. However, its biological roles in glioma remain to be elucidated.

LRIG1 (leucine-rich repeats and immunoglobulin-like domains1) is a pan-negative regulator of membrane-bound receptor tyrosine kinases (RTKs) [9-13]. Previous studies showed that LRIG1 was down-regulated in gliomas, whereas ectopic over-expression of LRIG1 was capable of inhibiting glioma cell growth. Accordingly, LRIG1 was proposed to be a tumor suppressor in glioma [13, 14]. Although decreased LRIG1 levels were found to be correlated with malignancy of glioma [14, 15], we failed to establish an association between the mRNA levels of LRIG1 and the World Health Organization (WHO) grades of glioma in our preliminary study (unpublished results). So, we hypothesized that the decreased levels of LRIG1 protein might attribute to a variety of post-transcriptional regulatory mechanisms.

In present study, we found that miRNA-19a was up-regulated in gliomas. Down-regulation of miRNA-19a decelerated glioma cell growth and increased the expression of LRIG1. Moreover, we identified that LRIG1 was a functional target of miRNA-19a in glioma.

Materials and methods

Tissue specimens

This study was approved by the Ethics Committee of Wuhan University. Informed consent was obtained from each patient. A total of 29 glioma samples were collected at the Department of Neurosurgery, Renmin Hospital of Wuhan University. According to the WHO categories, 14 grade I-II tumors, 7 grade III tumors,

Table 1. QPCR primer sequences

	Forward primer (5'-3')	Reverse primer (5'-3')
miR-19a	TGCGCTGTGCAAATCTATGCAAA	CCAGTGCAGGGTCCGAGGT
U6	CTCGCTTCGGCAGCACA	CCAGTGCAGGGTCCGAGGT
LRIG1	GGTGAGCCTGGCCTTATGTGAATA	CACCACCATCCTGCACCTCC
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

and 8 grade IV tumors were included in our study. Five normal brain tissues were obtained from patients who underwent internal decompression surgery for severe traumatic brain injury. Fresh samples were immediately frozen in liquid nitrogen for subsequent RNA extraction.

Cell culture

Human U251, U87, A172, and U118 glioma cell lines were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. These cell lines were cultured in Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37°C in a 5% CO_2 atmosphere.

Oligonucleotides transfection

Synthetic cholesterol-conjugated miRNA-19a inhibitor (antagomir-19a: 5'-UCAGUUUUGCAUAGAUUUGCACA-3') and negative control (antagomir-NC: 5'-CAGUACUUUUGUGUAGUACAA-3') were purchased from Genepharma (Shanghai, China). The small interfering RNA targeting LRIG1 (siLRIG1: 5'-ACTCTCTGAGATTGACCCT-3') and its negative control oligonucleotides (siNC: 5'-ACTACCGTTGTTATAGGTG-3') were synthesized by RiboBio (GuangZhou, China). The previously reported sequences were used [16]. Transfection of oligonucleotides was performed using HiPerFect Transfection Reagent (Qiagen, Germany) according to the manufacturer's instructions. The oligonucleotides were used at a final concentration of 100 nM.

RNA extraction and real-time RT-PCR

Total RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The expression of mature miRNA-19a was determined by quantitative real-time RT-PCR (qRT-PCR) using the SYBR PrimeScript miRNA RT-PCR Kit (TaKaRa, Ohtsu, Japan) according to the manufacturer's protocol. Its expression was normalized to U6 small nuclear RNA. The quantitative LRIG1 detection was performed as described previously [16]. GAPDH was used as an internal control. Their relative expression levels were measured in triplicate on a Prism 7500 Real-Time PCR monitor (Applied Biosystems). All primers were listed in **Table 1**.

Western blot

Western blot was performed as described previously [16]. The following primary antibodies were used: anti-LRIG1 antibody (1:1,000; Cell Signaling Technology, USA) and anti-GAPDH antibody (1:2,000; Santa Cruz Biotechnology, USA).

CCK-8 cell viability assay

Cells were harvested at 48 h post-transfection. They were cultured in 96-well plates (6×10^3 cells per well). Cell viability was determined using Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's protocol.

5-ethynyl-2'-deoxyuridine (EdU) incorporation assay

Proliferating cells were determined using the Cell-Light EdU imaging detecting kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. In brief, cells were incubated with 10 μM EdU for 24 h, then fixed with 4% formaldehyde for 30 min at room temperature. After washing, cells were incubated with 1 × reaction cocktail for 30 min. Subsequently, cell nuclei were stained with Hoechst 33342 (5 μg/ml) for 30 min and visualized under an inverted fluorescence microscope.

Luciferase reporter assay

The putative binding site of miRNA-19a in LRIG1 3'UTR was retrieved from TargetScan (http://www.targetscan.org) and miRanda (http://www.microrna.org/microrna/home.do). A LRIG1 3'UTR fragment containing wild or mutant type of miRNA-19a-binding sequence was clonesd into Xho I/Not I site of psiCHECK-2 (Promega). Cells were cultured in 24-well plates and co-transfected with 100 nM miRNA inhibitors and 1 μg reporter plasmids using Li-

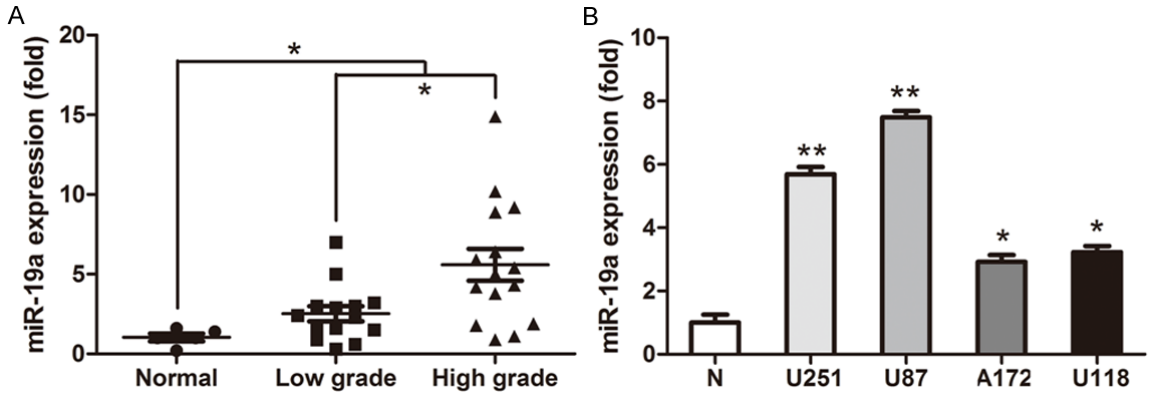

Figure 1. The expression of miRNA-19a in glioma tissues and cell lines. The endogenous expression of miRNA-19a was determined by qRT-PCR in 29 glioma specimens (A) and 4 glioma cell lines (B). The expression of miRNA-19a was normalized to U6 with respect to normal brain tissues. Each reaction was performed in triplicate. Date were represented as mean ± SD. *$P < 0.05$ and **$P < 0.01$.

pofectmine 2000 (Invitrogen). Cells were harvested at 72 h post-transfection and luciferase activity were measured by the Dual-Luciferase Reporter Assay System (Promega).

Tumorigenicity assays in nude mice

All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication Nos. 80-23, revised 1996) and the protocol was approved by Animal Experimentation Ethics Committee, Wuhan University. Female BALB/c nude mice (4~5 weeks old) were purchased from the Center of Experimental Animal, Wuhan University. A total of 2×10^6 U87 cells transfected with antagomir-19a or antagomir-NC were suspended in 100 μl serum free DMEM and then were subcutaneously injected into the right flank of the mice. After 4 weeks, the mice were euthanized. The tumors were removed and weighed.

Statistical analysis

The data were presented as mean ± SD and analyzed using Student's *t* test or one-way ANOVA test. A *P* value < 0.05 was denoted as statistical significance. Statistical analyses were performed using SPSS 19.0 software (SPSS, Chicago, IL, USA).

Results

miRNA-19a was up-regulated in human glioma tissues and cell lines

To investigate whether miRNA-19a was up-regulated in gliomas, we evaluated its levels in 29 glioma samples and 5 normal brain tissues by qRT-PCR. As shown in **Figure 1A**, miRNA-19a significantly increased in high-grade gliomas (WHO III and IV), compared with low-grade gliomas (WHO I and II) as well as normal brain tissues ($P < 0.05$). Next, we examined the levels of miRNA-19a in 4 glioma cell lines (U87, U251, A172, and U118). The data showed that it was aberrantly over-expressed in all 4 glioma cell lines (**Figure 1B**). Taken together, these results suggested that miRNA-19a was frequently up-regulated in human glioma tissues and cell lines.

Down-regulation of miRNA-19a inhibited glioma cell growth

Given that miRNA-19a was significantly up-regulated in glioma, it might function as an oncogene. To explore whether down-regulation of miRNA-19a affected the growth of glioma cells, its loss-of-function study was performed. U87 and U251 cells, which had a relatively high basal level of miRNA-19a, were transfected with miRNA-19a inhibitor (or negative control). The transfection efficiency was determined by qRT-PCR (**Figure 2A**). Then, the transfected cells were subjected to CCK-8 assay. As shown in **Figure 2B**, the glioma cells transfected with antagomir-19a exhibited a remarkable reduction of cell viability as compared to control group ($P < 0.01$). Moreover, EdU proliferation assay indicated that down-regulation of miRNA-19a led to a noticeable decreased EdU-positive cells compared with NC-transfected cells (**Figure 2C**; $P < 0.05$). Furthermore, pre-transfection of antagomir-19a significantly dimin-

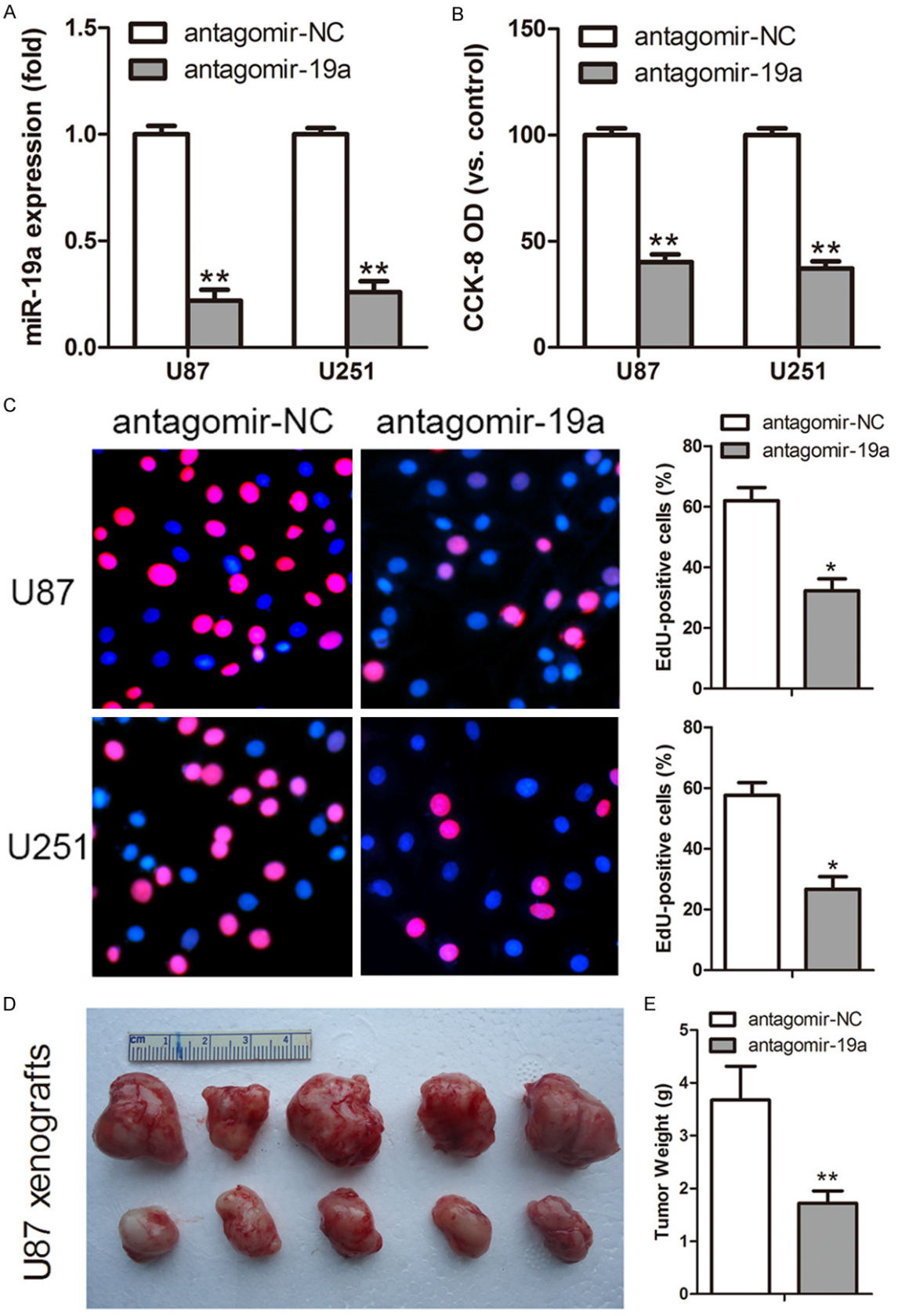

Figure 2. Down-regulation of miRNA-19a inhibited the growth of glioma cells. A. U87 and U251 cells were transfected with 100nM antagomir-19a or 100 nM antagomir-NC. After 48 h, miRNA-19a expression was determined by

qRT-PCR. Data were normalized to U6 with respect to control group. B. Cell viability was analyzed using CCK-8 assay at 72 h post-transfection. Optical density (OD) of control group was set to 100% and the data were normalized to control group. C. Proliferating cells were examined using EdU assay. Representative images were shown (original magnification, 400 ×). The histogram represented the percentage of EdU-positive cell (pink) in each group. At least 200 cells were quantified per well. Data were means ± SD of three independent experiments. D. Photograph illustrated the gross morphology of U87 xenografts. Upper panel: antagomir-NC group (n = 5). Lower panel: antagomir-19a group (n = 5). E. Average weight of tumors was plotted as mean ± SD. *P < 0.05 and **P < 0.01.

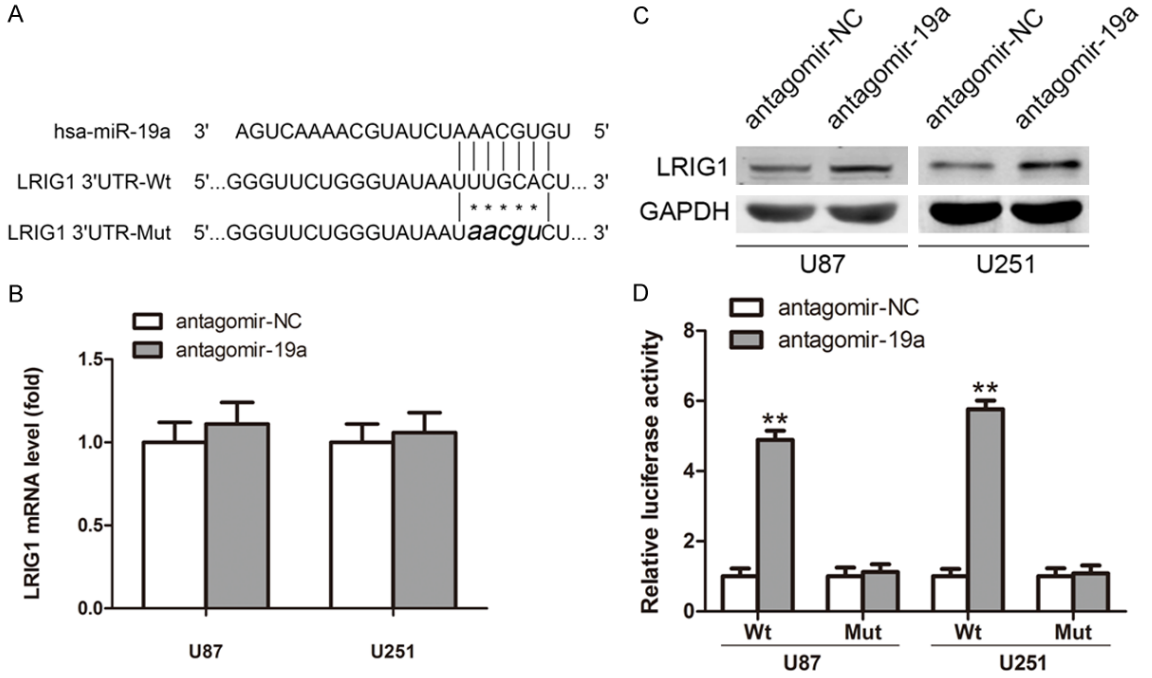

Figure 3. LRIG1 was a direct target of miRNA-19a. A. The binding site of miRNA-19a was predicted at position 1065-1071 of 3'UTR of LRIG1. Mutation was generated at the seed region of LRIG1 3'UTR (asterisks). B. Glioma cells were transfected with 100nM antagomir-19a (or negative control). After 48 h, qRT-PCR was performed to detect the level of LRIG1 mRNA. Data were normalized to GAPDH with respect to control group. C. Total proteins were analyzed by immunoblotting. Representative bands were represented. GAPDH was used as a loading control. D. Cells were co-transfected with antagomirs and reporter plasmids as indicated. After 72 h, the relative luciferase activity (firefly/Renilla) was measured. Values were normalized to that of control group. Each experiment was repeated at least three times. Data were represented as mean ± SD. *P < 0.05 and **P < 0.01.

ished the growth of U87 xenografts in nude mice (**Figure 2D**). The tumor weight of antagomir-19a group was dramatically decreased as compared to that of control group (**Figure 2E**; P < 0.05). These data provided strong evidence that suppression of miRNA-19a decelerated glioma cell growth both *in vitro* and *in vivo*.

LRIG1 was a direct target of miRNA-19a

To investigate the mechanisms by which down-regulation of miRNA-19a suppressed glioma cell growth, putative targets of miRNA-19a were retrieved from TargetScan and miRanda database. Among them, LRIG1 attracted our attention as it was a pan-negative regulator of receptor tyrosine kinases [9-13]. The potential binding site of miRNA-19a in LRIG1 3'UTR was illustrated in **Figure 3A**. To determine whether the expression of LRIG1 was affected by miRNA-19a, we examined the levels of LRIG1 mRNA and protein in glioma cells when miRNA-19a was down-regulated. As shown in **Figure 3B**, no obvious change of LRIG1 mRNA was observed in U87 or U251 cells following miRNA-19a knockdown, however, the protein levels of LRIG1 were obviously increased (**Figure 3C**). Furthermore, luciferase reporter assay was performed to identify whether miRNA-19a directly interacted with the 3'UTR of LRIG1. For this purpose, reporter plasmids containing the

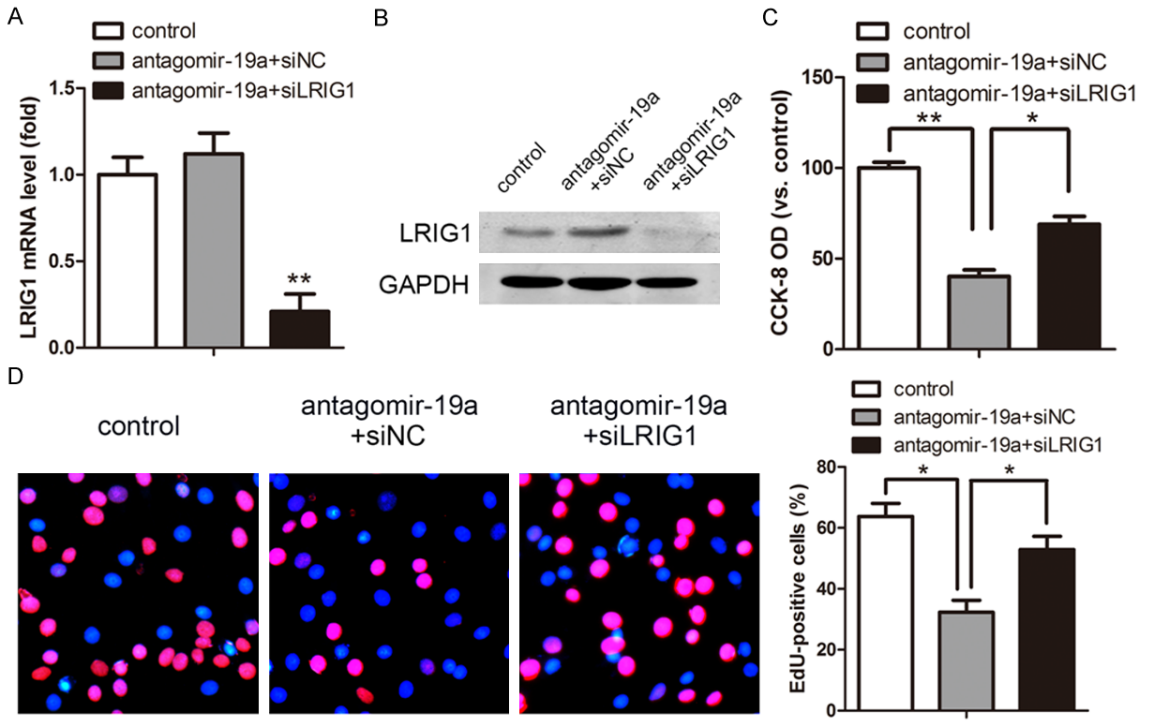

Figure 4. LRIG1 contributed to the suppressive effect of antagomir-19a on U87 cells. A. The levels of LRIG1 mRNA were examined by qRT-PCR at 48 h post-transfection. Data were normalized to GAPDH with respect to control group. B. The protein levels of LRIG1 in transfected U87 cells were detected by western blot analysis. GAPDH was used as an internal control. C. Cell viability was analyzed using CCK-8 assay at 72 h post-transfection. D. The cell proliferation of U87 cells after transfection was determined by EdU assay. Representative photographs were shown (original magnification, 400 ×). At least 200 cells were counted per well. Data were means ± SD of three independent experiments. *$P < 0.05$ and **$P < 0.01$.

predicted binding site and its mutant form were constructed (**Figure 3A**). As shown in **Figure 3D**, down-regulation of miRNA-19a significantly increased the luciferase activity of the reporter containing the wild type (Wt) of putative binding site ($P < 0.01$), whereas the luciferase activity of mutant reporter (Mut) was nearly unaffected. These data revealed that miRNA-19a suppressed the expression of LRIG1 by directly binding to its 3' UTR.

Knockdown of LRIG1 could attenuate the inhibitory effect of antagomir-19a

Because LRIG1 was a direct target of miRNA-19a, we wondered whether LRIG1 contributed to the suppressive effect of antagomir-19a on glioma cells. To address this issue, antagomir-19a and siLRIG1 were co-transfected into U87 cells. After 48h, the mRNA and protein levels of LRIG1 were confirmed by qRT-PCR and western blot analysis, respectively. As shown in **Figure 4A**, **4B**, the expression of LRIG1 was significantly down-regulated in the U87 cells that were co-transfected with antagomir-19a and siLRIG1. Meanwhile, both cell viability and cell proliferation assays indicated that the inhibitory effect of antagomir-19a was attenuated in the presence of siLRIG1 (**Figure 4C**, **4D**; $P < 0.05$). Similar results were obtained when U251 cells were co-transfected with antagomir-19a and siLRIG1 (data not shown). These results suggested that LRIG1 contributed to the growth-inhibitory effect of antagomir-19a.

Discussion

The oncogenic miRNA-19a has been reported to be frequently over-expressed in various types of cancers, such as lymphoma, breast cancer, cervical carcinoma [17-19]. In present study, we demonstrate that miRNA-19a is aberrantly up-regulated in gliomas and its knockdown could inhibit glioma cell growth *in vitro* and *in vivo*. Our results support that it is a potential oncogene in gliomas.

It is well recognized that miRNAs are potent negative regulators of protein coding genes.

Previously, PTEN was identified as a target of miRNA-19a in lymphoma [17]. As we known, PTEN is a pivotal tumor suppressor gene which modulates EGFR pathway by repressing PI3K [20]. However, the mutation of PTEN is observed in half of malignant gliomas [21]. Interestingly, down-regulation of miRNA-19a is able to suppress the growth of U87 and U251 cells even though PTEN is mutated in them [22], suggesting that the growth-inhibitory effect of antagomir-19a may be independent of PTEN.

Malignant gliomas are characterized by EGFR amplification and mutations. In primary glioblastoma, aberrant growth factor signaling is reported in approximately 40~50% of cases [21]. In normal tissues, the strength and duration of EGFR signals are tightly restrained by several negative regulators, such as LRIG1, RALT (receptor-associated late transducer), and SOCS4/5 (suppressor of cytokine signaling 4/5) [23]. In present study, we focus on LRIG1 as it is frequently decreased in gliomas [14]. We have identified that miRNA-19a negatively regulates the expression of LRIG1 by directly binding to its 3'UTR. Our results suggest that miRNA-19a may compromise the negative feedback loop in EGFR pathway by repressing LRIG1, contributing to a permissive environment for gliomagenesis.

Previous study indicated that ectopic overexpression of LRIG1 suppressed glioma cell growth by inhibiting EGFR signaling pathway [14]. Moreover, the soluble extracellular segment of LRIG1 (sLRIG1) was found to have a potently suppressive effect on glioma cell growth *in vitro* and *in vivo* [24, 25]. More recently, He *et al*. has reported that gambogic acid could repressed U87 cell growth by inducing LRIG1 [26]. These studies suggest that restoration of LRIG1 may be a potential treatment for gliomas. In our study, we demonstrate that LRIG1 can be induced following miR-19a knockdown, while silencing LRIG1 is able to attenuate the growth-inhibitory effect of antagomir-19a, indicating that this effect may be partially mediated by LRIG1. To clarify the full mechanisms behind it, however, additional targets of miRNA-19a remain to be identified.

In conclusion, our study demonstrates that miRNA-19a knockdown represses glioma cell growth, at least in part, through up-regulating the level of LRIG1.

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Disclosure of conflict of interest

None.

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