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Knockdown of Long Noncoding RNA LUCAT1 Inhibits Cell Viability and Invasion by Regulating miR-375 in Glioma

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Recently, long noncoding RNAs (lncRNAs) have emerged as new gene regulators and prognostic markers in several cancers, including glioma. Here we focused on lncRNA LUCAT1 on the progression of glioma. qRT-PCR was used to determine the expression of LUCAT1 and miR-375 in glioma tissues and cells. MTT and Transwell invasion assays were performed to determine the function of LUCAT1 in glioma progression. The bioinformatics tool DIANA was used to predict the targets of LUCAT1. Pearson's correlation analysis was performed to explore the correlation between LUCAT1 and miR-375. In the present study, we showed that LUCAT1 was substantially upregulated in glioma tissues and cells. LUCAT1 inhibition significantly suppressed the proliferation and invasion of glioma cells. Subsequently, DIANA showed that miR-375 was predicted to contain the complementary binding sites to LUCAT1. Luciferase reporter assay showed that miR-375 directly targeted LUCAT1. In addition, we found that miR-375 was downregulated in glioma tissues and negatively correlated with LUCAT1 expression in glioma tissues. Furthermore, the results showed that miR-375 could rescue the function of LUCAT1 in glioma progression. The lncRNA LUCAT1 was critical for the proliferation and invasion of glioma cells by regulating miR-375. Our findings indicated that LUCAT1 might offer a potential novel therapeutic target for the treatment of glioma.

Key words: Long noncoding RNAs (lncRNAs); LUCAT1; miR-375; Glioma; Progression

INTRODUCTION

Glioma is the most common malignant tumor of the nervous system, accounting for about 40%–60% of all intracranial tumors[1]. According to the WHO pathologic grading system, gliomas are further categorized into four grades (I to IV)[2]. Despite the technical advances in surgery, chemotherapy, and radiotherapy, the prognosis of glioma is still poor. The survival time of glioblastoma multiforme (GBM; grade IV) patients is only approximately 1 year[3,4]. Therefore, it is urgent to understand the molecular mechanism of glioma carcinogenesis in order to unearth reliable diagnostic and therapeutic targets for glioma.

Recently, accumulating data have indicated that the vast majority of genomes are transcribed into noncoding RNAs (ncRNAs), which are classified as small ncRNAs (less than 200 nucleotides) and long ncRNAs (lncRNAs; >200 nucleotides) based on their length[5,6]. lncRNAs are defined as a class of evolutionarily conserved ncRNAs with no or limited protein-coding potential[7]. Aberrant expression levels of lncRNAs have been correlated to various malignant biological processes, including carcinogenesis[8]. For example, Zhang et al. showed that increased expression of lncRNA MALAT1 promoted renal cancer cell proliferation and metastasis and correlated with poor prognosis[9]. Li et al. revealed that CASC2 suppressed the proliferation of gastric cancer cells by regulating the MAPK signaling pathway[10]. Sun et al. showed that LUCAT1 was associated with poor prognosis in human non-small cell lung cancer and regulated cell proliferation via epigenetically repressing p21 and p57 expression[11]. However, the expression and function of lncRNA LUCAT1 are still unclear in glioma's progression.

As a class of small ncRNAs, microRNAs (miRNAs), which have been extensively studied, are involved in carcinogenesis of various tumors[12]. Among these miRNAs, miR-375 was found to play important roles in tumor progression. For example, Fu et al. showed that miR-375

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was downregulated in esophageal cancer, and low miR-375 expression could predict unfavorable prognosis in esophageal cancer patients[13]. Ding et al. showed that miR-375 was frequently downregulated in gastric cancer and inhibited cell proliferation by targeting JAK2[14]. Moreover, Chang et al. showed that miR-375 was significantly decreased in glioma and associated with advanced clinical features and poor overall survival of patients[15]. Recently, competing endogenous RNA (ceRNA) hypothesis showed that lncRNAs served as miRNA sponges to modulate the expression of miRNA target genes, thus participating in the pathogenesis and development of cancers[16]. However, whether LUCAT1 could interact with miR-375 to regulate the tumorigenesis of glioma remains unknown.

In the present study, we explored expression and function of LUCAT1 in glioma. Our data showed that LUCAT1 expression was remarkably upregulated in glioma tissues and cells. LUCAT1 inhibition suppressed glioma cell viability and invasion in vitro, implying a possible role of lncRNA LUCAT1 as an oncogene in glioma. Moreover, we demonstrated that LUCAT1 was involved in the progression of glioma by targeting miR-375.

MATERIALS AND METHODS

Patient Samples

Thirty-eight glioma specimens of varying grades were collected from patients who were treated at the Department of Neurosurgery, Zhumadian Central Hospital (Henan, P.R. China). None of them received chemoradiotherapy before the surgery. There were 21 males and 17 females, including 13 cases of low-grade glioma (WHO I–II grade) and 25 cases of high-grade glioma (WHO III–IV grade). In addition, seven normal brain tissues were collected from intracranial decompression for traumatic brain injury. All cases received surgeries and were confirmed pathologically. The resected tissues were immediately preserved in liquid nitrogen and then transferred to −80°C. The tumor tissues were histologically confirmed by the pathologists. All patients signed informed consent, and the experimental protocol was approved by the hospital's ethics committee.

Cell Culture

Normal human astrocytes (NHAs) and human glioma cell lines (LN229, U251, SNB19, U87, and H4) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 μg/ml streptomycin (Sigma-Aldrich) at 37°C in a humidified incubator with 5% CO_2.

Cell Transfection

miR-375 mimics, miR-375 inhibitors, siRNA specifically targeting LUCAT1 (si-LUCAT1), and scrambled negative control siRNA (si-NC) were chemically synthesized from GenePharma Co., Ltd. (Shanghai, P.R. China). Glioma cells in logarithmic phase were transfected with miR-375 mimics, miR-375 inhibitors, si-LUCAT1, and their controls using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative Real-Time PCR

Total RNA was isolated from tumor specimens and cancer cell lines using TRIzol reagent (Invitrogen). The purity of RNA was examined by spectrophotometry, and the first-strand cDNA was synthesized using reverse transcription reagents (Applied Biosystems, Carlsbad, CA, USA) or the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. qRT-PCR was performed using SYBRH Select Master Mix for CFX (Invitrogen) and the CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA). All results were normalized to the expression of GAPDH. Quantitative analysis was calculated by using the $2^{-\Delta\Delta}$Ct method.

MTT Assay

Transfected cells (1×10^4 /well) were seeded into 96-well plates and incubated for 24, 48, 72, and 96 h, respectively. Then 20 μl of 5 mg/ml MTT reagent (Sigma-Aldrich) was added to each well and incubated for 4 h. Absorbance was measured at 450 nm on a microplate reader (Bio-Rad). A growth curve was generated with time and absorbance on the horizontal and vertical axes, respectively.

Transwell Invasion Assay

Transfected cells were seeded onto Transwell 24-well plates (BD Biosciences, San Jose, CA, USA) coated with diluted Matrigel (Sigma-Aldrich), with serum-free media containing 5% FBS used as a chemoattractant. After 48 h of incubation, the medium was removed, and the chambers were washed twice with PBS. The cells on the upper side of the inserts were softly scraped; those migrated to the lower surface were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Microphotographs were obtained on an immunofluorescence microscope (Olympus, Tokyo, Japan).

Luciferase Reporter Assays

The interaction between LUCAT1 and miR-375 was predicted by DIANA tools. To test the binding site of

LUCAT1 and miR-375, wild-type and mutated LUCAT1 (wt-LUCAT1 and mut-LUCAT1 containing mutation in the predicted binding sites of miR-375) luciferase reporter plasmids were constructed. Dual-luciferase reporter assay was performed on the wt-LUCAT1 or mut-LUCAT1 in cells using the dual-luciferase reporter assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and normalized against *Renilla* luciferase activity 48 h after transfection.

Statistical Analysis

Data analysis was performed using SPSS17.0 (IBM, Chicago, IL, USA). All data were represented as means ± standard deviation (SD). The significance of differences between groups was assessed by Student's *t*-test and chi-square test as appropriate. A value of $p < 0.05$ was considered as statistically significant.

RESULTS

LUCAT1 Was Increased in Human Glioma Tissues

To determine the role of lncRNA LUCAT1 in glioma progression, we explored the expression of LUCAT1 in glioma tissues and normal brain tissues via qRT-PCR. The results revealed that LUCAT1 expression was significantly upregulated in glioma tissues compared to normal tissues and correlated with tumor stage of glioma patients ($p < 0.05$) (Fig. 1A). In addition, we explored the expression of LUCAT1 in the NHAs and human glioma cell lines (LN229, U251, SNB19, U87, and H4). Our data showed that the expression of LUCAT1 was considerably increased in the glioma cell lines compared to NHAs ($p < 0.05$) (Fig. 1B). These data suggested that LUCAT1 might be involved in the development of glioma.

LUCAT1 Inhibition Suppressed Glioma Cell Viability and Invasion

To examine the role of LUCAT1 in glioma progression, U87 cells were transfected with si-LUCAT1 or control siRNA (si-NC). siRNA knockdown efficiency of LUCAT1 was confirmed by qRT-PCR (Fig. 2A). Cell proliferation was then measured by MTT. The MTT assay showed that LUCAT1 suppression remarkably suppressed cellular viability of U87 cells compared to the si-NC groups (Fig. 2B). To explore the effects of LUCAT1 on glioma cell invasion, we performed Transwell invasion assay. The results showed that knockdown of LUCAT1 significantly decreased the invasion abilities of U87 cells (Fig. 2C). These finding suggested that LUCAT1 inhibition suppressed glioma cell viability and invasion in vitro.

miR-375 Expression Was Directly Regulated by LUCAT1

Increasing evidence has shown that lncRNA might serve as a ceRNA or molecular sponge to regulate the expression and function of miRNA[17]. Thus, the bioinformatics tool DIANA was used to predict target miRNA regulated by LUCAT1 in glioma. As shown in Figure 3A, miR-375 was selected as the potential target of LUCAT1. The luciferase reporter assay validated the binding between miR-375 and LUCAT1 3′-UTR (Fig. 3B). Furthermore, we determined miR-375 expression in glioma examples. The results showed that miR-375 expression was significantly downregulated in glioma samples and inversely correlated with the expression of LUCAT1 in the glioma samples (Fig. 3C and D). We further analyzed miR-375 expression in LUCAT1 knockdown glioma cells and found that miR-375 was boosted after LUCAT1 downregulation (Fig. 3E), whereas ectopic overexpression of miR-375

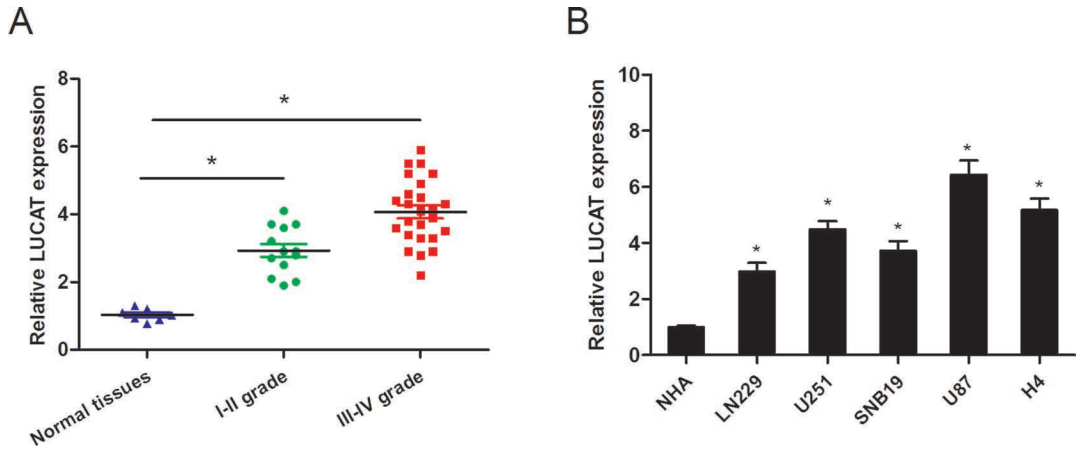

Figure 1. Long noncoding RNA (lncRNA) LUCAT1 expression was upregulated in glioma tissues and cell lines. (A) Quantitative real-time (qRT)-PCR analysis of LUCAT1 expression in 38 clinical glioma tissues (13 grades I–II, 25 grades II–IV) compared with 7 normal brain samples. (B) qRT-PCR analysis of LUCAT1 expression in normal human astrocytes (NHAs) and five glioma cell lines (LN229, U251, SNB19, U87, and H4). *$p < 0.05$.

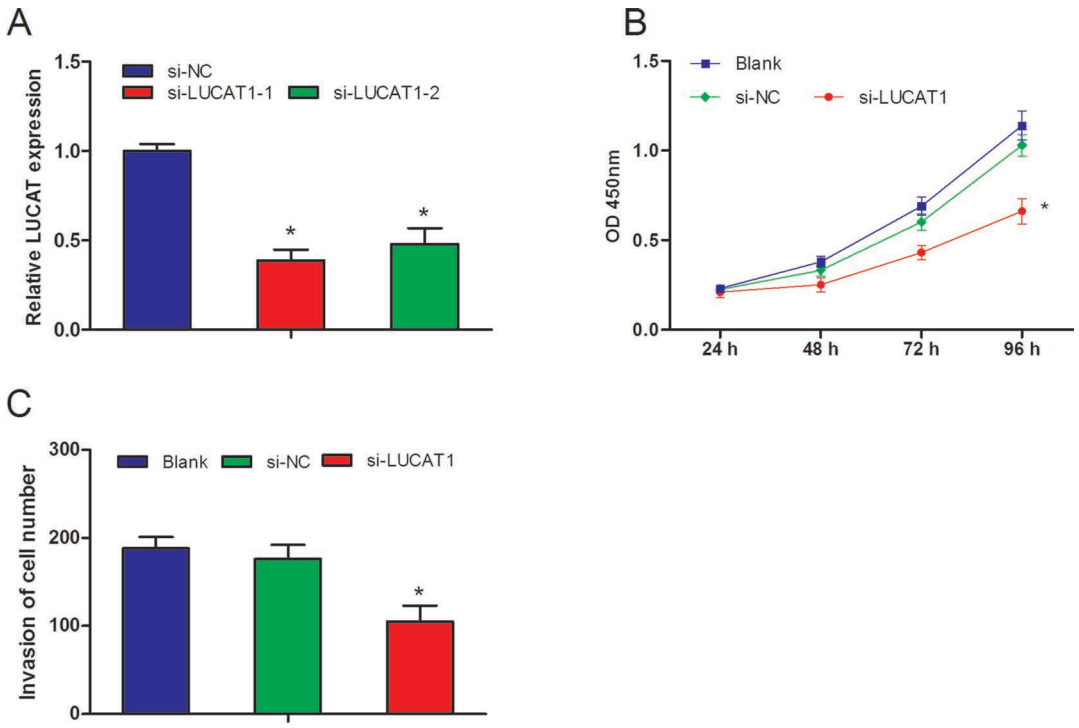

Figure 2. Downregulation of LUCAT1 inhibited glioma cell proliferation and invasion in vitro. (A) qRT-PCR analysis of LUCAT1 expression in U87 cells after transfection by si-LUCAT1 or si-NC. (B) Cell proliferation of U87 cells after si-LUCAT1 or si-NC transfection was determined by MTT assay. (C) Cell invasion of U87 cells after si-LUCAT1 or si-NC transfection was determined by Transwell invasion assay. *$p < 0.05$.

did not affect LUCAT1 expression (Fig. 3F). Thus, these findings suggested that LUCAT1 directly interacted with miR-375 and negatively regulated miR-375 expression in glioma cells.

miR-375 Reversed the Effect of LUCAT1 on Glioma Cell Proliferation and Invasion

To explore whether LUCAT1 exerts biological functions through miR-375, we performed rescue experiments by inhibiting miR-375 expression in LUCAT1-inhibited cells (Fig. 4A). As shown in Figure 4B, the MTT assay showed that LUCAT1 knockdown significantly inhibited U87 cell proliferation, whereas miR-375 inhibition partially rescued the reduction of proliferation. Furthermore, the miR-375 inhibitor reversed the tumor suppression of LUCAT1 knockdown on glioma cell invasion (Fig. 4C). Collectively, those data revealed that inhibition of miR-375 abrogated the suppression of proliferation and invasion of glioma cells induced by depletion of LUCAT1.

DISCUSSION

Accumulating evidence has revealed the critical functions of lncRNAs in regulating many physiological and pathological processes of tumors including glioma, and elucidated the underlying molecular mechanisms by which lncRNAs function in numerous malignancies[18]. For example, Wang et al. reported that upregulation of the lncRNA CRNDE contributed to glioma progression and promoted glioma cell growth and invasion through mTOR signaling[19]. Gao et al. showed that lncRNA ZFAS1 was an unfavorable prognostic factor and promoted glioma cell progression by activation of the Notch signaling[20]. Recently, LUCAT1 was reported to exert oncogenic properties in the progression of NSCLC[11]. However, the function and molecular mechanism of LUCAT1 in glioma progression remain unclear. In our study, we found that LUCAT1 expression was significantly increased in glioma tissues and cell lines. LUCAT1 suppression decreased glioma cell proliferation and invasion in vitro.

Recently, ceRNA hypothesis has been extensively proposed, and an increasing number of studies have confirmed the interaction between lncRNA and miRNA in cancers. For example, Li et al. revealed that lncRNA NEAT1 promoted human clear cell kidney carcinoma progression through the negative regulation of miR-129-5p[21]. Cui et al. found that lncRNA HOXA11-AS functioned as a ceRNA to regulate ROCK1 expression by sponging miR-124-3p in osteosarcoma[22]. In our study, based on a bioinformatics prediction analysis, we found there was an interaction

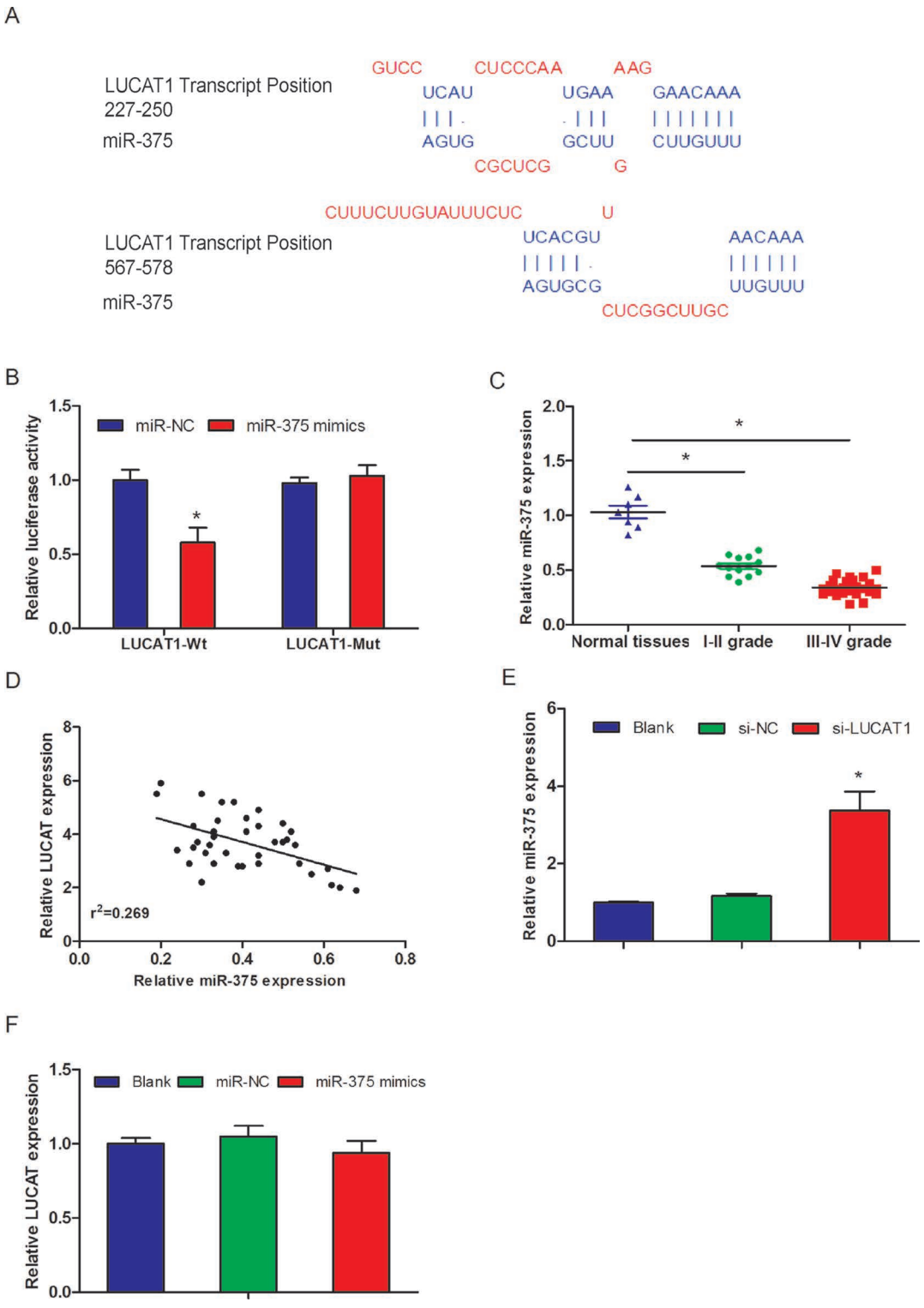

Figure 3. miR-375 expression was directly regulated by LUCAT1. (A) The predicted binding sites between LUCAT1 and miR-375. (B) Luciferase reporter assay validated the binding between miR-375 and LUCAT1 3′-UTR. (C) miR-375 was decreased in glioma tissues compared to normal brain tissues. (D) Pearson's correlation analysis revealed the correlations between LUCAT1 and miR-375 expression in glioma tissues. (E) miR-375 expression in LUCAT1-inhibited U87 cells was determined by qRT-PCR. (F) LUCAT1 expression in miR-375-overexpressed U87 cells was determined by qRT-PCR. *$p<0.05$.

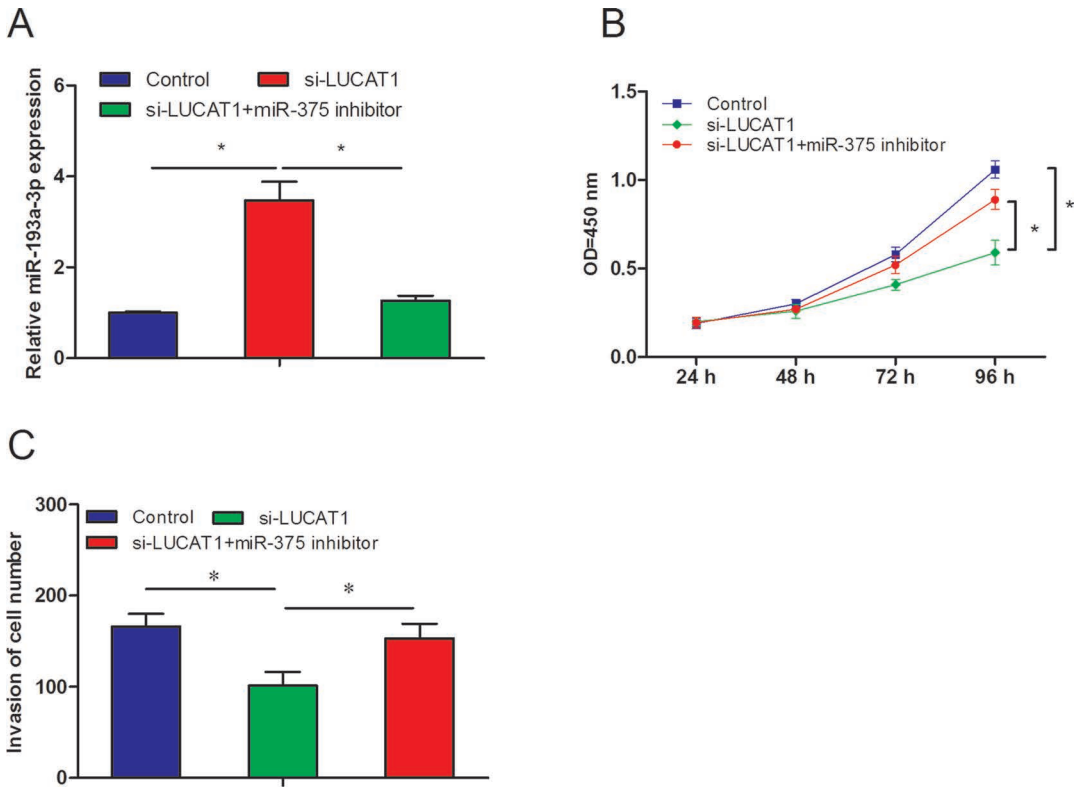

Figure 4. miR-375 reversed the effect of LUCAT1 on glioma cell proliferation and invasion. (A) miR-375 expression was determined by qRT-PCR in U87 cells transfected with si-LUCAT1 and miR-375 inhibitor. (B) MTT assay in U87 cells transfected with si-LUCAT1 and miR-375 inhibitor. (C) Transwell invasion assays in U87 cells transfected with si-LUCAT1 and miR-375 inhibitor. *$p<0.05$.

between LUCAT1 and miR-375. The luciferase reporter assay validated the binding between miR-375 and LUCAT1 3′-UTR. qRT-PCR showed that miR-375 was downregulated in glioma tissues and negatively correlated with LUCAT1 expression in glioma tissues. Furthermore, our data showed that miR-375 expression was negatively regulated by LUCAT in glioma cells. In addition, functional analysis revealed that miR-375 could reverse the effect of LUCAT1 on glioma cell proliferation and invasion. These results suggested that LUCAT1 acted as a molecular sponge of miR-375 to regulate glioma cell viability and invasion.

In summary, the present study demonstrated that lncRNA LUCAT1 could act as an oncogenic lncRNA that promotes glioma tumorigenesis via regulating miR-375, suggesting LUCAT1 could act as a novel therapeutic target for the treatment of glioma.

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