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miR-135a inhibits glioma cell proliferation and invasion by directly targeting FOXO1

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Abstract. – OBJECTIVE: To investigate the potential function of miR-135a in glioma.

PATIENTS AND METHODS: A total of 50 pairs of glioma tissue samples and para-carcinoma tissue samples were collected. Human glioma cell line (U251) and normal human astrocyte (NHA) were cultured. The expression of RNA and protein was detected by quantitative Real time-polymerase chain reaction (qRT-PCR) and Western blot, respectively. Cell proliferation assay and transwell assay were used to detect the activities of proliferation and invasion. Luciferase reporter assays were carried out to determine the binding efficiency between forkhead box O1 (FOXO1) and miR-135a in U251 cells.

RESULTS: qRT-PCR results showed that miR-135a expression was significantly reduced while FOXO1 was up-regulated in glioma tissues. miR-135a overexpression in U251 cells could prominently inhibit proliferation and invasion according to the transwell assays. Moreover, FOXO1 was recognized as the target for miR-135a and may partially reverse the functions of miR-135a in U251 cells.

CONCLUSIONS: We showed that miR-135a inhibits glioma cell proliferation and invasion by down-regulating the target gene FOXO1.

Key Words:
miR-135a, Proliferation, Invasion, FOXO1, Glioma.

Introduction

Glioma, an aggressive kind of brain cancers, has high rates of mortality. Glioma is characterized by necrosis, aggressive growth, dismal prognosis and so on[1]. Currently, the main therapies for glioma include surgical resection, oral alkylating agents and concurrent radiation with temozolomide (TMZ)[2]. Despite of great advances in therapeutic interventions of glioma, the prognosis of patients with glioma still remains poor[3]. The effects of these strategies are not very satisfactory due to low sensitivity to the therapeutic agents, high invasiveness and uncontrolled cell proliferation of glioma cells[4]. So far, the mechanism and etiology of glioma are still understood poorly. Thus, further researches aimed to explore the underlying mechanism of glioma and overcome the therapeutic challenges are necessary. Previous studies[5,6] have demonstrated that the progression of many malignant tumors has been related to aberrant microRNA (miRNA) expression. MiRNAs, a series of endogenous small non-coding RNAs, could modulate the expressions of target gene *via* base pairing with the 3'UTR of miRNA[7]. Accumulating evidence has indicated that altered miRNA expressions can influence tumor progression. Sun et al[8] found that miR-4465 suppressed cell proliferation and metastasis in non-small cell lung cancer by targeting the oncogene EZH2 directly. Wei et al[9] revealed that miR-215 could enhance cell migration and invasion via regulating retinoblastoma tumor suppressor gene 1 in glioma. Zhu et al[10] found that miR-30a functioned as tumor suppressor in prostate cancer and inhibited the cell proliferation and invasion by down regulating SIX1. However, the effects of miR-135a on modulating glioma cell proliferation and invasion need to be fully elucidated. Considering the link between the different miRNAs with the development of various tumors, miRNAs have been suggested as potential targets of anticancer therapeutic strategies.

Forkhead box O1 (FOXO1), one of the members of the forkhead box (FOX) family, is also known as the forkhead rhabdomyosarcoma transcription factor (FKHR)[11,12]. FKHR plays important roles in the regulation of various fundamental biological processes, including cell apoptosis, differentiation, proliferation and energy metabolism[13,14].

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Recently, the molecular regulations of FOXO1 are widely studied in multiple tumors. Previous studies[15] indicated that FOXO1 could suppress tumorigenesis of pancreatic cancer; however, the inverse results were discovered in glioblastoma[16]. Although there have been several studies that linked the expression of FOXO1 to glioma[17], no one of them investigated the complex functions of FOXO1 and miR-135a in the occurrence and development of glioma. Our current research aimed to investigate the correlation between miR-135a and FOXO1 in glioma.

Patients and Methods

Patient Specimens

50 pairs of glioma tissue samples and para-carcinoma tissue samples were collected from Jining No. 1 People's Hospital from 2015 to 2017. All the obtained specimens were pathologically confirmed and all the patients received no anticancer therapy. Liquid nitrogen was used to snap-freeze the tissue samples. Next, the samples were stored at -80°C for further investigations. Additionally, each of the involved patients offered the written informed consent for research purposes. This study was approved by the Ethical Committee of Jining No. 1 People's Hospital.

Cell Lines and Cell Cultures

Human glioma cell line (U251) and normal human astrocyte (NHA) were bought from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All the cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). In addition, all the incubations were in an atmosphere with 5% CO_2 at 37°C.

Cell Transfection

MiR-135a mimics, inhibitors and scramble negative control (NC) as well as the specific FOXO1 siRNA were purchased from RiboBio (Guangzhou, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was applied to transfect them in U251 respectively for further research purposes.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA in all of the tissues and cells. Then, Revert Aid First Strand complementary Deoxyribose Nucleic Acid (cDNA) Synthesis kit (Thermo Fisher, Waltham, MA, USA) was used to synthesize cDNA. SYBR Premix Ex Taq II kit (TaKaRa, Otsu, Shiga, Japan) was used to carry out qRT-PCR. The conditions for the cycling were as follows: 95°C (5 min), denaturation for 40 cycles at 95°C (15 s), 60°C (40 s), and 72°C (30 s). MiR-135a and FOXO1 miRNA relative expressions were normalized to U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. The relative expression levels of genes were evaluated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were shown in Table I.

Cell Proliferation Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) was performed to determine the proliferation ability of U251 cells. The U251 cell lines were seeded in 96-well plates before that culture medium with 10% fetal bovine serum (FBS) was added in each well. 24 h after the incubation, the culture medium was removed and the serum-free medium was added instead. Subsequently, miR-135a mimics or inhibitor were

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-135a forward	5'-TTTCAGCTGGGGAGTGATTG-3'
miR-135a reverse	5'-GCAATCTCTGTGAATGGGTCA-3'
U6 forward	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse	5'-AACGCTTCACGAATTTGCGT-3'
FOXO1 forward	5'-CCAGCCCAAACTACCAAAAATA-3'
FOXO1 reverse	5'-GAGGAGAGTCAGAAGTCAGCAAC-3'
GAPDH forward	5'-GACTCATGACCACAGTCCATGC -3'
GAPDH reverse	5'-AGAGGCAGGGATGATGTTCTG-3'

U6: small nuclear RNA, snRNA; FOXO1: Forkhead box O1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

respectively transfected into the U251 cells. After the cells grew for 12, 24, 48, and 72 h, respectively, MTT solution was added into each well. After further incubation for 4 h, supernatants were discarded. Thereafter, dimethyl sulfoxide (DMSO) (AppliChem, Darmstadt, Germany) was added into each well for 10 min incubation. The growth of U251 cell as a function of time was plotted by detecting the absorbance at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell Assay

The invasion capacity of U251 cells treated with miR-135a mimics or inhibitor was detected by transwell assays. In brief, matrigel was added to upper chamber for invasion assay to separate the two chambers of the transwell chambers (Corning, Corning, NY, USA). U251 cells with serum-free DMEM medium were placed onto top chamber while medium containing 10% fetal bovine serum (FBS) was placed in each bottom chamber as chemoattractant. Then, the above system was incubated for 48 h at 37°C with 5% CO_2. After that, the uninvaded cells on the top chambers were discarded with cotton swabs and the cells that invaded to the bottom side were fixed and stained with ethanol and crystal violet respectively. The number of the invaded U251 cells at least five random fields was counted under optical microscope (Olympus, Tokyo, Japan).

Western Blot

Total protein from glioma tissues and cell samples were lysed and extracted using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). The RIPA cell lysis buffer contained phosphatase inhibitors and proteinase. The concentrations of protein samples were evaluated by a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Afterwards, the obtained total proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Before that, the membrane was blocked in TBST for 2 h with 5% non-fat dry milk. Then, it was incubated with primary anti-bodies at 4°C overnight, and washed with Tris-buffered saline-Tween (TBST), followed by secondary antibody incubation for 2 h at room temperature. Primary antibodies were listed as follows: anti-FOXO1 (1:500; ab70208; Abcam, Cambridge, MA, USA); anti-GAPDH (1:500; ab181603; Abcam, Cambridge, MA, USA). The secondary antibody was Anti-Rabbit IgG (1:4000; ab191866; Abcam, Cambridge, MA, USA). Protein levels were measured by chemiluminescent detection system (Beyotime, Shanghai, China). GAPDH was used as an internal reference.

Luciferase Reporter Assay

Luciferase reporter assays were carried out to determine the binding efficiency between FOXO1 and miR-135a in U251 cells. The wild-type FOXO13'-UTR containing the target site of miR-135a was amplified. Meanwhile, the mutant FOXO1 3'-UTR was synthesized and amplified as well. The amplified FOXO1-3'-UTR-WT or corresponding FOXO1-3'-UTR-MUT was inserted into a pGL3 luciferase vector (Promega, Madison, WI, USA). U251 cells were co-transfected with FOXO1-3'-UTR-WT or FOXO1-3'-UTR-MUT luciferase reporter vector and miR-135a mimics by Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The luciferase activities were assessed by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) at 48 h post-transfection.

Statistical Analysis

The experiments involved in the current study were carried out in three independent duplicates. Statistical Product and Service Solutions (SPSS) version 18.0 (SPSS Inc., Chicago, IL, USA) were used for statistical analysis with Student's *t*-tests. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Data are presented as the mean ± SD. The differences were identified as statistically significant when $p<0.05$.

Results

MiR-135a Expression Reduced and FOXO1 Expression is Up-regulated in Glioma

First of all, we detected the miR-135a and FOXO1 expressions in 50 paired glioma tissue samples and the adjacent normal tissue samples as well as one glioma cell line respectively. The qRT-PCR results showed that the miR-135a expressions in glioma tissue samples were significantly decreased in contrast with that in the matched para-carcinoma tissues (Figure 1A). At the same time, we also compared the miR-135a expression in glioma cell line to that in the normal

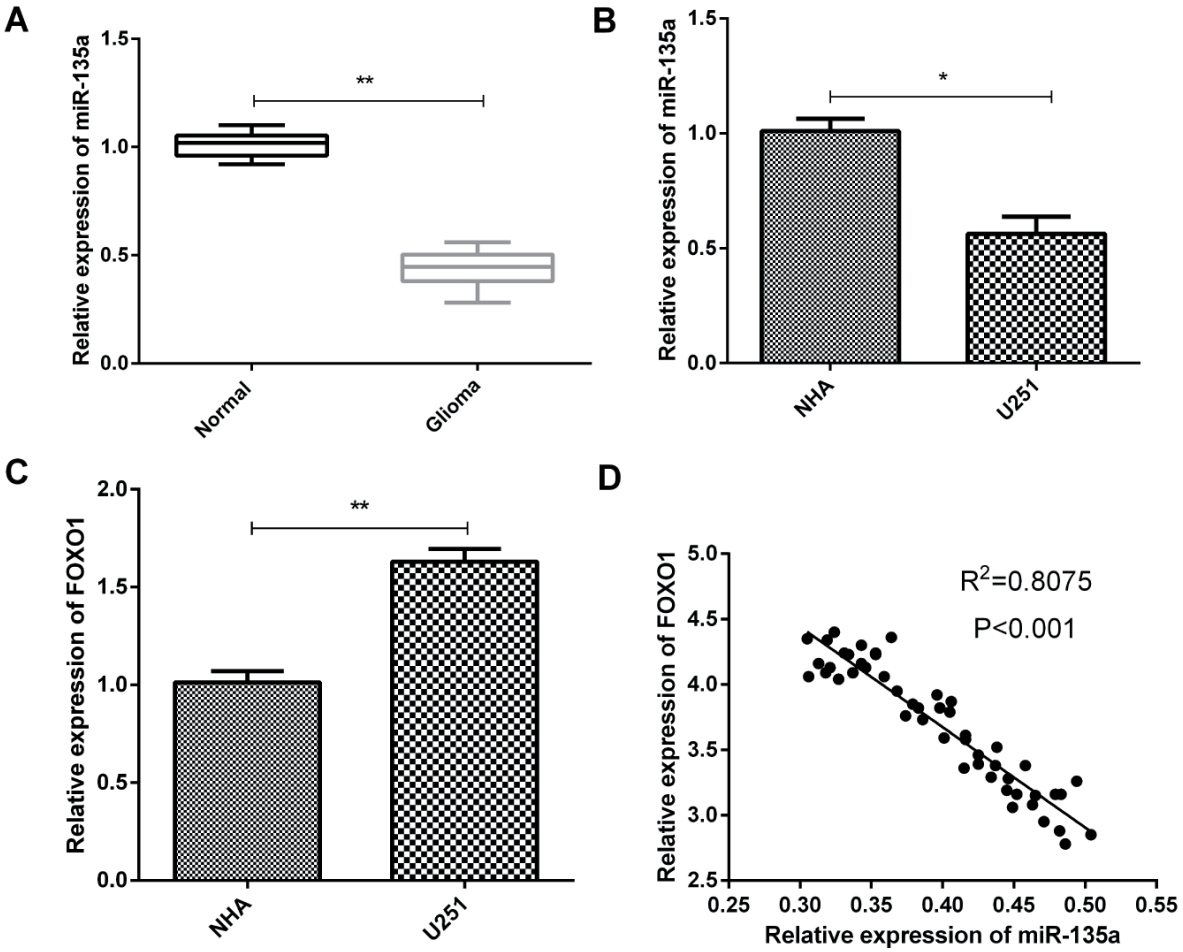

Figure 1. miR-135a expression reduced andFOXO1 expression enhanced in glioma. ***A,*** miR-135a expressions in glioma tissues (n=50) and matched normal tissues (n=50) were evaluated by qRT-PCR (**p<0.01). ***B,*** qRT-PCR was carried out to measure miR-135a expressions in U251 cells (*p<0.05). ***C,*** The miRNA expression levels of FOXO1 were evaluated using qRT-PCR in glioma cells (**p<0.01). ***D,*** Regression analysis of correlation between miR-135a and FOXO1 expressions.

human astrocyte (NHA). The results revealed that miR-135a expression in U251 cells was significantly lower than that in NHA (Figure 1B). Then, we further measured the FOXO1 miRNA expressions in U251 cells; inversely, the results indicated that there was a significantly higher FOXO1 miRNA expression in U251 cells than that in NHA (Figure 1C). In addition, we investigated the correlation between miR-135a and FOXO1 expression to better understand their functions in glioma. The results demonstrated that the expression of miR-135a had a negative correlation to the expression of FOXO1 in glioma (Figure 1D).

MiR-135a Inhibited Glioma Cell Proliferation and Invasion

We investigated the functions of miR-135a in glioma cell proliferation and invasion. Firstly, miR-135a mimics or inhibitor were transfected into U251 cell lines and the efficiency was assessed through performing qRT-PCR. Findings indicated that miR-135a was over-expressed in the cells with the transfection of miR-135a mimics when it was lowly expressed in cells transfected with miR-135a inhibitor in contrast with the control group, respectively (Figure 2A). Thereafter, MTT assay was conducted to observe the proliferation abilities of U251 cell lines with different transfections. The results manifested that the U251 cells with the transfection of miR-135a mimics had weaker proliferation ability than the U251 cells with the miR-135a inhibitor transfection (Figure 2B). The findings suggested that miR-135 inhibited U251 cell proliferation. Additionally, we performed the transwell assays to explore the function of miR-135a in U251 cell invasion. The

results indicated that miR-135a overexpression decreased the invasion ability of U251 cells and miR-135a silencing increased the invasion capacity of U251 cells (Figure 2C and 2D).

MiR-135a Inhibited FOXO1 Gene Expression in Glioma by Targeting its 3'-UTR

Afterwards, we investigated the relationship between FOXO1 and miR-135a to fully understand the function of miR-135a in regulating glioma. Firstly, we used Target Scan to find out the target sites of miR-135a in the sequence of FOXO1 (Figure 3A). The wild type or mutant FOXO1 3'UTR was cloned into luciferase vectors. Then, miR-135a mimics and the luciferase reporter vectors contained the FOXO1 3'-UTR-WT or FOXO1 3'-UTR-MUT were co-transfected into U251 cells. Subsequently, the luciferase reporter gene assay was conducted and the results demonstrated that the fluorescence activity of U251 cells treated with miR-135a mimics and the FOXO1 3'-UTR-WT were significantly decreased in contrast with the control group, while there was no significant difference between the cells treated with the miR-135a and FOXO1 3'-UTR-MUT vector and the control group (Figure 3B). Next, we detected the FOXO1 expressions not only at miRNA level but also at protein level in U251 cell lines with transfections of miR-135a mimics or inhibitor. Both qRT-PCR and Western blot results revealed that miR-135a suppressed FOXO1 expression in U251 cells (Figure 3C and 3D).

Knockdown of FOXO1 Markedly Reversed miR-135a-Medicated Inhibition of Glioma Cell Proliferation and Invasion

To confirm whether FOXO1 was needed in regulating miR-135a functions of inhibiting glio-

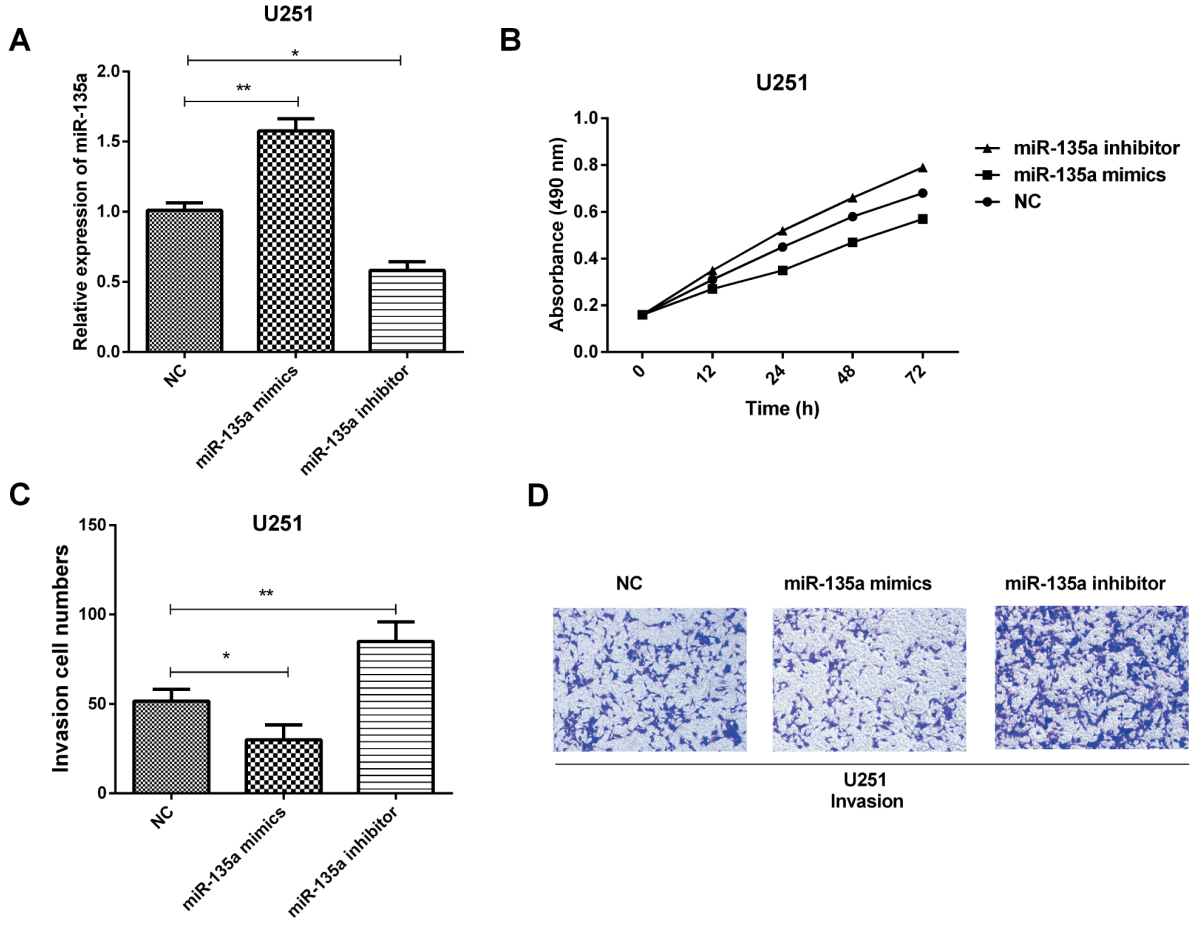

Figure 2. miR-135a suppressed glioma cell proliferation and invasion. ***A,*** The miR-135a expressions in transfected U251 cells were assessed using qRT-PCR (*p<0.05, **p<0.01). ***B,*** The MTT assay was carried out to investigate proliferation of treated U251 cells. ***C,*** The invasion cell numbers of U251 cells were counted (*p<0.05, **p<0.01). ***D,*** Cell invasion was observed by the transwell assay in treated U251 cells.

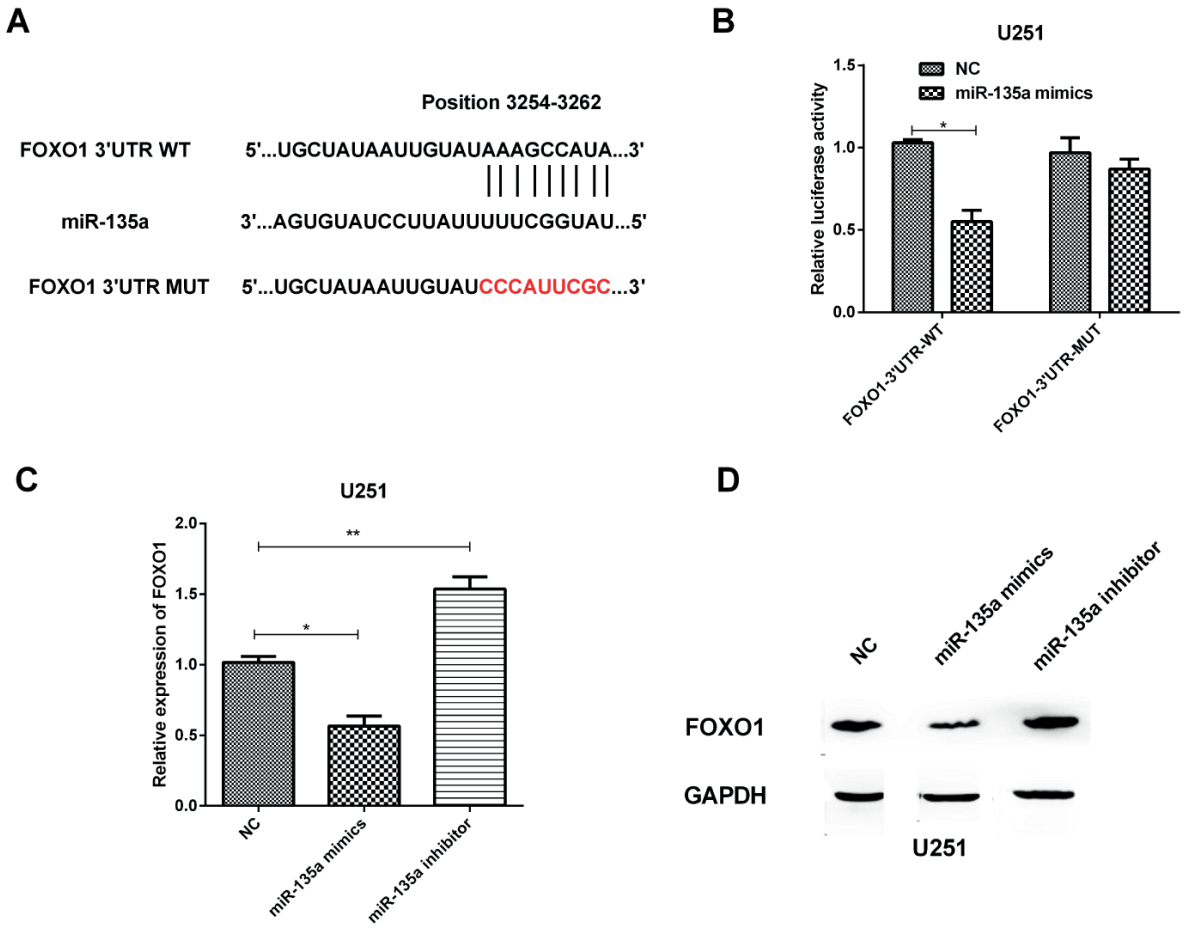

Figure 3. miR-135a de-regulated FOXO1 expression through binding its 3'-UTR directly. ***A,*** The target site of miR-135a in the 3'-UTR of FOXO1. ***B,*** The luciferase reporter gene assays were carried out to detect the fluorescence activities of the FOXO1 3'UTR in U251 cells which were cotransfected with wild-type FOXO1 3'UTR or mutational type FOXO1 3'UTR and miR-135a, respectively (*p<0.05). ***C,*** The FOXO1 miRNA expressions in treated U251 cells were measured by qRT-PCR (*p<0.05, **p<0.01). ***D,*** The FOXO1 protein expression in treated U251 cells was evaluated by Western blotting.

ma cell proliferation and invasion, we knocked down endogenous FOXO1 of U251 cells. Firstly, we co-transfected FOXO1 siRNAs and miR-135a inhibitor into U251 cells and determined the FOXO1 expressions on miRNA level and protein level, respectively by qRT-PCR and Western blots assays. Findings indicated that the FOXO1 expression in U251 cells with the treatment of FOXO1 siRNAs and miR-135a inhibitor was significantly decreased in contrast with that in U251 cells with the treatment of control siRNAs and miR-135a inhibitor (Figure 4A and 4B). Thereafter, MTT assay was performed to investigate the function of FOXO1 in modulating the miR-135a-medicated inhibition effect on U251 cell proliferation. The results revealed that FOXO1siRNA abrogated the function of miR-135a inhibitor on cell proliferation (Figure 4C). Moreover, we also observed the invasion capacity of U251 cells treated with FOXO1 siRNA and miR-135a inhibitor using transwell assay. The results also indicated that the deletion of FOXO1 markedly deprived miR-135a inhibitor-medicated function in U251 cell invasion (Figure 4D and 4E). The above findings together suggested that FOXO1 may reverse partial function of miR-135a in U251 cells.

Discussion

As a lethal and aggressive kind of brain tumors, glioma has become a major challenge for public health. The glioma cell proliferation, invasion, and metastasis make glioma therapies inef-

fective[18]. Currently, the mechanism that drives the initiate and development of glioma is still largely unclear. Emerging evidence shows that the investigations of molecular mechanisms about glioma initiate, development, invasion, migration and recurrence are quite necessary to explore novel and efficient therapeutic strategies. Recent studies[19] have suggested that miRNAs might be new therapeutic approach for the treatment of glioma.

Increasing studies showed that miRNA may function as oncogene or tumor suppressor in kinds of tumors. miR-135a was previously reported to play suppressive roles in prostate cancer[20] and lung cancer[21]. At the same time, miR-135a was found to be an oncogene in gastric cancer[22] and colorectal cancer[23]. In the present research, we further explored the functions of miR-135a in glioma and the results showed that miR-135a expressions were decreased significantly in glioma tissues; additionally, we also confirmed that miR-135a expressions were related to glioma cell proliferation and invasion. All findings in this research together indicated that miR-135a had essential functions in the processes of tumor proliferation and invasion. There were several studies supported our findings. For example, Tribollet et al[24] found that miR-135a inhibited the invasion of cancer cells via down-regulation of ERR alpha; Dang et al[25] reported that miR-135a inhibited pancreatic ductal adenocarcinoma cell proliferation via targeting Bmi1.

FOXO1 has complex functions in various tumors. Its role in glioma still needs to be fully elucidated. FOXO1 regulates several biological functions of cancer cells[26]. Previous research revealed that FOXO1 expression was down-regulated in liver cancer, resulting in abnormal cell apoptosis and proliferation[27]. The current study

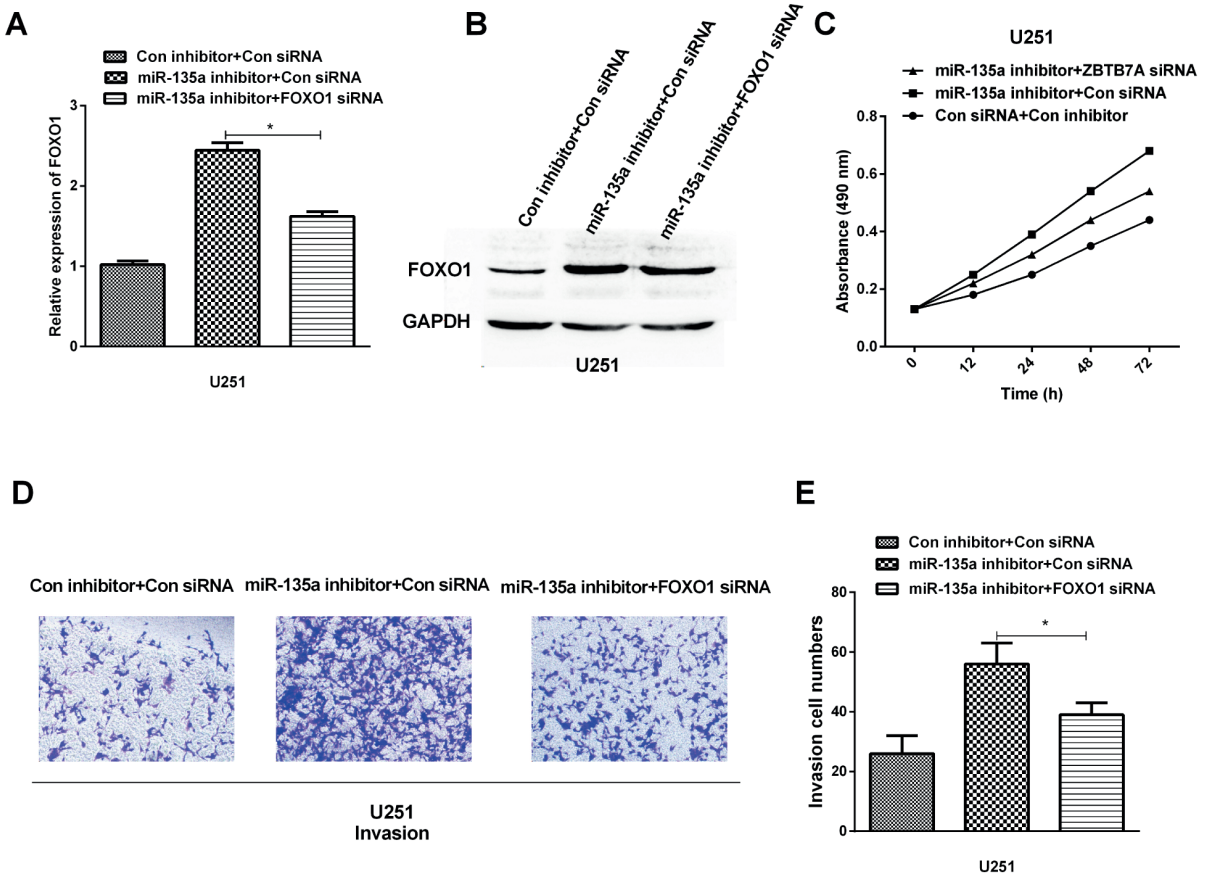

Figure 4. Depletion of FOXO1 could reverse partial function of miR-135a in glioma cells. ***A, B,*** The FOXO1 miRNA or protein expression level was detected using Western blot or qRT-PCR in U251 cells treated with FOXO1 siRNA and miR-135a inhibitor (*p<0.05). ***C,*** MTT assays were conducted to investigate proliferation of U251 cells treated with FOXO1 siRNA and miR-135a inhibitor. ***D, E,*** Transwell assay was carried out to detect invasion capacity of U251 cells co-transfected with FOXO1 siRNA and miR-135a inhibitor (*p<0.05).

indicated that FOXO1 was a target of miR-135a; additionally, the findings of the current study also found that knockdown of FOXO1 significantly inhibited human glioma cell proliferation and invasion, and miR-135a over-expression inhibited the expression of FOXO1.

Conclusions

We revealed that the miR-135a expression reduced significantly in glioma tissues and cells. Our study also demonstrated that miR-135a inhibited glioma cell proliferation and invasion by down regulating the target gene FOXO1. Therefore, we provided new insights into the mechanisms exerting the tumor-suppressor effects of miR-135a, and suggested that miR-135a might be an effective biomarker and therapy for glioma treatment in the future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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