Int J Clin Exp Pathol 2019;12(8):2972-2980
www.ijcep.com /ISSN:1936-2625/IJCEP0095990

Original Article

MiR-28-5p promotes human glioblastoma cell growth through inactivation of FOXO1

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Received April 24, 2019; Accepted May 24, 2019; Epub August 1, 2019; Published August 15, 2019

Abstract: Objective: Glioblastomais is one of the main universal, primary brain cancers, in adults, that has an extremely poor clinical prognosis and a median living period of 12-15 months, accounting for nearly 3-4% of all cancer-related deaths. MicroRNAs (miRNAs) play key roles in cancer pathogenesis by binding the specific and complementary sequences of the 3'UTR of target mRNAs to regulate protein synthesis. Therefore, recognizing functional miRNAs and the fundamental molecular mechanisms will offer novel evidences for the progress of targeted malignancy interferences. Our current study intended to explore the function of miR-28-5p in the promotion of the glioblastoma. Methods: Human glioblastoma tissues, paired nearby normal/non-tumor tissues were accumulated from our hospital. Human glioblastoma SNB19 cells were infected by miR-28-5p mimics or miR-28-5p siRNA by lentivirus. Tumor spheres formation was used to evaluate the growth ability. MTT examine was applied for measuring viability. BrdU cell proliferation assay was applied to uncover the proliferation ability of SNB19 glioblastoma cells. Real-time PCR was conducted to identify miRNA expression. Western blot analysis was employed to measure protein expression. Dual-luciferase FOXO1-3'UTR reporter was used to determine the ability of miR-28-5p to regulate FOXO1. Results: Expression of miR-28-5p was explored to be increased in both human glioblastoma tissues and cell lines. Up-regulated miR-28-5p expression promotes tumor spheres formation, cell viability, and proliferation ability of glioblastoma cells. FOXO1 was found to be the target of miR-28-5p and the activity of FOXO1 was down-regulated by miR-28-5p in glioblastoma cells. Conclusions: MiR-28-5p is an oncogene and promotes the occurrence of glioblastoma by directly targeting the FOXO1.

Keywords: MiR-28-5p, FOXO1, glioblastoma cells, proliferation

Introduction

Glioblastoma (GBM), one of the main universal primary brain tumors in the adult population, is a deadly and highly infiltrative tumor This tumor remains uniformly lethal despite aggressive multimodal therapies, including maximal surgical resection, concomitant radiation, and chemotherapy. The prediction for GBM patients is particularly poor, showing a median period of 12-15 months of life remaining, thus accounting for about 3-4% of all cancer-related deaths [1-4].

Due to its aggressive nature and the availability of tissues for molecular interrogation, GBM was one of the first tumors to be characterized by the Cancer Genome Atlas [5]. These initial analyses revealed a landscape of molecular alterations and allowed for the association of genetic changes (expression levels and mutations) and patient prognosis. These analyses also revealed a high degree of molecular heterogeneity among patients. Due to the severity and notability of the illness, it is necessary to identify novel and additional valuable targets. However, the molecular mechanisms of GBM progress and invasiveness have become more and more obvious in latest years, many more continue to be explored [3]. This is predominantly correct about the function of microRNAs (miRNAs), since the implication of abnormal expression of miRNAs in human malignancies has materialized in latest years [3].

MiRNAs, the small size non-coding RNAs (~22 nucleotides), show high conservation to support the cleavage of mRNAs or inhibit transla-

tion by binding with the target complement sequences of miRNAs in the 3' untranslated region (UTR). At the posttranscriptional level, microRNAs down-regulate the expressions of multiple protein-encoding target genes and interact with numerous mRNA transcripts [6, 7].

More and more proof has shown that miRNAs function essential roles in diverse biological processes, such as development, differentiation, growth, and metabolism [8, 9]. Dysfunction of miRNAs has been reported in numerous categories of malignancies, such as lung cancer, liver cancer, gastric cancer, prostate cancer, bladder cancer, breast cancer, and glioblastoma, which can be tumor suppressive genes or oncogenes during malignancy occurrence and progress. It is currently obvious that miRNAs are physically powerful regulators of many oncogenetic courses, such as cell growth apoptosis, differentiation, invasion, and angiogenesis [10-12]. Consequently, miRNAs have lately fascinated awareness to be the prospective curative targets.

Abnormal miR-28-5p expression has been reported in different categories of malignancies, implying that miR-28-5p plays an important role in malignancy growth and progress. Studies have also indicated that the overexpression of miR-28-5p cause the decrease of cell growth, invasion, and migration in CRC cells [13]. LncRNA-UCA1 modifies development of colon cancer by changing the miR-28-5p/HOXB3 axis [14]. SSRP1 stimulates colorectal cancer progress and is negatively modified by miR-28-5p [15] which is a tumor suppressor in RCC for multiple antitumor effects by targeting RAP 1 B [16]. Inhibited miR-28-5p is associated with tumor growth and migration by targeting IGF-1 in human hepatocellular carcinoma [17]. On the other hand, some other reports specify that miR-28-5p is an oncogene to support malignancy proliferation. miR-28-5p stimulates the oncogenesis and progression of ovarian cancer by inhibiting N4BP1 [16].

Nevertheless, the specific role and fundamental mechanisms of miR-28-5p in the occurrence of glioblastoma still remain unclear.

In the current work, miR-28-5p was over-expressed or silenced in human glioblastoma SNB19 cells followed by Real-time qPCR, tumor spheres formation, MTT, BrdU cell proliferation assay, and western blot analysis, as well as the dual-luciferase reporter assay to discover the fundamental function and mechanism of miR-28-5p in the occurrence of glioblastoma.

Materials and methods

Glioblastomatissues

Tissues from glioblastoma patients were collected from the First Affiliated Hospital of Xinjiang Medical University. Normal brain tissues were collected from persons who died in traffic accidents without pre-existing pathologically evident diseases. All investigations were carried out in line with the Declaration of Helsinki standards.

Compounds and reagents

The RNA kit was bought from RiboBio Co., Ltd (Guangzhou, China). The Trizol reagent was obtained from Invitrogen (Rockville, MD, USA). Cell Lysis Buffer was from Cell Signaling Technology (Danvers, USA). Super Signal Chemiluminescent HRP Substrate was bought from Thermo Fisher scientific Inc (Rockford, USA). Antibodies against GAPDH, CyclinD1, pRb, and p-pRband FOXO1 were purchased from abcam (Cambridge, MA). 3-(4,5-dimetrylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (Saint Louis, USA). 5-Bromo-2-deoxy Uridine (BrdU) cell proliferation assay kit (Kit #6813) was from Cell Signaling Technology (Danvers, MA). Dual-Luciferase® Reporter Assay System was from Promega Corporation (Madison, USA).

Cells and culture

The normal human astrocytes (NHA), the human glioblastoma cells (SNB19, U251, LN18, A172, LN444, and U87) were bought from American Type Culture Collection (ATCC, Manassas, VA, USA). NHA (Sciencell) was incubated according to the condition as the manufacture instructed. Glioblastoma cell lines were regularly cultured in the DMEM medium (Invitrogen, Carlsbad, CA) including 10% fetal bovine serum (HyClone, Logan, UT).

Extraction of RNAs and analyzing by reverse transcription quantitative-PCR (RT-PCR)

Total RNA was extracted from cells with the Trizol reagent following the instruction of the

manufacturer. The cDNA was reverse transcribed using 2 μg of each sample RNA. The primer sequences used were as follows: U6-forward, 5'-GCTTCGGCAGCACATATACTAAAT-3', U6-reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; miR-28-5p-forward, 5'-GGTCCTTGCCCTCAAGGAGCTCACA-3' miR-28-5p-reverse, 5'-AGTGCCTGCCCTCCAGGAGCTCACA-3'. MiRNA expression was determined using the threshold cycle (Ct). 2-[(Ct of miR-28-5p)-(Ct of U6)] normalized by the internal reference expression of U6 nuclear RNA was used to calculate the relative expression levels of miRNA.

Sphere formation assays

One thousand cells were placed in a 6-well ultra low attachment plate (Corning, NY) or about 10 cells were placed in a 24-well ultra low attachment plate (Corning, NY) for 13 days to culture the spheres in DMEM/F12 serum-free medium (Invitrogen, Grand Island, NY) in the presence of 2% B27 (Invitrogen, Grand Island, NY), 20 ng/ml of bFGF (PeproTech, Offenbach, Germany), 20 ng/ml of EGF, 5 μg/ml insulin and 0.4% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA).

Cell viabilities assay

Cell viabilities were assessed by MTT assay. In brief, the wild type, the miR-28-5p over-expressed or the silenced SNB19 glioblastoma cells were placed in 96-well cell culture plates (1×10^5 cells/ml) and incubated overnight for attachment. The optical density (O.D.) values were examined at a wavelength of 570 nm.

BrdUcell proliferation assay

The 1×Wash Buffer was made by dilution of the 20×Wash Buffer supplied in the BrdU Kit using purified water. The 1×detection antibody solution was made by dilution of the BrdU Detection Antibody 1:100 using the Detection Antibody Diluent (green). The 1×HRP-conjugated secondary antibody solution was made by dilution of the Anti-mouseIgG, HRP-linked Antibody 1:100 using HRP-linked Antibody Diluent (red). Finally, the 10×BrdU solution was made by dilution of BrdU 1:100 using cell culture medium. Incorporation of BrdU: the wild type, the miR-28-5p over-expressed, or the silenced SNB19 glioblastoma cells were placed in the 96-well cell culture plates (5000 cells/ml) and incubated overnight for attachment. Prepared 10× BrdU solution was added into each plate well for a final 1×concentration and incubated for 4 hours in an incubator at 37°C with 5% CO_2. The medium was removed, 100 μl of the Fixing/Denaturing Solution was added into each well and incubated for 30 min at room temperature. The solution was removed and 100 μl of 1×detection antibody solution was added in each well, incubated for 1 hour at room temperature. We then removed the solution and washed the plates 3 times using 1×Wash Buffer. A 100 μl of 1×HRP-conjugated secondary antibody solution was added in each well and incubated for 30 min at room temperature. The solution was removed and the plates were washed 3 times using 1×Wash Buffer, then 100 μl of TMB Substrate was added and incubated at room temperature for 30 min. Finally, we added 100 μl STOP Solution. The images and numbers of the BrdU positive cells were obtained under the florescence microscope.

Western blotting analysis

The wild type, miR-28-5p over-expressed or silenced SNB19 glioblastoma cells were collected and rinsed using cold PBS, then lysed in Cell Lysis Buffer for 30 min on ice to isolate total proteins. The protein concentration was detected by bicinchoninic acid assay. Equal amounts of proteins were separated using SDS-PAGE and transferred onto the PVDF membranes to identify the protein expression levels after incubation of the PVDF membranes, using the primary antibodies. They were then developed with the ECL reagents.

Luciferase reporter assay

The LAR II, Stop & GloR Reagent and test samples were heated to room temperature before execution of the Dual-Luciferase Assay. The anticipated number of DLR™ assay was conducted by pre-dispensing 100 μl of LAR II to the right number of luminometer tubes. A 2-second pre-measurement delay was conducted by programming the luminometer, then a 10-second examination period for each reporter evaluation. A 20 μl of cell lysate was carefully transferred into the luminometer tube comprising LAR II and mixed 2 or 3 times by pipetting without vortexing. We recorded the firefly luciferase activity measurement. The sample tube

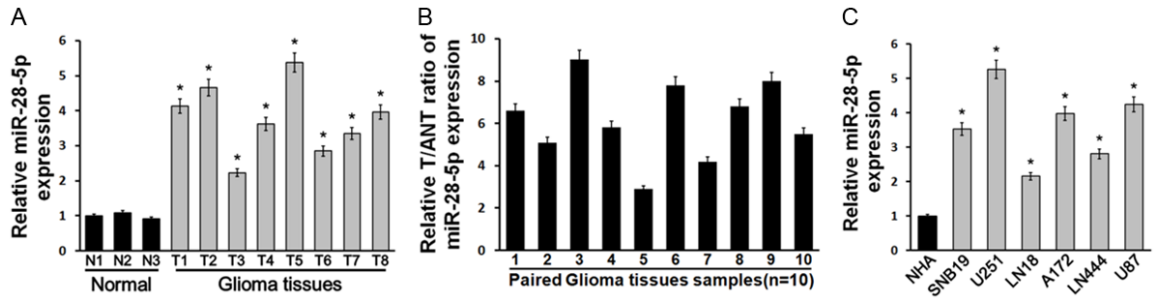

Figure 1. MiR-28-5p is up-regulated in glioblastoma tissues and cell lines confirmed by Real-time PCR analysis. A. Expression of miR-28-5p in freshly-frozen human glioblastoma tissues was statistically more significant than that in the nearby normal tissues. B. Relative miR-28-5p expression between the paired glioblastoma tissues/nearby non-tumor tissue (T/ANT ratio). C. Up-regulated expression of miR-28-5p was further confirmed in 6 different glioblastoma cell lines versus the normal human astrocytes (NHA). Transcript levels were normalized to *U6* expression. Error bars represent the mean ± s.d. of three independent experiments. **P* < 0.05.

was removed from the luminometer, and 100 μl of Stop & GloR Reagent was added and briefly vortexed for mixture. We replaced the sample in the luminometer and initiated the reading. The measurement of Renilla luciferase activity was recorded.

Statistical analysis

Mean ± standard deviation was used for all data. SPSS statistical software (SPSS Inc., Chicago, IL, USA) was applied for the statistical analysis. The significance of the data differences between groups was evaluated using a 2-tailed Student's t-test. P < 0.05 was statistically significant.

Results

Human glioblastoma shows up-regulated miR-28-5p expression

To identify whether miR-28-5p was essential during the pathogenesis of glioblastoma, a healing target or prospective biomarker of glioblastoma, the expression of miR-28-5p in GBM tissues from 8 patients versus that in 3 normal brain tissues was detected by real-time qPCR. The results showed that miR-28-5p levels were differentially increased in 8 GBM tissues compared to that in 3 normal brain tissues (**Figure 1A**). To further validate the expression of miR-28-5p in human glioblastoma, we compared the relative miR-28-5p expression between the 10 paired glioblastoma tissues (T) and nearby non-tumor tissue (ANT) and the ratio between the paired glioblastoma tissue and the nearby non-tumor tissue (T/ANT ratio) was calculated. Our results showed that each T/ANT ratio was over 1, which indicated that the miR-28-5p expression was more in the glioblastoma tissue than that in the nearby non-tumor tissue (**Figure 1B**). Meanwhile, up-regulated miR-28-5p expression was also confirmed in 6 different glioblastoma cell lines (SNB19, U251, LN18, A172, LN444, and U87) versus the normal human astrocytes (NHA) (**Figure 1C**).

Jointly, our results recommend that miR-28-5p is up-regulated and might be associated with the progression of human GBM.

MiR-28-5p promotes the growth of SNB19 human glioblastoma cells

As miR-28-5p was up-regulated in human glioblastoma tissues, we assumed that miR-28-5p may stimulate the pathogenesis of glioblastoma. Lentivirus infection was used to over-express or knock down the expression of miR-28-5p in human glioblastoma SNB19 cells. Real-time qPCR confirmed that miR-28-5p was either successfully over-expressed or knocked down in SNB19 human glioblastoma cells (**Figure 2A**). MiR-28-5p over-expression increased the tumor spheres formation at about two-fold and a ~2-6-fold higher cell number versus the wild type cells. On the other hand, silence of miR-28-5p in SNB19 human glioblastoma cells generated about 3-fold less tumor spheres with a ~2-4-fold less cell number versus the wild type cells (**Figure 2B**). Next, MTT assay demonstrated that miR-28-5p mimics obviously elevated, whereas miR-28-5p inhibitor evidently declined SNB19 cell viability

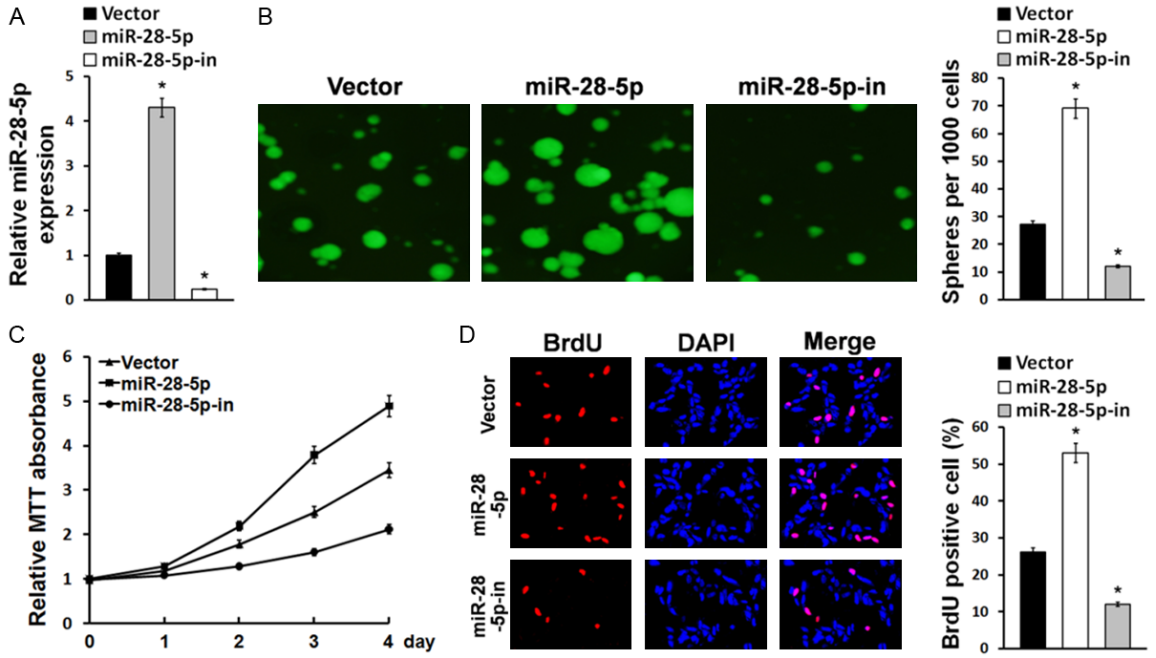

Figure 2. Over-expression of miR-28-5p promoted glioblastoma cell growth. A. Real-time PCR analysis confirmed that miR-28-5p was successfully over-expressed or knocked down after miR-28-5p or miR-28-5p-in containing lentivirus infection. B. Symbolic micrographs of neural tumor spheres formed by indicated cells, green is GFP fluorescence. C. MTT assays revealed that miR-28-5p up-regulation promoted the viability, while miR-28-5p down-regulation inhibited viability of SNB19 glioblastoma cells. D. BrdU cell proliferation assay, cells were seeded at 5000 cells/well in a 96-wellplate incubated overnight. A total of 10 μm BrdU was added to the plate and the cells were incubated for 4 hr. The images (left panel) and numbers (right panel) of the BrdU positive cells were obtained under the florescence microscope.

after 96 h incubation (**Figure 2C**). Furthermore, we performed the BrdU cell proliferation assay, which indicated that miR-28-5p over-expression stimulated the proliferation ability of the SNB19 cells, while miR-28-5p knock down decreased the proliferation ability of SNB19 and calculated numbers (**Figure 2D**). The results mentioned above specified that miR-28-5p promoted the growth of SNB19 human glioblastoma cells.

MiR-28-5p regulates the key factors involved in cell growth

To further investigate if the key factors involved in cell growth were also adjusted by miR-28-5p, CyclinD1, p-pRb, and pRb expressions in the glioblastoma SNB19 cells were first identified by western blot assay. The expression of GAPDH was used as the internal reference. Our results displayed that miR-28-5p over-expression in the glioblastoma SNB19 cells up-regulated the expressions of CyclinD1 and p-pRb, but miR-28-5p silence in the glioblastoma SNB19 cells down-regulated the expressions of proteins mentioned above, which were evidenced by both the bands and the quantitative analysis of each target band (**Figure 3A**). Then, the mRNA expressions of Bim, p21, FASL, p27, Bax, and Casepase-9 were determined in miR-28-5p-in or miR-28-5p transfected SNB19 glioblastoma cells by Real-time PCR. Our results revealed that miR-28-5p over-expression in the glioblastoma SNB19 cells down-regulated the expressions of Bim, p21, FASL, p27, Bax, and Casepase-9, while miR-28-5p silence in the glioblastoma SNB19 cells up-regulated the mRNA expressions (**Figure 3B**). Real-time PCR assay exposed that the expressions of AXIN2, Oct4, CD133, MMP2, Twist, LEF1, MMP2, Tiam1, and SOX9 were up-regulated in the miR-28-5p over-expressing glioma cells but down-regulated in the miR-28-5p silencing cells (**Figure 4C**). These results all recommended that up-regulated miR-28-5p induced occurrence of glioblastoma by regulation of the key factors involved in cell growth.

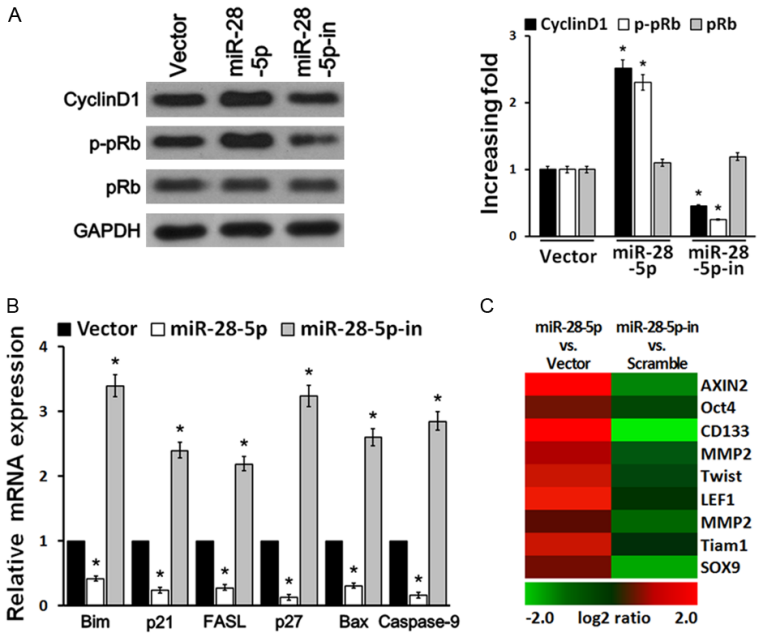

Figure 3. Key factors involved in cell growth were regulated by miR-28-5p. A. The bands (left panel) and quantitative analysis (right panel) of test protein expression levels in SNB19 glioblastoma cells with miR-28-5p or miR-28-5p-in transfection were detected by western blot analysis. B. Real-time PCR detection of cell proliferation-related gene expressions in miR-28-5p or miR-28-5p-in transfected SNB19 glioblastoma cells. C. Real-time PCR analysis revealed that miR-28-5p regulates the expression levels of AXIN2, Oct4, CD133, MMP2, Twist, LEF1, MMP2, Tiam1, and SOX9. The pseudo colors represent the intensity scale of expression in miR-28-5p vs. vector cells, or antagomiR-28-5p vs. control cells generated by log2 transformation.

MiR-28-5p promoted occurrence of glioblastoma by targeting FOXO1

Using the publicly available algorithms miRBase, we found that the FOXO1 might be prospective targets of miR-28-5p (**Figure 4A**). The western blotting assay discovered that over-expression of miR-28-5p vigorously diminished FOXO1 expression, but miR-28-5p knockdown amplified it, proposing that miR-28-5p was negatively correlated with the protein (**Figure 4B**). Furthermore, luciferase analysis disclosed that over-expression of miR-28-5p diminished. However, knock down of miR-28-5p raised the reporter activities motivated by the 3'UTRs of FOXO1 (**Figure 4C**). Furthermore, neither over-expression nor knock down of miR-28-5p displayed influences on the reporter activities of FOXO1 with mutant 3'UTRs (**Figure 4D**). The data mentioned above designated that miR-28-5p inhibited FOXO1 expression by directly targeting the element of mRNA 3'UTR.

Concurrently, the counted numbers of the BrdU positive cells were also increased in SNB19 glioblastoma cells co-transfected by miR-28-5p-in and FOXO1-siRNAs. This suggests that FOXO1-siRNA abolishes miR-28-5p knockdown and reduced the proliferation ability of SNB19 (**Figure 4E**). Correlation analysis demonstrated that expression of FOXO1 was negatively related with the expression of miR-28-5p in glioblastoma tissues (**Figure 4F**), indicating that both the miR-28-5p and FOXO1 are closely related during the occurrence of glioblastoma.

Discussion

FOXO1, known in layman's terms as the fork head rhabdomyosarcoma transcription factor (FKHR), is the fork head box (FOX) family [18, 19]. The initial research of FOXO family was achieved in Drosophila [20]. Genomic transformation of fork head, the location-specific homeotic gene, results in disordered embryonic development [21, 22]. Therefore, fardauer formation-16 is the single FOX member that has been recognized in Caenorhabditis elegans, the invertebrates [23]. However, the Fox family in vertebrates has 4 members of FOXO1, 3, 4, and 6 [24]. In normal tissues/organs, both the C-terminal and amino-locations of FOXO1 are vital for the alteration of linker histones. FOXO1 depletion could cause embryonic lethal and defective angiogenesis. Besides, damage or revival of FOXO1 in human controls various functions in cells during oncogenesis, progress, and metastasis as different malignancies. These include rostate cancer, digestive malignancy, breast cancer, alveolar rhabdomyosar coma, ovarian cancer, and pancreatic cancer [25-29]. FOXO1 can augment the efficiency of chemotherapy in cancers. Consequently, studies have been conducted to combine FOXO1 with anti-tumor medications. Regardless of 5-FU being the first line of treatment for progressive colorectal cancer, the efficacy is frequently hindered by dose-dependent toxicity and drug resistance [25, 30]. However, the function of FOXO1 in glioblastoma has not been revealed.

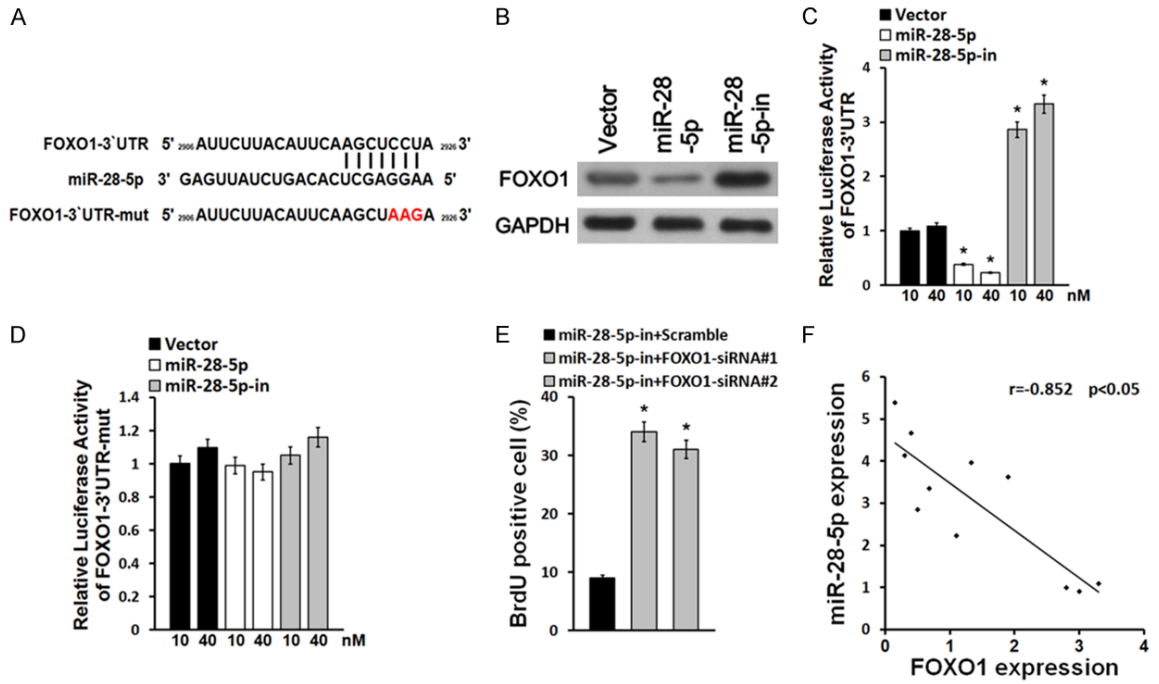

Figure 4. MiR-28-5p promoted the occurrence of glioblastoma by targeting FOXO1. A. The predicted target sequence of miR-28-5p in the 3'UTR of FOXO1. B. FOXO1 expression in SNB19 glioblastoma cells with miR-28-5p or miR-28-5p-in transfection detected by western blot analysis. C. Dual-luciferase reporter assay of the SNB19 glioblastoma cells transfected with the WT-FOXO1-3'UTR reporter and miR-28-5p or miR-28-5p-in. D. Dual-luciferase reporter assay of the SNB19 glioblastoma cells transfected with the mutation FOXO1-3'UTR reporter and miR-28-5p or miR-28-5p-in. E. Representative micrographs (left) and quantification of BrdU-incorporating cells after transfection with miR-28-5p, miR-28-5p inhibitor or vector. Each bar represents the mean of three independent experiments. *P < 0.05. F. Correlation analysis of miR-28-5p and FOXO1 expression in SNB19 glioblastoma tissues. *P < 0.05.

MiRNAs demonstrate a significant role in the transcriptional level by acting on the target gene 3'UTR which contributes to numerous biological processes. MiRNAs negatively or positively influence cancer development by managing the expression of tumor suppressors and oncogenes. The down-regulated miR-28-5p has been revealed in numerous malignancies, including CRC, RCC, and HCC [31-33]. In the central nervous system, subclasses of GBM were demonstrated attractive relations to diverse lineages clinically and hereditarily [34]. Besides, profiling researches have exposed that miRNAs are differentially expressed in brain cancers versus normal tissues. The abnormally expressed miRNA divisions would show diagnostic propositions [35]. Some miRNA expressions are related to clinical prognosis for certain GBM patients [36, 37]. Various abnormal miRNAs have been distinguished independently regarding their targets and function in GBM [37, 38].

In this study, our results presented that miR-28-5p expression was increased in glioblastoma tissues and cell lines. Advanced studies disclosed that miR-28-5p over-expression further stimulated the colony formation, cell viability, and proliferation ability of SNB19 glioblastoma cells. FOXO1 was found to be the target of miR-28-5p and the activity of FOXO1 was inhibited by miR-28-5p in glioblastoma cells.

In the future, it would be important to investigate the involvement and specific mechanism of miR-28-5p in animal models and patients with glioblastoma. The association between FOXO1 and miR-28-5p was only confirmed in SNB19 cells. More studies are still required to verify these findings, which will provide further strong evidence supporting the role of miR-28-5p and FOXO1 in glioblastoma occurrence, metastasis, and progression.

In conclusion, our study demonstrated that miR-28-5p expression was up-regulated in human glioblastoma tissues and cell lines. Colony formation, cell viability, and proliferation ability of glioblastoma cells were significantly further increased with miR-28-5p over-expres-

sion. Moreover, FOXO1 was found to be a target of miR-28-5p and negatively related with miR-28-5p during glioblastoma progression, proposing that miR-28-5p is a tumor suppressor in glioblastoma. Therefore, it would be a potential strategy to inhibit miR-28-5p expression of or promote the expression of FOXO1 for the glioblastoma therapy.

Disclosure of conflict of interest

None.

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