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ORIGINAL ARTICLE

miRNA-429 Inhibits Astrocytoma Proliferation and Invasion by Targeting BMI1

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Abstract Glioblastoma multiforme (GBM), the most common primary brain cancer in adults, is usually the most lethal type of brain tumor. MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules that deeply involves with the regulation of gene expression and cellular processes, including proliferation, apoptosis, migration and invasion. The objective of the study is to investigate the effect of miRNA-429 on human glioblastoma tissues and cell lines. miRNA-429 expressions in human glioblastoma, normal brain tissue samples, and human malignant glioma cell lines (U87, U251 and LN229) were compared using reverse transcription-quantitative PCR and western blot methods. U251 cell lines were transfected with miRNA-429 mimics, and then the effects of miRNA-429 on cell proliferation and invasion were investigated by CCK8 and Transwell invasion assay, respectively. It was found that miRNA-429 expression was significantly reduced in the examined Glioblastoma samples and human glioma cell lines. Overexpression of miRNA-429 inhibited Glioblastoma cell proliferation and invasion. Additionally, the present study also showed that BMI1 was a functional target of miRNA-429. Overexpression of BMI1 undermined the inhibition effect of miRNA-429 in glioblastoma and U251 cell lines. The current study demonstrated that miRNA-429, as a tumor suppressor gene, was capable of negatively regulating the expression of BMI1 in U251 cells.

Keywords Glioblastoma · miRNA-429 · BMI1 · Proliferation · Invasion

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Introduction

Glioblastoma multiforme (GBM) is the most common and most aggressive intrinsic malignant cancer arising in the central nervous system. It involves with glial cells and constitutes around 52 % of tissue tumor in the brain and approximately 20 % of all kinds of tumors inside human skull. The molecular and cellular mechanism of GBM is little understood, treatment strategy normally involves combination of surgery, radiotherapy and chemotherapy. However, mortality and morbidity rate of GBM remains high [1, 2]. About 50 % of the patients diagnosed with GBM will die within one year, while 90 % within three years [2]. Thus, it is necessary to broaden the knowledge of GBM, and to develop more effective treatment for this horrible disease.

Previous studies indicated that microRNAs (miRNAs) can be used for diagnostic and therapeutic purposes of cancer. Ectopic expression of microRNAs has been found in a wide variety of human malignancies [3, 4], such as breast cancer, lung cancer and colon carcinoma, in which cases miRNAs contribute to cancer initiation, development and progression. The tissue-specific expression pattern of microRNA suggested that microRNA can be developed for diagnostic and prognostic purposes [3–5]. microRNAs has been demonstrated to function as tumor suppressor gene or oncogene. Previous study reported that microRNA-25 expression level was significantly enhanced in astrocytoma tissues and in GBM cell lines, and the overexpression of microRNA-25 is capable of stimulating cell proliferation and invasion in U251 cells by targeting NEFL directly [6]. Jennifer A. Chan et al. demonstrated that microRNA-21 was strongly overexpressed in human GBM cells and the knockdown of microRNA-21 resulted in the increased apoptotic cells [7].

MiRNA-429 was reported to involved in hepatocellular carcinoma (HCC), endometrioid endometrial carcinoma

(EEC), oral squamous cell carcinoma [8–12], these findings suggested that miRNA-429 was down-regulated in the above mentioned carcinomas, indicating its potential role of tumor suppressor gene. The present study confirmed that inhibitive effect of miRNA-429 on human GBM tissues, NHA (normal human astrocytoma) and glioblastoma cell lines, such as U87, U251, and LN229, suggesting that miRNA-429 could be uses as a novel therapeutic tool for GBM treatment.

Materials and Methods

Tissue Sample and Cell Lines

Human glioblastoma and normal brain tissue samples were collected from patients who underwent surgery at our hospital. The current study complies with the regulations of the Ministry of Health, and the Declaration of Helsinki on the Ethical Principles for Medical Research Involving Human Subjects. All patients included in the study have provided informed consent according to the protocols approved by the ethnic committee of our hospital. Human malignant GBM cell lines, including U87, U251 and LN229, were purchased from the American Type Culture Collection (Manassas, VA, USA). Patients have not undergone any local or systemic treatment prior to the operation. All tissue samples were collected, and immediately frozen in liquid nitrogen, and stored at −80 °C until the extraction of RNAs and proteins. The cell lines (U87, U251, and LN229) were cultured in RPMI 1640 medium (Hyclone, USA) supplemented with 10 % FBS, and were maintained in a 5 % CO_2 incubator at 37 °C.

miRNA Transfection

Synthesized and modified RNA mimics, including miRNA-429 mimics, control RNA mimics, control micro-RNA inhibitor, and miRNA-429 inhibitor were purchased from Sangon Biotechnology (Shanghai, China). The mimic co-transfections were conducted using Lipofectamine 2000 (Invitrogen Life Technologies, USA) at a working concentration of 50 Nm according the manufacturer's instructions. At 24-h post-transfection, the cells were plated for proliferation and invasion assays. The cells were harvested for RNA and protein extraction at 48 h post-transfection.

RNA Extraction, Reverse Transcription, and Real-Time PCR Analysis

Total RNAs from cultured cells and human astrocytoma issues were isolated using Trizol reagent (Invitrogen, CA, US.) according to the manufacturer's instruction. microRNA expression levels were measured using qRT-PCR. RNA was converted into cDNA using the PrimeScript™ RT-PCR Kit (Takara). Real-time PCR reaction was performed using SYBR Premix DimerEraser (Takara). Human GAPDH and U6 were used as endogenous controls. Melting curves were used to evaluate non-specific amplification. Relative expression of each gene was quantified by measuring Ct values and normalized using using the $2^{-\Delta\Delta Ct}$ method t relative to U6 snRNA or GAPDH.

Western Blotting Analysis

Primary antibody against BMI1 was purchased from Abcam (ab14389, 1: 1000, Cambridge, UK); GAPDH antibody as internal control was obtained from Cell Signaling Technology (1: 4000, USA). Cells were washed three times with cold PBS buffer, scraped from the dishes, and centrifuged at 1500 rpm, 4 ◦ C for 15 min. Cell lysates were prepared using RIPA buffer supplemented with protease inhibitors (Millipore, Billerica, MA, USA). The supernatants were collected, and protein concentration was measured using a BCA assay kit (Thermo Fisher, USA). Subsequently, SDS-PAGE was used for gel electrophoresis, then gels were transferred to polyvinylidene fluoride membranes (Millipore, USA). After blocking the membrane with 5 % non-fat dry milk, the membranes were incubated with primary antibody BMI, followed by incubation with HRP-conjugated secondary antibody. Reactions were developed with ECL (Millipore, Billerica, MA, USA) for signal detection.

Luciferase Reporter Assay

The 3′-UTR of BMI1 was amplified, and individually inserted into the MluI and HindIII sites of pMIR-reporter luciferase vector, downstream of the stop codon of the gene for luciferase. Similarly, the fragment of BMI1 3′-UTR mutant was inserted into the pMIR-reporter luciferase vector at the same sites. Cells were seeded into a 24-well plate and cultured overnight prior to co-transfection with wild-type or mutation-type plasmid, pRL-TK plasmid, and equal amounts of miRNA-429 or miRNC. After 48 h, Luciferase activity was measured using dual-glo luciferase reporter system according to the manufacturer's instructions (Promega, Madison, WI, USA). Firefly luciferase units were normalized against Renilla luciferase units to monitor transfection efficiency. Experiments were repeated in triplicate independently.

Cell Viability Assay

Cell growth was measured by performing the CCK8 assay. After plating cells into 96-well plates at the density of 2000 cells/well, and treating cells of each well with microRNA mimics, 10 μL of CCK8 was added to each well at harvest time. Cellular viability was determined by measuring the absorbance of the converted dye at the wavelength of 450 nm using a CCK8 kit (R&D Systems, M.N., USA), according to the manufacturer's instruction. Data were collected from three separate experiments each time.

Transwell Invasion Assay

To measure cell invasion, 8 mm pore 24-well Matrigel invasion chambers (Corning Inc., NY, USA) was used according to the manufacturer's instructions. 2×10^4 cells were seeded into each well. In the upper chamber, DMEM with 0.1 % FBS was added, while in the lower chamber well DMEM supplemented with 10 % FBS was added to promote cell invasion. After 24 h of incubation at 37 °C with 5 % CO_2, non-invading cells were removed from the top well, while migrated cells were quantified by photographing in 3 independent visual fields under the microscope using a ×20 magnifications, and was stained with 0.1 % crystal violet. Independent experiments were repeated at least three times.

Statistical Analysis

Differences between the variables of each group were tested using the Student's t test or one-way ANOVA, with the SPSS 17.0 (Chicago, Illinois, USA). All experiments were repeated in triplicate, and the correlation between miRNA-429 and BMI1 expression pattern in GBM cells was tested using Spearman's rank test. $p < 0.05$ was considered of statistical significance.

Results

miRNA-429 Expression Was down-Regulated and BMI1 Expression Was up-Regulated in Human Astrocytoma Samples and Cell Lines

To determine whether miRNA-429 was down-regulated in human astrocytoma samples and other cell lines, we compared miRNA-429 expression in 34 astrocytoma samples and in 16 normal brain tissues using the qRT-PCR. U6 was used for normalization. As showed in Fig. 1a, the expression of miRNA-429 were significantly down-regulated in the astrocytoma samples compared with that in the normal brain tissues ($P < 0.001$). Next, we examined the expression of miRNA-429 in glioblastoma cell lines of U251, LN229, and U87. The control cell line NHA was the cells derived from normal human astrocytes (NHA). The results showed a significantly lower expression of miRNA-429 in U251, LN229, and U87 cell lines (Fig. 1b) compared with that of NHA ($P < 0.001$).

BMI1 Is one of the Targets of miRNA-429

BMI1 expression was significantly increased in GMB cell lines of U87, U251 and LN229 compared with that in NHA. However, it remains uncertain whether there is a correlation

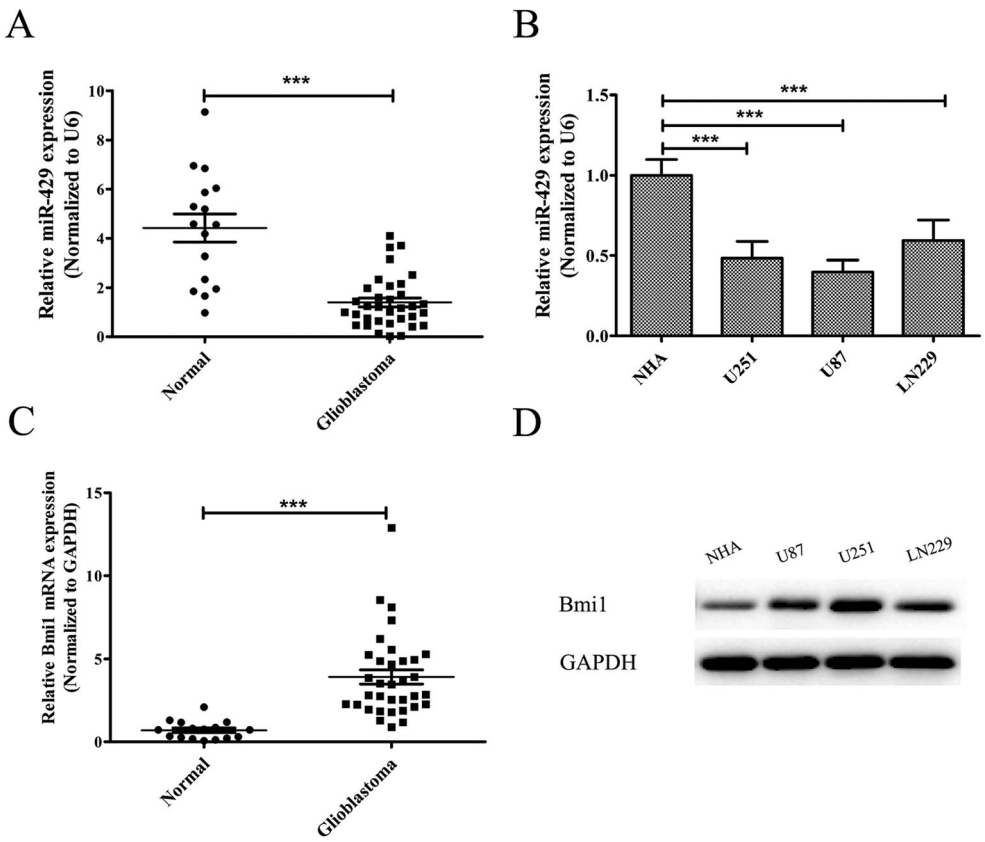

Fig. 1 miRNA-429 expression level was decreased in human glioblastoma samples and cell lines compared with that in normal brain tissues. **a** qRT-PCR analysis showed that miRNA-429 expression was significantly lower in glioblastoma tissues compared with that in normal brain tissues. **b** The expression of miRNA-429 is significantly higher in the normal human astrocytes (NHA) compared with that in cell lines of U251, U87 and LN229. The data represent the mean ± SDs in triplicate. **c** qRT-PCR analysis suggested that BMI1 expression was significantly higher in astrocytoma compared with that in normal brain tissues. **d** Western blot analysis showed that BMI1 expression in NHA was significantly decreased compared with that in cell lines of U87, U251 and LN229. GADPH was used as internal control. ***$p < 0.001$

between the up-regulation of BMI1 and the down-regulation of miRNA-429. To investigate the mechanism by which the proliferation of astrocytoma cells were regulated by miRNA-429, the targeting gene of miRNA-429 was predicted using miRNA target prediction software. It was identified that the potential binding site of miRNA-429 was the BMI1 3′-UTR (Fig. 2a).

Subsequently, wild-type (WT) BMI1 3′-UTR and mutation-type (MT) BMI1 3′-UTR luciferase reporter vectors were co-transfected into HEK293 and U251 cells, along with miRNA-429 mimic or miRNA-NC (negative control) overnight. The results showed that transfection of the miRNA-429 mimics in HEK293 and U251 cell lines with BMI1 3′-UTR vector significantly reduced the luciferase activity compared with that with miRNA-NC, both in HEK293 and U-251 cell lines ($P < 0.05$). However, transfection of the miRNA-429 mimics with mutant-type (MT) BMI1 3′-UTR showed no significant difference in luciferase activity, both in HEK293 and U-251 cell lines ($P > 0.05$, Fig. 2b). After that, we measured the BMI expression level in U251 cells transfected with miRNA-429 mimics and miRNA-NC, using Quantitative real-time PCR and western blotting. Our findings suggested that the expression levels of BMI1 in U251 cells transfected with miRNA-429 mimics were significantly lower than that of the miRNA-NC group ($P < 0.001$). Conversely, the expression levels of BMI in U251 cells transfected with miRNA-429 inhibitor were significantly increased that that transfected with the anti-miRNA-NC ($P < 0.001$). All these data indicated that BMI1 was regulated by miRNA-429 in astrocytoma cells post-transcriptionally. The correlation between BMI1 and miRNA-429 expression in astrocytoma cells was calculated using Spearman's rank test. The data showed an inverse correlation existed between the expression of miRNA-429 and BMI1 in astrocytoma cells (Spearman's correlation, $r = -0.586$) (Fig. 2e).

The Effect of miRNA-429 Expression on the Proliferation and Invasion of Glioblastoma Cells

To elucidate the biological significance of miRNA-429 in glioblastoma tumorigenesis, we up-regulated (Fig. 3a) and down-regulated (Fig. 3b) the expression of miRNA-429 in U251 cells, respectively. The effect of miRNA-429 on the proliferation of U251 cells was investigated using CCK-8 assay (Fig. 3c, d). As indicated in Fig. 3C, in the group of miRNA-429 overexpression, the proliferation of U251 cells at 24 h post-transfection was significantly inhibited compared with miRNA-NC group (Fig. 3c). To the contrary, in the group

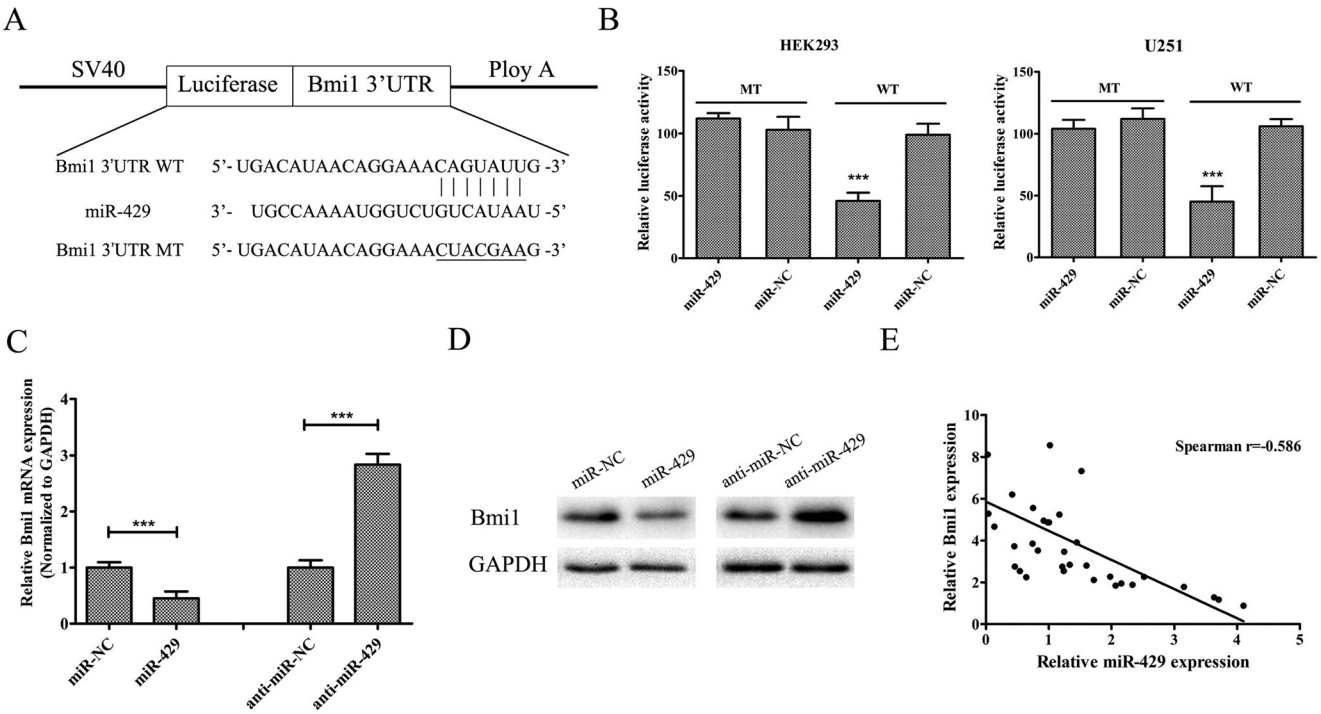

Fig. 2 BMI1 is one target gene of miRNA-429. **a** The BMI1 3′-UTR interaction site was targeted by miRNA-429; **b** Luciferase activity in HEK293 and U251 cells was measured by co-transfected cells with pMIR-REPORT- WT BMI1 3′-UTR, pMIR-REPORT-MT BMI1 3′-UTR, along with miRNA-429 or the miRNA-NC; **c** qRT-PCR analysis showed the mRNA level of BMI1 in U251 cell line transfected with miRNA-429 mimics, and transfected with miRNA-429 mimic inhibitor; **d** Western blot analysis showed the protein level of BMI in U251 cell line transfected with miRNA-429 mimics, and transfected with miRNA-429 mimic inhibitor; GAPDH was used as an internal control. **e** Spearman's correlation analysis demonstrated the correlation between the miRNA-429 and BMI1 expression level in human astrocytoma; Spearman's correlation, $r = -0.586$. ($n = 34$). ***$p < 0.001$

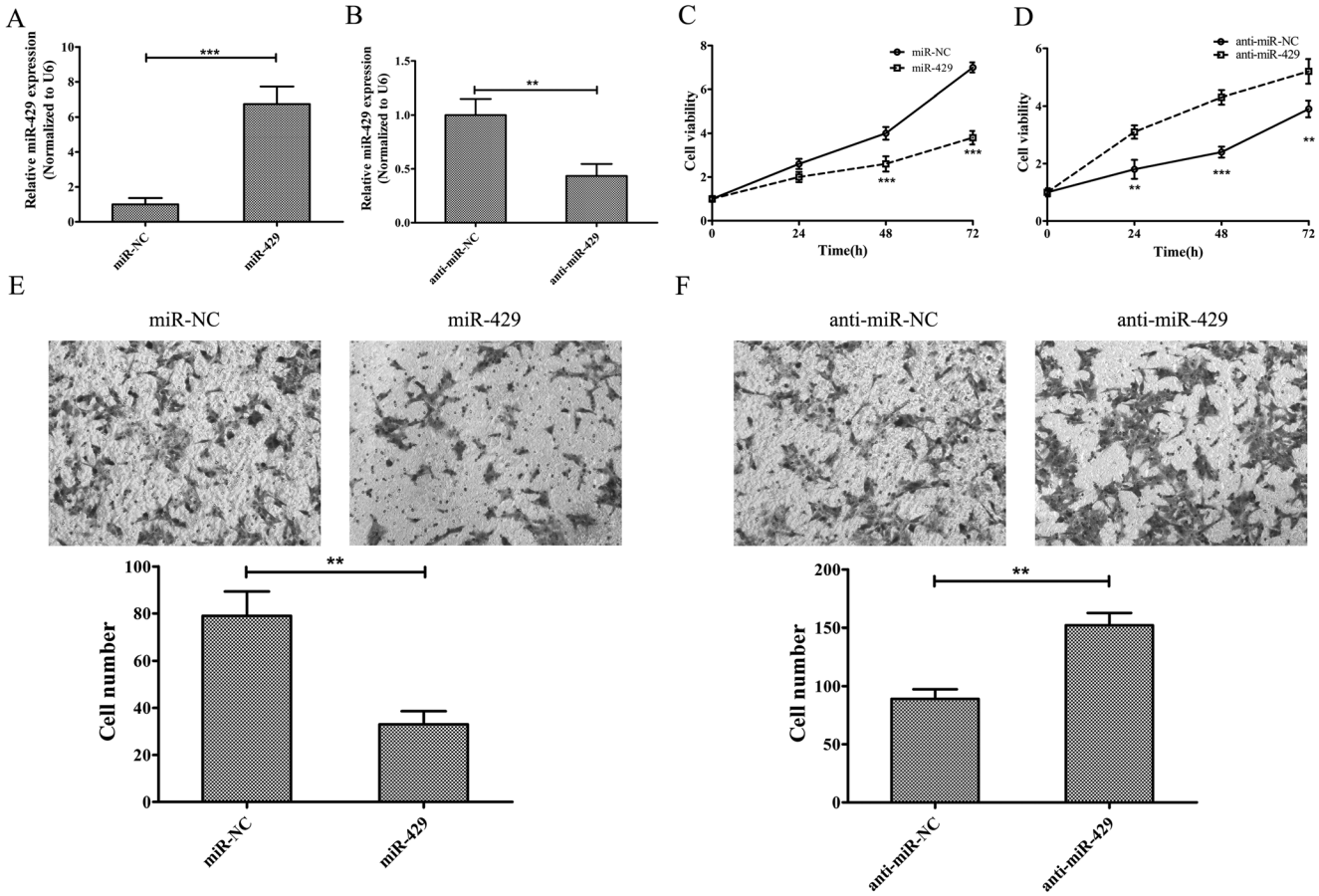

Fig. 3 miRNA-429 inhibited the proliferation and invasion of glioblastoma. **a** miRNA-429 expression was significantly higher in U251 cell line post-transfection of miRNA-429 mimic ($P < 0.001$); **b** miRNA-429 expression is significantly lower in U251 cell line post-transfection of miRNA-429 mimic inhibitor ($P < 0.01$); **c** CCK8 assay revealed that the proliferation of U251 cells was decreased post-transfection with miRNA-429 mimics; **d** CCK8 assay revealed the proliferation of U251 cells was increased post-transfection with miRNA-429 mimic inhibitor; **e** Transwell invasion assay showed that the invasion of U251 cells was reduced post-transfection with miRNA-429 mimics. **f** Transwell invasion assay showed that cell invasion was enhanced post-transfection with miRNA-429 mimics. The data represent the mean ± SDs repeated in triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$

of miRNA-429 knockdown by transfecting U251 cell line into a miRNA-429 inhibitor, cell growth was significantly increased compared with miRNA-NC group (Fig. 3d). It was also observed that overexpression of miRNA-429 suppressed cell colony formation and invasion, while the knockdown of miRNA-429 induced cell colony formation and invasion, compared with that of the miRNA-NC (Fig. 3e, f). These data provide sufficient and concrete evidence that over-expression of miRNA-429 effectively reduced the survival rate of glioblastoma cells, while the knockdown of miRNA-429 considerably increased the survival rate of glioblastoma cells.

BMI1 Reverses the Effects of over-Expression of miRNA-429 in U251 Cells

To further explore the role of BMI1 on the biological effects of miRNA-429, the impact of BMI1 expression with miRNA-429 expression was conducted. As shown in Fig. 4a, over-expression of miRNA-429 repressed the BMI1 expression, indicating that BMI1 expression was promoted by repressing miRNA-429. And CCK-8 assay was used to investigate U251 cells viability, as indicated in Fig. 4b, proliferation of U251 cells transfected with miRNA-429 mimics (overexpression of miRNA-429) was significantly inhibited, while enhanced BMI1 expression significantly undermined the inhibitory effect of miRNA-429 on cell proliferation. Moreover, as shown in Fig. 4c, invasion of U251 cells was measured by conducting transwell chamber invasion experiment, after the U251 cells were transfected with miRNA-429, which inhibits the expression of BMI1, U251 cells decreased significantly. The over-expression of BMI1 with plasmid vector could attenuate the inhibitory effect of miRNA-429 on the invasion and proliferation of U251 cells. These results demonstrated that BMI1 is capable of reversing the overexpression effect of miRNA-429 in U251 cell lines.

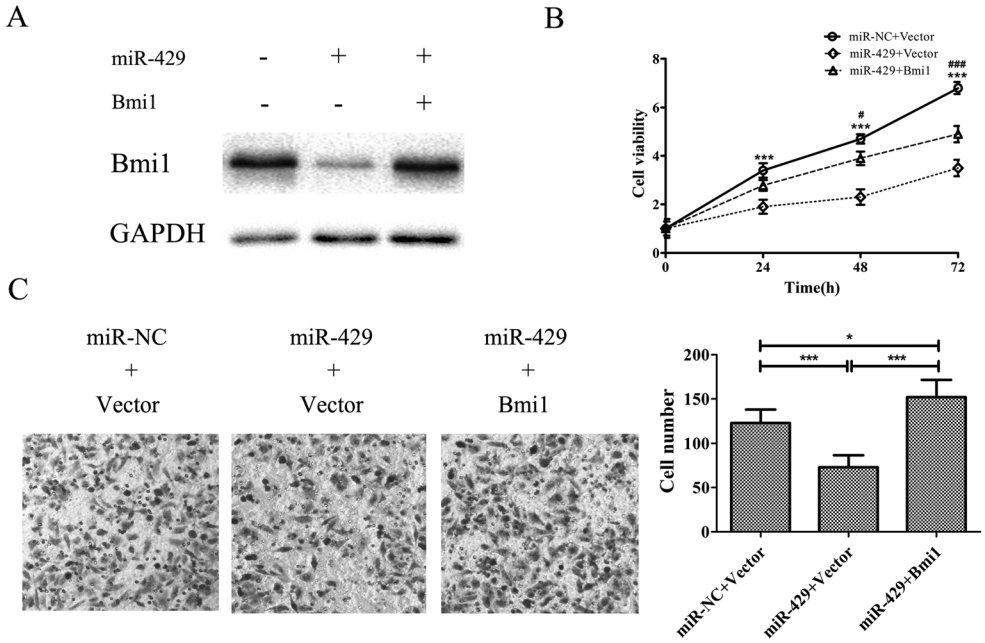

Fig. 4 miRNA-429 repressed the proliferation and invasion of astrocytoma by targeting BMI1, the overexpression of BMI could inverse the inhibitory effect of miRNA-429. **a** Western blot analysis showed the protein expression level of BMI1, in U251 transfected with miRNA-429 mimics or BMI1-vector; **b** CCK8 assay showed cell proliferation transfected with miRNA-429 mimics repressed or BMI1-vector; **c** Transwell invasion assay showed that cell invasion was decreased significantly after miRNA-429 mimic transfection, so was cell number. However, BMI1-vector weakened the suppressive effect of miRNA-429. The data were based on experiments conducted independently three times. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Discussion

miRNAs have been reported to regulated the expression of around one thirds of human genes [3–5]. Therefore, the expression patterns of microRNAs are associated with human diseases, especially with cancer. In most case, miRNAs might function as either oncogene or tumor suppressor gene [3, 4, 13, 14]. Investigation on the effects of miRNA in cancer specimens and cell lines has been enormously fruitful in helping deepen the understanding of carcinogenesis.

In the present study, we focused on gliblastoma. Known for high rate of mortality, glioblastoma multiforme (GBM) is regarded as one of the most dangerous type of cancers in a global scale. GBM accounts for around 52 % of all types of brain tissue tumor, as well as about 20 % of tumors arising inside human skull [15, 16]. Previous studies reported that dysfunction of miRNAs is associated with several types of human tumor, hence modulating miRNA activities may provide promising opportunities for cancer treatment and therapy. The better understanding of the carcinogenic mechanisms may also help identify biomarker for cancer or develop therapeutic strategies for cancer treatment [17].

MicroRNAs are a growing cluster of small, non-coding RNAs predicted to involve in regulating human genes expression at post-transcriptional and translational levels [18]. Deregulation of miRNAs has been frequently detected in cells. miRNAs regulate and modulate a vast variety of biological processes and cellular activities, including but not limited to cell proliferation, invasion, apoptosis, metastasis and development [4, 5, 10, 19, 20].

A few miRNA-429 targets have been predicted and identified using TargetScan. Zhang M et al. reported that miRNA-429 function as tumor suppressor in gastric cancer cells, confirming that FSCN1 is one target gene, knockdown of FSCN1 mimics the effect of miRNA-429 overexpression [11]. Qiu M et al. focused on renal cell carcinoma, indicated the inhibitive effect of miRNA-429 and two targets BMI1 and E2F3 [21].

In agreement with these findings, the current study demonstrated the tumor suppressive effect of miRNA-429, whose expression level was significantly down-regulated both in patients' astrocytoma samples and cancer cell lines of U251, U87 and LN229, compared with that in normal brain tissue. Moreover, we also confirmed that BMI is a target of miRNA-429. Protein and miRNA expression levels of BMI1 were significantly higher in human glioma tumor samples, as well as in cell lines (U251, U87 and LN229), compared with matched non-tumor tissues. And the knockdown of BMI1 significantly decreased the tumor cell survival rate and tumor cells number.

Previous studies reported that miRNAs function in vivo by binding to the characteristic sites in the 3′-UTRs of target gene

[4, 13, 22]. In the present study, the BMI 3′-UTR was observed to be targeted by miRNA-429 using microRNA Targetscan software. Our study confirmed a negative correlation between miRNA-429 and BMI1 expression. 24 h post-transfection of the miRNA-429 inhibitor into U251 cell line resulted in the enhancing of BMI1 expression, while the over-expression of miRNA-429 by mimic transfection reduced BMI1 expression. Spearman's correlation analysis calculated the correlation between the expression levels of miRNA-429 and BMI1 in patients' astrocytoma. The result indicated that an inverse correlation, $r = -0.586$ ($n = 34$). BMI1 is a direct target of miRNA-429 in GBM tumorigenesis.

Cell proliferation and invasion are notably characteristics for tumor cells. In the current study, cell proliferation was investigated by CCK8 assay, which indicated that the inhibition of miRNA-429 promoted cell viability. Invasion assay was conducted to measure cell invasion, revealing that colony formation of U251 cells decreased significantly after being transfected with miRNA-429. The overexpression of miRNA-429 suppressed the proliferation, and invasion of GBM tumor cells; on the other hand, the down-regulation of miRNA-429 promoted tumor cell growth and invasion.

Therefore, our study demonstrated the tumor suppressive effects of miRNA-429 in GBM progression [11, 17, 23–28]. As a potential oncogene, the overexpression of BMI1 considerably promoted the proliferation and invasion of tumor cells, weakening the inhibitive effect of miRNA-429.

In conclusion, the present manuscript focused on miRNA-429 expression pattern and its role in regulating GBM tumor cell proliferation and invasion at cellular level. Our findings demonstrated that the up-regulation of miRNA-429 enormously inhibited the growth and invasion of GBM tumor cells and its expression was inversely associated with BMI1 in human GMB tissues and cell lines. The study contributes to identify the tumor suppressive effect of miRNA-429, implying its potential application in GBM treatment.

Conflict of Interest This manuscript was supported by China hunan provincial science & technology department, grant No: 2013FJ4102.

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