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Original Article

MicroRNA miR-1249 downregulates adenomatous polyposis coli 2 expression and promotes glioma cells proliferation

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Abstract: Continuous activation of the Wnt/β-Catenin signaling has been reported to play important roles in multiple process of tumor progression, leading to uncontrolled cancer cell proliferation, growth, and survival. However, the mechanism underlying the regulation of the Wnt/β-Catenin pathway remains largely unknown. Determining the molecular mechanism of the Wnt/β-Catenin pathway's aberrant activation in glioma carcinogenesis might improve therapeutic strategies for patients with glioma. In this study, we showed that the expression of microRNA miR-1249 was markedly upregulated in glioma cell lines and tissues. Upregulation of miR-1249 enhanced, whereas downregulation inhibited, the proliferation of glioma cells both *in vitro* and *in vivo*. Furthermore, bioinformatic and experimental approaches showed that miR-1249 targets and suppresses APC2 expression, an important Wnt/β-Catenin pathway-regulated factor. These data suggested that miR-1249 could be a novel therapeutic target of microRNA-mediated cell proliferation in glioma.

Keywords: MiR-1249, APC2, Wnt/β-catenin, proliferation, glioma

Introduction

Glioma, which accounts for approximately 70% of all brain tumor cases, is the most aggressive tumor in the central nervous system, and is considered to be one of the deadliest human cancers [1-3]. Although significant improvements have been made in neurosurgical techniques and the development of new chemotherapeutic agents, the overall prognosis for glioma patients remains poor. The 1-year overall survival rate of patients with glioma is less than 30%, and the median survival time of patients with high-grade gliomas is only 15 months [4-6]. Evidence suggests that progression of glioma depends on the rate of cell proliferation. Therefore, there is an urgent clinical challenge to understand the key regulatory mechanisms of sustained cell growth and proliferation in glioma, as well as to develop novel and effective therapeutic strategies for this deadly disease.

Molecular pathways controlling cell proliferation, such as the Wnt/β-catenin pathways, have been shown to play important roles in tumor progression [7-9]. Constitutive activation of β-catenin signaling plays key roles in the development and progression of glioma, and contributes to the therapeutic failure and poor prognosis of patients with glioma [10-12]. For instance, Wickström and colleagues found that activated Wnt/β-catenin signaling results in resistance of glioma cells to chemotherapy and promotes cell survival; however, inhibition of Wnt/β-catenin signaling dramatically reduced the proliferation of glioma cells [13]. Negative regulation of Wnt/β-catenin signaling by membrane-associated guanylate kinase inverted 3 (MAGI3) suppresses malignant phenotypes of glioma cells [14]. It is reported that β-Catenin is overexpressed in malignant glioma and plays an important role in proliferation and apoptosis in glioblastoma cells [15]. Conversely, blockade of wnt signaling contributed to the inhibition of proliferation and tumor sphere formation in glioblastoma cells [16]. In addition, the pharmacological downregulation of Wnt reduced the proliferation, survival, and clonogenicity of glioblastoma cells [17].

Therefore, the discovery of novel molecules that regulate the aberrant activation of the Wnt/β-catenin signaling pathway could be important for clinical glioma therapy.

Adenomatous polyposis coli 2 (APC2), a well-known homolog of the adenomatous polyposis coli (APC) tumor suppressor gene, regulates the Wnt/β-catenin signaling pathway negatively [18-21]. The abundant and specific expression of APC2 in the central nervous system indicated an important role in neuronal proliferation and differentiation [22, 23]. Nakagawa *et al.* showed that APC2 expression was reduced significantly in most glioma tissues and cell lines compared with that in normal brain tissue [23]. However, single-strand conformation polymorphism (SSCP) analysis and DNA sequencing of the entire coding region of APC2 detected no mutations in any of the glioma cell lines. These data suggested that an epigenetic mechanism is involved in the decrease in APC2 expression in glioma.

Given that one miRNAs that can simultaneously interact with multiple targets, we hypothesis it might be the molecular to modulate negative regulators of the β-catenin pathway at different layers. In current study, we found that the microRNA miR-513a-5p was significantly overexpressed in ovarian cancer and enhanced the stem cell-like traits by downregulation of multiple negative modulators of Wnt/β-catenin pathway, including DKK1, Axin2 and ICAT. Therefore, our results suggest that miR-513a-5p might serve as a novel therapeutic target for ovarian cancer.

MicroRNAs (miRNAs) are endogenous non-coding small RNAs that modulate gene expression at the post-transcriptional level [24-26]. Accumulating evidence has shown that, miRNAs are altered aberrantly in diverse cancers, including breast cancer, colon cancer, glioblastoma, and glioma by inducing tumor proliferation and metastasis [27-30]. Based on the clinical relationship between miRNAs and carcinogenesis, miRNAs are considered as potential targets for cancer diagnosis and anti-cancer therapies. In current study, we found that the microRNA miR-1249 was significantly overexpressed in glioma cell lines and tissues and enhanced the proliferation ability in glioma cells by targeting *APC2* mRNA expression. Therefore, our results suggest that miR-1249 might serve as a novel therapeutic target for glioma.

Material and methods

Cell culture

Primary normal human astrocytes (NHA) were purchased from Sciencell Research Laboratories (Carlsbad, CA) and cultured as manufacturer suggested. Glioma cell lines, including U87MG, LN-18, LN-443, LN-340, A172, LN-229, D27MG were cultured in Dulbecco's modified Eagle's medium in the presence of 10% FBS, 100 units/ml penicillin, and 100 g/ml streptomyc in a humidified 5 % (v/v) atmgliomaphere of CO_2 at 37°C incubator.

Tissue specimens

Twenty freshly dissected glioma tumor specimens were clinically and histopathologically diagnosed at Qilu Hospital of Shandong University. Four normal brain tissues were obtained by donation from individuals who died intraffic accidents and confirmed to be free of any pathologically detectable lesions. Prior consents of donors and approvals from the Institutional Research Ethics Committee were obtained.

RNA extraction, reverse transcription (RT) and real-time PCR

Total cellular RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. cDNAs were amplified and quantified in an ABI Prism 7500 Sequence Detection System (Applied Bigliomaystems, Fgliomater City, CA, USA) using dye SYBR Green I (Molecular Probes, Invitrogen). The primers were follows: *c-myc* forward: 5'-TCAAGAGGCGAACACACAAC-3'; *c-myc* reverse: 5'-GGCCTTTTCATTGTTTTCCA-3'; *LEF* forward: 5'-CACTGTAAGTGATGAGGGGG-3', *LEF* reverse: 5'-TGGATCTC TTTCTCCACCCA-3'; *TCF4* forward: 5'-CCAACTTCTTTGGCAAGTGG-3', *TCF4* reverse: 5'-TCTCCATAGT TCCTGGACGG-3'; *Cyclin D1* forward: 5'-AACTACCTGGACCGCTTCCT-3', *Cyclin D1* reverse: 5'-CCACTT GAGCTTGT-TCACCA-3'.

Expression levels of genes were normalized to that of the housekeeping gene *GAPDH* as the control (*GAPDH* forward primer, 5'-GACTCA-TGACCACAGTCCA TGC-3'; reverse primer, 3'-AG-

AGGCAGGGATGATGTTCTG-5'), and calculated as $2^{-[(Ct\ of\ Cyclin\ D1,\ MYC,\ TCF4,\ LEF1)\ -\ (Ct\ of\ GAPDH)]}$, where C_t represents the threshold cycle for each transcript. The expression of the miRNA was defined based on the threshold cycle (Ct), and relative expression levels were calculated as $2^{-[(Ct\ of\ miR\text{-}1249)\ -\ (Ct\ of\ U6)]}$ after normalization with reference to expression of U6 small nuclear RNA.

Western blotting

Western blotting analysis was performed according to standard methods. The membranes were probed with polyclonal rabbit antibodies against anti-APC2 (1:500; Abcam, Cambridge, MA, USA), anti-cyclinD1, anti-β-Catenin and anti-Myc (1:1,000; Cell Signaling, Danvers, MA, USA). The membranes were stripped and re-probed with an anti-α-Tubulin mouse monoclonal antibody (1:1,000; Sigma, Saint Louis, MO, USA) as a loading control.

Plasmid, oligonucleotides, siRNA and transfection

The region of the human APC2 3'-UTR, from 900 to 1700 bp, generated by PCR amplification from DNA of the 293FT cells, was cloned into vector pGL3 (Promega, Madison, WI, USA). The primers selected were as follows: APC2-3'UTR-wt-forward: 5'-GCCCCGCGGAAGCAGCCTAGCACAGAC-3', APC2-3'UTR-wt-reverse: 3'-GCCCTGCAGTCCTTAACAGGCAGGGAT-5'. For depletion of APC2, the siRNA was synthesized and purified by RiboBio. The APC2 siRNA sequences used were: AACATAGACATCACTGCTGGG. The miR-1249 anti-sense was cloned into miRZip plasmid purchased from System Biosciences (San Francisco, CA) and used according to previous report. To generate a mir-1249 expression vector, approximately 300-bp genomic fragment up and downstream of the pre-mir-1249 form was generated by PCR amplification from genomic DNA, and subcloned into the EcoRI and SpeI sites of the pSin-EF2-puro retroviral vector (Clontech Laboratories Inc., Mountain View, CA). The primers for amplifying miR-1249 were as the following: miR-1249-forward: 5'-GCCGAATTCGAGGGATGTGCTAGGGTCAC-3'; miR-1249-reverse: 3'-GCCACTAGTCCCCACTTCTAATCACGTTTG-5'. The miR-1249 inhibitor and negative control (NC) were purchased from RiboBio (RiboBio Co.Ltd, Guangzhou, Guangdong, China). Transfection of oligonucleotides and siRNA were performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Generation of stably engineered cell lines

pSin-EF2-miR-1249 and pSin-EF2-Vector were co-transfected with the PMD2g and PSPAX packaging plasmid into 293FT cells using the standard calcium phosphate transfection method. Twenty-four hours after co-transfection, supernatants were collected and incubated with cells to be infected for 24 hours in the presence of polybrene (2.5 μg/ml). After infection, puromycin (1.5 μg/ml) was used to select stably transduced cells over a 10-day period.

Luciferase assay

Glioma cells (3.5×10^4) were seeded in triplicate in 24-well plates. 24-hours later, indicated luciferase reporter plasmids plus 3 ng pRL-TK Renilla plasmid were transfected into the cells using Lipofectamine 2000 Reagent (Life Technologies, USA). 48 hours after transfection, Dual Luciferase Reporter Assay (Promega, USA) was performed according to the manufacturer's instructions. Three independent experiments were performed and the data were presented as the mean ± SD.

Flow cytometry analysis

All cells in a culture dish were harvested by trypsinization, washed in ice-cold PBS, and fixed in 80% ice-cold ethanol. Before staining, the cells were pelleted in a cooled centrifuge and resuspended in cold PBS. Bovine pancreatic RNAase (Sigma) was added at a final concentration of 2 μg/ml, and cells were incubated at 37°C for 30 min, followed by incubation in 20 μg/ml propidium iodide (Sigma) for 20 min at room temperature. 20,000 cells were analyzed on a flow cytometer (FACSCalibur; BD Biosciences, Bedford, MA, USA).

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

U87MG, LN-229 cells were seeded into 96 well plates and stained at the indicated time point with 100 μl sterile 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO) dye (at 0.5 mg/ml) for 4 h at 37°C, followed by removal of the culture

medium and the addition of 150 μl dimethyl sulfoxide (Sigma-Aldrich). The absorbance was measured at 570 nm, with 655 nm as the reference wavelength. All experiments were performed in triplicate.

Colony formation assays

U87MG, LN-229 cells were plated on 60 mm plates (0.5×10^3 cells per plate) and cultured for 10 days. The colonies were stained with 1.0% crystal violet for 30 s after fixation with 10% formaldehyde for 5 min.

Anchorage-independent growth ability assay

One thousand cells were trypsinized and suspended in 2 ml complete medium plus 0.33% agar (Sigma). The agar-cell mixture was plated on top of a bottom layer comprising 0.66% complete medium agar mixture. After 10 days, colony sizes were measured with an ocular micrometer and colonies greater than 0.1 mm in diameter were counted. The experiment was performed for independently three times for each cell line.

Five hundred cells were trypsinized and seeded into 0.33% top agar with DMEM and spread onto 6-well plates containing 1% bottom agar with DMEM. The cells were grown at 37°C for 10 days and viable colonies that contained more than 50 cells or were larger than 0.1 mm were counted. Colony size was measured with an ocular micrometer and colonies greater than 0.1 mm in diameter were counted. The experiment was performed for three independently times for each cell line.

Bromodeoxyuridine labeling and immunofluorescence

U87MG, LN-229 cells grown on coverslips (Fisher, Pittsburgh, PA) were incubated with bromodeoxyuridine (BrdUrd) for 1 h and stained with anti-BrdUrd antibody (Upstate, Temecula, CA) according to the manufacturer's instruction. Gray level images were acquired under a laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co. Ltd., Jena, Germany).

Xenografted tumor model, IHC, and H&E staining

BALB/c-nu mice (4-5 weeks of age, 18-20 g) were purchased from the Center of Experimental Animal of Guangzhou University of Chinese Medicine. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Shandong University. The BALB/c nude mice were randomly divided into two groups (n = 5/group). One group of mice was inoculated subcutaneously with U87MG/Vector cells (5×10^6) in the left dorsal flank and with U87MG/miR-1249 cells (5×10^6) in the right dorsal flank per mouse. Another group was inoculated subcutaneously with miRZip/vector cells (5×10^6) in the left dorsal flank and with U87MG/miRZip/miR-1249 cells (5×10^6) in the right dorsal flank. Tumors were examined twice weekly; length and width measurements were obtained with calipers and tumor volumes were calculated using the equation $(L*W^2)/2$. On day 40, tumors were detected by an IVIS imaging system, and animals were euthanized, tumors were excised, weighed and paraffin-embedded. Serial 5.0 μm sections were cut and subjected to IHC analyzed using an anti-Ki67 antibodies (Dako, Glostrup, Denmark). Proliferation index was quantized by counting proportion of Ki67-positive cells. Apoptotic index was measured by percentage of TUNEL-positive cells.

Statistical analysis

Statistical tests for data analysis included Fisher's exact test, log-rank test, Chi-square test, and Student's 2-tailed t test. Statistical analyses were performed using the SPSS 19.0 statistical software package. Data represent mean ± SD. $P < 0.05$ was considered statistically significant.

Microarray data process and visualization

Microarray data were downloaded from the GEO database: (http://www.ncbi.nlm.nih.gov/geo/).

Results

MiR-1249 expression is elevated in glioma cell lines and tissues

By analyzing a published microarray-based high-throughput assessment, miR-1249 was identified to be significantly upregulated in human malignant glioma tissues compared with glioma peritumoral tissue (n = 15; $P <$

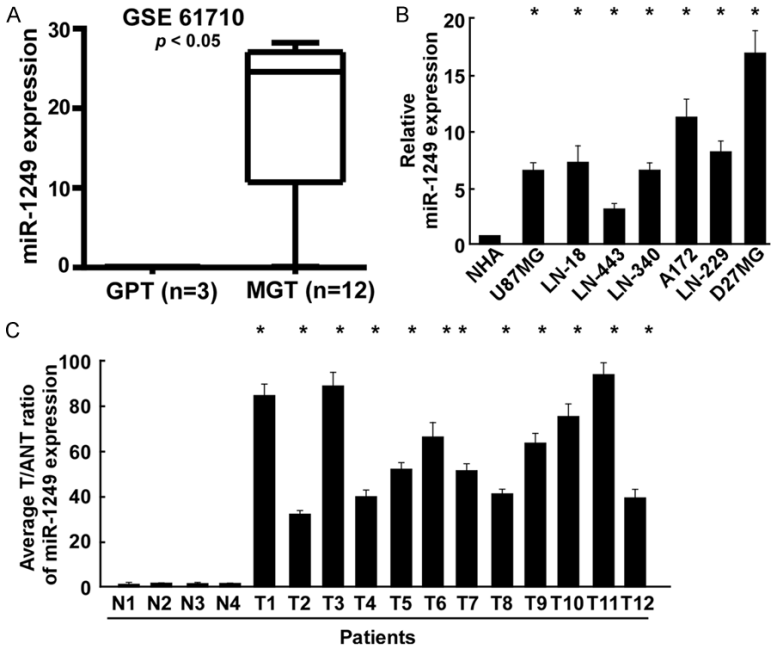

Figure 1. Expression of miR-1249 is increased in glioma tissues and cell lines. A. Expression profiling of miRNAs showing that miR-1249 is upregulated in human malignant glioma tissues compared with glioma peritumoral tissue (n = 15, P < 0.05; NCBI/GEO/GSE 61710). GPT, Glioma peritumoral tissue; MGT, Malignant glioma tissue. B. The expression of miR-1249 was examined in a normal human glial cell line and glioma cells, including U87MG, LN-18, LN-443, LN-340, A172, LN-229, and D27MG. NHA, normal human astrocytes. The average miR-1249 expression was normalized using U6 expression. C. Real-time PCR analysis of miR-1249 expression in 12 glioma tissues (T) and four noncancerous tissues (N). Each bar represents the mean ± SD of three independent experiments. *$P < 0.05$.

0.05; NCBI/GEO/GSE61710; **Figure 1A**). miR-1249 expression was markedly increased in all 7 glioma cell lines compared with primary normal human astrocytes NHA (**Figure 1B**). Moreover, Real-time PCR analysis reveal that miR-1249 was significantly overexpressed in 12 glioma tissues compared with the 4 normal brain tissues (**Figure 1C**). Collectively, these results suggested that miR-1249 was upregulated in glioma.

Ectopic expression of miR-1249 enhances proliferation of glioma cells in vitro

To investigate the effect of miR-1249 on the progression of glioma, gain-of-function studies using the miR-1249 expression vector pSin-EF2-miR-1249 were used to investigate the effect of endogenous miR-1249 on the proliferative properties of glioma cells (**Figure 2A**). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and colony formation assays showed that overexpression of miR-1249 increased the growth rate of U87MG and LN-229 glioma cell lines dramatically compared with that of control cells (U87MG and LN-229 cells transformed with the empty vector; NC) (**Figure 2B** and **2C**). Importantly, the anchorage-independent growth assay revealed that U87MG-miR-1249 cell and LN-229-miR-1249 developed more and larger-sized colonies than their corresponding control cells (**Figure 2D**). Furthermore, a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay showed that the percentage of cells in the DNA replication phase was increased dramatically in glioma cells overexpressing miR-1249 compared with the control cells (**Figure 2E**). Moreover, flow cytometry analysis of the cell cycle of U87MG-miR-1249 cell and LN-229-miR-1249 showed a significant decrease in the percentage of cells in the G1/G0 phase and an increase in the percentage of cells in the S phase (**Figure 2F**). Taken together, these results suggested that overexpression of miR-1249 promoted the proliferation of glioma cells.

Inhibition of miR-1249 repressed the proliferation of glioma cells in vitro

Loss-of-function studies using a miR-1249 antisense were used to further investigate the biological function of endogenous miR-1249 in the proliferation of glioma cells (**Figure 3A**). As shown in **Figure 3B-E**, suppression of miR-1249 in glioma by transfection with the miRZip-1249 decreased the growth rate of glioma cell lines significantly compared with that of the NC cells. Mechanically, flow cytometry showed a significant increase in the percentage of cells in the G1/G0 phase and a decrease in the percentage of cells in the S phase in U87MG-miRZip-1249 cells and LN-229-

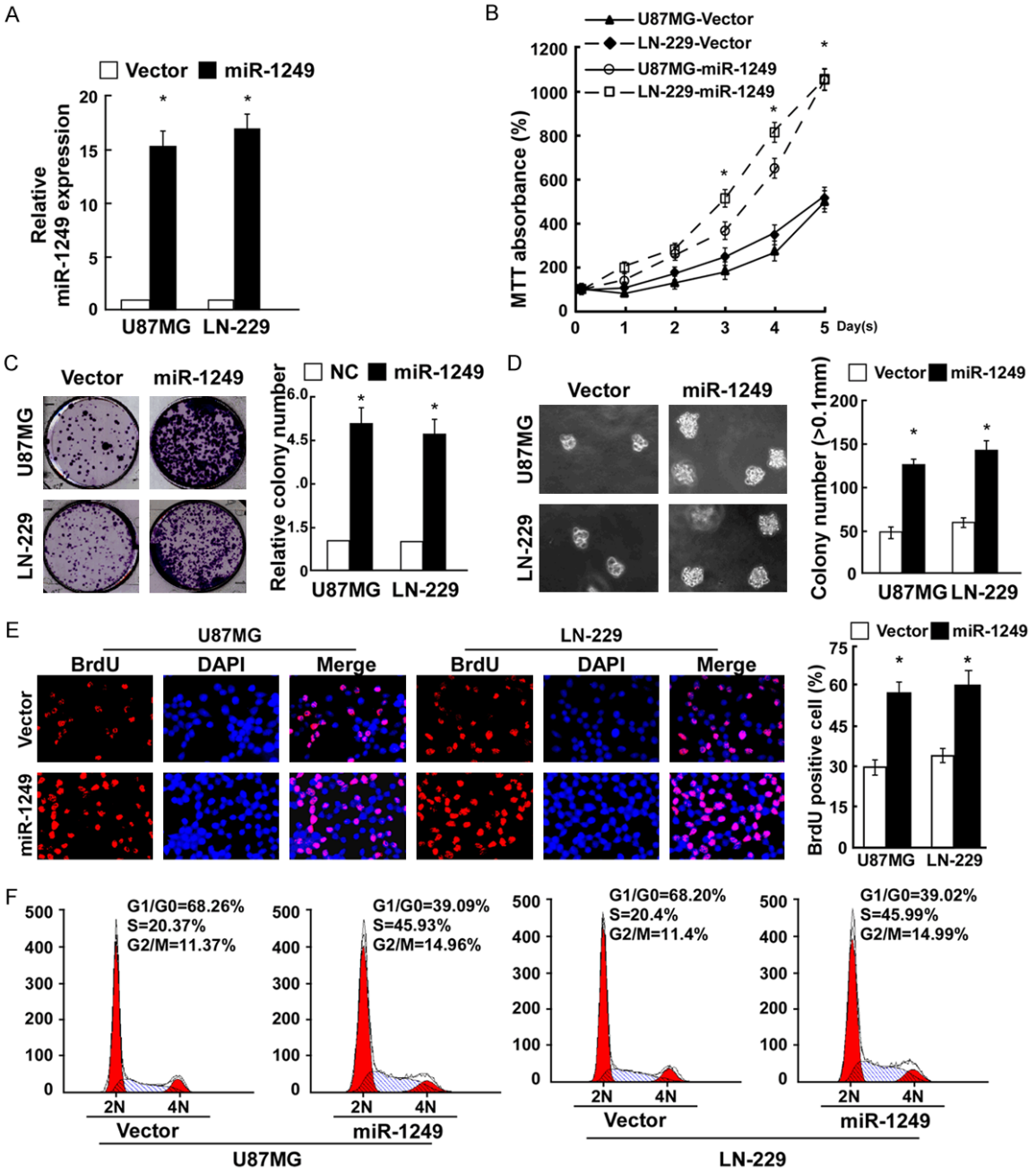

Figure 2. Upregulation of miR-1249 promotes the proliferation of glioma cells *in vitro.* A. Real-time PCR analysis of miR-1249 expression in U87MG and LN-229 cells expressing miR-1249 and control cells. B. Effects of ectopic miR-1249 expression on the proliferation of U87MG and LN-229 cells lines, as analyzed by the MTT assay. C. Representative micrographs (left) and quantification (right) of crystal violet stained cell colonies formed by the indicated glioma cell lines, 10 days after inoculation. D. Effects of ectopic miR-1249 on the proliferation of U87MG and LN-229 cell lines, as determined by an anchorage-independent growth ability assay. Colonies larger than 0.1 mm were scored. E. Representative micrographs (left) and quantification (right) of BrdU glioma signaling in the cells transfected with miR-1249 or Vector. F. Effects of miR-1249 overexpression on the cell cycle progression of glioma cells, as measured by flow cytometry analysis. Each bar represents the mean ± SD of three independent experiments. *$P < 0.05$.

miRZip-1249 cells compared with Vector cells (**Figure 3F**). These results suggested that downregulation of miR-1249 suppressed the proliferation of glioma cells.

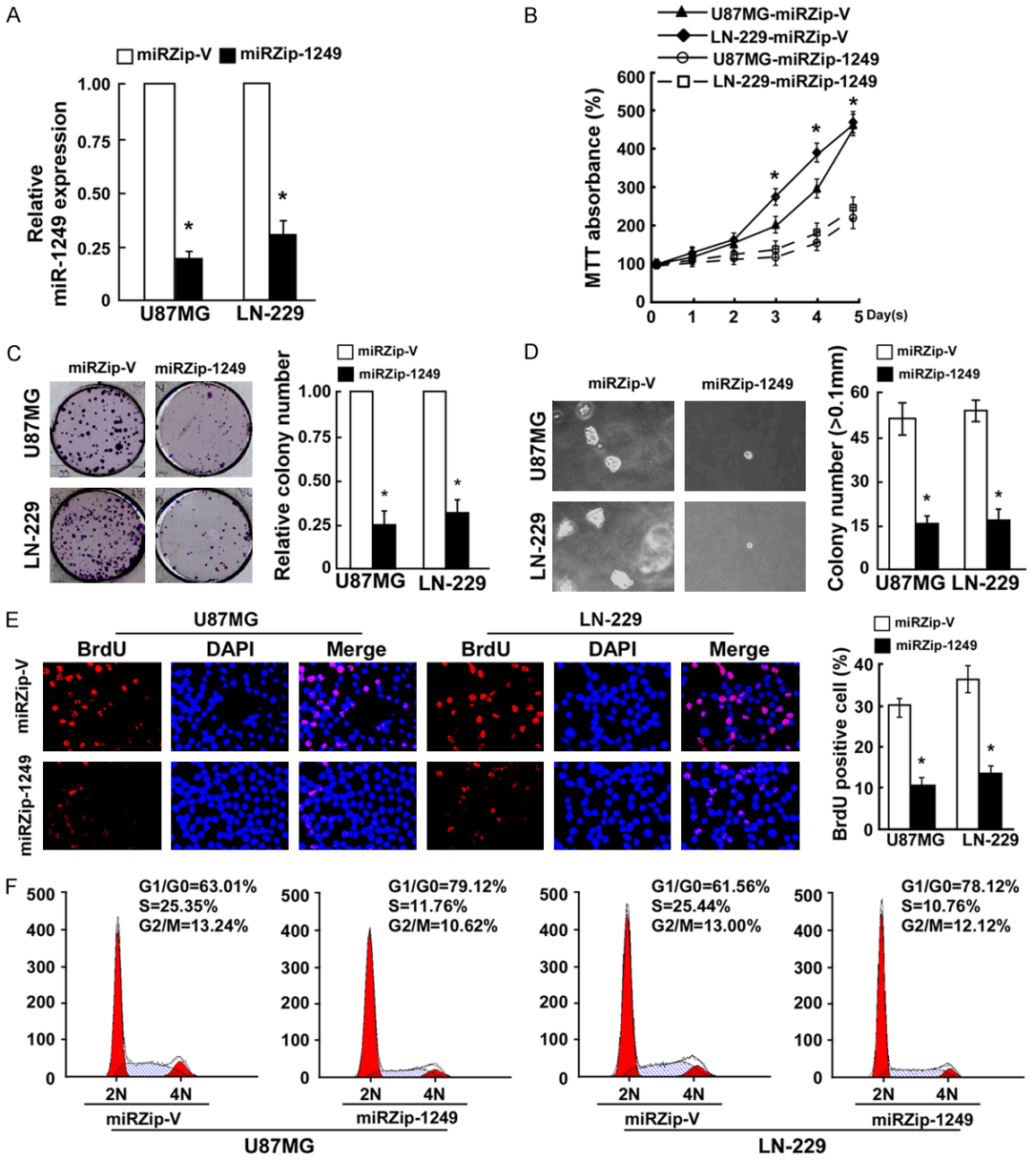

Figure 3. Inhibition of miR-1249 reduces glioma proliferation *in vitro*. A. Real-time PCR analysis of miR-1249 expression in U87MG and LN-229 cells expressing the miRZip-1249 and control cells (Vector). B. The proliferation ability of glioma cells U87MG and LN-229 cells transfected with miRZip-1249 or Vector measured by the MTT assay. C. Representative micrographs (left) and quantification (right) of crystal violet stained cell colonies formed by the indicated glioma cell lines, 10 days after inoculation. D. The proliferation of glioma cells U87MG and LN-229 cells transfected with miRZip-1249 or Vector, as measured by an anchorage-independent growth ability assay. Colonies larger than 0.1 mm were scored. E. BrdU indicated glioma cells transfected with miR-1249-inor NC. F. Effects of miRZip-1249 on the cell cycle progression of glioma cells, as measured by flow cytometry. Each bar represents the mean ± SD of three independent experiments. *$P < 0.05$.

Over-expression of miR-1249 contributes to glioma progression in vivo

The biological effects of miR-1249 overexpression on glioma progression were further examined using a xenograft tumor model. As showed in Supplemental **Figures 3** and **4A-C**, tumors formed by miR-1249-overexpressing cells exhibited a greater size and mass than tumors formed by the control cells. Conversely, tumors

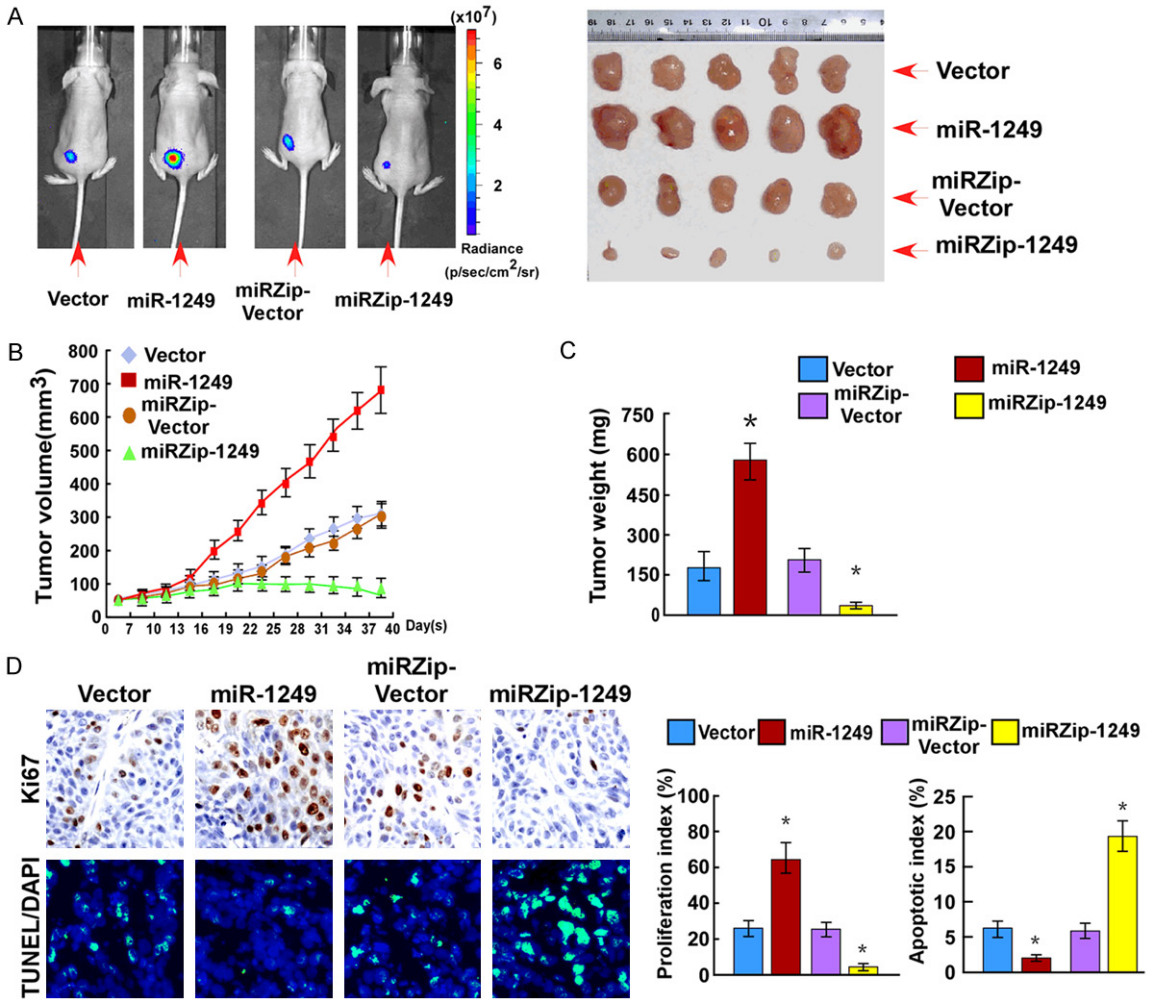

Figure 4. Upregulation of miR-1249 promotes the proliferation of glioma cells *in vivo*. A. Representative images of tumor-bearing mice (left) and tumors from all of the mice in each group (right). B. Tumor volumes were measured on the indicated days. C. Mean tumor weights. D. IHC staining demonstrated the expression of Ki67 as well as TUNEL-positive cells in the indicated tissues.

formed by miR-1249-silenced glioma cells were smaller and had lower tumor weights than control tumors. IHC analysis revealed that miR-1249-overexpressing tumors showed increased percentages of Ki67-positive cells and fewer TUNEL-positive cells, whereas miR-1249-silenced tumors displayed lower Ki67 proliferation index and a higher percentage of TUNEL-positive apoptotic cells (**Figure 4D**). Taken together, our findings indicate that miR-1249-overexpression contributes to glioma progression *in vivo*.

APC2 is a direct target of miR-1249 in glioma cells

The Wnt/β-catenin pathway is an important regulator of tumor initiation and progression, and palys an important role in cell proliferation. Therefore, we detect the effect of miR-1249 in regulating the wnt/β-catenin signaling. As shown in **Figure 5A**, miR-1249 overexpression markedly increased the luciferase activity of TOPflash or FOPflash reporter; conversely, transfection of miRZip-1249 decreased the luciferase activity of TOPflash or FOPflash reporter, compare with vector or negative control, respectively. To explore the mechanism underlying the promoted effect of miR-1249 on Wnt/β-catenin signaling, we used publicly available algorithms to predict the potential targets of miR-1249 in humans. By using publicly available algorithms to predict the target(s) of miR-1249 in humans, the results indicated that APC2 was one of the potential targets of miR-

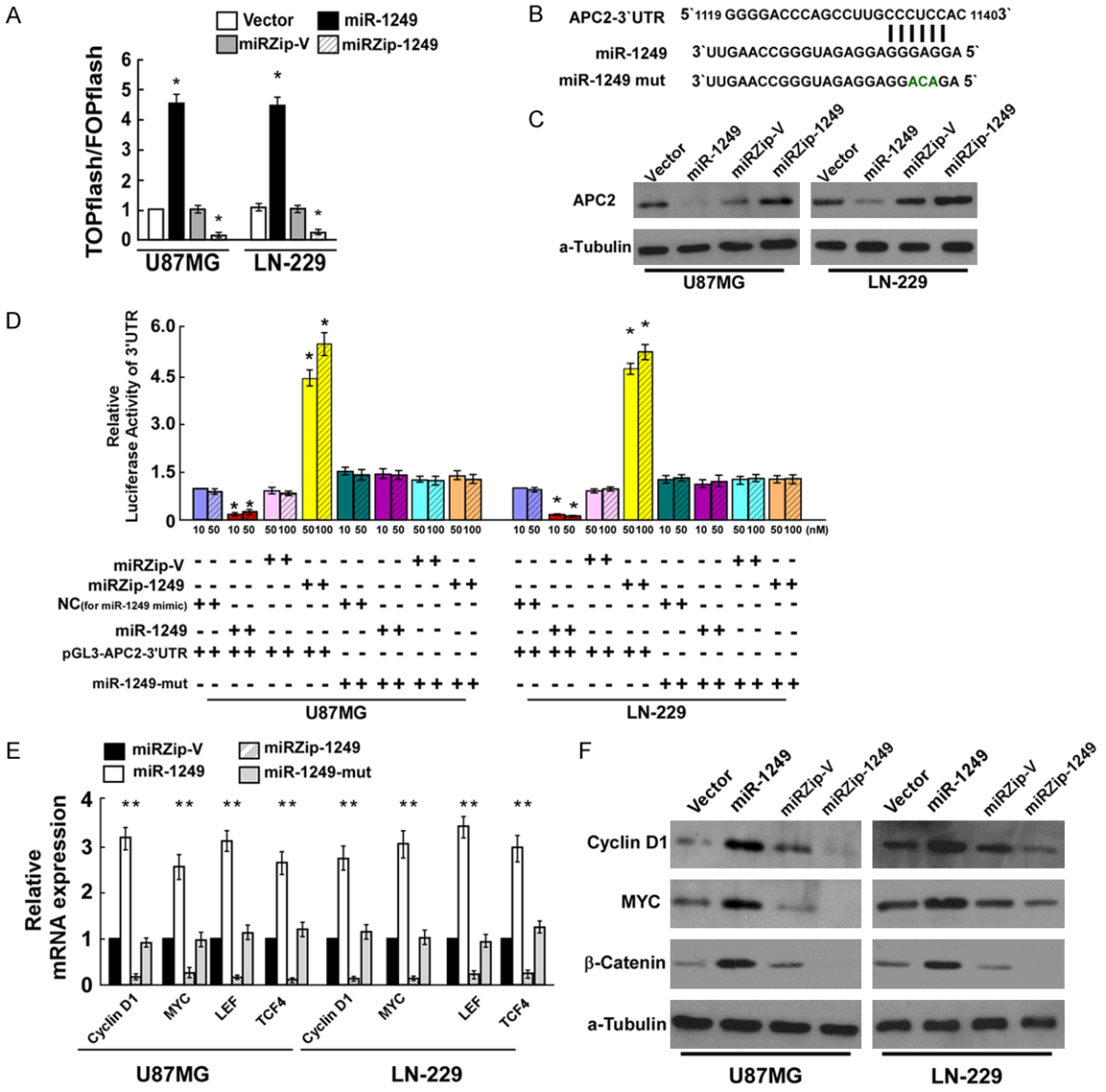

Figure 5. MiR-1249 directly targets the 3'UTR of *APC2* mRNA. A. Indicated cells transfected with TOPflash or FOPflash and Renilla pRL-TK plasmids were subjected to dual-luciferase assays 48 h after transfection. The detected reporter activity was normalized b using the Renilla luciferase activity. B. Schematic representation of the miR-1249 target sites in the 3'UTR of *APC2* mRNA and an miR-1249 mutant containing two altered nucleotides in the seed sequence (miR-1249-mut). C. The expression levels of APC2 protein in U87MG and LN-229 cells overexpressing or suppressing miR-1249, by western blotting 48 h after transfection. α-Tubulin served as the loading control. D. Luciferase assay of pGL3-APC2-3'UTR reporter co-transfected with different amounts (10 and 50 nM) of miR-1249 mimic in the indicated cells, or different amounts (50 and 100 nM) of miR-1249 inhibitor. E. Real-time PCR analysis of *APC2* downstream genes mRNA expression in the indicated glioma cells. F. Levels of β-Catenin, C-Myc, and CyclinD1 protein, as measured by western blotting, in the indicated glioma cells. α-Tubulin served as the loading control. Each bar represents the mean ± SD of three independent experiments. $^*P < 0.05$.

1249 (**Figure 5B**). As predicted, western blotting revealed that APC2 expression decreased in the U87MG and LN-229 cells overexpressing miR-1249 and increased in cells transfected with the miRZip-1249 (**Figure 5C**).

Futhermore, fragments of the the APC2-3'-UTR, containing the miR-1249 binding site, was subcloned into a pGL3 dual luciferase reporter vectors in order to examine whether miR-1249 mediated-APC2 downregulation was effected

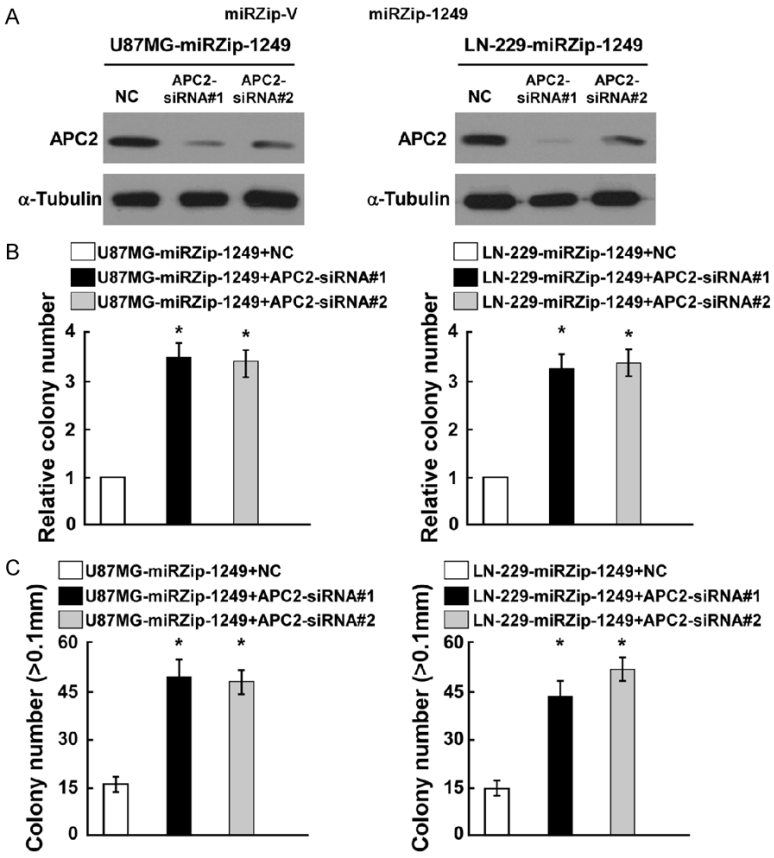

Figure 6. MiR-1249 promotes glioma progression by inhibiting APC2. A. The levels of APC2 in U87MG-miRZip-1249, and LN-229-miRZip-1249 transfected with APC2-siRNA, as measured by western blotting. α-Tubulin served as the loading control. B. Representative micrographs (left) and quantification (right) of crystal violet stained cell colonies formed by the indicated glioma cell lines 10 days after inoculation. C. Representative images (left) and quantification (right) of colony numbers of the indicated cells determined by an anchorage-independent growth assay. Colonies larger than 0.1 mm in diameter were scored. Error bars represent mean ± SD from three independent experiments. **P* < 0.05.

via the 3'-UTR of APC2. As shown in **Figure 5D**, ectopic expression of miR-1249 in U87MG and LN-229 cells dramatically decreased, and suppression of miR-1249 increased, the luciferase activity of the APC2 3'-UTR-luciferase reporter, however, point mutations in the miR-1249-binding seed region in APC2 3'-UTR abrogated the suppressive effect of APC2 mediated by miR-1249.

It has been well documented that APC2 could remarkably reduced Wnt/β-catenin signaling activity and the expression of its downstream genes, we further examined the expression of two Wnt/β-catenin downstream target genes, MYC LEF, TCF4 and Cyclin D1. The results showed that the mRNA of MYC, LEF, TCF4 and Cyclin D1 were significantly upregulated by ectopic miR-1249, whereas they were downregulated by inhibition of miR-1249; Moreover, the expression of β-Catenin, MYC and Cyclin D1 proteins were upregulated in miR-1249 overexpressing cells compared with the negative control cells (**Figure 5F**). Taken together, our results demonstrate that APC2 is a *bona fide* target of miR-1249.

Inhibition of APC2 is important for miR-1249-induced cell proliferation in glioma

The effect of APC2 reduction on glioma progression was examined by repressing endogenous *APC2* expression using an APC2-specific siRNA (**Figure 6A**). As showed in **Figure 6B** and **6C**, silencing *APC2* in miRZip-1249 transfected cells increased the ability of colony formation growth and anchorage-independent growth of glioma cells. The results suggested that further silencing *APC2* expression in U87MG-miRZip-1249 cells and LN-229-miRZip-1249 cells could reverse the inhibitory effect of the miRZip-1249 on glioma cells' proliferation. These data confirmed that miR-1249 promoted glioma cells proliferation by repressing endogenous *APC2* expression and that APC2 plays important role in miR-1249-mediated glioma cell proliferation.

Clinical relevance of miR-1249, β-catenin nuclear accumulation, and APC2 expression in Glioma

Finally, we examine whether miR-1249-mediated suppression of *APC2* and β-catenin nuclear accumulation in glioma tissues is clinically relevant. Using 10 freshly collected clinical glioma samples, we found that miR-1249 expression correlated inversely with the levels of APC2 (r =

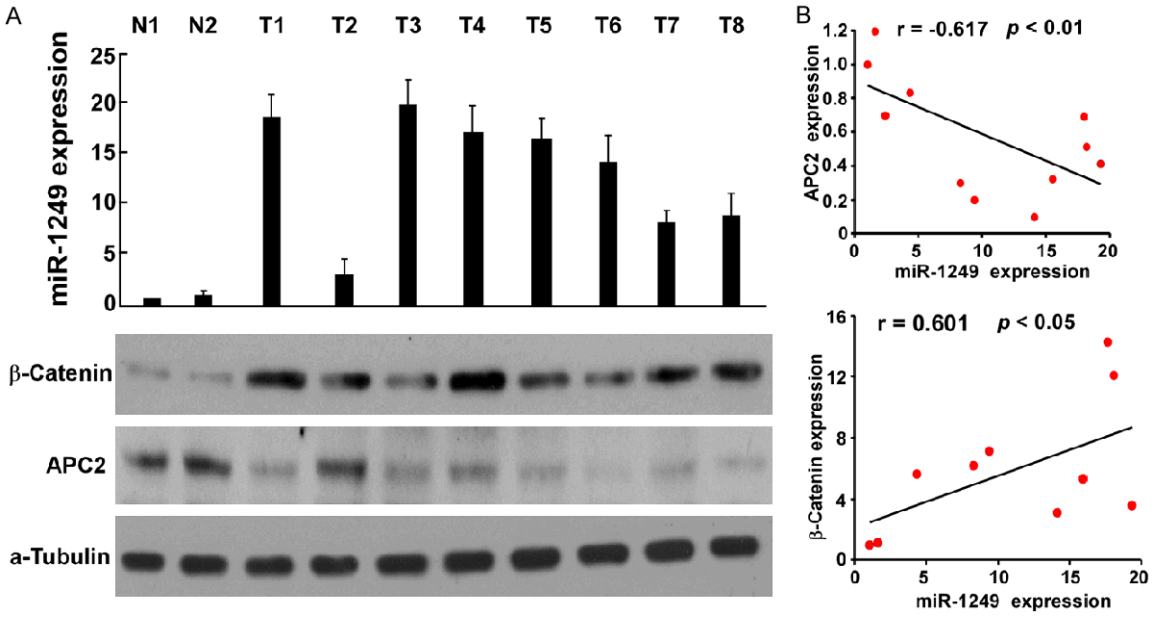

Figure 7. Clinical relevance of miR-1249 upregulation and APC2 protein expression in glioma. A. Expression of miR-1249, β-Catenin, and APC2 expression in glioma, as measured by real-time PCR (top) and western blotting (bottom), respectively. α-Tubulin served as the loading control. B. Correlations between miR-1249 and β-Catenin and APC2 protein levels were analyzed using SPSS software. Error bars represent the mean ± SD from of three independent experiments. *$P < 0.05$.

-0.617, $P < 0.01$), and correlated positively with the levels of β-catenin (r = 0.601, $P < 0.05$) (**Figure 7A** and **7B**). Collectively, these results support the hypothesis that miR-1249 upregulation promotes proliferation in glioma and activates the Wnt/β-catenin signaling pathway by repressing the important regulator of this pathway, APC2.

Discussion

In the current study, we provided evidence for a novel functional link between miR-1249 and the oncogenic Wnt/β-catenin activity in glioma cells. MiR-1249 is upregulated in glioma cell lines and tissues. Overexpression of miR-1249 promoted, and inhibiting miR-1249 expression suppressed, the proliferation of glioma cells both *in vitro* and *in vivo*. Furthermore, we demonstrated that miR-1249 promoted the activity of Wnt/β-catenin signaling by post- transcriptional downregulation of *APC2* expression. Therefore, miR-1249 might act as an oncogene by regulating β-catenin signaling and could be an important target for clinical intervention in glioma.

MicroRNAs have been demonstrated to function as either tumor suppressors or oncogenes in tumor development, and are key regulators in a wide variety of oncogenic processes, such as cell proliferation, survival, and differentiation [25, 26]. Therefore, determining the mechanism of miRNAs in tumor malignant development could provide effective diagnostic and therapeutic strategies for malignancy. Although little is known about miR-1249 in human cancers, it should be noted that miR-1249 is either downregulated or upregulated in different tumor types. For example, Okumura *et al.* suggested that miR-1249 was upregulated in patients with small cell carcinoma of the esophagus and this upregulation showed significant correlation with tumor relapse [31]. Meanwhile, a pilot study by Qiu and colleagues showed that miR-1249 was upregulated remarkably in papillary thyroid carcinoma patients with non-131I and 131I-avid lungs metastases, and suggested it might function as a diagnostic and therapeutic target in PTC patients with non-(131) I-avid metastatic disease [32]. Interestingly, we found that miR-1249 was significantly upregulated in glioma, which promoted the proliferation of glioma cells dramatically and inhibited APC2 signaling, demonstrating an oncogenic role of miR-1249 in glioma. Consistent with previous studies, our findings indicated an impor-

tant role of miR-1249 in modulating tumor progression, which suggested that it could be a promising therapeutic target in glioma. However, expression profiling to identify pathogenesis-related microRNAs in hepatocellular carcinoma showed that miR-1249 was downregulated in HCC tumor tissues compared with non-tumor tissues [33]. Collectively, these findings suggested that miR-1249 expression and its biological functions are tumor-type dependent. Therefore, it would be interesting to further investigate the clinical significance and mechanism of miR-1249 upregulation in glioma.

APC2, a newly-identified homolog of the APC tumor suppressor gene, is located on chromosome 19p13.3 and encodes a protein of 2303 amino acids that is expressed specifically in the brain [19, 23, 34, 35]. Like APC, the APC2 tumor-suppressor protein controls the Wnt signaling pathway by forming a "destruction complex" with GSK-3beta, axin, and β-catenin. Complex formation induces the rapid degradation of β-catenin, which subsequently terminates Wnt signaling. Van Es showed that loss of APC2 leads to the accumulation of β-catenin in the nuclei of colon carcinoma cells, where it binds to and activates the Tcf-4 transcription factor [36]. Moreover, a study by De Jong showed that promoter methylation of *APC2* might contribute to non-small cell lung cancer (NSCLC) [37]. These studies suggested that APC2 play an important role in tumor development; however, the regulatory mechanism of APC2 in glioma remains unclear. Herein, we reported that miR-1249 promotes cell proliferation in glioma by binding directly to the 3'UTR of *APC2* and downregulating its expression. In addition, β-catenin nuclear accumulation was promoted robustly by miR-1249 overexpression in glioma. Taken together, our results represent a novel mechanism of APC2 upregulation in glioma, and a functionally and clinically relevant epigenetic mechanism of glioma pathogenesis.

In summary, we demonstrated that miR-1249 plays an important role in the progression of glioma. MiR-1249 upregulation promoted glioma cell proliferation drastically by inhibiting the expression a key regulator of the Wnt/β-catenin signaling pathway, *APC2*. We hypothesized that miR-1249 inhibitors could be used as a therapeutic agent for glioma and further study is required to identify the clinical relevance of miR-1249 upregulation in glioma diagnosis and therapy.

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Disclosure of conflict of interest

None.

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