MicroRNA-301a/ZNRF3/wnt/β-catenin signal regulatory crosstalk mediates glioma progression

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Abstract. MicroRNA (miR)-mediated mRNA and multiple signaling pathway dysregulations have been extensively implicated in several cancer types, including gliomas. Although previous studies have reported that miR-301a acts as an oncogene, the underlying mechanisms of miR-301a in the initiation and progression of glioma remain unknown. The present study aimed to investigate the involvement of miR-301a-mediated signaling pathway dysregulation in glioma. The results identified that miR-301a was significantly upregulated in gliomas and was associated with a poor prognosis based on The Cancer Genome Atlas and Chinese Glioma Genome Atlas databases. Moreover, zinc and ring finger 3 (ZNRF3) exerted a critical role in the miR-301a-mediated effects on the malignant phenotype, such as by affecting proliferation and apoptosis. Mechanistically, the TOP/FOP luciferase assay, western blotting and immunofluorescence results demonstrated that miR-301a knockdown inhibited the wnt/β-catenin signaling pathway, at least partially via ZNRF3, while ZNRF3 was a direct functional target of miR-301a, as indicated by luciferase reporter assay and western blot analysis. Furthermore, ZNRF3 could in turn repress miR-301a expression, which was dependent on the wnt pathway. Collectively, the present study identified a novel miR-301a/ZNRF3/wnt/β-catenin signaling feedback loop that serves critical roles in glioma tumorigenesis, and that may represent a potential therapeutic target.

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Abbreviations: GBM, Glioblastoma multiforme; ZNRF3, Zinc and ring finger 3; AS-miR-301a, miR-301a inhibtor; TCGA, The Cancer Genome Atlas Program; CGGA, Chinese Glioma Genome Atlas; LGG, low-grade gliomas

Key words: microRNA-301a, ZNRF3, transcription factor 4, wnt/β-catenin, apoptosis, glioma

Introduction

Glioblastoma multiforme (GBM) is a highly heterogeneous and incurable intracranial tumor (1). The occurrence of GBM is associated with various genetic alterations, such as PTEN loss and isocitrate dehydrogenase (IDH) mutations, as well as with multiple oncogene signaling pathways, including PI3K/AKT/mTOR and wnt/β-catenin (1,2). Recently, non-coding RNAs, including microRNAs (miRNAs/miRs), have been reported to be involved in glioma tumorigenesis (3,4). Although several novel target candidates and their complex network regulatory relationships have been comprehensively investigated using high throughput data and advanced experimental strategies, such as miR-4528/ETS transcription factor ELK4, nuclear paraspeckle assembly transcript 1/miR-107/CDK6 axis (3,4), the detailed molecular mechanisms remain poorly understood. Therefore, it is important to identify novel molecules and regulatory loops in order to provide additional insights for the combination targeted therapy of GBM.

In the last few decades, along with the extensive identification and study of novel non-coding RNAs, such as long non-coding RNAs and circular RNAs, miRNA-mediated network dysregulations have been found to serve significant roles in the development and progression of malignancies, including gliomas (5,6). Previous studies have reported that miR-301a is markedly upregulated in a variety of cancer types, including melanoma, cervical, breast, pancreatic and colorectal cancer (CRC), indicating that it has an oncogene potential (7-11). For instance, miR-301a mediates breast cancer proliferation and invasion by directly targeting forkhead box F2, BCL2 binding component 3, PTEN and collagen type II α 1 chain (9). In pancreatic cancer, miR-301a promotes *in vitro* migration and *in vivo* tumorigenicity by negatively regulating its target SMAD4 (12). Moreover, Yue *et al* (13) revealed that miR-301a was upregulated in gliomas and that it

enhanced the malignnat phenotype via its target gene, septin 7; however, the exact molecular mechanism of miR-301a on the glioma progression is yet to be fully elucidated.

Increasing evidence has confirmed that the wnt/β-catenin signaling is one of the most crucial mediators in the progression of GBM and forms a network crosstalk with other signaling pathways, including PI3KAKT/mTOR and Ras/MAPK (14,15). Zinc and ring finger 3 (ZNRF3) belongs to the E3 ubiquitin ligase family and represses wnt/β-catenin signaling by promoting the turnover of the Frizzled and LDL receptor related protein 6 (LRP6) receptors (16,17). Previous studies have reported that multiple miRNAs, such as miR-106b-3p and miR-93, directly target ZNRF3 and subsequently activate the wnt/β-catenin signal pathway and epithelial-mesenchymal transition (EMT) program (18,19). However, whether ZNRF3 serves a crucial role in miR-301a-mediated regulation of the wnt/β-catenin signaling pathway in gliomas remains unknown.

In the present study, a series of *in vitro* and *in vivo* experiments were performed to confirm whether mi-301a may function as oncogene via a ZNRF3-mediated wnt/β-catenin signaling pathway, based on the verification of ZNRF3 as a functional target of miR-301a, as indicated by luciferase reporter assay and western blotting. Additionally, the fact that ZNRF3, in turn, could inhibit miR-301a expression was identified via luciferase reporter assay and reverse transcription-quantitative (RT-q)PCR. Overall, the present study identified a new miR-301a/ZNRF3/wnt/β-catenin feedback loop in gliomas, which represents a novel therapeutic target.

Materials and methods

Patients and samples. Gene expression data and relevant clinicopathologic variable information were downloaded from TCGA database (http://cancergenome.nih.gov) (n=489). Additionally, miRNAs expression data and metadata containing survival information from the CGGA database (http://www.cgcg.org.cn) (n=198) were analyzed. Moreover, the ENCORI database (http://starbase.sysu.edu.cn/) was used to analyze the relationship between miR-301a and ZNRF3 expression in low-grade gliomas (LGG; n=525).

Furthermore, 39 clinical glioma specimens from 15 female and 24 male patients (age range, 19-76 years; mean age, 42.9±15.4 years) were obtained from the nerve tumor tissues bank at the Tianjin Institute of Neurosurgery, including 10 low grade (WHO I and II) and 29 GBMs (WHO IV), who were diagnosed by surgeons and pathologists at Tianjin Huan Hu Hospital (Tianjin, China) between January 2010 and July 2018. None of the patients had received any radiotherapy, chemotherapy or any other anticancer treatments prior to surgery. In total, five normal adult brain tissue samples were collected from 4 male and 1 female patient (age range, 58-74 years; mean age, 66±6.3 years) undergoing post-trauma surgery for severe traumatic brain injury. All the collected tissues were frozen immediately in liquid nitrogen and stored at -80˚C. This study was approved by the Institutional Review Board of Tianjin Huan Hu Hospital, and written informed consent was obtained from all participants.

Cell culture and transfection. The human glioblastoma cell lines U87, U251 and A172 and the normal astrocyte cell line were obtained from the Peking Union Medical College Cell Library. Human LN308, LN229 and T98G glioblastoma cell lines were obtained from the China Academia Sinica Cell Repository. The U87 cell line was authenticated using short tandem repeat profiling analysis; the U87 cell line (glioblastoma of unknown origin) used in the present study was of the American Type Culture Collection type. Cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). All cell lines were incubated at 37˚C with 5% CO_2.

The oligonucleotide sequencess of human miR-301a inhibitor (AS-miR; 5'- GCUUUGACAAUACUAUUGCACUG-3'), miR-301a mimics (5'-CAGUGCAAUAGUAUUGUCAAAGC-3'), miRNA-negative control (miR-NC; 5'-UUGUACUACACAAAAGUACUG-3'), ZNRF3 small interfering (si)RNA (5'- CACUGGGCCUAUGUAAUAATT-3') and transcription factor 4 (TCF4) overexpression plasmid were purchased from Shanghai GenePharma Co., Ltd. In brief, LN229 and U87 cells were incubated in a 6-well plate at a density of $2x10^5$ cells/well and then transfected with miRNA (2 μg) or siRNA (2 μg), or co-transfected both of the transfectants at room temperature using X-tremeGENE transfection reagent (Roche Diagnostics GmbH) according to the manufacturer's protocol. Subsequent experiments were performed after 72 h.

Lentivirus-transducing units carrying the ZNRF3 variant 3 (LV-ZNRF3) and Lentivirus vector control (LV-NC) were purchased from Shanghai Genechem Co., Ltd. For stable transfection, after seeding in 6-well plates ($2x10^5$ cells/well) for 24 h, cells were infected with LV-ZNRF3 or LV-NC at a multiplicity of infection of 10 in the Opti-DMEM (Gibco; Thermo Fisher Scientific, Inc.) and incubated at 37˚C. In order to establish stable overexpressing cell lines, infected cells were treated with puromycin (2 μg/ml for the LN229 and U87 cells) for 7 days. The overexpression efficiency was evaluated via western blot analysis.

Total RNA extraction and RT-qPCR. Total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and was reverse transcribed to cDNA to evaluate the mRNA expression levels of ZNRF3, c-myc, cyclin D1 and TCF4 using the PrimeScript RT Master mix (Takara Bio, Inc.) according to the manufacturer's instructions. The protocol of RT was 37˚C for 15 min, 85˚C for 5 sec and held at 4˚C. The PCR protocol was: Initital denaturation at 95˚C for 5 sec, followed by 40 cycles at 60˚C for 30 sec and by annealing and extension at 50˚C for 30 sec. To evaluate miR-301a, the protocol of miRNA reverse transcription was 37˚C for 60 min, 95˚C for 5 min and held at 4˚C, and stem-loop RT-PCR was performed with an RNA PCR kit (Qiagen GmbH) according to the manufacturer's instructions. qPCR was performed using the miScript SYBR Premix Green PCR kit (Qiagen GmbH) and the Roche LC480 quantitative Real-Time PCR system (Roche Diagnostics). The amplification conditions were: Initital denaturation at 95˚C for 15 min, followed by 40 cycles at 94˚C for 15 sec and by annealing and extension at 55˚C for 30 sec. GAPDH or U6 levels were selected as internal controls, and fold changes were calculated using the relative quantification ($2^{-\Delta\Delta Cq}$) method (20). The primer sequences are presented in Table SI.

Data were analyzed from three independent experiments and are presented as the mean ± SD.

Dual-luciferase reporter assay. StarBaseV2 (http://starbase.sysu.edu.cn/), TargetScan (http://www.targetscan.org/vert_72/) and Pictar (http://www.pictar.org/) algorithms were applied to identify targets of miR-301a, and the seed sequence of miR-301a was identified to match the 3' untranslated region (3'UTR) of the ZNRF3 gene. The 3'UTR of ZNRF3 containing the miR-301a conserved binding sites and the corresponding mutant (MT) sites were inserted into the pMIR-REPORT vector (Promega Corporation). LN229 and U87 cells were cultured in 96-well plates, and co-transfected with the wild-type (WT) or MT luciferase reporters (0.15 μg) and the AS-miR-301a (0.15 μg) using the X-tremeGENE transfection reagent (Roche Diagnostics GmbH) at room temperature.

To analyze the transcriptional activity of β-catenin/TCF4, TOP-FLASH and FOP-FLASH luciferase reporter constructs were constructed. The TOP-FLASH (with repeats of the Tcf-binding site) or FOP-FLASH (with repeats of the MT Tcf-binding site) plasmids (EMD Millipore) (0.15 μg) were transfected into LN229 and U87 cells treated with the miR-301a inhibitor (0.15 μg) in 96-well plates at room temperature. After 72 h incubation, luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega Corporation), and *Renilla* luciferase activity was used as an internal control.

The PGL3-WT-miR-301a-promoter-Luc reporter or pGL3-MT-miR-301-promoter-Luc reporter constructs (0.15 μg; Promega Corporation) were transfected into LN229 and U87 cells or stable ZNRF3 overexpressing cells, following the co-transfection of the TCF4 overexpression plasmid or siTCF4 using the X-tremeGENE transfection reagent (Roche Diagnostics) at 37˚C for 72 h. The luciferase activity was detected after 72 h as aforementioned.

Western blotting and immunohistochemistry (IHC) analysis. The ExKine Total Protein Extraction kit was purchased from Abbkine Scientific Co., Ltd. and used to extract proteins. Protein concentrations were determined using a BCA Protein assay kit (Thermo Fisher Scientific, Inc.). Then, 40 μg protein lysate from each sample was resolved via 10% SDS-PAGE and subsequently transferred to PVDF membranes (EMD Millipore). After blocking in 5% skimmed milk for 1 h at room temperature, the membranes were incubated with diluted primary antibodies at 4˚C overnight. After washing with TBS-Tween-20 (1% Tween-20), membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. nos. ab222772 and ab222759; Abcam) for 1 h at room temperature. The immune-reactive bands were visualized using ECL Western Blot Detection reagents (EMD Millipore) and semi-quantified using ImageJ software (version 1.47v; National Institutes of Health). The primary antibodies used were as follows: ZNRF3 (1:1,000; cat. no. A16026; ABclonal Biotech Co., Ltd.), cyclin D1 (1:10,000; cat. no. ab134175; Abcam), c-myc (1:1,000; cat. no. ab39688; Abcam), TCF4 (1:10,000; cat. no. ab76151; Abcam) and β-actin (1:5,000; cat. no. ab6276; Abcam), which was used as the control.

IHC assays were performed using antibodies against ZNRF3 (1:200; cat. no. A16026, ABclonal Biotech Co., Ltd.), β-catenin (1:500; cat. no. ab32572; Abcam), c-myc (1:100; cat. no. ab39688; Abcam), TCF4 (1:500; cat. no. ab76151; Abcam) and caspase-3 (1:100; cat. no. 9662; Cell Signaling Technology, Inc.). The protocols used were performed as described previously (21). Briefly, the animal tumor samples were fixed with 10% formalin for 48 h at room temperature, embedded with paraffin and sliced into 4-μm sections, followed by dewaxing. Subsequently, these sections were incubated with 3% hydrogen peroxide for 20 min at room temperature to block endogenous peroxidase. Following antigen retrieval, the slides were blocked with 10% normal goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at room temperature and then incubated at 4˚C overnight with appropriate primary antibody. After washing with PBS, the slides were incubated with biotinylated secondary antibody (cat. no. Sp-9001; 1:1,000; OriGene Technologies, Inc.) for 1 h at room temperature. A DAB Horseradish Peroxidase Color Development kit (Beyotime Institute of Biotechnology) was used for color development, and neutral gum (Sinopharm Chemical Reagent Co., Ltd.) was used for sealing. Finally, five randomly selected fields were imaged using the Olympus X71 inverted microscope (Olympus Corporation; magnification, x200), and semi-quantitatively analyzed in the form of mean optical density using Image-Pro Plus 6.0 (Media Cybernetics, Inc.). The signal density of the tissue areas from five randomly selected fields were counted in a blinded manner. The significance of the differences between two groups was determined using Student's t-test.

Immunofluorescence staining. To examine the regulatory role of miR-301a on β-catenin/TCF4 activity, LN229 and U87 cells were transfected with AS-miR-301a or miR-scramble (miR-NC) or were co-transfected with AS-miR-301a and siZNRF3, followed by immunofluorescence analysis. The procedures were conducted as previously described (21). In brief, cells were fixed with 0.1% paraformaldehyde for 20 min at room temperature and were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) at room temperature for 2 h. Cells were incubated with anti-β-catenin primary antibody (1:200; cat. no. ab32572; Abcam) overnight at 4˚C. After washing with PBS at room temperature, Goat anti-rabbit fluorescence conjugated second antibody (1:10,000; cat. no. A21245; Invitrogen; Thermo Fisher Scientific, Inc.) was added for 1 h at room temperature and imaged using a confocal microscope (magnification, x50).

Cell viability assay. Cell viability was analyzed using a Cell-Counting Kit 8 (CCK-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Following transfection with AS-miR-301a or co-transfection with AS-miR-301a and siZNRF3, LN229 and U87 cells were incubated at 37˚C for 24, 48, 72 and 96 h. CCK-8 solution (10 μl) was added to each well, and the absorbance at 490 nm was measured after incubation at 37˚C for 2 h to estimate the number of viable cells.

Apoptosis assay. Apoptosis was quantified after transfection, using Annexin V labelling and caspase-3/7 activity. For the Annexin V assay, the Annexin V-FITC-labelled Apoptosis Detection kit (Abcam) was used according to

the manufacturer's protocol. In brief, cells were harvested, washed and resuspended, and then 5 μl Annexin V-FITC and 5 μl PI were added and maintained for 15 min at room temperature in the dark. Within 1 h, the stained cells were analyzed with a flow cytometry system (BD FACSCalibur™ flow cytometer; BD Biosciences) equipped with CellQuest software (version-5.1; BD Biosciences). The apoptotic rate was calculated as the percentage of early + late apoptotic cells.

Caspase-3/7 activity was analyzed using the Caspase-Glo-3/7 reagent (cat. no. G8091; Promega Corporation). Caspase-Glo-3/7 reagent (100 μl) was added to a white-walled 96-well plate, which was gently mixed using a plate shaker at 50 x g for 30 sec at room temperature. Following incubation at room temperature for 1-2 h, the luminescence of each sample at 510 nm was measured in a plate-reading luminometer.

Xenograft model with nude mice. All animal protocols were performed following the approval of the Animal Care and Used Committee of Tianjin Huanhu Hospital. A total of 30 female BALB/c-A nude mice (Age, 5 weeks; weight,18-21 g) were purchased from the Animal Center of the Cancer Institute, Chinese Academy of Medical Science. Animal welfare was considered and all the mice were housed with filtered air, a 12-h light/dark cycle, regulated temperature (25±2˚C) and humidity (50±10%), and with *ad libitum* access to sterilized food and water. Then, ~5x10^6 U87 GBM cells in 100 μl PBS were subcutaneously injected into the left flank region of nude mice. When the tumor volume reached 100 mm^3, the mice were randomly divided into two groups, namely NC group and AS-miR-301a group (5 mice per group). Each group was treated with oligonucleotides (10 μg AS-miR-301a or miR-NC) with a mixture of 10 μl Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) via local injection into the xenograft tumor at multiple sites on all sides. The treatment was performed once every 3 days for 27 days, and the tumor volume was monitored with a caliper, using the formula: Volume = length x $width^2/2$.

To evaluate the miR-301a-mediated role of ZNRF3 in tumor inhibition *in vivo*, 5x10^6 U87 cells stably expressing LV-ZNRF3 or LV-NC in 150 μl PBS were injected into the left flank region of nude mice. When all the groups formed a tumor at ~4 weeks, the LV-NC and LV-ZNRF3 injected mice were separated into two groups (5 mice per group), respectively. One of the LV-NC groups was treated with miR-scramble (miR-NC), while one of LV-ZNRF3 groups was treated with miR-301a mimics and another one was treated with miR-NC every 3 days for 30 days. The tumor injection of oligonucleotides (10 μg AS-miR-301a or miR-NC) with a mixture of 10 μl Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was also performed at multiple sites. At the end of the experiment, the mice were anesthetized by injecting 1% pentobarbital sodium (100 mg/kg body weight) and sacrificed via cervical vertebra dislocation. Finally, tumor weight was calculated. The removed tumor tissues were subjected to RNA extraction and immunohistochemistry assays.

Statistical analysis. All statistical analyses were performed using GraphPad software version 6.0 (GraphPad Software, Inc.) or IBM SPSS Statistics 23.0 (IBM Corp.). Data are presented as the mean ± SD of ≥3 independent experiments. The unpaired Student's t-test, and one-way ANOVA were performed to analyze statistically significant differences between two groups or multiple groups, respectively. Additionally, a Tukey's test was used for multiple comparisons following ANOVA. The Kaplan-Meier method was used to evaluate the differences in survival rates, which were analyzed using the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Relationship between miR-301a and clinicopathological characteristics expression of patients with glioma.

		miR-301a expression		
Variable	No. of cases	Low	High	P-value
Sex				0.3332
Female	15	6	9	
Male	24	14	10	
Age, years				0.7524
<42	20	9	11	
≥42	19	10	9	
Histology				0.0084
Astrocytoma	10	9	1	
Glioblastoma	29	11	18	

Fisher's exact test was conducted to determine the statistical significance of the relationship between miR-301a expression and different variables. miR, microRNA.

Results

miR-301a is significantly upregulated in glioma specimens and cell lines. To evaluate the potential role of miR-301a in gliomagenesis, miR-301a expression was analyzed in gliomas with different grades, including 10 LGG and 29 GBM cases, and seven malignant glioma cell lines. miR-301a was significantly upregulated in the clinical samples and GBM cell lines (Fig. 1A and B), and its expression level was positively associated with the WHO grade (Fig. 1A). The clinicopathological characteristics of the 39 glioma specimens are summarized in Table I. There was a statistically significant statistically correlation between miR-301a expression and histology, but not for sex and age. Additionally, analysis of the data from the CGGA database indicated that miR-301a expression was positively associated with the WHO grade, which was in accordance with the data from the clinical specimens (Fig. 1C), and a higher level of miR-301a predicted a poor prognosis (Fig. 1D). Furthermore, the relationship between the miR-301a expression and prognosis from TCGA database was analyzed, and the results were consistent with the analysis of the CGGA data (Fig. S1). These findings indicated that miR-301a may act as a critical oncogene in gliomas.

ZNRF3 is a direct target of miR-301a. miRNAs serve as oncogenes or tumor-suppressors by regulating gene expression by either mRNA degradation or translational inhibition (22).

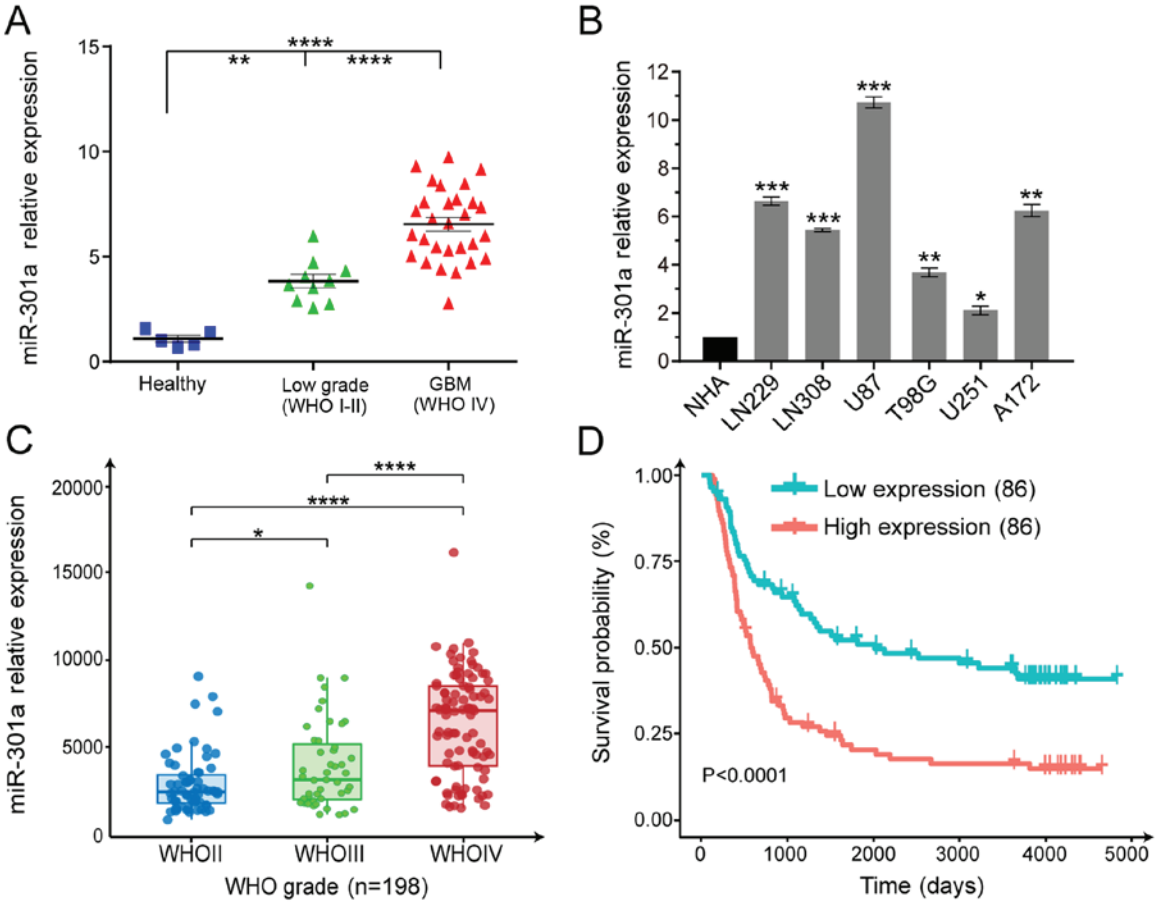

Figure 1. miR-301a is significantly upregulated in glioma samples and cell lines, and is negatively correlated with patient survival. (A) RT-qPCR was used to evaluate the miR-301a expression in clinical specimens of different grades compared with five healthy brain tissues. **P<0.01, ****P<0.0001. (B) RT-qPCR analysis of GBM cell lines (LN229, LN308, U87, T98G, U251 and A172) compared with the normal astrocytes. *P<0.05, **P<0.01, ***P<0.001 vs. NHA. (C) miR-301a expression from WHOII, WHOIII and WHOIV cases as detected via RT-qPCR from the CGGA database. *P<0.05, ****P<0.0001. (D) Kaplan-Meier analysis of survival of the miR-301a low-expressing (n=86) and high-expressing glioma cases (n=86) from the CGGA database. miR-301a, microRNA-301a; GBM, glioblastoma multiforme; WHO, World Health Organization; CGGA, Chinese Glioma Genome Atlas; RT-qPCR, reverse transcription-quantitative PCR.

Thus, StarBaseV2, TargetScan and Pictar algorithms were used to verify the downstream effectors of miR-301a. Based on the database evaluation, the seed sequence of miR-301a that matched the 3'UTR of the ZNRF3 was identified (Fig. 2A).

To verify whether ZNRF3 expression was downregulated by miR-301a, the changes in ZNRF3 mRNA and protein expression levels were detected in LN229 and U87 cells after knocking down miR-301a expression. The results demonstrated that ZNRF3 expression was significantly increased at both the mRNA and protein levels after miR-301a knockdown (Fig. 2B and C). Additionally, to assess whether ZNRF3 was the direct functional target of miR-301a, the p-MIR-WT-ZNRF3-3'UTR and p-MIR-MT-ZNRF3-3'UTR reporter plasmids were constructed. Reporter assay results indicated that AS-miR-301a triggered a significantly increase of p-MIR-WT-ZNRF3-3'UTR luciferase activity, but no change in luciferase activity was observed for the MT reporter plasmid (Fig. 2D). Moreover, ZNRF3 expression was significantly downregulated in GBM tissues compared with healthy tissues (Fig. 2E and F).

The public database ENCORI was analyzed, and it was identified that miR-301a expression was weakly negatively associated with ZNRF3 expression in 525 LGG (r=-0.148; P<0.001; Fig. 2G). However, the correlation coefficient was very small, which may due to the LGG or the sample size. These data suggested that miR-301a directly modulated ZNRF3 expression at both the transcriptional and post-transcriptional levels via binding to the 3'UTR.

ZNRF3 is required for the biological effects of miR-301a in the GBM malignant phenotype. To further identify the functional relationship between miR-301a and its target ZNRF3, the ZNRF3-mediated role on proliferation and apoptosis exerted by miR-301a was investigated. The verification of miR-301a knockdown is presented in Fig. 3A. The successful establishment of lentivirus-mediated ZNRF3 overexpression is presented in Fig. 3B, and the successful establishment of ZNRF3 knockdown in LN2229 and U87 cells singly transfected with siZNRF3 is presented in Fig. S2.

When ZNRF3 expression was knocked down by siZNRF3 in LN229 and U87 cell lines co-transfected with AS-miR-301a, the ZNRF3 protein expression was significantly downregulated compared with cells transfected with AS-miR-301a alone (Fig. 3B), indicating that siZNRF3 transfection could effectively abrogate the effect of AS-miR-301a-mediated ZNRF3 upregulation. CCK-8, Annexin V and caspase-3/7 activity analyses demonstrated that the inhibition of proliferation and the promotion of apoptosis induced by AS-miR-301a

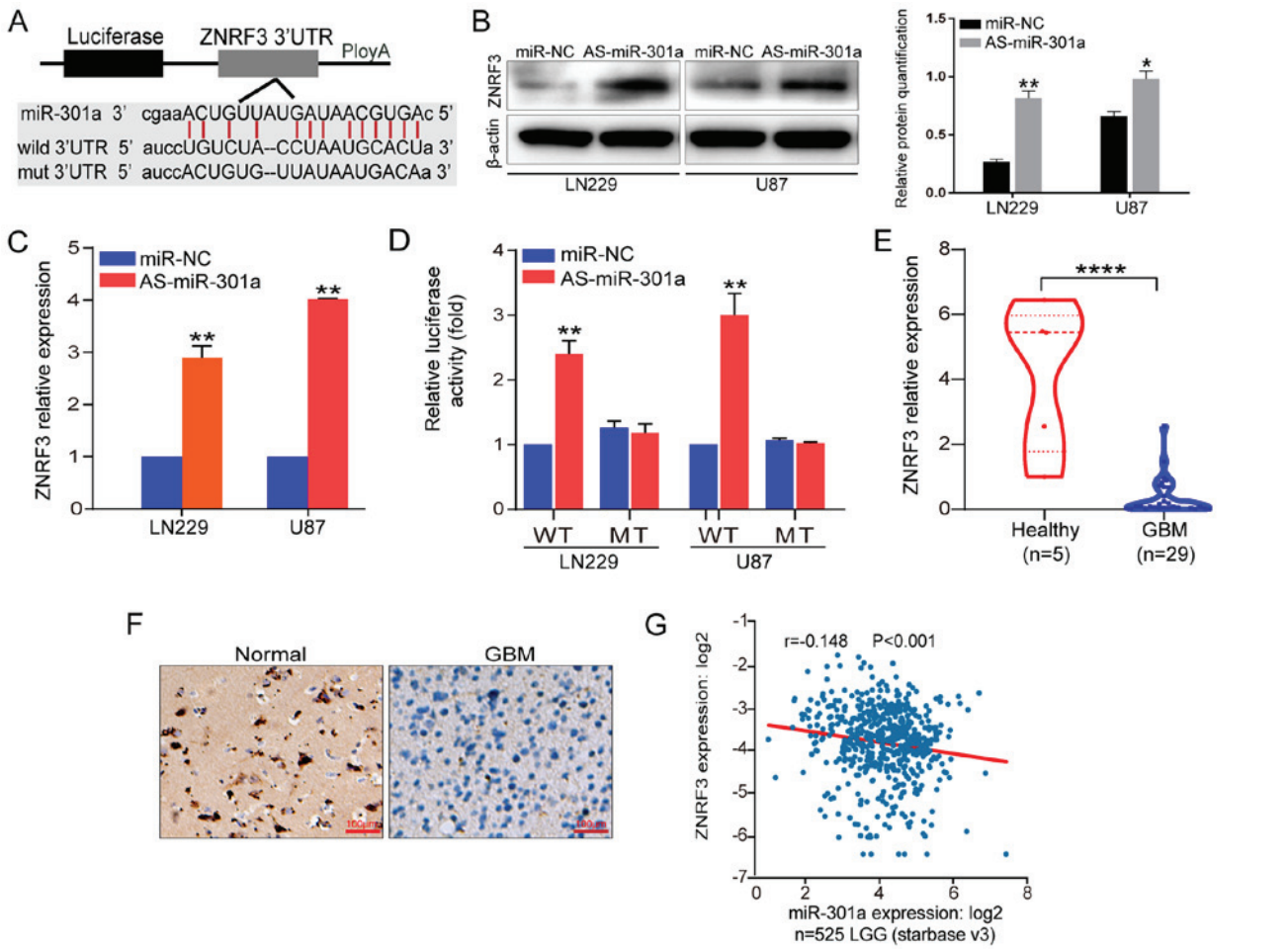

Figure 2. ZNRF3 is a direct functional target of miR-301a. (A) A schematic diagram of the seed sequence of miR-301a matching the ZNRF3 3'UTR, and the design of the WT or MT ZNRF3 3'UTR including reporter constructs. (B) ZNRF3 protein expression in LN229 and U87 cells transfected with miR-NC or miR-301a inhibitor was analyzed via western blotting. β-actin was used as a loading control. The relative protein semi-quantification was performed with ImageJ software. *P<0.05, **P<0.01 vs. miR-NC. (C) ZNRF3 mRNA expression in LN229 and U87 cells transfected with miR-NC or AS-miR-301a was analyzed using RT-qPCR. (D) Luciferase reporter assays were performed in LN229 and U87 cells following co-transfection with the WT or MT 3'UTR of ZNRF3 and AS-miR-301a or miR-NC. The data present the fold changes as the mean ± SD of three independent experiments. **P<0.01 vs. miR-NC. (E) ZNRF3 expression was examined via RT-qPCR in GBM cases compared with the healthy controls. ****P<0.0001. (F) ZNRF3 expression in patients with GBM was determined via immunohistochemistry. Scale bar, 100 μm. (G) Negative correlation analysis from 525 LGG cases from the public database ENCORI. ZNRF3, Zinc and ring finger 3; miR-301a, microRNA-301a; 3'UTR, 3'untranslated region; NC, negative control; WT, wild-type; MT, mutant; LGG, low-grade glioma; RT-qPCR, reverse transcription-quantitative PCR; AS-miR-301a, miR-301a inhibtor; GBM, glioblastoma multiforme.

were partially abolished by siZNRF3 (Fig. 3C-E). Furthermore, ZNRF3 overexpression could significantly enhance caspase-3/7 activity compared with its NC (Fig. 3F), consistent with the effect mediated by AS-miR-301a. These results suggested that AS-miR-301a inhibited the GBM malignant phenotype, partially via ZNRF3.

Knockdown of miR-301a inhibits the wnt/β-catenin signaling pathway, at least partially via ZNRF3. Our previous studies have reported that ZNRF3 can repress the wnt/β-catenin signaling pathway (unpublished), and combined with the fact that ZNRF3 is a direct target of miR-301a, it was further examined whether miR-301a also regulates the wnt/β-catenin signaling pathway via ZNRF3. Firstly, a TOP/FOP luciferase assay was used to analyze the β-catenin/TCF4 transcriptional activity mediated by miR-301a, and the results indicated that AS-miR-301a suppressed TOP luciferase activity with no apparent change in FOP activity (Fig. 4A). Additionally, AS-miR-301a could decrease the mRNA and protein expression levels of TCF4, c-myc and cyclin D1, as demonstrated by RT-qPCR and western blotting, respectively (Fig. 4B and C). The immunofluorescence results indicated that AS-miR-301a repressed the expression of β-catenin both in the nucleus and cytoplasm (Fig. 4D). It was found that knockdown of ZNRF3 via siRNA following the transfection with AS-miR-301a could significantly abrogate the effects mediated by AS-miR-301a (Fig. 4A-D). Taken together, these data indicated that AS-miR-301a could attenuate the wnt/β-catenin signaling pathway, at least partially via ZNRF3, in LN229 and U87 cells.

AS-miR-301a represses tumor growth and wnt signaling in vivo. Based on the *in vitro* experimental findings, the effects of AS-miR-301a on tumor growth and wnt/β-catenin signaling were assessed *in vivo*. U87 cells were implanted into the left flanks of nude mice via subcutaneous injection. AS-miR-301a and miR-NC were treated in a multisite manner every 3 days. After 27 days of treatment, tumors were excised and subjected to further analysis. The verification of miR-301a knockdown in

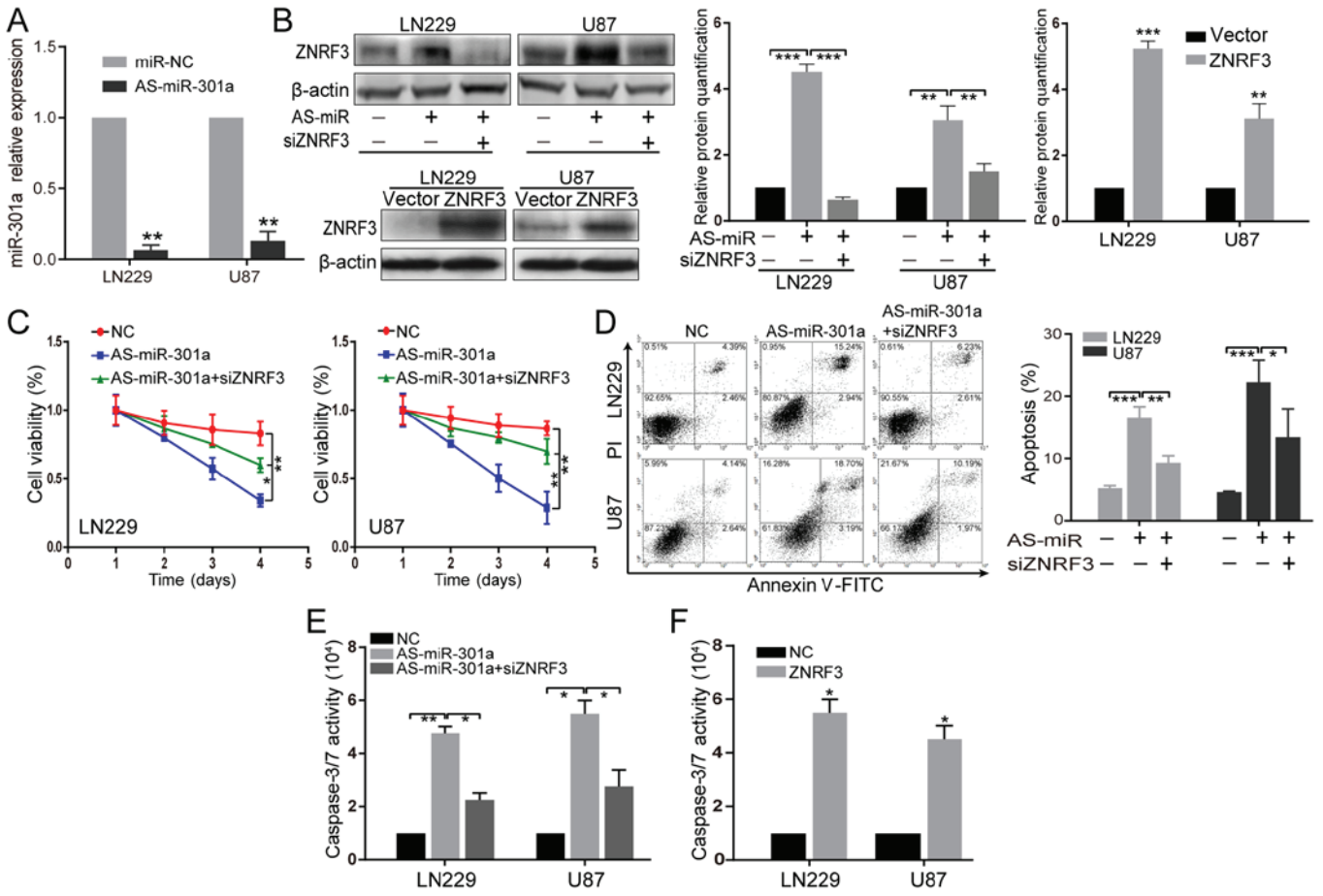

Figure 3. AS-miR-301a inhibits the GBM malignant phenotype, partially via ZNRF3. (A) Verification of miR-301a knockdown in LN229 and U87 cells transfected with miR-NC or miR-301a inhibitor. **P<0.01 vs. miR-NC. (B) ZNRF3 protein expression was examined in LN229 and U87 cells transfected with AS-miR-301a or co-transfected with AS-miR-301a and siZNRF3 (upper), and in stably ZNRF3-overexpressing LN229 and U87 cells (below) via western blot analysis. β-actin was used as an internal loading control. The relative protein semi-quantification was performed with ImageJ software. **P<0.01, ***P<0.001. (C) LN229 and U87 cells were transfected with AS-miR-301a or co-transfected with both AS-miR-301a and siZNRF3, and 72 h later a Cell Counting Kit-8 assay was used to detect the ZNRF3-mediated effect on proliferation by AS-miR-301a. *P<0.05, **P<0.01. (D) Annexin V-PI and (E) caspase-3/7 activity assays were utilized to detect the ZNRF3-mediated effect on apoptosis by AS-miR-301a in LN229 and U87 cells at 72 h after transection of AS-miR-301a or co-transfection of both AS-miR-301a and siZNRF3. *P<0.05, **P<0.01, ***P<0.001. (F) Caspase-3/7 activity assays were performed in LN229 and U87 cells stably overexpressing ZNRF3. *P<0.05 vs. NC. ZNRF3, Zinc and ring finger 3; miR-301a, microRNA-301a; NC, negative control; siRNA, small interfering RNA; AS-miR-301a, miR-301a inhibtor.

tumor tissues is presented in Fig. 5A. The tumor growth curve indicated that AS-miR-301a significantly inhibited the tumorigenicity of GBM cells, as observed in AS-miR-301a treated mice (Fig. 5B). Additionally, a significant decrease in tumor weight was identified in AS-miR-301a-treated tumors (Fig. 5C). The IHC assay demonstrated that AS-miR-301a significantly increased ZNRF3 expression, and decreased β-catenin, c-myc and cyclin D1 expression levels (Fig. 5D), which was consistent with the *in vitro* results.

ZNRF3 is downregulated in glioma and is associated with patient prognosis based on the CGGA dataset. Based on the miR-301a-mediated regulatory effect on ZNRF3, ZNRF3 expression and its relationship with patient prognosis was evaluated in the CGGA dataset (CGGA Mseq325). The mRNA expression of ZNRF3 was significantly downregulated in high-grade gliomas compared with the LGG, and ZNRF3 expression was negatively associated with the WHO grade (Fig. 6A). Analysis of ZNRF3 expression and its relevant clinical characteristics indicated that ZNRF3 expression was associated with IDH1 gene mutation status, 1p/19q codeletion status (Fig. 6B and C) and age (Fig. S3B). No statistical significance was observed for ZNRF3 expression and sex or progression status (Fig. S3A and C). Furthermore, Kaplan-Meier survival analysis identified that ZNRF3 upregulation conferred prolonged overall survival (Fig. 6D). In combination with the ZNRF3 expression data of the clinical specimens, it was concluded that ZNRF3 may serve as a key tumor suppressor and is involved in GBM progression.

ZNRF3 can attenuate the miR-301a expression level dependent on wnt/β-catenin activity. A previous study revealed that miR-301a is activated by the wnt/β-catenin pathway by direct binding of TCF4 to the miR-301a promoter region (13). Thus, it was hypothesized that ZNRF3 restoration could, in turn, affect miR-301a expression via TCF4. Firstly, ZNRF3 could inhibit the wnt/β-catenin transcriptional activity, as verified by the TOP/FOP assay, and the ectopic restoration of miR-301a in stable ZNRF3-overexpressing LN229 and U87 cells could partially abrogate this effect mediated by ZNRF3 (Fig. 7A). The

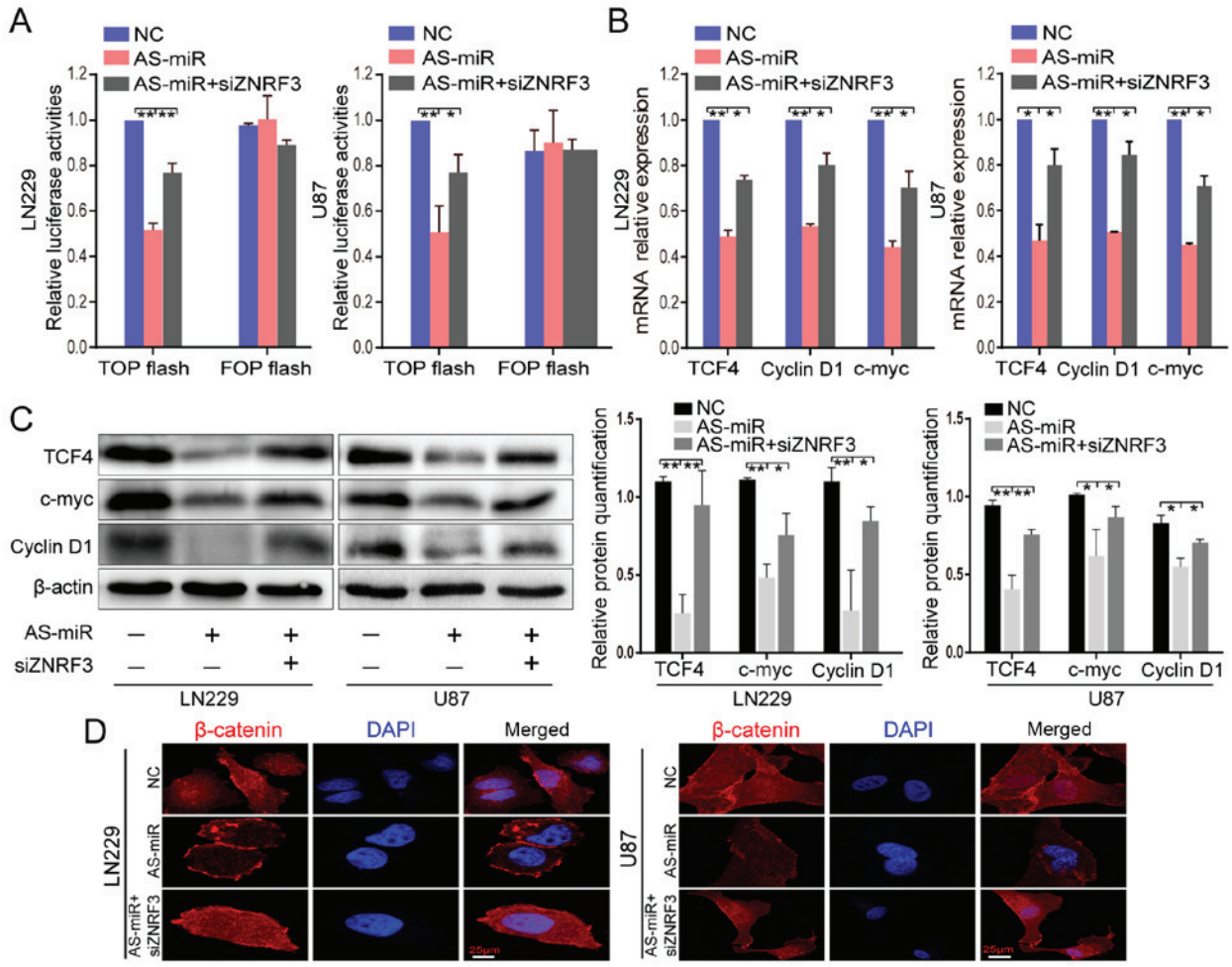

Figure 4. AS-miR-301a suppresses β-catenin/TCF4 transcriptional activity partially via ZNRF3. (A) LN229 and U87 cells were co-transfected with TOP/FOP FLASH luciferase plasmids, AS-miR-301a or siZNRF3. After 72 h, the luciferase reporter assays were conducted. *P<0.05, **P<0.01. (B) Reverse transcription-quantitative PCR and (C) western blotting were performed to detect TCF4, c-myc and cyclin D1 expression levels in LN229 and U87 cells transfected with AS-miR-301a or co-transfected with AS-miR-301a and siZNRF3. *P<0.05, **P<0.01. (D) Immunofluorescence detection of β-catenin in the nucleus after transfection with AS-miR-301a or co-transfection with AS-miR-301a and siZNRF3 (magnification, x50). ZNRF3, Zinc and ring finger 3; miR-301a, microRNA-301a; NC, negative control; siRNA, small interfering RNA; AS-miR-301a, miR-301a inhibtor; TCF4, transcription factor 4.

successful transfection efficiency of miR-301a overexpression via RT-qPCR is presented in Fig. S4A. ZNRF3 overexpression could significantly inhibit miR-301a expression, and the knockdown of TCF4 (siTCF4) decreased the miR-301a expression. Moreover, the TCF4 overexpression plasmid in stable ZNRF3-overexpressing LN229 and U87 cells could reverse the ZNRF3-mediated inhibitory effect (Fig. 7B). The successful transfection efficiency of TCF4 overexpression and siTCF4 detected via RT-qPCR is illustrated in Fig. S4B and C. Furthermore, the luciferase assay results with the constructed miR-301a promotor reporter plasmid indicated that ZNRF3 overexpression or the knockdown of TCF4 alone could significantly decreased the luciferase activities, while the TCF4 restoration in ZNRF3 stably overexpressing cells could abrogate this inhibitory effect mediated by ZNRF3 in WT groups as compared with the MT groups (Fig. 7C), indicating that TCF4 transcriptionally regulated miR-301a expression by binding to the promotor of miR-301a. These data demonstrated that ZNRF3 could repress TCF4-depedent miR-301a expression.

To further evaluate the miR-301a-mediated role of ZNRF3 on apoptosis, Annexin V-PI and caspase-3/7 activity assays were performed and the results demonstrated that the transfection of miR-301a mimics in stable ZNRF3-overexpressing LN229 and U87 cells reversed the ZNRF3-induced effect on apoptosis (Fig. 7D and E). Furthermore, *in vivo* experiments indicated that ZNRF3 overexpression significantly inhibited tumor growth and that the miR-301a mimics treatment partially reversed this effect mediated by ZNRF3 (Fig. 7F). The ZNRF3 expression from different treatment groups is presented in Fig. 7G, it was found that ZNRF3 expression in the ZNRF3 + miR-301a group was decreased compared with the ZNRF3 + miR-NC group (P<0.01; Fig. 7G). The IHC assay results for caspase 3 expression were in line with the *in vitro* results (Fig. 7H). In summary, the results indicated that the miR-301a/ZNRF3/wnt signal feedback loop may be involved in gliomagenesis.

Discussion

Previous studies have reported that miR-301a acts as a crucial oncogene for diagnosis and prognosis evaluation in various malignant cancer types. For example, Karimi *et al* (23) suggested that upregulation of exosomes miR-301a and

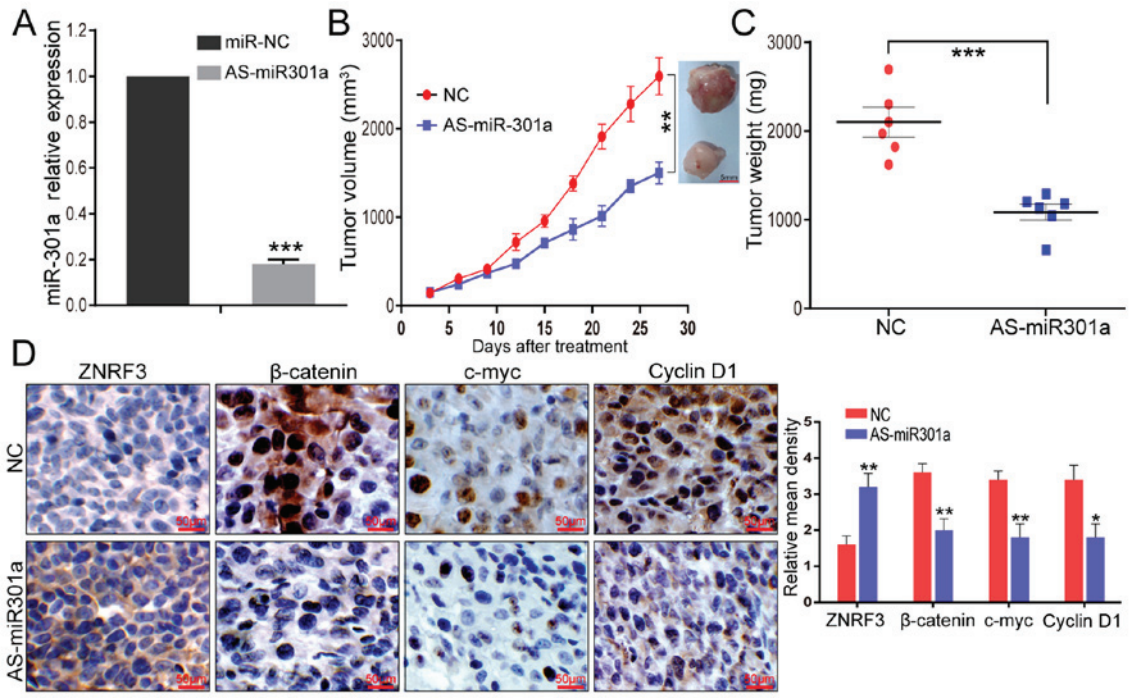

Figure 5. AS-miR-301a inhibits the GBM malignant phenotype and the wnt signal pathway *in vivo*. (A) miR-301a knockdown identification from resected tumor tissues was detected via reverse transcription-quantitative PCR. ***P<0.001 vs. miR-NC. (B) U87 cells were subcutaneously injected into nude mice. When tumors were established uniformly in the two groups, AS-miR-301a was injected in a multisite injection every 3 days. Tumor volume was evaluated every 3 days during treatment. **P<0.01. (C) At the end of the experiment, tumor weight was measured. ***P<0.001. (D) Immunohistochemistry assays were performed to evaluate ZNRF3, β-catenin, c-myc, and cyclin D1 expression levels from xenograft tumor sections. Scale bar, 50 μm. *P<0.05, **P<0.01. miR-301a, microRNA-301a; NC, negative control; AS-miR-301a, miR-301a inhibtor.

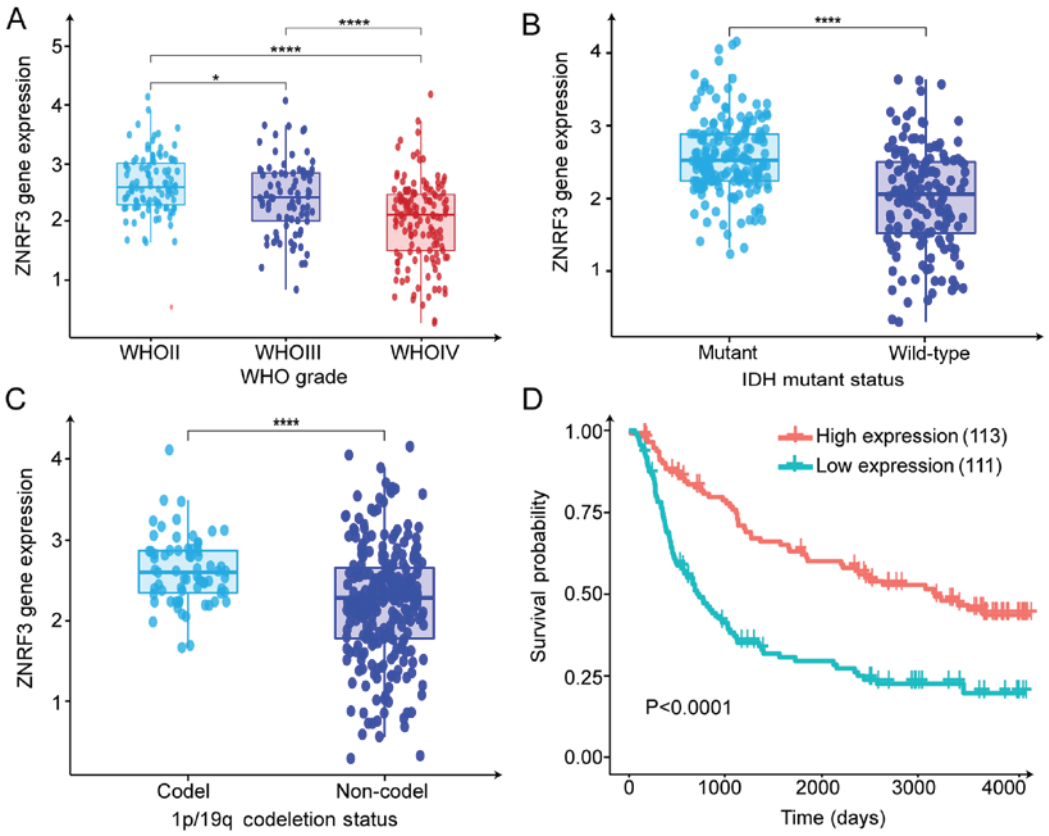

Figure 6. ZNRF3 downregulation is negatively associated with the glioma grade and predicts a poor outcome according to the CGGA (mRNAseq325) analysis. (A) ZNRF3 expression in gliomas with various WHO grades was determined. Clinical parameters of ZNRF3 expression were analyzed based on (B) IDH gene mutation status or (C) 1p/19q codeletion status. (D) Kaplan-Meier survival curves for ZNRF3 expression with low or high expression for primary gliomas. Patients with low levels of ZNRF3 predicted a significantly worse outcome. *P<0.05, ****P<0.0001. ZNRF3, Zinc and ring finger 3; WHO, World Health Organization; IDH, isocitrate dehydrogenase.

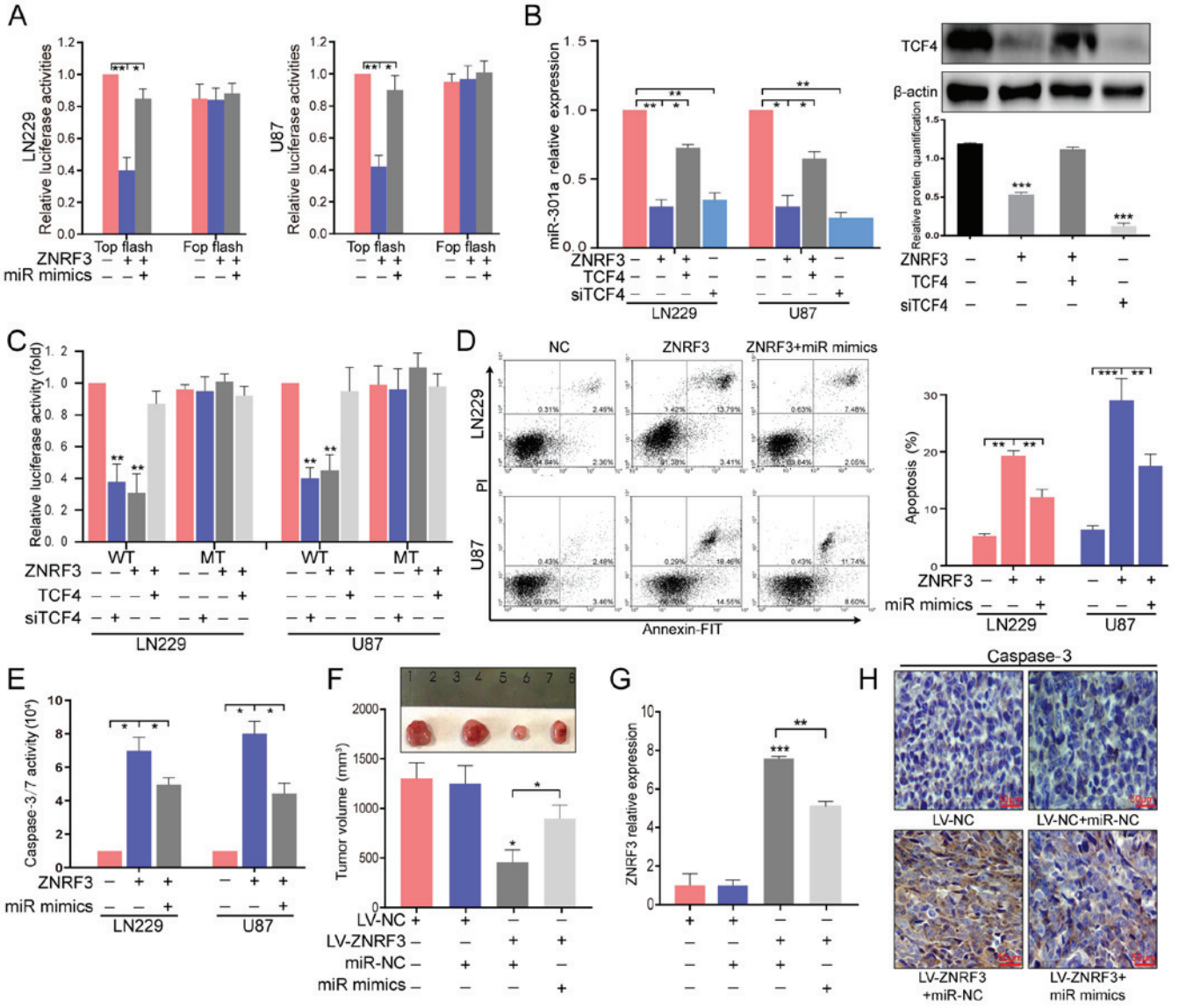

Figure 7. ZNRF3 can inhibit miR-301a expression that is partially dependent on TCF4. (A) TOP/FOP flash was used to detect wnt/β-catenin transcriptional activity in ZNRF3 stably overexpressing LN229 and U87 cells with or without co-transfection of miR-301a mimics. *P<0.05, **P<0.01. (B) RT-qPCR was performed to analyze miR-301a expression in L229 and U87 cells transfected with siTCF4, ZNRF3 stable overexpressing LN229 and U87 cells or those co-transfected with TCF4 overexpression plasmid compared with the cells co-transfected with NC-siRNA and empty vector (left). Western blotting was used to identify TCF4 expression after different treatments (right). The relative protein semi-quantification was performed with ImageJ software. *P<0.05, **P<0.01, ***P<0.001. (C) pGL3-WT-miR-301a-promotor-luc reporter and pGL3-MT-miR-301a-promotor-luc reporter were constructed and co-transfected into LN229 and U87 cells (LV-ZNRF3 or not) with TCF4 overexpression plasmid or siTCF4. Luciferase activity was further analyzed after 72 h. **P<0.01 compared with NC. (D) Annexin V-PI via flow cytometry and (E) caspase-3/7 activity assays were used to detect apoptosis in LN229 and U87 cells (LV-ZNRF3 or not) co-transfected with miR-301a mimics or miR-NC. *P<0.05, **P<0.01, ***P<0.001. *In vivo* assays investigating the miR-301a mimic-mediated role on the tumor-suppressive function of ZNRF3 were performed. (F) Tumor volume and representative images of excised tumors from different treatment group at the end of the experiment. *P<0.05. (G) ZNRF3 expression was determined via RT-qPCR in resected tumor tissues of different treatment groups. **P<0.01, ***P<0.001. (H) Representative photomicrographs of immunohistochemistry of tumor sections for caspase-3 in different groups. Scale bar, 50 μm. ZNRF3, Zinc and ring finger 3; siRNA, small interfering RNA; miR, microRNA; WT, wild-type; MT, mutant; NC, negative control; TCF4, transcription factor 4; RT-qPCR, reverse transcription-quantitative PCR.

miR-23a in serum samples could discriminate patients with CRC from healthy controls, highlighting their role as non-invasive biomarkers for the early detection of CRC. Moreover, Zheng *et al* (24) revealed that the expression of miR-301a was associated with decreased overall survival in breast cancer. It was also observed that the miR-301a upregulation could predict a poor outcome in pancreatic cancer and hepatocellular carcinomas (25,26). In addition, a study on glioma demonstrated that a higher serum exosome miR-301a level was closely correlated with the WHO grade and lower Karnofsky scores (27). Results from both univariate and multivariate Cox regression analysis have revealed that miR-301a could act as a biomarker for glioma diagnosis and prognosis, especially in advanced grades (27). By analyzing the public datasets, the present results also verified the prognostic potential of miR-301a. Moreover, miR-301a expression was measured in glioma clinical specimens and it was found that miR-301a expression was positively associated with the WHO grade, which was consistent with previous reports (13,27). The present study demonstrated that the knockdown of miR-301a inhibited cell

proliferation, as detected via CCK-8, and promoted apoptosis as shown via Annexin V-FITC assay and caspase-3/7 activity detection. However, a limitation of the current study was that some significant apoptosis-related proteins were not been detected, including Bcl-2 and poly(ADP-ribose) polymerase 1.

miRNAs are short non-coding RNAs that modulate gene expression by either mRNA degradation or translational inhibition (22). miR-301a participates in the cancer progression by directly targeting a large number of tumor suppressor genes, such as PTEN, RUNX family transcription factor 3 (RUNX3) and septin 7 (7,11,13). Accumulating evidence has demonstrated that miR-301a is innately associated with cancer cell proliferation, invasion and drug resistance by targeting PTEN in cervical cancer, malignant melanoma and pancreatic cancer (7,8,10). In the present study, according to bioinformatic predictions and experimental assays, it was identified that ZNRF3 was a functional target of miR-301a in GBM cell lines. Moreover, the results suggested a key function of ZNRF3 in miR-301a-mediated promotion of the glioma malignant phenotype, indicating a novel molecular mechanism for ZNRF3 inactivation.

Mechanistically, the present data indicated that miR-301a could activate the wnt/β-catenin signaling pathway, at least partially via ZNRF3, which is a negative regulator of the wnt pathway that acts by promoting the ubiquitination, internalization and degradation of the Wnt receptors Frizzled and LRP5/6 (28,29). This finding was also reported in various previous cancer studies. For example, Wang *et al* (30) revealed that ZNRF3 inhibited the invasion and EMT by inactivating the wnt/β-catenin pathway in nasopharyngeal carcinoma. Our previous data also suggested that ZNRF3 could repress the glioma proliferation and invasion, as well as promote apoptosis via the inactivation of the wnt/β-catenin signaling pathway (unpublished data), which supports the theoretical feasibility of the miR-301a/ZNRF3/wnt pathway. Additionally, Yue *et al* (31) reported that exo-miR-301a secreted by hypoxic glioma cells could suppressed radiation sensitivity by activating the wnt/β-catenin pathway via targeting transcription elongation factor A like 7. It has also shown been that RUNX3 is a direct target of miR-301a in CRC and lung cancer (11,32), and our previous study demonstrated that RUNX3 repressed the β-catenin/TCF4 transcriptional activity in gliomas (33), indicating that miR-301a may regulated the wnt pathway via RUNX3. However, this hypothesis requires further investigation.

In addition to the wnt/β-catenin signaling, other important oncogene pathways are also regulated by miR-301a. For instance, Hu *et al* (34) indicated that miR-301a could decrease the expression of suppressor of cytokine signaling 5 and subsequently result in JAK/STAT3 signal activation. Lu *et al* (35) reported that miR-301a reduced NKRF repressing factor expression, leading to NF-κB activation in pancreatic cancer. Yin *et al* (36) also revealed that miR-301a could activate ERK/cAMP-response element binding protein signaling in triple-negative breast cancer. Moreover, based on PTEN target gene identification, miR-301a participates in biological processes of cancer via AKT/mTOR signaling (7,9). Hence, the knockdown of miR-301a may disrupt multiple significant carcinogenic signaling pathways, and it represents a key candidate target for cancer treatment.

It is well known that various transcriptional factors may, to some extent, contribute to miRNA deregulation (37). Currently, several transcriptional factors that affect the miR-301a expression have been identified, including NF-κB, forkhead box M1 and E2F transcription factor 1, which have been proven to be key oncogene molecules in the majority of malignancies (34,36,38). Additionally, Yue *et al* (13) found that miR-301a was activated by the wnt/β-catenin pathway, and that TCF4 enhanced miR-301a expression by directly binding to its promoter region, as demonstrated by Chromatin immunoprecipitation and luciferase reporter assays, indicating that the miR-301a-mediated wnt pathway feedback loop is implicated in glioma tumorigenesis. The present study investigated whether ZNRF3 overexpression could attenuate the expression of miR-301a. The results demonstrated that ZNRF3 overexpression significantly inhibited miR-301a expression and that overexpression of TCF4 in GBM cells stably expressing ZNRF3 could abrogate the inhibitory effect mediated by ZNRF3. Therefore, these findings also suggested that the miR-301a/ZNRF3/wnt/β-catenin signaling feedback loop exists. However, the miR-301a-mediated regulatory network is complicated and should be further investigated.

In conclusion, to the best of our knowledge, the present study demonstrated for the first time that miR-301a promoted glioma progression via the ZNRF3-mediated wnt/β-catenin signaling pathway. Additionally, ZNRF3 could repress miR-301a expression that is dependent on TCF4 transcriptional activity. The present study identified a novel miR-301a/ZNRF3/wnt/β-catenin signaling feedback loop that serves critical roles in glioma tumorigenesis, and it may provide further insights for understanding the molecular mechanism of glioma.

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Availability of data and materials

All the datasets generated and analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

JS, JWa and CS conceived and designed the experiments. JS, JX, BL and QM performed the *in vitro* experiments. QM, JLi, JLiu and JWu collected the clinical specimens. JS, JX, JLiu and JWu performed the *in vivo* experiment. CS, SZ and JLi helped with the bioinformatics analysis and data analysis. JS and QM interpreted the data and co-wrote the manuscript. JS, SZ and JWa reviewed and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was reviewed and approved by Institutional Review Board of Tianjin Huanhu Hospital (Tianjin, China; approval no. CK19-190318), and written informed consent was obtained from patients in all cases. Animal studies were approved by Animal Ethical and Welfare Committee of Tianjin Huanhu Hospital of Nankai University ((Tianjin, China; approval no. SYXK2019-002).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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