Long non-coding RNA FLVCR1-AS1 promotes glioma cell proliferation and invasion by negatively regulating miR-30b-3p

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Abstract. Glioblastoma (GBM) is the most common and malignant primary brain tumor in adults that originates from glial cells. The prognosis of patients with high-grade glioma is poor. It is therefore crucial to develop effective therapeutic strategies. Long non-coding RNAs (lncRNAs) have been reported as potential inducers or suppressors of tumor progression. Previous studies have indicated that the lncRNA Feline Leukemia Virus Subgroup C Cellular Receptor 1 Antisense RNA 1 (FLVCR1-AS1) is involved in the development and progression of gastric and lung cancer, as well as hepatocellular carcinoma and cholangiocarcinoma; however, the biological effect of FLVCR1-AS1 in glioma is not completely understood. The aim of the present study was to investigate how FLVCR1-AS1 modulates cell proliferation and invasion in glioma. FLVCR1-AS1 expression was significantly upregulated in GBM tissues compared with adjacent normal brain samples, and was higher in GBM cell lines compared with normal human astrocyte cells. Furthermore, the microRNA (miR)-30b-3p was revealed to be a putative target of FLVCR1-AS1, and the suppressive effects of miR-30b-3p on cellular proliferation and invasion were reversed following FLVCR1-AS1-knockdown. The results from Cell Counting Kit-8 and Transwell assays confirmed that FLVCR1-AS1-knockdown inhibited GBM cell proliferation and invasion ability. In addition, FLVCR1-AS1 was found to directly interact with miR-30b-3p, and a rescue experiment further established that FLVCR1-AS1 contributed to glioma progression by inhibiting miR-30b-3p. The results from the present study demonstrated that FLVCR1-AS1 may serve an oncogenic role in GBM and promote disease progression by interacting with miR-30b-3p. These findings suggested that FLVCR1-AS1 may be considered as a novel therapeutic target and diagnostic biomarker for GBM.

Introduction

Glioblastoma (GBM) is one of the commonest solid tumors and the leading cause of central nervous system-associated mortality worldwide (1). Statistical analysis has demonstrated that glioblastoma accounts for ~75% of all malignant tumors associated with the brain (2). Conventional therapeutic approaches, including surgery, chemotherapy and radiotherapy, have greatly improved patient survival; however, the treatment efficacy remains poor, with an overall survival of 12-14 months following surgical resection (3-5). Tumor resistance and recurrence are particularly problematic and require further investigation (6-8). It is therefore crucial to determine the underlying mechanisms of glioma occurrence and progression, in order to develop novel therapeutic targets.

Long non-coding RNAs (lncRNAs) are defined as transcripts of 200-1,000 nucleotides in length that are not translated into proteins (9), and which modulate various biological processes, including cellular migration, invasion and apoptosis (10). Increasing evidence has indicated that lncRNAs are promising biomarkers for tumor diagnosis and prognosis, including in GBM (11-12). Matrix metallopeptidase (MMP)-2 and MMP-9 are members of the MMP family, which promote ECM degradation to allow cancer cells to migrate out of the primary tumour to form metastases during cancer progression (13). These studies revealed the importance of lncRNAs and suggested a novel potential therapeutic strategy for the treatment of GBM.

The lncRNA Feline Leukemia Virus Subgroup C Cellular Receptor 1 Antisense RNA 1 (FLVCR1-AS1) is a novel tumor suppressor located on chromosome 1q32.3 (13). Previous studies have identified impaired expression of FLVCR1-AS1 in hepatocellular carcinoma, lung cancer and ovarian cancer (14-17); however, the underlying mechanism of FLVCR1-AS1 in glioma remains unknown.

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MicroRNAs (miRNAs) are a class of non-coding RNAs that play pivotal roles in cellular proliferation, migration, invasion and apoptosis. Numerous miRNAs have been confirmed as potential biomarkers for glioma development (18,19). Previous studies reported a decrease in miR-30b-3p expression in various types of cancer, including glioma (20,21). However, the molecular mechanism by which miR-30b-3p deregulation contributes to glioma tumorigenesis remains elusive.

Materials and methods

Cell lines and clinical samples. The human GBM cell lines U251, T98G, LN229 and SHG44 were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Normal Human Astrocyte (NHA) cells were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37°C (5% CO_2) in a humidified atmosphere.

Human GBM samples (n=50) and adjacent normal brain samples (n=50; 0.5-1 cm from the tumor margin) ere resected from patients with GBM between June 2013 and June 2017 at The Second Affiliated Hospital of Harbin Medical University (Harbin, China). All GBM samples and adjacent normal tissues were confirmed by two senior pathologists. Then the samples were immediately frozen at the Department of Pathology, and the clinicopathological characteristics of all patients were collected. Patients consisted of 22 men and 28 women with age ranging from 20 to 74 years (median age, 46 years). None of the patients had received any therapy before surgery. All GBM samples were confirmed by two senior pathologists. The present study was approved by The Institutional Review Board of Harbin Medical University, and all patients provided written informed consents.

Bioinformatics and Gene Ontology (GO) term enrichment analysis. The edgeR software package (R Studio3.5.1; Bioconductor; https://www.rstudio.com) was used to analyze the aberrantly expressed lncRNAs among normalized gene expression profile data from The Cancer Genome Atlas (TCGA) GBM database (http://cancergenome.nih.gov) (22,23). Samples from moderate to severe TCGA-GBM cases were compared with those from healthy individuals. A log fold change >2 and false-discovery rate (P-value) <0.01 were used as the cutoff values for significance, for which aberrantly expressed candidate lncRNAs were detected. Clinical data were obtained from the Gene Expression Profiling Interactive Analysis (GEPIA) dataset (http://gepia.cancer-pku.cn/), and GO term enrichment analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery version 6.8 (DAVID; https://david.ncifcrf.gov/).

Reverse transcription-quantitative (RT-q) PCR). RT-qPCR was used to detect the expression levels of FLVCR1-AS1 and miR-30b-3p. Total RNA was extracted from clinical samples (250 mg) or cell lines (1x10⁶) using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The concentration of eluted RNA was determined with the NanoDrop spectrophotometer (Applied Biosystems; Thermo Fisher Scientific, Inc.). cDNA was synthesized using the PrimeScript™ RT kit, and qPCR was conducted using SYBR® Green PCR Master Mix (both Takara Biotechnology Co., Ltd.) according to the manufacturer's protocols. The thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, and 60°C for 60 sec. GAPDH was used as an endogenous control to normalize lncRNA FLVCR1-AS1 expression level. U6 was used as an endogenous control to normalize miR-30b-3p expression level and the results were calculated using the $2^{-\Delta\Delta Cq}$ method (24). The sequences of the primers are presented in Table I.

Cell transfection. Small interfering RNAs (siRNAs) targeting FLVCR1-AS1 and the corresponding negative control (si-NC; both Shanghai GenePharma, Co., Ltd.) were used to generate the FLVCR1-AS1-knockdown model. In order to increase or decrease the level of miR-30b-3p, the miR-30b-3p mimic or inhibitor and corresponding negative control (miR-NC; all Shanghai GenePharma, Co., Ltd.) were used to up- and downregulate the expression of miR-30b-3p, respectively. LN229 and T98G cells (5x10⁵) were transfected with 20 μM of each construct using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol and incubated at 37°C for 6 h. The culture medium was then changed with fresh DMEM containing 10% FBS, and subsequent experimentation was conducted 24 h post-transfection. The sequences of the siRNA are listed in Table II.

Dual-luciferase reporter assay. TargetScan (www.targetscan.org/) and StarBase (http://starbase.sysu.edu.cn/) databases were used to predict the potential target miRs of FLVCR1-AS1. Among the statistically relevant miRs, the top three were selected in terms of their prediction score, and included miR-30b-3p, miR-96-5p and miR-513. As the highest scoring miR in both databases, miR-30b-3p was selected for further experimentation. The 3'-untranslated region of FLVCR1-AS1 was cloned into the human FLVCR1-AS1 Luc-reporter plasmid (GenePharm, Inc.), which was then used to transfect LN229 cells as aforementioned. Briefly, cells were seeded into 6-well plates at 3x10⁵ cells/well, and the recombinant vectors were co-transfected with miR-30b-3p mimics or miR-NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Luciferase activity was measured 48 h post-transfection using the Dual Luciferase Reporter Assay System (Promega Corporation), and firefly luciferase activity was normalized to that of *Renilla* luciferase.

Cell proliferation and colony formation assays. LN229 and T98G cells were collected 24 h post-transfection and were seeded into 96-well plates at the density of 1x10⁴ cells/well. Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology) at days 1, 2 and 3, according to the manufacturer's protocol. Briefly, 10 μl CCK8 solution was added to each well. After incubation at 37°C for 4 h, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad laboratories, Inc.).

Table I. Sequences of the primers used for reverse transcription-quantitative PCR.

Gene	Sequence
FLVCR1-AS1-sense	5'-GTGGCTCTCTCGTTCCC-3'
FLVCR1-AS1-antisense	5'-CCGTCCTTCGGTAGTGTC-3'
miR-30b-3p-sense	5'-UGUAAACAUCCUACACUCAGCU-3'
miR-30b-3p-antisense	5'-ACAUUUGUAGGAUGUAGUCGA-3'
MMP-9-sense	5'-AGACCTGGGCAGATTCCAAAC-3'
MMP-9-antisense	5'-CGGCAAGTCTTCCGAGTAGT-3'
MMP-2-sense	5'-CAGGACATTGTCTTTGATGG-3'
MMP-2-antisense	5'-TGAAGAAGTAGCTATGACCA-3'
GAPDH-sense	5'-TCCTCTGACTTCAACAGCGACAC-3'
GAPDH-antisense	5'-CACCCTGTTGCTGTAGCCAAATTC-3'
U6-sense	5'-CTCGCTTCGGCAGCACA-3'
U6-antisense	5'-AACGCTTCACGAATTTGC-3'

FLVCR1-AS1, Feline Leukemia Virus Subgroup C Cellular Receptor 1 Antisense RNA 1; miR, microRNA; MMP, matrix metalloproteinase.

Table II. Sequences of the siRNAs used in the present study.

Gene	Sequence
si-FLVCR1-AS1-sense	5'-GGUAAGCAGUGGCUCCUCUAA-3'
si-FLVCR1-AS1-antisense	5'-AAUUCUCCGAACGUGUCACGU-3'
miR-30b-3p mimics sense	5'-UGUAAACAUCCUACACUCAGCU-3'
miR-30b-3p mimics antisense	5'-UCACAACCUCCUAGAAAGAGUA-3'
miR-inhibitor mimics sense	5'-AGCUGAGUGUAGGAUGUUUAC-3'
miR-inhibitor mimics antisense	5'-GGUAAGCAGUGGCUCCUCUAA-3'
siRNA-NC-sense	5'-UUCUCCGAACGUGUCACGUUU-3'
siRNA-NC-antisense	5'-ACGUGACACGUUCGGAGAAUU-3'
miR-mimics NC-sense	5'-ACAGUCGCGUUUGCGACUGUU-3'
miR-mimics NC-antisense	5'-UUG UCAGCGCAAACGCUGACC-3'
miR-inhibitor NC-sense	5'-CAGUACUUUUGUGUAGUACAA-3'
miR-inhibitor NC-antisense	5'-UUAACUAAUAUUUCAUCCAUA-3'

FLVCR1-AS1, Feline Leukemia Virus Subgroup C Cellular Receptor 1 Antisense RNA 1; miR, microRNA.

LN229 and T98G cells (~300 cells/well) were seeded into 6-well plates in fresh DMEM with 10% FBS and cultured at 37˚C. The medium was replaced every 3 days. After 14 days, cells were fixed with 4% polyoxymethylene for 10 min at room temperature and stained with 10% Giemsa (Sigma-Aldrich; Merck KGaA) at room temperature for 30 min. Colonies >50 cells were counted under a light microscope (Nikon Corporation).

Invasion assays. LN229 and T98G cells ($5x10^4$) were seeded into the upper chambers of Transwell inserts pre-coated with Matrigel® (pore size, 8.0 μm; Corning Life Sciences), and incubated at 37˚C (5% CO_2) in a humidified atmosphere for 24 h. Serum-free DMEM was added to the upper chamber whereas the lower chamber contained DMEM with 20% FBS as a chemoattractant. The invasive cells were fixed for 10 min using 4% paraformaldehyde and stained with hematoxylin and eosin for 5 min, both at room temperature. The stained cells were counted in five random fields under a light microscope (Nikon Corporation; magnification, x100).

Western blotting. Total protein was extracted from patient tissue samples (250 mg/sample) and cell lines (LN229 and T98G) using RIPA lysis buffer (Pierce; Thermo Fisher Scientific, Inc.) containing Protease Inhibitor Cocktail (Complete™ Mini; Roche Applied Science) at 4˚C for 10 min. Protein concentration was measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Proteins (40 μg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Beyotime Institute of Biotechnology). Membranes were blocked with tris-buffered saline (TBS) containing 5% non-fat milk (w/v) for 1 h at room temperature. Subsequently, membranes were incubated with primary antibodies targeted against MMP-2 (rabbit polyclonal;

1:1,000; cat. no. 409494; Cell Signaling Technology, Inc.), MMP-9 (rabbit polyclonal; 1:1,000; cat. no. 13667; Cell Signaling Technology, Inc.) and GAPDH (mouse monoclonal; 1:1,000; cat. no. SC-47724; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit (cat. no. ab6721 and ab6728; 1:2,000; Abcam) secondary antibodies were added separately for 1 h at room temperature. The bands were visualized using enhanced chemiluminescence detection (Beyotime Institute of Biotechnology) with a ChemiDoc™ MP Imaging System and analyzed by Image Lab software (version 3.0; Bio-Rad Laboratories, Inc.).

Immunofluorescence staining. LN229 and T98G cells (1x10^5) were cultured on glass slides treated overnight with 0.1% poly-L-Lysine, fixed with 4% paraformaldehyde (5% BSA; cat. no. 9048-46-8; Sigma Aldrich; Merck KGaA) for 20 min, and washed with 0.1% Triton X-100 for 10 min at room temperature. Slides were incubated with a primary antibody against MMP-2 (rabbit polyclonal; 1:1,000; cat. no. 40094s; Cell Signaling Technology, Inc.) at 4˚C for 1 h, and a fluorescence-labeled rabbit secondary antibody [Rhodamine (TRITC)-conjugated goat anti-rabbit immunoglobulin G (IgG); 1:100; cat. no. SA00007-2; ProteinTech Group, Inc.] at room temperature for 1 h. Cell nuclei were stained with DAPI (1 μg/ml; cat. no. 4083s; Cell Signaling Technology, Inc.) for 15 min and images were captured using a fluorescence microscope (Nikon Corporation; magnification, x400).

Anti-AGO2 RNA-binding protein immunoprecipitation (RIP) assay. RIP assay was conducted by using the Magna RIP RNA-Binding Protein Immunoprecipitation kit (cat. no. 17-701; EMD Millipore). As a core element of RISC complex, AGO2 directly initiates the degradation of target mRNAs via its catalytic activity in gene silencing processes guided by siRNAs or miRNAs. AGO2 is associated with tumorgenesis via miRNAs-dependent or independent pathways (25). LN229 and T98G cells transfected with miR-30b-3p were lysed using RIPA lysis buffer and 100 μl of the cell lysate was collected for RIP experiments using an anti-AGO2 antibody (Abcam) according to the manufacturer's instructions. The RNA fraction isolated by RIP was subjected to RT-qPCR analysis to detect the direct binding between FLVCR1-AS1 and miR-30b-3p.

Statistical analysis. Data were expressed as the means ± standard deviation. Statistical analysis was conducted using SPSS v20.0 (IBM Corp.). One-way ANOVA with Tukey's post hoc test and Student's t-test were used to determine the level of significance between groups. The Kaplan-Meier method and the log-rank test were used for survival analyses using GraphPad Prism v5.0 (GraphPad Software, Inc.). The association between FLVCR1-AS1 expression and the clinicopathological characteristics of patients with GBM was evaluated using χ^2 or Fisher's exact tests. The correlation between the expression of FLVCR-AS1 and the expression of miR-30b-3p, MMP-2 and MMP-9 was analyzed using Pearson's correlation. Experiments were independently performed three times. P<0.05 was considered to indicate a statistically significant difference.

Results

FLVCR1-AS1 is upregulated in GBM tissues and is associated with poor prognosis. The top 20 differentially expressed lncRNAs were identified from TCGA based on the Benjamini-Hochberg method (26) [log2-fold change (FC) of >2 and a false discovery rate, P<0.01; Fig. 1A]. FLVCR1-AS1 was the most highly expressed differentially expressed lncRNAs among the GBM tissue samples, and its expression was significantly increased in GBM tissues compared with adjacent normal brain tissue (Fig. 1A and B). To investigate the potential function of FLVCR1-AS1, the associated gene expression profiles collected from the GO database were analyzed. 'Cell invasion', 'Cell adhesion' and 'Zinc-finger' were the most prominent biological processes (Fig. 1C). In addition, clinical data collected from the GEPIA database indicated that increased FLVCR1-AS1 was associated with worse overall survival in patients with GBM, according to the median patient survival time (Fig. 1D). Among the clinical samples collected for the present study, the expression level of FLVCR1-AS1 was significantly associated with mean tumor diameter (n=50; Table III; P=0.030). Furthermore, the results from RT-qPCR demonstrated that FLVCR1-AS1 expression level was significantly increased in GBM tissues compared with adjacent normal brain tissues (Fig. 1E). These results suggested that FLVCR1-AS1 may be involved in the development of GBM.

FLVCR1-AS1-knockdown inhibits GBM cell proliferation, colony formation and invasive ability. To determine FLVCR1-AS1 function in GBM, its expression level in GBM (U251, LN229, T98G and SHG44) and NHA cells was assessed using RT-qPCR (Fig. 2A). The results demonstrated a significant increase in FLVCR1-AS1 expression level in all GBM cells compared with NHA cells, in particular in LN229 and T98G cell lines. To determine the effect of FLVCR1-AS1 on the proliferative and invasive abilities of GBM cells, FLVCR1-AS1 was knocked down in LN229 and T98G cells. The transfection efficiency was confirmed by RT-qPCR, where FLVCR1-AS1 was significantly decreased in transfected cells compared with siRNA-Negative Control (si-NC; Fig. 2B). Furthermore, the results from CCK-8 assay demonstrated that the proliferation of GBM cells transfected with si-FLVCR1-AS1 was decreased compared with cells transfected with si-NC (Fig. 2C). In addition, FLVCR1-AS1-knockdown significantly decreased the colony formation (Fig. 2D) and invasion abilities of GBM cells (Fig. 2E). These findings suggested that GBM cell proliferation and invasive ability may be inhibited by FLVCR1-AS1-knockdown *in vitro*.

Data from TCGA database were used to confirm whether reduced FLVCR1-AS1 expression affected the invasive capacity of GBM. The results suggested a positive correlation between FLVCR1-AS1 expression and the invasion-associated markers MMP-2 (r=0.3428; P=0.0027) and MMP-9 (r=0.2928; P=0.0004; Fig. 3A). Furthermore, RT-qPCR and western blotting were performed to assess the mRNA and protein expression levels of MMP-2 and MMP-9 in samples from patients with GBM. MMP-2 levels were also determined by immunofluorescence detection. The results demonstrated that FLVCR1-AS1-knockdown decreased MMP-2 and

Table III. Clinical characteristics of patients with glioblastoma patients according to FLVCR1-AS1 tissue level (n=50).

Variable	No. of cases	FLVCR1-AS1 expression		P-value
		Low	High	
Age, years				
<60	23	6	17	0.781
≥60	27	8	19	
Sex				
Male	22	7	15	0.594
Female	28	7	21	
Karnofsky performance status				
<60	21	5	16	0.991
≥60	29	9	20	
Mean tumor diameter, cm				
<5	27	11	16	0.030[a]
≥5	23	3	20	
Necrosis on magnetic resonance imaging				
Yes	31	8	23	0.659
No	19	6	13	
Seizure				
Yes	10	4	6	0.345
No	40	10	30	

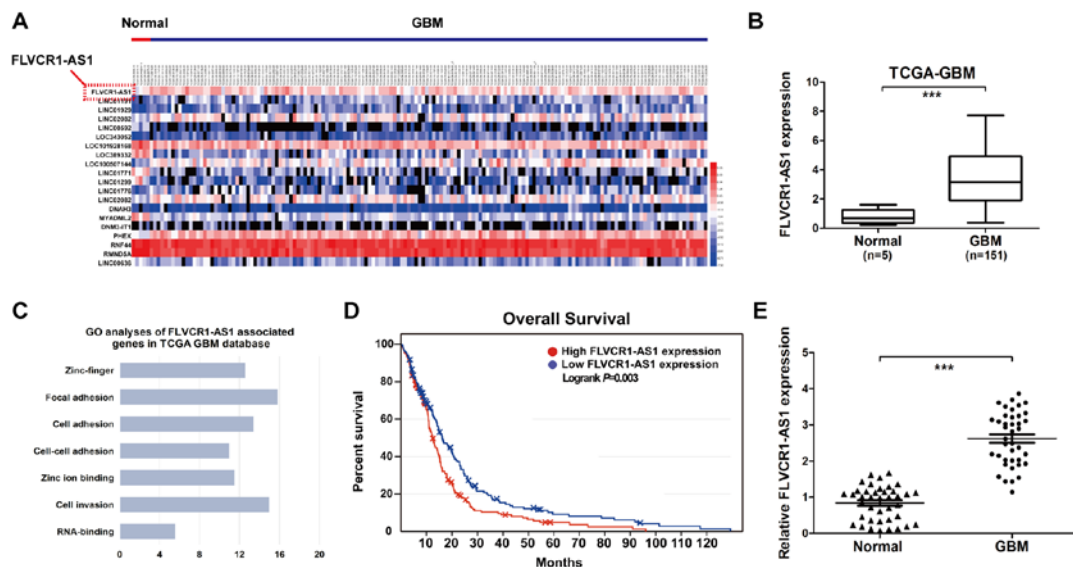[a]$P<0.05$. FLVCR1-AS1, Feline Leukemia Virus Subgroup C Cellular Receptor 1 Antisense RNA 1.

Figure 1. lncRNA FLVCR1-AS1 expression is increased in human GBM samples and is associated with poor prognosis. (A) Heat map demonstrating the differential expression of lncRNAs from TCGA GBM database. (B) Gene expression levels of FLVCR1-AS1 in TCGA GBM cohort compared with the normal cohort. (C) GO analysis of genes positively associated with FLVCR1-AS1 from TCGA GBM datasets. (D) Kaplan-Meier curve of overall survival in patients with GBM; high levels of FLVCR1-AS1 are associated with poorer prognosis (n=151). (E) High expression levels of FLVCR1-AS1 in patients with GBM (n=50), compared with matched adjacent normal tissues (reverse transcription-quantitative PCR analysis). Each experiment was performed in triplicate. ***$P<0.001$. FLVCR1-AS1, Feline Leukemia Virus Subgroup C Cellular Receptor 1 Antisense RNA 1; GBM, glioblastoma; TCGA, The Cancer Genome Atlas; GO, Gene Ontology.

MMP-9 expression in GBM tissues (Fig. 3B and C). In addition, the results from immunofluorescence staining confirmed that FLVCR1-AS1-knockdown inhibited the expression of MMP-2 in GBM cell lines (Fig. 3D). Taken together, these findings suggested that FLVCR1-AS1 may be associated with GBM cell proliferation and invasive ability.
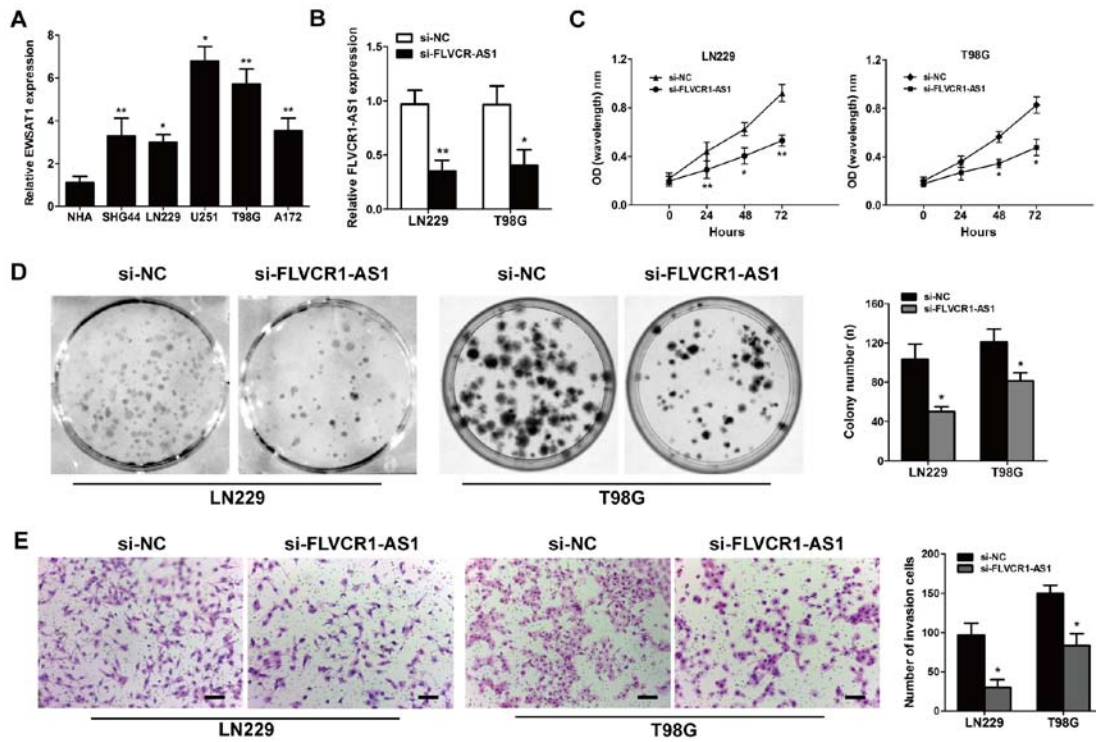
Figure 2. Knockdown of FLVCR1-AS1 inhibits the proliferation and invasive ability of GBM cell lines *in vitro*. (A) FLVCR1-AS1 gene expression levels in GBM cell lines (U251, LN229, T98G and SHG44) vs. normal NHA cells were assessed by RT-qPCR analysis. (B) FLVCR1-AS1 was efficiently knocked down by siRNA in LN229 and T98G cells, compared with the si-NC group, (detected by RT-qPCR). (C) GBM cell proliferation was assessed using the Cell Counting Kit-8 assay (si-FLVCR1-AS1 vs. si-NC). (D) GBM cells transfected with si-NC or si-FLVCR1-AS1 were incubated for 14 days and colonies >50 cells were counted. (E) Transwell assay assessed invasive ability; magnification, x100. $^*P<0.05$ and $^{**}P<0.01$ vs. si-NC. Data are presented as the mean ± standard deviation of three independent experiments. FLVCR1-AS1, Feline Leukemia Virus Subgroup C Cellular Receptor 1 Antisense RNA 1; GBM, glioblastoma; NHA, Normal Human Astrocyte; RT-qPCR, reverse transcription-quantitative PCR; si-, small interfering; NC, negative control.
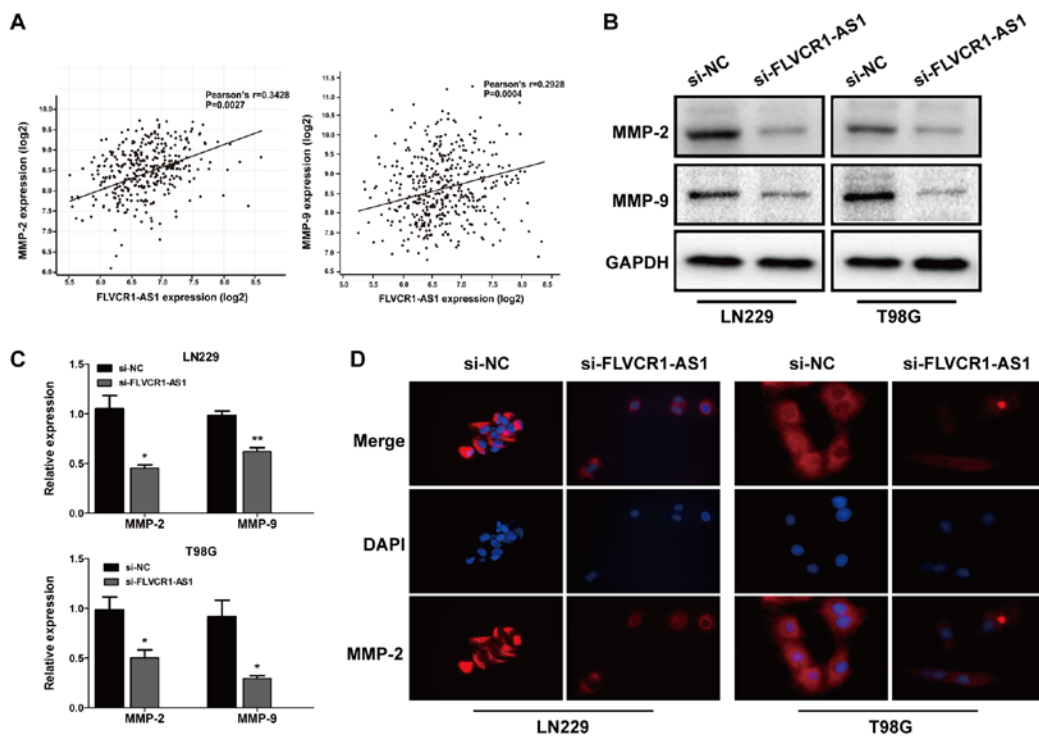

Figure 3. Expression of proliferation- and invasion-related markers in GBM cells. (A) Correlation between FLVCR1-AS1 and MMP-2/MMP-9 expression in sample data from TCGA GBM database. Following transfection with si-FLVCR1-AS1 or si-NC, the (B) protein and (C) mRNA expression levels of PCNA, MMP-2 and MMP-9 were determined. (D) Immunofluorescence staining revealed a decreased level of MMP-2 in si-FLVCR1-AS1-transfected cells (magnification, x400). $^*P<0.05$ and $^{**}P<0.01$ vs. si-NC. Experiment was repeated three times. GBM, glioblastoma; FLVCR1-AS1, Feline Leukemia Virus Subgroup C Cellular Receptor 1 Antisense RNA 1; MMP, matrix metalloproteinase; TCGA, The Cancer Genome Atlas; si-, small interfering; NC, negative control; si, small interfering RNA; TCGA, The Cancer Genome Atlas.
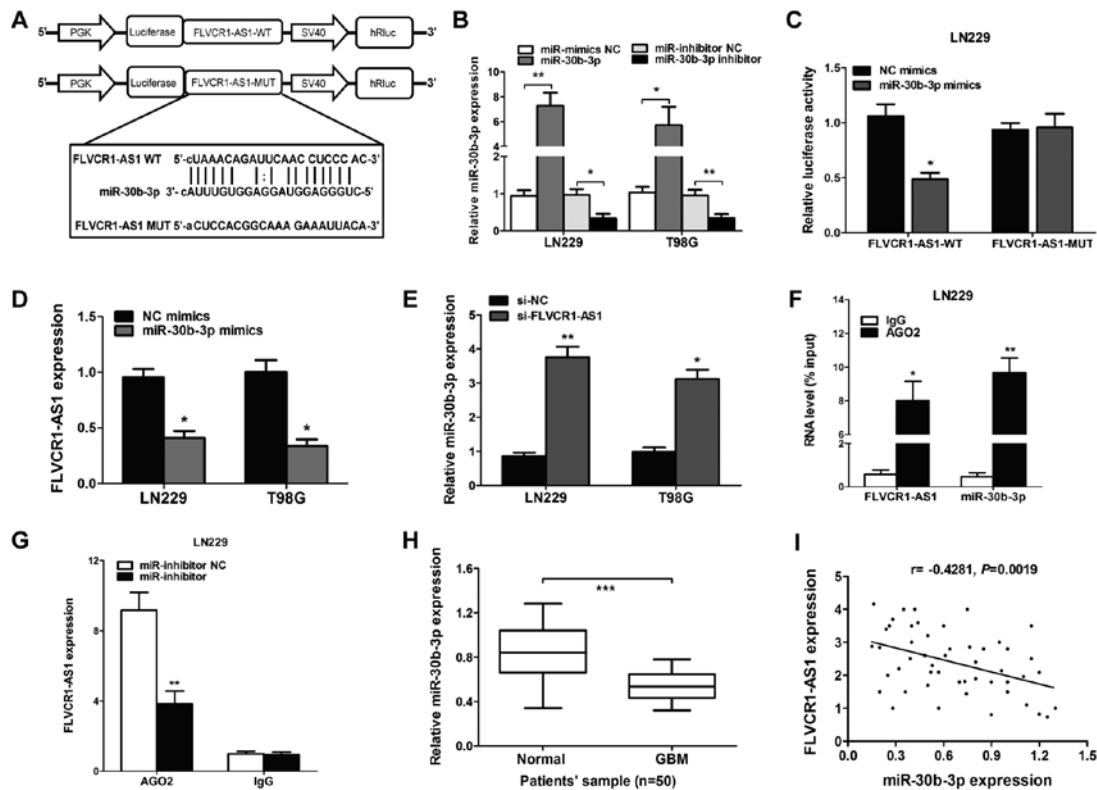
Figure 4. FLVCR1-AS1 is targeted by miR-30b-3p at the 3'UTR. (A) Target site of miR-30b-3p in the 3'UTR region of FLVCR1-AS1. (B) miR-30b-3p expression was upregulated by miR-30b-3p mimics compared with the miR-mimic NC (**P<0.01 in LN229 and T98G cells), or inhibited by an miR-30b-3p inhibitor compared with the miR-inhibitor NC (*P<0.05 and **P<0.01), respectively, in LN229 and T98G cells. (C) Relative luciferase activity was determined following co-transfection of LN229 cells with miR-30b-3p mimics or miR-NC and FLVCR1-AS1-WT or FLVCR1-AS1-MUT. (D) After transfection with miR-30b-3p mimics, the level of FLVCR1-AS1 was decreased in LN229 cells, as detected by RT-qPCR. (E) Expression levels of miR-30b-3p in GBM cell lines following transfection with si-FLVCR1-AS1 or si-NC. (F) Association between FLVCR1-AS1 and miR-30b-3p with AGO2. (G) Change in FLVCR1-AS1 level in glioma cells transfected with an miR-30b-3p inhibitor. AGO2 RNA level determined by RT-qPCR. (H) Expression level of miR-30b-3p in GBM compared with adjacent normal tissues. (I) Pearson's correlation coefficient analysis between FLVCR1-AS1 and miR-30b-3p expression level. *P<0.05, **P<0.01 and ***P<0.001. Experiments were repeated three times. FLVCR1-AS1, Feline Leukemia Virus Subgroup C Cellular Receptor 1 Antisense RNA 1; miR, microRNA; NC, negative control; UTR, untranslated region; WT, wild-type; MUT, mutant; siRNA, small interfering RNA; IgG, immunoglobulin G; RT-qPCR, reverse transcription-quantitative PCR; GBM, glioblastoma.

Correlation between FLVCR1-AS1 and miR-30b-3p expression. Using the TargetScan and StarBase databases, a complementary sequence was identified between FLVCR1-AS1 and miR-30b-3p (Fig. 4A). Luciferase reporter assays were subsequently used to confirm this putative miR-30b-3p target site. miR-30b-3p mimics or inhibitor mimics were used to assess transfection efficiency in the LN229 and T98G cell lines (Fig. 4B), and luciferase reporter vectors carrying either the predicted miR-30b-3p wild-type binding site (FLVCR1-AS1-WT) or its mutant fragment (FLVCR1-AS1-MUT) were constructed. The luciferase activity of the LN229 cells was significantly decreased following co-transfection with the miR-30b-3p mimics and FLVCR1-AS1-WT, but not with FLVCR1-AS1-MUT (Fig. 4C).

The interaction between FLVCR1-AS1 and miR-30b-3p was subsequently assessed in GBM tissues and cell lines, in particular whether miR-30b-3p could decrease FLVCR1-AS1 expression. The results demonstrated that increased miR-30b-3p expression inhibited FLVCR1AS1 expression in LN229 cells (Fig. 4D). To determine whether miR-30b-3p was negatively regulated by FLVCR1-AS1, FLVCR1-AS1 was knocked down in GBM cells. The results demonstrated that miR-30b-3p was upregulated following FLVCR1-AS1-knockdown in LN229 cells (Fig. 4E).

miRNAs have been reported to function by interacting with the RNA-induced silencing complex (RISC), which is required for miRNA-mediated gene silencing. As a core element of RISC complex, AGO2 directly initiates the degradation of target mRNAs via its catalytic activity in gene silencing processes guided by siRNAs or miRNAs. A RIP assay was therefore conducted with LN229 cells and an anti-AGO2 antibody, followed by RT-qPCR analysis. As demonstrated in Fig. 4F, compared with the NC (anti-IgG), FLVCR1-AS1 and miR-30b-3p were both preferentially increased in AGO2 antibody-incubated group. In addition, the miR-30b-3p inhibitor suppressed the interaction between AGO2 and FLVCR1-AS1 in LN229 cells (Fig. 4G). The results indicated that FLVCR1-AS1 and miR-30b-3p reciprocally repressed one another in GBM cells.

miR-30b-3p inhibits the effect of FLVCR1-AS1 in GBM cells. The expression level of miR-30b-3p in the 50 GBM and adjacent normal brain samples was determined by RT-qPCR. A significant decrease in miR-30b-3p expression was observed in GBM tissues compared with adjacent normal tissues (P<0.001; Fig. 4H). Furthermore, results from Pearson's correlation analysis revealed that FLVCR1-AS1 expression was negatively correlated
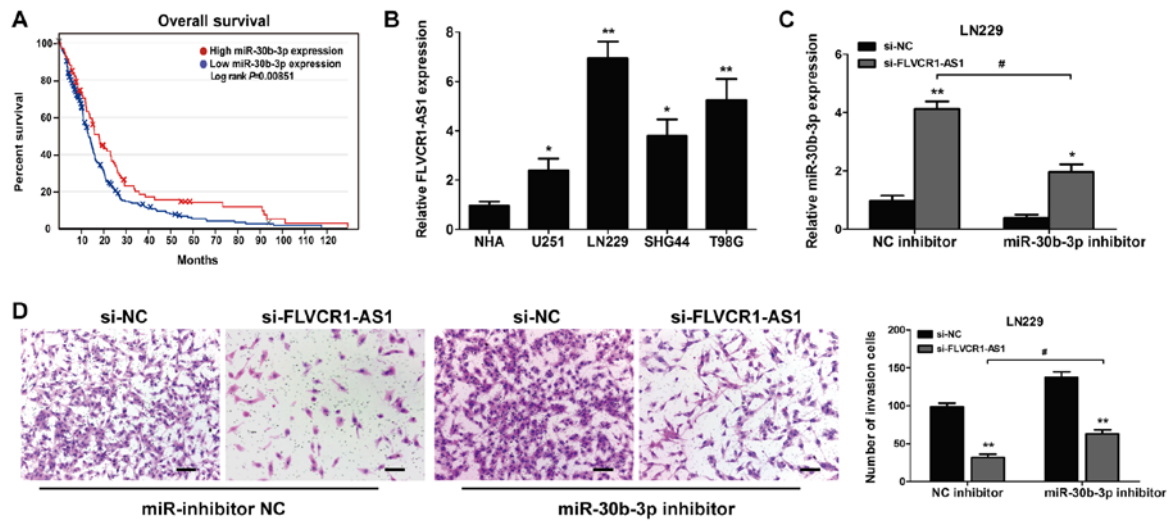
Figure 5. miR-30b-3p suppressed the effect of FLVCR1-AS1 in GBM cells. (A) Kaplan-Meier survival analysis of patient data from The Cancer Genome Atlas database with high or low levels of miR-30b-3p (n=151). (B) Expression level of miR-30b-3p in GBM cells compared with NHA cells assessed by reverse transcription-quantitative PCR. (C) Expression level of miR-30b-3p determined in LN229 cells co-transfected with si-FLVCR1-AS1 + miR-30b-3p inhibitor, and with si-FLVCR1-AS1 + miR-inhibitor NC (*P<0.05 vs. si-NC); and si-FLVCR1-AS1 + miR-30b-3p inhibitor compared with si-FLVCR1-AS1 + miR-inhibitor NC (#P<0.05 vs. si-NC). (D) Invasive ability assessed using Transwell assay (si-FLVCR1-AS1 or si-NC) + the miR-30b-3p inhibitor; and (si-FLVCR1-AS1 or si-NC) + miR-inhibitor NC; and in si-FLVCR1-AS1 + miR-30b-3p inhibitor compared with si-FLVCR1-AS1 + miR-inhibitor NC. *P<0.05, **P<0.01 and #P<0.05. Experiment was repeated three times. miR, microRNA; FLVCR1-AS1, Feline Leukemia Virus Subgroup C Cellular Receptor 1 Antisense RNA 1; GBM, glioblastoma; NHA, Normal Human Astrocyte; NC, negative control; si, small interfering RNA.

with miR-30b-3p expression in GBM tissues (n=50; r=-0.4281; P=0.0019; Fig. 4I). These data suggested that FLVCR1-AS1 may interact with miR-30b-3p in GBM cells. Furthermore, the results from Kaplan-Meier analysis demonstrated that miR-30b-3p expression was associated with the overall survival of patients with glioma from the GEPIA database (Fig. 5A). Furthermore, miR-30b-3p expression level in GBM cells was significantly downregulated compared with that in NHA cells (Fig. 5B). miR-30b-3p expression level was increased following transfection with si-FLVCR1-AS1, compared with si-NC. However, following treatment with miR-30b-3p inhibitor, miR-30b-3p level was decreased in the si-FLVCR1-AS1-transfected group compared with the miR-inhibitor NC group (Fig. 5C). miR-30b-3p inhibitor also attenuated the decrease in cell invasive ability induced by FLVCR1-AS1-knockdown (Fig. 5D). These findings confirmed that miR-30b-3p may be considered as a crucial mediator of FLVCR1-AS1-regulated proliferation and invasive ability in GBM.

Discussion

GBM is one of the most common tumors of the central nervous system, with a high mortality rate (1). Although great progress has been made, there is no efficient therapy for GBM. It has been reported that lncRNAs are key regulators in the progression and development of various types of cancer type. lncRNAs can act as potential tumor oncogenes or suppressors, and altered lncRNA expression has been associated with tumorigenesis (27-30).

The mechanism by which FLVCR1-AS1 functions as an oncogene in GBM remains unknown. In the present study, FLVCR1-AS1 expression was significantly increased in GBM tissues compared with adjacent normal tissues. Furthermore, miR-30b-3p expression was downregulated in GBM tissues,

and miR-30b-3p expression was negatively correlated with FLVCR1-AS1 expression. Results from bioinformatics analysis was used to validate miR-30b-3p as a potential target of FLVCR1-AS1. In addition, FLVCR1-AS1-knockdown inhibited GBM cell proliferation and invasive ability, which was reversed by miR-30b-3p inhibitor. Taken together, these findings suggested that FLVCR1-AS1-knockdown may inhibit GBM progression, indicating a potential therapeutic method for GBM.

Numerous studies reported that the interaction between lncRNA and miRNA regulates gene expression during tumorigenesis (31-34). A previous study illustrated that lncRNA maternally expressed 3 was downregulated in cervical cancer, and altered cell proliferation and apoptosis by regulating miR-21 (35). Similarly, the lncRNA H19 was demonstrated to act as a competing endogenous RNA (ceRNA) for miR-29b-3p, which may promote epithelial-mesenchymal transition and bladder cancer metastasis (36). In non-small cell lung cancer, the lncRNA small nucleolar RNA host gene 1 was reported to regulate cell proliferation and invasive ability by increasing the expression of metadherin via an interaction with miR-145-5p (37).

It has been reported that aberrant expression of miRNAs serves a vital role in the biological processes of various types of cancer (38). For example, Kumar *et al* (39) reported that miR-30b-3p acts as a direct regulator of androgen receptor signaling in prostate cancer. It was also reported that FLVCR1-AS1 may interact with miR-513, miR-573 and the Wnt/β-catenin signaling pathway in hepatocellular carcinoma, lung cancer and ovarian cancer (14-17). Furthermore, Liu *et al* (40) demonstrated that FLVCR1-AS1 can act as a sponge for miR-155, promoting therefore gastric cancer tumorigenesis by targeting c-Myc. As such, FLVCR1-AS1 may be used as a ceRNA to indirectly regulate the proliferation,

migration and invasive ability of cholangiocarcinoma cells (41). Yan *et al* (42) reported that FLVCR1-AS1 aggravates the biological behaviors of glioma cells via targeting the miR-4731-5p/E2F2 axis. Bioinformatics analysis identified FLVCR1-AS1 as a sponge for miR-4731-5p, which resulted in E2F2 upregulation. Rescue assays indicated that FLVCR1-AS1 can modulate the expression of E2F2 during glioma progression. This study demonstrated that FLVCR1-AS1 may be considered as a potential target for tumor therapy. lncRNAs can function as ceRNAs, sequestering miRNAs and subsequently preventing their expression. The present study aimed therefore to investigate whether FLVCR1-AS1 could act as a ceRNA for miR-30b-3p in GBM.

In the present study, the putative binding site between FLVCR1-AS1 and miR-30b-3p was confirmed by using a luciferase reporter assay system. The results validated FLVCR1-AS1 as a potential tumor suppressor regulating miR-30b-3p and subsequently inhibiting GBM cell proliferation and invasive ability. The suppressive effect of si-FLVCR1-AS1 was impeded by co-transfection with miR-30b-3p inhibitor. Taken together, the results from the present study described the role and underlying mechanism of FLVCR1-AS1 in the proliferation and invasive ability of GBM cells, which may provide some essential information on its role in GBM tumorigenesis.

In conclusion, the present study demonstrated that FLVCR1-AS1 may act as an oncogenic lncRNA that could promote the development and progression of GBM through miR-30b-3p, suggesting that FLVCR1-AS1 may be considered as a potential biomarker and therapeutic target for GBM.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WG designed the study, performed experiments, analyzed the data and wrote the manuscript. HL and YL performed the *in vitro* experiments. YZ and FL analyzed the data and drafted the manuscript. FL and HZ designed and supervised the study and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients and the study was approved by the Ethics Committee of Harbin Medical University (Harbin, China). All procedures were performed in accordance with national (D.L.n.26, March 4th, 2014) and international laws and policies (directive 2010/63/EU).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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