Downregulation of lncRNA SATB2-AS1 facilitates glioma cell proliferation by sponging miR-671-5p

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Abstract. The antisense transcript of SATB2 protein (SATB2-AS1) is a novel long non-coding RNA (lncRNA) which is involved in the development of colorectal cancer, breast cancer and hepatocellular carcinoma. In the present study, it was aimed to investigate the consequent situation of SATB2-AS1 in tissue and cell lines of glioma. The expression of SATB2-AS1 in glioma cases was analyzed in The Cancer Genome Atlas datasets. The glycolytic metabolism was determined in glioma cells by detection of extracellular glucose level, oxygen consumption rate and extracellular acidification rate. Cell Counting Kit-8 assay and flow cytometry were used to assess cell proliferation and apoptosis in glioma cells. The interaction between SATB2-AS1 and microRNA (miR)-671-5p was verified by bioinformatic analysis, reverse transcription-quantitative PCR, dual luciferase reporter assay and RNA immunoprecipitation assay. The expression levels of the downstream targets of SATB2-AS1 were studied by western blotting. Results demonstrated that SATB2-AS1 was a downregulated lncRNA in low grade glioma and glioblastoma. Gain-of-function assay demonstrated that SATB2-AS1 inhibited cell proliferation, and glycolytic metabolism, while induced cell apoptosis in glioma cells. SATB2-AS1 sponged and suppressed the expression of an oncogenic miRNA miR-671-5p. By regulation of miR-671-5p, SATB2-AS1 upregulated cerebellar degeneration related protein 1 (CDR1) and Visinin-like 1 (VSNL1) expression in glioma cells. miR-671-5p overexpression partially reversed the antitumor effect of SATB2-AS1 in glioma. In conclusion, the current study demonstrated that there was a downregulation of SATB2-AS1 in glioma, and SATB2-AS1 regulated miR-671-5p/CDR1 axis and miR-671-5p/VSNL1 axis in glioma.

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Introduction

Glioma is the most prevalent, lethal and highly aggressive primary brain tumor with a poor prognosis (1). Pathologically, glioma can be divided into 4 grades with the characteristics of increased aggressive nature and decreased 5-year overall survival (OS) rate (2). For example, glioblastoma (GBM) is the grade IV glioma with the lowest OS (5-year OS rate <5%) (3). Known as Warburg effect, tumor cells mainly rely on glycolysis to utilize glucose and facilitate malignant cell proliferation (4). At present, treatment against glycolysis is a promising strategy for patients with glioma (5). Therefore, further study is needed towards improved understanding of the molecular mechanism underlying glycolysis of glioma.

Long non-coding RNAs (lncRNAs) are single-stranded transcripts longer than 200 nucleotides with no protein coding potential (6). Previously, accumulating studies have reported that numerous lncRNAs were differentially expressed in glioma compared with normal brains; in addition, lncRNAs have been reported to function as tumor suppressors or oncogenes during the progression of glioma (7-9). According to analysis of The Cancer Genome Atlas (TCGA)-GBM dataset, >300 lncRNAs were aberrantly expressed between GBM and normal brain (10); a large proportion of these lncRNAs acted as competing endogenous RNAs (ceRNAs) by sponging microRNAs (miRNA or miR) to regulate the signaling network in glioma (11-13). For instance, lncRNA PVT1 promoted glioma progression by sponging tumor suppressor miR-128-3p (14). In 2017, SATB2-AS1 was identified as a novel antisense lncRNA with 3197 nucleotides residing on chromosome 2 (15). Subsequently, the tumor suppressive role of SATB2-AS1 has been reported in three types of cancers, including colorectal cancer (16), breast cancer (17), and hepatocellular carcinoma (18). However, the expression and function of SATB2-AS1 in glioma has not been revealed yet.

In the present study, SATB2-AS1 was identified as a downregulated lncRNA in gliomas, especially in GBM. The biological role of SATB2-AS1 was examined in two glioma cell lines by the gain-of-function assay. The present study suggested that SATB2-AS1 regulated the miR-671-5p/CDR1 axis and miR-671-5p/VSNL1 axis in glioma. Taken together, the data demonstrated that SATB2-AS1 is a novel tumor suppressor in glioma.

Materials and methods

Patients. All glioma tissues (n=45) were collected from patients (35 men and 10 women) that underwent surgical resection at the Affiliated Hospital of Xuzhou Medical University (Xuzhou, China) during June 2019 to July 2021. Tissues were clinicopathologically confirmed as glioma (WHO I/II: 14 cases, WHO III/IV: 31 cases). These patients did not receive chemotherapy or radiotherapy before the surgery.

The 12 normal brain tissues were obtained from patients (8 men and 4 women) with non-glioma diseases that underwent treatment at the Affiliated Hospital of Xuzhou Medical University June 2019 to July 2021.

These samples were immediately stored at -80˚C before experiments. All patients provided a written, signed and dated informed consent form. The protocol was reviewed and approved by the Ethics Committee of The Affiliated Hospital of Xuzhou Medical University (Xuzhou, China; approval no. 20190508003). Written informed consent was obtained from all patients. The detailed clinical characteristic information of patients is presented in Table I.

Cell culture. Glioma cell lines SHG44 [WHO II/III astrocytoma cell line (19)], U251MG [WHO III/IV GBM cell line (20)], A172 [WHO III/IV GBM cell line (20)] and normal human astrocytes (NHA) were purchased from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's Medium containing 10% FBS (both from Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator with 5% CO_2.

Cell transfection. Full length of SATB2-AS1 was synthesized by GenScript (Nanjing) Co., Ltd. and inserted into pcDNA3.1 expression vector. Overexpression of SATB2-AS1 was achieved by transfection of 2 μg pcDNA3.1-SATB2-AS1 into cells in six-well plates by Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C following the manufacturer's protocol.

miR-671-5p mimic (5'-ACUCUUUCCCUGUUGCAC UAC-3') and miR-negative control (NC, 5'-CUGAACUGC UAGGACGCGUA-3') were synthesized and purchased from Suzhou GenePharma Co., Ltd. In brief, 100 nM miR-671-5p mimic or miR-NC was transfected into cells in six-well plates using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C following the manufacturer's protocol. The transfection efficiency was determined at 48 h after transfection by reverse transcription-quantitative (RT-q) PCR.

Cell proliferation assay. Cells (1x10^5/well) were seeded and incubated in 96-well plates, at the time point of 0, 1, 2 and 3 days, the Cell Counting Kit-8 solution (10 μl; Dojindo Laboratories, Inc.) was added into each well and maintained for 2 h at 37˚C. After that, the absorbance from each well was measured at a wavelength of 450 nm by a Microplate Reader (Bio-Rad Laboratories, Inc.).

Determination of extracellular glucose levels and extracellular acidification rate (ECAR). Lactate levels were measured by the Lactate Colorimetric/Fluorometric Assay kit (BioVision, Inc.) following the protocol of the manufacturer. The ECAR was detected by the XFp Extracellular Flux Analyzer (Seahorse Bioscience) with a Seahorse XFp Glycolysis Stress Test kit (Agilent Technologies, Inc.). Briefly, cells (1x10^5/well) were seeded in a Seahorse XF 96 cell culture microplate, thereafter, sequentially added with glucose (1 μM), oligomycin (1 μM) and 2-DG (500 mM). The data of each timepoint was acquired and calculated with Seahorse XF-96 Wave software.

Cell apoptosis assay. Flow cytometric analysis was used to detect percentage of apoptotic cells in each group. In brief, cells were cultured for 2 days, then harvested and suspended in Annexin V binding buffer provided by the Dead Cell Apoptosis kit with Annexin V Alexa Fluor™ 488 & Propidium Iodide (PI) kit (Invitrogen; Thermo Fisher Scientific, Inc.). Thereafter, cells were stained with Annexin V and PI. Finally, cells were subjected to flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences). The results were analyzed by FlowJo software (Tree Star, Inc.). Annexin V positive cells with or without PI staining were regarded as apoptotic cells.

RNA extraction and RT-qPCR. Total RNA was extracted from cells and tissues by the RNAiso™ Plus reagent (Takara Bio, Inc.) following manufacturer's protocol. Frist stranded cDNA was synthesized from RNA by the PrimeScript™ 1st Strand cDNA Synthesis kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was performed with the TB Green® Premix Ex Taq™ kit (Takara Bio, Inc.) on a CFX-96 Touch PCR system (Bio-Rad Laboratories, Inc.). The thermocycling conditions included initial denaturation at 95˚C for 1 min; followed by 42 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 31 sec and elongation at 72˚C for 30 sec; and a final extension step at 72˚C for 5 min. U6 and β-actin were internal controls for miRNA and mRNA/lncRNA, respectively. Relative gene expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (21). The sequences for qPCR forward and reverse primers are listed in Table II.

RNA immunoprecipitation (RIP) assay. After preparation of U251MG lysates with RIPA lysis buffer containing RNaseOUT (100 U/ml; Thermo Fisher Scientific, Inc.), the U251MG lysates (250 μl) were collected by centrifugation at 15,000 x g for 10 min at 4˚C. The lysates (250 μl) were then incubated with streptavidin magnetic beads (245 μl; cat. no. 5947; Cell Signaling Technology, Inc.) conjugated with Ago2 antibody (cat. no. 2897; 1:50; 5 μl; Cell Signaling Technology, Inc.) or rabbit IgG control (cat. no. 3900; 1:50; 5 μl; Cell Signaling Technology, Inc.). RNAs were extracted by RNAiso™ Plus reagent (Takara Bio, Inc.) and studied by RT-qPCR as aforementioned.

Protein extraction and western blotting. Proteins were extracted from cells using the RIPA lysis buffer (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The concentration of lysates was determined by a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Lysates (20 μg) were separated by SDS-PAGE on 10% gels and transferred to PVDF membranes. Subsequently, the PVDF membranes were blocked with 5% BSA (Thermo Fisher Scientific, Inc.) for 1 h at room temperature. After sequential incubation of the membranes in primary antibodies overnight at 4˚C and secondary antibodies

Table I. Clinicopathological features of 45 patients with glioma and the expression of SATB2-AS1.

Clinicopathological features	Number of cases	Expression level of SATB2-AS1		P-value
		Low (n=23)	High (n=22)	
Sex				0.2837
Male	35	16	19	
Female	10	7	3	
Age, years				0.2078
<30	14	5	9	
≥30	31	18	13	
Tumor size (mm)				0.007
<30	25	8	17	
≥30	20	15	5	
Pathological stage				0.047
WHO I-II	12	3	9	
WHO III-IV	33	20	13	

SATB2-AS1, antisense transcript of SATB2 protein.

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

Gene name	Primer sequence (5'-3')
SATB2-AS1	F: ATCAAGGCCTCTTGAAAGAGA
SATB2-AS1	R: TCTCTTTCAAGAGGCCTTGAT
U6	F: CTCGCTTCGGCAGCACATATAC
U6	R: GGAACGCTTCACGAATTTGC
GAPDH	F: TGCACCACCAACTGCTTAGC
GAPDH	R: GGCATGGACTGTGGTCATGAG
miR-671-5p	F: AGGAAGCCCTGGAGGGGCTGGAG
miR-671-5p	F: CTCCTGCCCCTCCAGGGCTTCCT
miR-190a-3p	R: CTATATATCAAACATATTCCT
miR-190a-3p	F: AGGAATATGTTTGATATATAG
miR-130b-5p	F: ACTCTTTCCCTGTTGCACTAC
miR-130b-5p	R: GTAGTGCAACAGGGAAAGAGT
CDR1	F: TGCTGGAAGACTTGATTTACTGG
CDR1	R: CCAGTAAATCAAGTCTTCCAGCA
VSNL1	F: GTTTGAATTTTCAAAGGCTTCCA
VSNL1	R: TGGAAGCCTTTGAAAATTCAAAC

F, forward; R, reverse; SATB2-AS1, antisense transcript of SATB2 protein; miR, microRNA; CDR1, cerebellar degeneration related protein 1; VSNL1, Visinin-like 1.

at room temperature for 2 h, the blots were developed by an ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.). CDR1 (cat. no. AB45874; 1:2,000) and β-actin (cat. no. AB21800; 1:2,000) antibodies were purchased from AbSci. VSNL1 (cat. no. ab180141; 1:2,000) antibody was a product of Abcam. Secondary antibodies [goat anti-rabbit IgG H&L (HRP; cat. no. ab7090; 1:5,000) and goat anti-mouse IgG H&L (HRP; cat. no. ab97040; 1:5,000) were obtained from Abcam. ImageJ (version 1.8.0; National Institutes of Health) was used for the analysis of densitometry.

Bioinformatics analysis. The expression of SATB2-AS1 in low grade gliomas (LGG), GBM and normal brains were retrieved from TCGA-LGG, TCGA-GBM and The Genotype-Tissue Expression (GTEx) projects using GEPIA software (http://gepia.cancer-pku.cn/). The software was also used to study the association between SATB2-AS1 and CDR1, or VSNL1 expression in expression data of TCGA-LGG and TCGA-GBM projects. The potential target miRNAs of SATB2-AS1 were predicted by miRDB software (http://mirdb.org/).

Nucleocytoplasmic separation. Cells were harvested and resuspended in RLN buffer (50 mM Tris-HCl pH 7.4, 0.14 M NaCl, 1.5 mM $MgCl_2$, 0.5% IGEPAL CA-630, 1 mM DTT) and incubated on ice for 10 min. After homogenization, the supernatant was used as cytoplasmic fraction. The pellet was resuspended in RSB buffer (0.25 M sucrose, 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF). The solution was subjected to homogenization and centrifugation at 15,000 x g for 10 min at 4˚C. Then, the nuclear fraction was resuspended in 2M RSB buffer (2 M sucrose, 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF). RNA was extracted from cytoplasmic fraction and nuclear fraction using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

Dual luciferase reporter assay. SATB2-AS1-wild type (WT) or SATB2-AS1-mutant (M, two mutations in the putative miRNA responsive element) was inserted into pmirGLO vector (Promega Corporation). Cells were transfected with pmirGLO-SATB2-AS1-WT or pmirGLO-SATB2-AS1-M in combination with miR-NC or miR-671-5p mimic using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following manufacturer's protocol. After 2 days, the relative luciferase activity of each group was measured

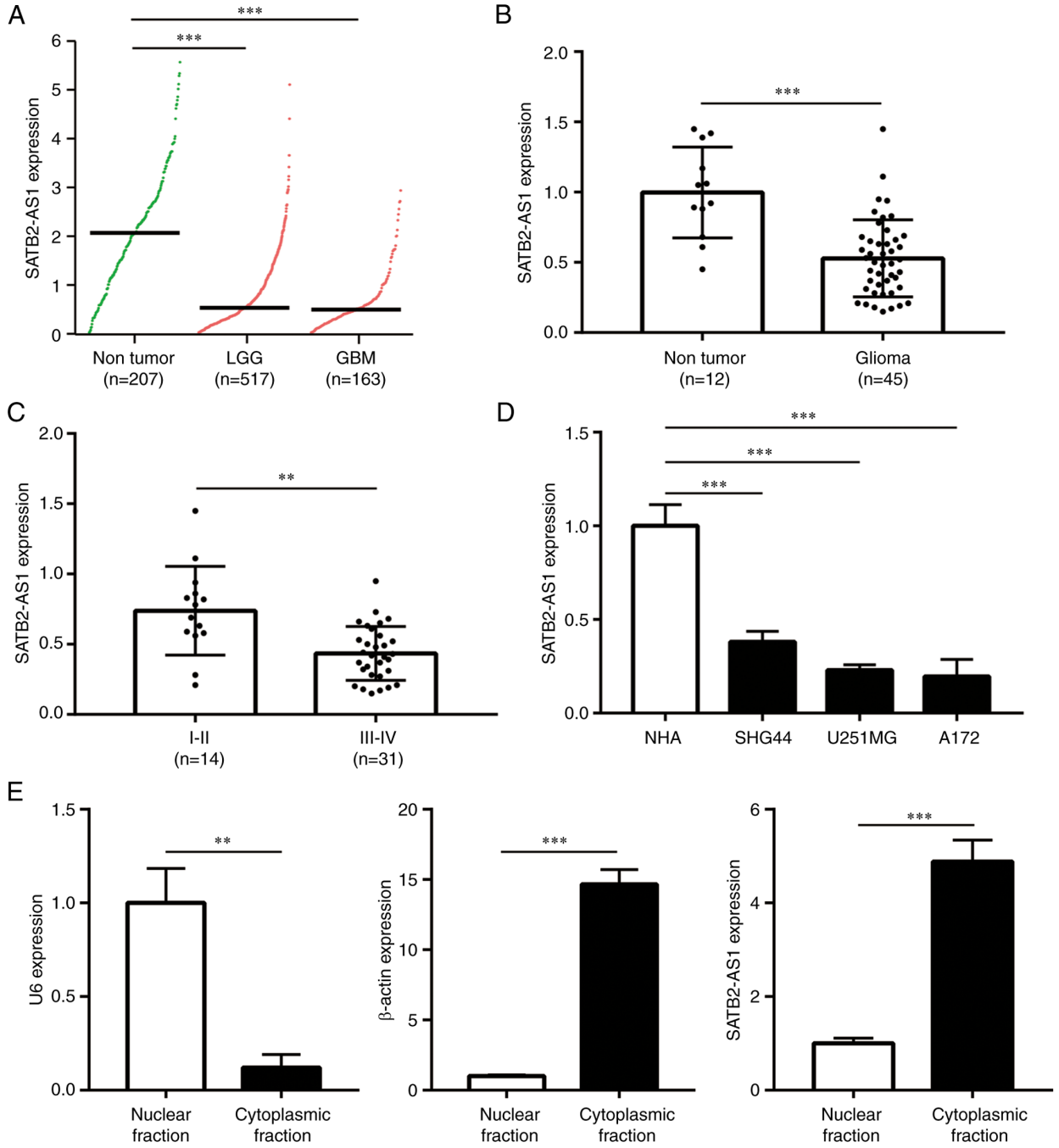

Figure 1. SATB2-AS1 is a downregulated lncRNA in glioma. (A) By retrieving expression data from TCGA-LGG, TCGA-GBM and GTEx, SATB2-AS1 expression was compared in normal brains (n=207), LGG (n=517) and GBM (n=163). (B) RT-qPCR analysis on the difference of STAB2-AS1 expression between 12 normal brains and 45 gliomas. (C) RT-qPCR analysis on the difference of STAB2-AS1 expression between LGG (I/II, n=14) and high-grade glioma (III/IV, n=31). (D) RT-qPCR analysis on the difference of SATB2-AS1 expression among NHA and glioma cell lines (SHG44, U251MG and A172). (E) U6, β-actin and SATB2-AS1 expression levels were detected in cytoplasmic and nuclear fractions of A172 cells by RT-qPCR. **P<0.01 vs. I/II stage or nuclear fraction; ***P<0.001 vs. non-tumor, NHA, or nuclear fraction. SATB2-AS1, antisense transcript of SATB2 protein; TCGA, The Cancer Genome Atlas; LGG, low-grade glioma; GBM, glioblastoma; RT-qPCR, reverse transcription-quantitative PCR; NHA, normal human astrocytes.

using the Dual Luciferase Reporter Assay System (Promega Corporation) with *Renilla* luciferase activity as the control.

Statistical analysis. Data were analyzed with GraphPad Prism 6.0 (Dotmatics) and presented as the mean ± standard deviation (SD). All experiments were repeated three times. P<0.05 was considered to indicate a statistically significant difference. Pearson's correlation analysis was performed to study the association between SATB2-AS1 and miR-671-5p in tumor samples. Two groups were compared by unpaired Student's t-test, whereas multiple groups were compared using one-way ANOVA followed by Tukey's post hoc test.

Results

SATB2-AS1 is downregulated in glioma, especially in high grade glioma. To investigate the relevance of SATB2-AS1 in glioma progression, SATB2-AS1 expression data in LGG

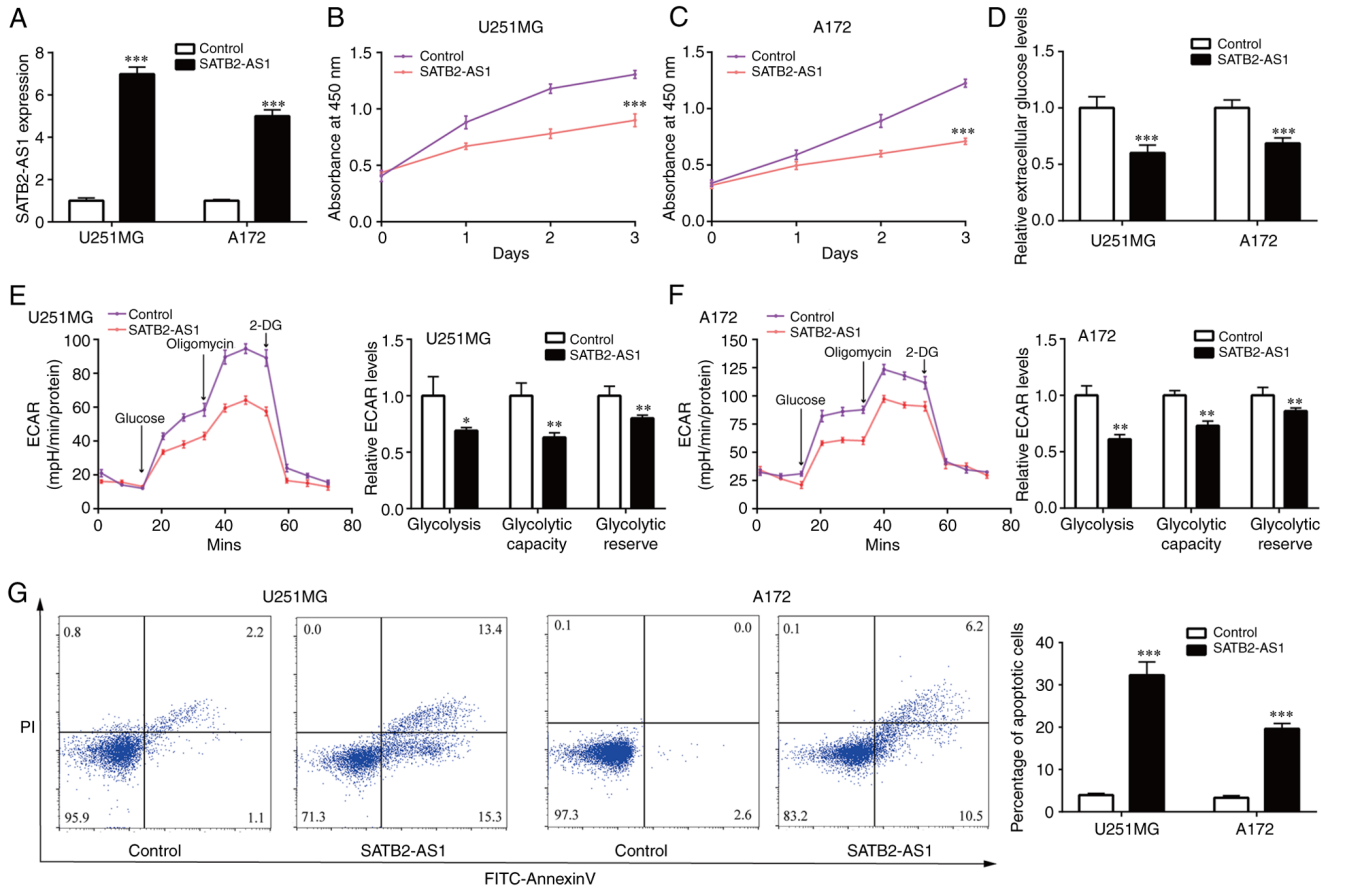

Figure 2. SATB2-AS1 inhibits glioma cell proliferation and glycolysis, while it promotes cell apoptosis. pcDNA3.1 (control group) or pcDNA3.1-SATB2-AS1 (SATB2-AS1 group) were transfection into U251MG and A172 cells. (A) SATB2-AS1 expression was determined by reverse transcription-quantitative PCR. (B and C) Cell proliferation rates were detected by Cell Counting Kit-8 at day 0, 1, 2 and 3 days, respectively. (D) Colorimetric/fluorometric assay was performed to detect extracellular glucose levels. (E and F) ECAR was detected. (G) Cell apoptosis was detected by flow cytometric analysis. *P<0.05, **P<0.01 and ***P<0.001 vs. control. SATB2-AS1, antisense transcript of SATB2 protein; ECAR, extracellular acidification rate.

and GBM were retrieved from TCGA-LGG and TCGA-GBM projects, and normal brains from GTEx project. SATB2-AS1 exerted low expression in glioma compared with normal brains; specifically, SATB2-AS1 was significantly down-regulated in GBM compared with LGG (Fig. 1A), indicating that SATB2-AS1 was associated with the grade of glioma. In the current study, 45 glioma tissues and 12 healthy brain tissues were collected from patients during surgery and were subjected to RT-qPCR for the detection of SATB2-AS1 expression. Consistently, lower expression of SATB2-AS1 transcript was observed in glioma compared with non-tumor brain tissues (Fig. 1B). In addition, SATB2-AS1 was decreased in high grade (III-IV) glioma compared with that in low grade (I-II) glioma (Fig. 1C). Subsequently, the association between SATB2-AS1 and clinicopathological factors of patients were examined, exerting that SATB2-AS1 was associated with tumor size and pathological grade but not age or gender (Table I). Thereafter, SATB2-AS1 expression in a panel of glioma cell lines (SHG44, U251MG and A172) and NHA was detected, exerting that SATB2-AS1 was decreased in all glioma cell lines compared with NHA (Fig. 1D). Furthermore, the cellular distribution of SATB2-AS1 in A172 was evaluated, showing that SATB2-AS1 was mainly localized in the cytoplasmic fraction compared with nuclear fraction (80 vs. 20%, Fig. 1E).

SATB2-AS1 inhibits cell proliferation, glycolysis and cell apoptosis of glioma. Afterwards, the gain-of-function studies were performed by pcDNA3.1 vector containing SATB2-AS1. In the two cell lines (U251MG and A172) with relatively lower expression of SATB2-AS1, pcDNA3.1-SATB2-AS1 induced 5-fold increase of SATB2-AS1 levels (Fig. 2A). Upregulation of SATB2-AS1 significantly suppressed cell proliferation of U251MG and A172 (Fig. 2B and C). Reprogramming of glycolytic metabolism contributed to robust cell growth of glioma cells (22). Extracellular glucose levels in glioma cells were measured after overexpression of SATB2-AS1, exerting that SATB2-AS1 decreased glucose content in U251MG and A172 (Fig. 2D). ECAR assay revealed that SATB2-AS1 overexpression inhibited glycolysis, glycolytic capacity and glycolytic reserve in U251MG and A172 (Fig. 2E and F). Flow cytometric analysis demonstrated that SATB2-AS1 overexpression induced cell apoptosis in U251MG and A172 (Fig. 2G). Collectively, overexpression of SATB2-AS1 inhibited glioma cell proliferation and glycolysis.

SATB2-AS1 sponges oncogenic miR-671-5p in glioma. The cytoplasmic localization of SATB2-AS1 suggested that SATB2-AS1 may exert its function via sponging miRNAs, next, miRNAs that may interact with SATB2-AS1 were predicted using miRDB. There are 62 potential targets of SATB2-AS1

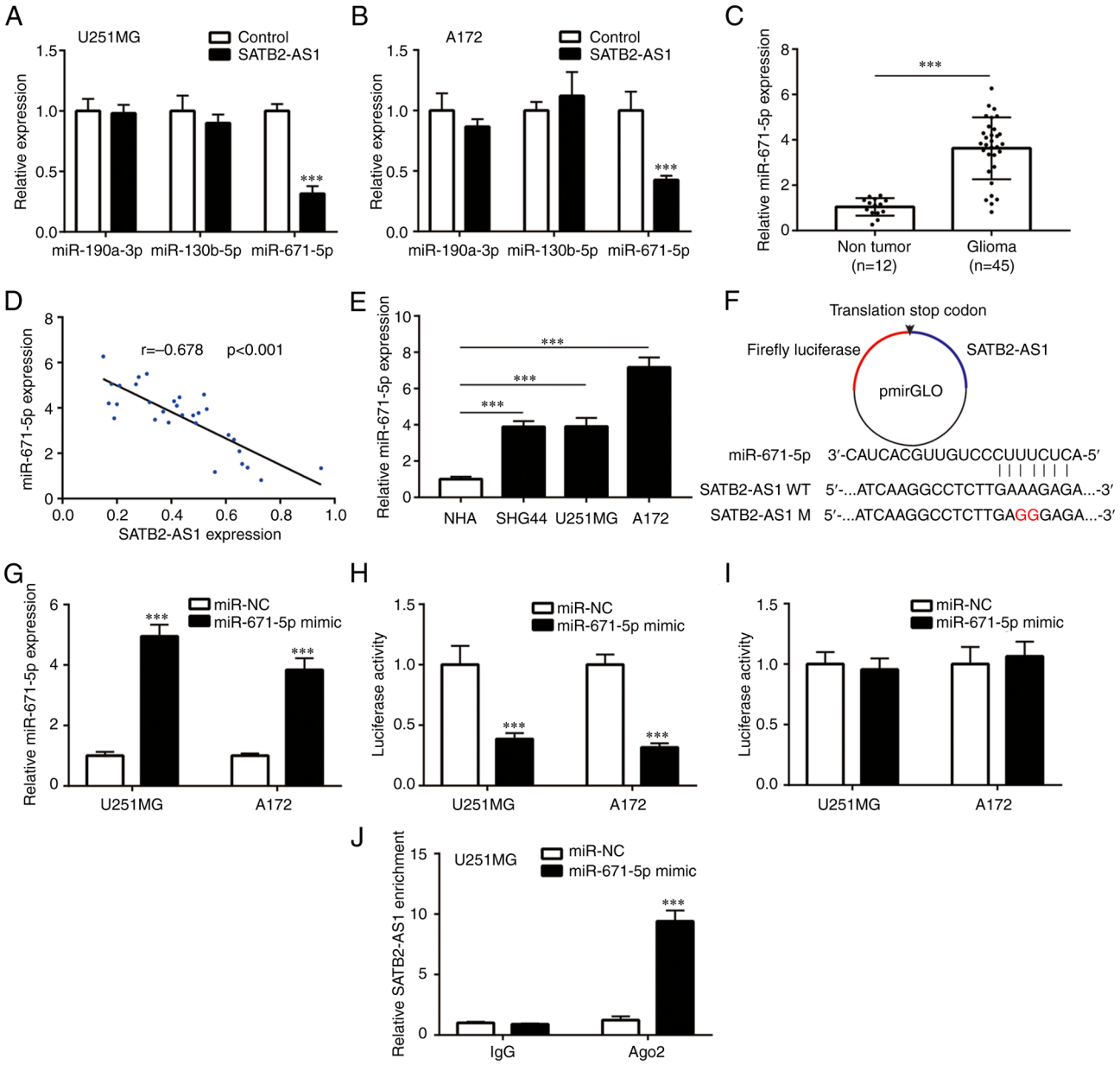

Figure 3. SATB2-AS1 sponges and suppresses miR-671-5p in glioma cells. (A and B) After transfection of pcDNA3.1 or pcDNA3.1-SATB2-AS1, the expression levels of miR-190a-3p, miR-130b-5p and miR-671-5p were detected in U251MG and A172 by RT-qPCR. (C) RT-qPCR analysis on the difference of miR-671-5p expression between 12 normal brains and 45 gliomas. (D) Pearson's correlation analysis was applied to study the association between SATB2-AS1 and miR-671-5p in 45 gliomas. (E) RT-qPCR detection on the difference of miR-671-5p expression among NHA and glioma cell lines (SHG44, U251MG and A172). (F) SATB2-AS1 WT or SATB2-AS1 M was inserted into pmirGLO. (G) After transfection of miR-671-5p mimic or miR-NC in U251MG and A172, miR-671-5p expression was detected by RT-qPCR. (H and I) Relative luciferase activity was determined by dual luciferase reporter assay. (J) RNA immunoprecipitation assay was performed in U251MG with transfection of miR-NC or miR-671-5p mimic, to detect the relative enrichment of SATB2-AS1 by RT-qPCR. ***P<0.001 vs. control, non-tumor, NHA, or miR-NC. SATB2-AS1, antisense transcript of SATB2 protein; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; NHA, normal human astrocytes; WT, wild-type; M, mutant; NC, negative control.

(Table SI). Among these miRNAs, miR-190a-3p, miR-130b-5p and miR-671-5p are well-known oncogenic miRNAs in glioma (23-25), and their expression levels in glioma cells were detected with overexpression of SATB2-AS1. Among them, only miR-671-5p level was significantly decreased by SATB2-AS1 in U251MG and A172 cells (Fig. 3A and B). In the collected samples of the current study, miR-671-5p was elevated in glioma tissues compared with normal brains (Fig. 3C). Pearson's correlation analysis demonstrated that miR-671-5p was negatively correlated with SATB2-AS1 expression in the collected glioma tissues (r=-0.678, P<0.001) (Fig. 3D). Moreover, miR-671-5p was increased in glioma cell lines (SHG44, U251MG and A172) compared with NHA (Fig. 3E).

Next, SATB2-AS1 (WT) and SATB2-AS1 (M) were inserted into luciferase vector pmirGLO (Fig. 3F). miR-671-5p mimic was transfected into U251MG and A172 cells to elevate miR-671-5p expression (Fig. 3G). As expected, miR-671-5p mimic suppressed luciferase activity of pmirGLO-SATB-AS1-WT in U251MG and A172 cells (Fig. 3H). By contrast, miR-671-5p mimic did not affect the luciferase activity of pmirGLO-SATB2-AS1-M in U251MG

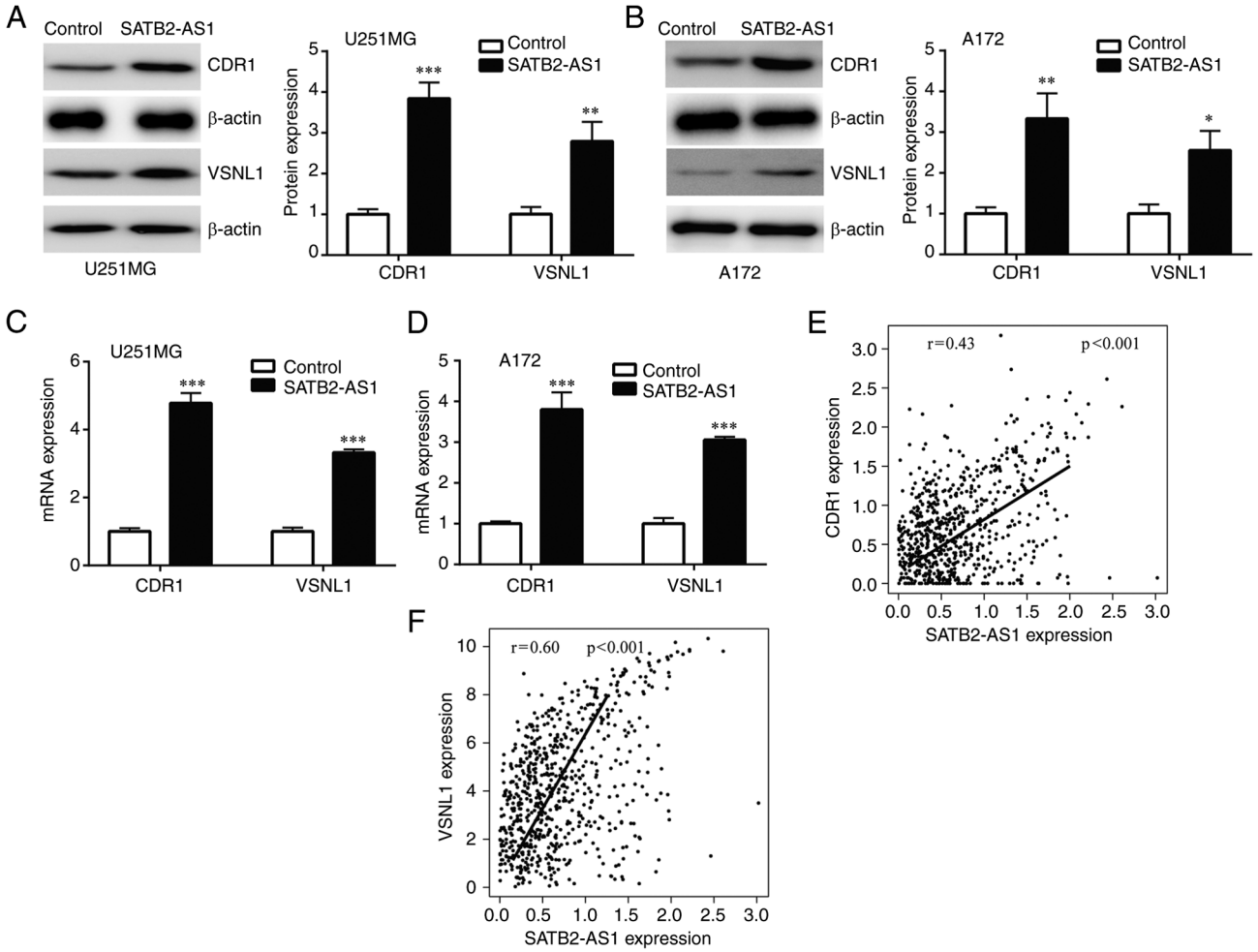

Figure 4. SATB2-AS1 promotes CDR1 and VSNL1 expression in glioma cells. (A-D) Western blotting and reverse transcription-quantitative PCR were performed to detect CDR1 and VSNL1 (A and B) protein expression and (C and D) mRNA expression in U251MG and A172 cells with transfection of pcDNA3.1 or pcDNA3.1-SATB2-AS1. (E and F) By retrieving expression data from TCGA-low grade glioma and TCGA-glioblastoma, the association between SATB2-AS1 and CDR1 or VSNL1 was studied using the Pearson's correlation analysis. *P<0.05, **P<0.01 and ***P<0.001 vs. control. SATB2-AS1, antisense transcript of SATB2 protein; CDR1, cerebellar degeneration related protein 1; VSNL1, Visinin-like 1; TCGA, The Cancer Genome Atlas.

or A172 cells (Fig. 3I). For further validation, RIP assay was conducted in U251MG, demonstrating that anti-Ago2 antibody could enrich significantly more SATB2-AS1 in U251MG cells transfected with miR-671-5p mimic compared with miR-NC (Fig. 3J), indicating their direct interaction in Ago2 complex.

SATB2-AS1 regulates the miR-671-5p/CDR1 axis and miR-671-5p/VSNL1 axis in glioma. miR-671-5p plays a pivotal role in progression of glioma by suppression of CDR1 and VSNL1 (24). Subsequently, CDR1 and VSNL1 expression levels were evaluated in glioma cells with transfection of SATB2-AS1. It was found that SATB2-AS1 overexpression increased CDR1 and VSNL1 protein expression in U251MG and A172 cells (Fig. 4A and B). In addition, SATB2-AS1 elevated CDR1 and VSNL1 mRNA levels in U251MG and A172 cells (Fig. 4C and D). To examine the association between SATB2-AS1 and miR-671-5p/CDR1axis and miR-671-5p/VSNL1 axis in clinical setting, expression levels of SATB2-AS1, CDR1 and VSNL1 in LGG and GBM were retrieved from TCGA-LGG and TCGA-GBM projects to analyze their association. Pearson's correlation analysis showed that SATB2-AS1 was significantly positively correlated with CDR1 (r=0.43, P<0.001) and VSNL1 (r=0.60, P<0.001) expression (Fig. 4E and F).

SATB2-AS1 regulates CDR1 and VSNL1 expression by sponging miR-671-5p. Next, the involvement of miR-671-5p in the function of SATB2-AS1 was further studied. Western blotting revealed that SATB2-AS1 overexpression induced upregulation of CDR1 and VSNL1 protein expression, which was partially reversed by miR-671-5p mimic in both U251MG and A172 cells (Fig. 5A and B). Consistently, SATB2-AS1 overexpression induced elevation of CDR1 and VSNL1 mRNA expression, which was also partially reversed by miR-671-5p mimic in both U251MG and A172 cells (Fig. 5C and D).

SATB2-AS1 regulates cell proliferation, glycolysis and cell apoptosis of glioma by sponging miR-671-5p. The proliferation assay demonstrated that miR-671-5p mimic attenuated the inhibitory effect of SATB2-AS1 on cell proliferation in U251MG and A172 cells (Fig. 6A and B). miR-671-5p mimic also attenuated the inhibitory effect of SATB2-AS1 on glucose content in U251MG and A172 cells (Fig. 6C). In addition,

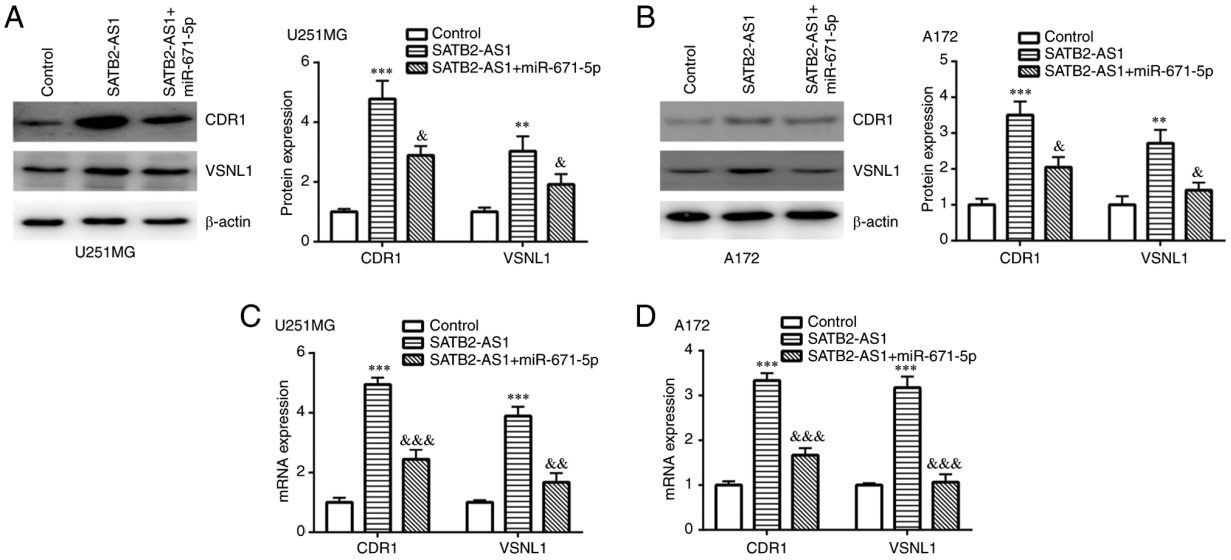

Figure 5. SATB2-AS1 promotes CDR1 and VSNL1 expression by sponging miR-671-5p. (A-D) After transfection of pcDNA3.1 + miR-NC (control group) or pcDNA3.1-SATB2-AS1+ miR-NC (SATB2-AS1 group) or pcDNA3.1-SATB2-AS1 + miR-671-5p mimic (SATB2-AS1 + miR-671-5p group) in U251MG and A172 cells, western blotting and reverse transcription-quantitative PCR were performed to detect CDR1 and VSNL1 (A and B) protein expression and (C and D) mRNA expression in U251MG and A172 cells. **P<0.01 and ***P<0.001, vs. control (pcDNA3.1 + miR-NC); $^{\&}$P<0.05, $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. SATB2-AS1 (pcDNA3.1-SATB2-AS1 + miR-NC). SATB2-AS1, antisense transcript of SATB2 protein; CDR1, cerebellar degeneration related protein 1; VSNL1, Visinin-like 1; miR, microRNA; NC, negative control.

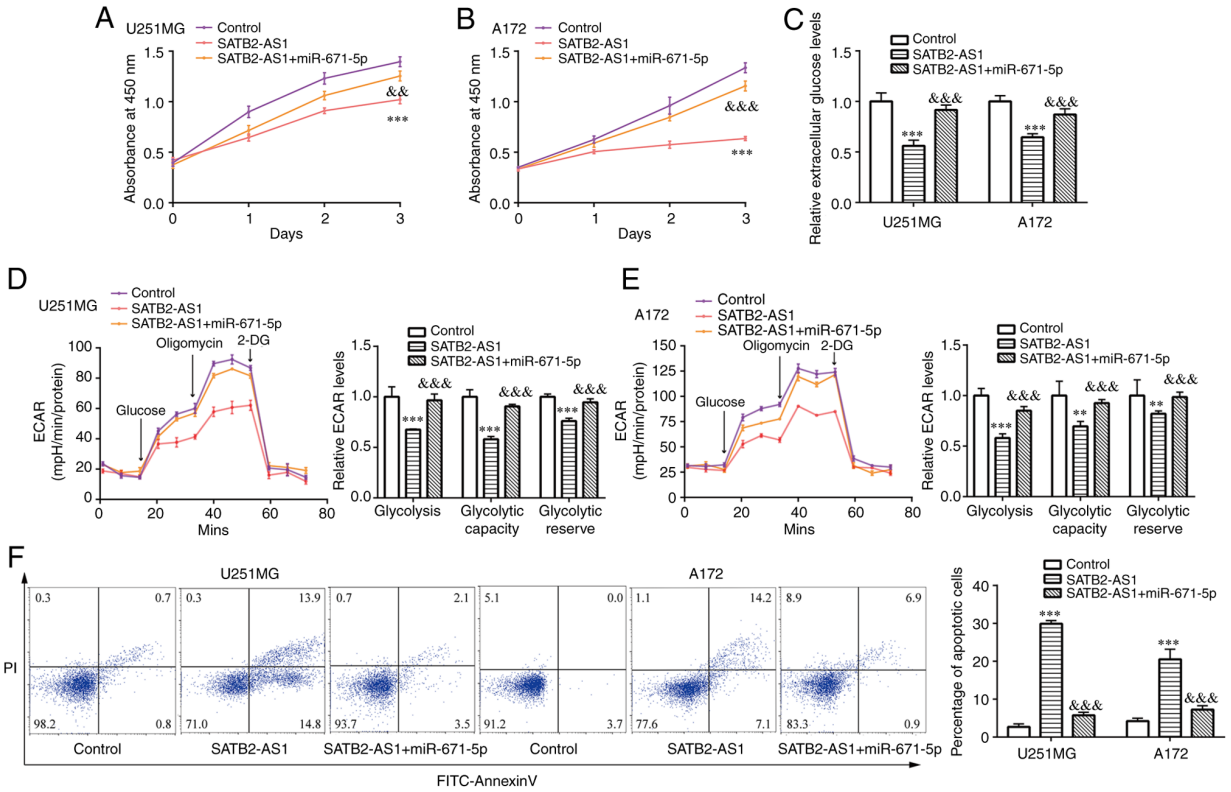

Figure 6. The SATB2-AS1/miR-671-5p axis reduces glioma cell proliferation and glycolysis, while it enhances cell apoptosis. After transfection of pcDNA3.1 + miR-NC (control group) or pcDNA3.1-SATB2-AS1 + miR-NC (SATB2-AS1 group) or pcDNA3.1-SATB2-AS1 + miR-671-5p mimic (SATB2-AS1 + miR-671-5p group) in U251MG and A172 cells, (A and B) cell proliferation rates were detected by the Cell Counting Kit-8 assay at the time point of 0, 1, 2 and 3 days, respectively; (C) colorimetric/fluorometric assay was performed to detect extracellular glucose levels; (D and E) ECAR was detected; (F) flow cytometric analysis was used to detect percentage of apoptotic cells. **P<0.01 and ***P<0.001, vs. control (pcDNA3.1 + miR-NC); $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001, vs. SATB2-AS1 (pcDNA3.1-SATB2-AS1 + miR-NC). SATB2-AS1, antisense transcript of SATB2 protein; miR, microRNA; NC, negative control.

ECAR assay indicated that miR-671-5p mimic reversed the inhibition of glycolysis, glycolytic capacity and glycolytic reserve induced by SATB2-AS1 in U251MG and A172 cells (Fig. 6D and E). Furthermore, SATB2-AS1- induced cell

apoptosis was partially reversed by miR-671-5p mimic in U251MG and A172 cells (Fig. 6F).

Discussion

A large number of lncRNAs have been identified to be aberrantly expressed and could serve as promising biomarkers in glioma (26-28). For instance, an analysis on lncRNA profiling in GBM and normal brains suggested that a survival model consisted of 9 lncRNAs could accurately predict the OS of patients with GBM (29). It is well-characterized that hyperactivation of glycolysis leads to the progression of glioma (30). The dysregulation of glycolysis pathway facilitates glioma cell proliferation and resistance to cell death (31). Previous studies have revealed that several lncRNAs tightly control the glycolytic metabolism in glioma cells by targeting key enzymes (32,33). By investigating TCGA and GTEx data, numerous differentially expressed lncRNAs were identified in LGG and GBM. In the present study, it was found that SATB2-AS1 was a downregulated lncRNA in both LGG and GBM. RT-qPCR confirmed that SATB2-AS1 was decreased in glioma and its expression was associated with tumor size and pathological grade. Regarding the function of SATB2-AS1 in cancers, it has been reported to suppress cell proliferation, cell cycle and metastasis of osteosarcoma (15), and inhibit epithelial mesenchymal transition of colorectal cancer (16). Whereas, the role of SATB2-AS1 in glioma has not been identified yet. In the present study, forced overexpression of SATB2-AS1 was identified to inhibit cell proliferation of glioma cells. The impact of SATB2-AS1 on cell metabolism has not been studied in cancer cells yet. In the present study, to the best of the authors' knowledge, it was revealed for the first time that SATB2-AS1 suppresses glycolysis and induces cell apoptosis in glioma. Altogether, the data revealed a critical role of SATB2-AS1 in glioma.

By direct interaction with WDR5, GADD45A and p300, SATB2-AS1 upregulated tumor suppressor SATB2 expression in a cis-activating manner in colorectal cancer (16,34). In the present study, miR-671-5p was predicted to interact with SATB2-AS1. miR-671-5p which is encoded by the gene located in 7q36.1, is frequently amplified in GBM, and promotes proliferation and metastasis of GBM cells (24,35). As for the oncogenic role of miR-671-5p in glioma, there are other 2 related studies; for instance, circ_0001946 was found to inhibit GBM progression by sponging miR-671-5p (36) and circDLC1 was revealed to suppress the malignant proliferation of glioma by competitively binding to miR-671-5p (37). Currently, miR-671-5p was proved to be sponged by SATB2-AS1 and its expression was suppressed by SATB2-AS1 in glioma cells.

As for the target mRNAs for miR-671-5p, CDR1 and VSNL1 were selected, as identified by a previous study in glioma (25), since miR-671-5p could degrade CDR-AS1 and downregulate CDR1 and VSNL1 expression in glioma cells (24). CDR1 which was highly expressed in cerebral hemisphere cortex (38), suppressed cell proliferation, colony forming, migration and invasion, while induced cell apoptosis in glioma cells U251 and U87 (36). VSNL1 was known to inhibit cell proliferation and behaved as a tumor suppressor in several cancer types, including squamous carcinoma and skin carcinogenesis (39,40). In the current study, the data suggested that SATB2-AS1 positively regulated CDR1 and VSNL1 protein expression. In addition, elevation of miR-671-5p reversed the effect of SATB2-AS1 on CDR1 and VSNL1 protein expression in glioma cells. Furthermore, the potential implication of CDR1 and VSNL1 in glycolysis was demonstrated in the present study for the first time, to the best of the authors' knowledge. Consequently, the present study revealed SATB2-AS1 as a novel regulator of miR-671-5p and demonstrated the SATB2-AS1/miR-671-5p/CDR1 axis and SATB2-AS1/miR-671-5p/VSNL1 axis in glioma.

Collectively, it was demonstrated in the current study that SATB2-AS1 is decreased in patients with glioma, especially in those of high grade, indicating a biomarker potential of SATB2-AS1 in glioma. In addition, in the *in vitro* models, SATB2-AS1 inhibited glioma cell proliferation and glycolysis, while induced cell apoptosis, identifying the SATB2-AS1/miR-671-5p/CDR1 axis and SATB2-AS1/miR-671-5p/VSNL1 axis in glioma. However, further study needs to be performed to investigate the molecular mechanism underlying downregulation of SATB2-AS1 in glioma, and explore the role of SATB2-AS1 in the initiation, metastasis and tumor growth in the *in vivo* models.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

JG, YY, RS, WZ and RY contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, read and approved the final manuscript, and agree to be accountable for all aspects of the work. JG and RY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All experiments performed in the present study involving human participants were approved by the Ethical Committee of the Affiliated Hospital of Xuzhou Medical University (Xuzhou, China; approval no. 20190508003). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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