

# Knockdown long noncoding RNA SLC8A1-AS1 attenuate cell invasion and migration in glioma via suppression of Wnt- $\beta$ catenin signaling pathways

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## Research

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# Abstract

**Background:** Glioma, as the most common aggressive malignant tumor in the central nervous system, is still an insurmountable disease in neural system. The potential mechanism of its carcinogenesis remains largely unclear.

**Methods:** In the present study, we identified dysregulated lncRNA solute carrier family 8 member A1 antisense RNA 1 (SLC8A1-AS1) as associated with glioma based on The Cancer Genome Atlas [TCGA] data. Validation experiment was conducted to confirm a high expression level of lncRNA SLC8A1-AS1 in glioma tissues.

**Results:** Down-regulation of lncRNA SLC8A1-AS1 suppressed proliferation, clone formation, migration and invasion of glioma cells *in vitro* and *in vivo*. Moreover, lncRNA SLC8A1-AS1 silencing decreased the activity of the Wnt/ $\beta$ -catenin pathway and suppressed the epithelial to mesenchymal transition (EMT) in glioma cells.

**Conclusions:** Collectively, these findings provide a novel insights into the function and mechanism of lncRNA SLC8A1-AS1 in the pathogenesis of glioma and highlight its potential as a therapeutic target for glioma intervention.

## Background

Glioma is the most common and aggressive tumor in the nervous system, and accounts for about 80% of primary malignant brain tumors[1,2]. Although there have been advances in glioma therapy in the past decades, overall survival of most patients with glioma have not changed significantly, for which the median survival time is only approximately 14 months[3]. Therefore, it is an urgent need to clarify molecular mechanisms and identify new therapeutic targets for glioma.

Recent integrative genomic studies have revealed that 98% of the human genome transcripts are non-coding RNA (ncRNA) with limited or no protein-coding capacity [4]. Long non-coding RNAs (lncRNAs), transcripts >200 nucleotides in length are important new members of the ncRNA family[5]. The dysregulated lncRNAs expression has been implicated in glioma[6,7], however, most latent functions of these lncRNAs are still unknown.

In the present study, a high expression level of lncRNA solute carrier family 8 member A1 antisense RNA 1 (SLC8A1-AS1) was found in glioma tissues by analysis of the gene-expression dataset of TCGA. We assessed the expression of lncRNA SLC8A1-AS1 in 67 glioma samples and 7 histologically normal tissues, and confirmed the up-regulation of lncRNA SLC8A1-AS1 in glioma. Functional studies were further performed and the results suggested that knockdown of lncRNA SLC8A1-AS1 could result in the inhibition of cell proliferation, clone formation, invasion and migration *in vitro* and tumor growth *in vivo*. More over, we demonstrated that lncRNA SLC8A1-AS1 is involved in glioma cells progression by activating of Wnt/ $\beta$ -catenin signaling pathway.

# Materials And Methods

## Tissue samples

In this study, tumor tissues were obtained from 67 glioma patients, and non-neoplastic brain samples were obtained from 7 traumatic brain injury patients at the Department of Neurosurgery of The first affiliated Hospital of Wannan Medical College from February 2014 to October 2017. Two pathologists evaluated all specimens according to the World Health Organization (WHO) guidelines. No local or systemic treatments were administered to these patients before surgery. The tissues were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. All of the patients provided signed, informed consent before the use of these clinical materials for research purposes. The use of these archival tissues in this study was approved by the Ethics Committee of The first affiliated Hospital of Wannan Medical College.

## Cell culture

Human U87MG and LN382 glioblastoma cell lines were obtained from ATCC (American Type Culture Collection, USA). All cell lines were routinely cultured at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  humidified atmosphere in Dulbecco's-modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS, Gibco).

## Real-time RT-PCR

Total RNA was extracted from the transfected cells with TRIzol (Invitrogen) and 0.4 $\mu\text{g}$  RNA was used to synthesize cDNA using a first strand cDNA synthesis kit (Termo scientific, USA). The RNA concentration was examined using a NanoDrop 2000 spectrophotometer (Termoscientific, USA). Real-time RT-PCR analysis was performed using the CFX-96 (BioRad, USA) according to the manufacturer's instructions. Data were normalized according to the level of GAPDH expression in each sample. GAPDH was used as an internal control, and  $2^{-\Delta\Delta\text{Ct}}$  values were used to assess the relative expression of the target gene. The primers of GAPDH were synthesized by RiBoBio (Guangzhou, China)

qRT-PCR primers for lncRNA SLC8A1-AS1 were as follows:

Forward 5'-3': CAGTCGTGTTCTCGCACTT

Reverse 5'-3': GCTGCCCGTGACGTTACCTAT

## Colony formation assay

For the colony formation assay, the cells were plated in 6-well plates at  $2 \times 10^2$  cells per well and maintained in DMEM containing 10% FBS for 2 weeks. After 2 weeks, the cells were washed two times with PBS, fixed with methanol and stained with crystal violet at the end of the time course prior to the capture of the representative images via camera. The number of colonies was counted under a microscope. All experiments were performed in triplicate.

## Cell cycle assay

Cell cycle analysis was performed by determining the DNA content with propidium iodide (PI) staining (BD Biosciences; San Jose, CA, USA). Briefly, U87MG and LN382 glioma cells were harvested, re-suspended and stained with propidium iodide (PI; BD Biosciences) in the presence of RNase A for 20 min. Cells were analysed using a flow cytometer (BD Biosciences) according to the manufacturer's instructions.

## Wound healing assay

Culture and transfection conditions for U87MG and LN382 cells were optimized to ensure a homogeneous and viable cell monolayer prior to wounding. One day before transfection, equal numbers of U87MG and LN382 cells ( $5.0 \times 10^5$ ) were seeded into 6-well tissue culture plates without antibiotics. Cells were then transfected with Noncontrol (100nM) and SLC8A1-AS1 siRNA (100 nM) using riboFECT™ CP RiBoBio (Guangzhou, China), respectively. When the cell confluence reached about 90 % at 24h post-transfection, an artificial homogenous wound was created onto the monolayer with a sterile plastic 200μL micropipette tip. After wounding, the debris was removed by washing the cells with serum-free medium. Migration of cells into the wound was observed at 0 and 24h, respectively. Cells that migrated into the wounded area or cells with extended protrusion from the border of the wound were visualized and photographed under an inverted microscope (Nikon Ti-u). Migration was quantified by counting the total number of cells that migrated toward the original wound field. A total of three areas were selected randomly from each well and the cells in three wells of each group were quantified in each experiment.

## Invasion assay

Invasion assay was tested on the newer technique of real time migration monitoring using the CIM devices and the xCELLigence DP system (ACEA Biosciences, USA). Before experiment going on, Matrigel was coated on wells respectively. In this system,  $8 \times 10^3$  treated either with siRNA SLC8A1-AS1 or Noncontrol, then seeded in the upper chamber in the normal culture medium of the respective cell line without FBS. This upper chamber was then placed on the lower part of the CIM-device containing growth medium supplemented with 10% FBS as an attractant. Invasion of the cells was followed over a time period of up to 120h by changes of the impedance signal in a CIM-plate (ACEA Bio) measured on the backside of the membrane and cell growth was monitored in a 16-well e-plate (ACEA Bio) as described for the xCELLigence DP system. Invasion assay was also tested by using Tumor Invasion System (BD BioCoat, BD, NJ) in matrigel coated 24-well inserts. A sample of  $3 \times 10^4$  cells treated with Noncontrol or SLC8A1-AS1 siRNAs was placed on this system in DMEM-medium without FCS. The inserts were set into DMEM-medium with 10% FCS as an attractant. After 48 h, the cells were stained with 0.1% crystal violet for 30 min and observed under a light microscope.

## Migration assay

Cell migration assay were performed by using the newer technique of real time migration monitoring using the CIM devices and the xCELLigence DP system (ACEA Bio). In this system,  $8 \times 10^3$  cells treated either with siRNA SLC8A1-AS1 or Noncontrol, then seeded in the upper chamber in the normal culture medium of the respective cell line without FBS. This upper chamber was then placed on the lower part of the CIM-device containing growth medium supplemented with 10% FBS as an attractant. Migration of the cells was followed over a time period of up to 120h by changes of the impedance signal in a CIM-plate (ACEA Bio) measured on the backside of the membrane and cell growth was monitored in a 16-well e-plate (ACEA Bio) as described for the xCELLigence DP system. Cell migration assay was also tested by using Transwell insert chambers (8µm pore size, Corning, USA). A total of  $3 \times 10^4$  concentration cells were seeded into the upper chambers in serum-free medium. The lower chamber of the Transwell was filled with 500µl culture media containing 10% FBS as a chemo-attractant. After the chambers were incubated at 37°C for 48 h, non-invaded cells on the top of the Transwell were scraped off with a cotton swab. Successfully migrated cells were fixed with 10% formalin. Then, they were stained with 0.1% crystal violet for 30 min and counted under a light microscope.

### Western blot analysis

Cell lysates were prepared using sample buffer, laemmli (Sigma, USA) for protein extraction. Protein lysates were separated by 10% SDS-PAGE, transferred to 0.22-µm NC membranes (Milipore, USA) and incubated with antibody. β-actin was used as a control. Antibodies (1:1000) against claudin, vimentin, E-cadherin, N-cadherin, GSK-3β, phosphor GSK-3β, β-catenin, phosphor β-catenin were purchased from Abcam. β-actin (1:1000) were purchased from Sigma. The band intensity was measured by densitometry using the Image J. The protein levels were normalized with that of β-actin. All experiments were repeated in triplicate, and the representative results were shown.

### Statistical analysis

The SPSS 16.0 statistical analysis software was used for the statistical analysis of the experimental data. The significance of differences between groups was estimated by Student's t-test. A p value less than 0.05 were considered significant.

## Results

### LncRNA SLC8A1-AS1 is up-regulated in glioma.

The expression levels of lncRNAs in human 156 glioma tissues and 5 adjacent normal tissues downloaded from The Cancer Genome Atlas (TCGA, <https://tcga-data.nci.nih.gov/tcga/>) was profiled to explore the functions of lncRNAs in glioma. The results showed that lncRNA SLC8A1-AS1 expression level is significantly up-regulated in glioma tissues ( $P < 0.01$ ) compared with noncancerous tissues (**Figure 1A**). We further used real-time RT-PCR to verify the lncRNA SLC8A1-AS1 expression in 67 glioma samples and 7 histologically normal tissues. As shown in **Figure 1B**, the lncRNA SLC8A1-AS1 expression was significantly over-expressed in glioma tissues ( $P < 0.001$ ). In addition, receiver operating characteristic

(ROC) curve analysis was determined to evaluate the diagnostic value of lncRNA SLC8A1-AS1 for glioma. Notably, lncRNA SLC8A1-AS1 displayed the highest accuracy for predicting glioma, because the area under the curve (AUC) was 0.891 in TCGA data ( $P = 0.0029$ , **Figure 1C**) and 0.99 in our validation queue ( $P < 0.0001$ , **Figure 1D**).

### **Knockdown of lncRNA SLC8A1-AS1 inhibited glioma cell proliferation and clone formation by inducing cell-cycle arrest *in vitro*.**

To assess the roles of lncRNA SLC8A1-AS1 in glioma, we down-regulated expression of lncRNA SLC8A1-AS1 in human glioma cell lines U87MG and LN382 by transfection with siRNAs. Real time cellular analysis (RTCA) and colony-formation assay were performed to investigate the effects of lncRNA SLC8A1-AS1 on the glioma cells proliferation. The results revealed that down-regulation of lncRNA SLC8A1-AS1 markedly inhibited U87MG and LN382 cells growth (**Figure 2A**) and decreased the number of colonies formed (**Figure 2B**). Dysregulation of cell cycle is a vital reason for tumor cell proliferation, to further explore whether lncRNA SLC8A1-AS1 promoted proliferation by influence cell cycle progression in glioma, we examined cell cycle kinetics by using flow cytometric analysis. The results revealed that U87MG and LN382 cells with siRNAs had an obvious cell cycle arrest in the G2 phase and the population of cells in the S phase was decreased, whereas the G2/M phase fractions increased significantly (**Figure 2C**). These results confirmed that lncRNA SLC8A1-AS1 is involved in glioma cells progression by inducing cell-cycle arrest.

### **lncRNA SLC8A1-AS1 regulated glioma cell invasion and migration *in vitro*.**

We further assessed the effects of lncRNA SLC8A1-AS1 on the invasion and migration capacity of U87MG and LN382 cells. The transwell assay (with or without matrigel coated in the upper chambers) demonstrated that invasion and migration ability of U87MG and LN382 cells was notably decreased with lncRNA SLC8A1-AS1 knockdown (**Figure 3A**). The wound-healing assay also authenticated this observation and revealed that wounds scraped in confluent cultures of SLC8A1-AS1 siRNA transfected cells closed only partially, whereas nearly full wound closure was observed in the control cells. As shown in **Figure 3B**, transfection of lncRNA SLC8A1-AS1 siRNA obviously inhibited the time-dependent migration profile of U87MG and LN382 cells. The epithelial mesenchymal transition (EMT) pathway play crucial role in migration and invasion of tumor cells [8,9]. Consequently, we determined the effects of lncRNA SLC8A1-AS1 silencing on EMT pathway. As shown in **Figure 3C**, down-regulation of lncRNA SLC8A1-AS1 with siRNA treatment increased the protein expression of E-cadherin and Claudin (markers of epithelial phenotype) and decreased expression of N-cadherin and Vimentin (markers of mesenchymal phenotype) in U87MG and LN382 cells. These results confirmed that SLC8A1-AS1 plays a crucial role in glioma cells progression by regulating EMT.

### **Down-regulation of lncRNA SLC8A1-AS1 suppressed glioma cell Wnt/ $\beta$ -catenin pathway.**

To determine the possible mechanism by which SLC8A1-AS1 regulated the migration and invasion of glioma cells, Western-blot analysis was performed to investigate the effects of knockdown of SLC8A1-

AS1 on the Wnt/ $\beta$ -catenin pathway, which is often aberrantly activated in human cancers and contributes to enhanced cell invasion and migration. Western-blot analysis showed that down-regulation of lncRNA SLC8A1-AS1 significantly reduced the levels of phosphorylated GSK-3 $\beta$  (p-GSK3 $\beta$ ) and phosphorylated  $\beta$ -catenin (p- $\beta$ -catenin), while no detectable changes were observed in the total levels of GSK-3 $\beta$  and  $\beta$ -catenin. Furthermore, the Hippo pathway are known to play a significant role in glioma proliferation, motility and invasiveness. Our study corroborate that the Hippo pathway doesn't participate in the glioma development (Figure 4A). Moreover, inhibition the Wnt/ $\beta$ -catenin signaling pathway with XAV939 partly impaired SLC8A1-AS1-induced invasion and metastasis of glioma cells (Figure 4B). Taken together, these data suggest that lncRNA SLC8A1-AS1 facilitate the progression of glioma by activating the Wnt/ $\beta$ -catenin signaling pathway.

### Silencing of lncRNA SLC8A1-AS1 repressed glioma growth *in vivo*.

Finally, we validated the effects of lncRNA SLC8A1-AS1 using a nude mouse model with glioma xenografts *in vivo*. Figure 5A displayed the morphology of tumor xenografts after injection of U87MG cells into the right axilla of nude mice at 35 days. Figure 5B and C showed the tumor size and average tumor weight in the lncRNA SLC8A1-AS1 silencing group was obviously decreased compared with the control group.

## Discussion

Glioma is the most common primary brain tumors of adults, and represents one of the most aggressive and lethal human cancer types [1]. At present, the prognosis of glioma patients is very poor and the 5-year survival rate less than 10% [10], even when multimodal treatment strategies are used. Hence, it is an urgent need to clarify the molecular mechanisms underlying progression of glioma and provide evidence for novel therapeutic targets. Many important efforts have been made to identify prognostic molecular biomarkers that could provide explanations regarding glioma formation and progression [11]. Studies have shown that lncRNAs are of great importance in the diagnosis and treatment of tumors. lncRNA-PLACT1, which was significantly upregulated in tumor tissues and correlated with progression and poor survival in pancreatic cancer patients [12]. lncRNA NEAT1 contributes to glioblastoma progression through the Wnt/ $\beta$ -catenin pathway by scaffolding EZH2 [13]. In the present study, we first analyze the data from the TCGA database and found a novel lncRNA SLC8A1-AS1 was associated with glioma.

lncRNA SLC8A1-AS1 is oriented in an antisense direction to the protein-coding gene *SLC8A1*, which is located on chromosome 2 of the human genome [14]. *SLC8* gene is a member of CaCA (Ca<sup>2+</sup>/Cation Antiporter) superfamily, encoding Na<sup>+</sup>/Ca<sup>2+</sup> exchangers (NCX), mainly expressed on cell membrane, mediate Na<sup>+</sup>/Ca<sup>2+</sup> fluxes across the cell-membrane. Three mammalian genes (*SLC8A1*, *SLC8A2*, and *SLC8A3*) and their splice variants are expressed in different tissues, they all significantly contribute to regulation of Ca<sup>2+</sup>-dependent events in many cell types [15]. We also observed a marked up-regulation of lncRNA SLC8A1-AS1 in clinical glioma samples detected by real-time RT-PCR. ROC curves were used to evaluate the sensitivity and specificity of lncRNA SLC8A1-AS1 expression in predicting glioma tissues

from normal tissues, and the results displayed the highest accuracy for predicting glioma. Our results implied that lncRNA SLC8A1-AS1 might act as “oncogene” to promote the progression of glioma and might provide imperative clinical significance in glioma diagnosis.

We then investigated the effects of aberrant lncRNA SLC8A1-AS1 expression on the cellular biological behavior of glioma cells. Abnormal cell proliferation and growth are characteristics of human gliomas [16]. Importantly, lncRNA SLC8A1-AS1 silencing obviously inhibited the proliferation, clone formation, by inducing cell cycle arrest. The results show that knockdown of lncRNA SLC8A1-AS1 leads to reduced cell proliferation in vitro, and in vivo data suggest that it is a potential molecular target for therapy. Cell migration and invasion are significant aspects of cancer progression [17], we found that inhibit the expression of lncRNA SLC8A1-AS1 can inhibited the migration and invasion of U87MG and LN382 cells significantly. EMT is a critical biological process in tumor cell migration and invasion [18]. We found that lncRNA SLC8A1-AS1 siRNA treatment could increase the expression of E-cadherin and claudin, however, decrease the expression of N-cadherin and vimentin at the protein level in U87MG and LN382 cells. lncRNA SLC8A1-AS1 silencing appeared to specifically suppress the progression of EMT in glioma cells, which reduced their invasion abilities. These results indicated that lncRNA SLC8A1-AS1 may serve as a crucial regulator of invasion and migration by inducing EMT in gliomas.

To elucidate the possible mechanism by which lncRNA SLC8A1-AS1 regulates the proliferation, clone formation, migration and invasion of glioma cell, Western-blot analysis of the key molecular factors of cancer-related pathways, such as Hippo pathway, Wnt/ $\beta$ -catenin pathway was performed. The Wnt/ $\beta$ -catenin pathway plays a key role in tumor EMT[19-21]. GSK-3 $\beta$  is one of the components of the destruction complex in the Wnt/ $\beta$ -catenin signaling [22]. In this study, down-regulation of lncRNA SLC8A1-AS1 significantly reduced the levels of p-GSK-3 $\beta$  and p- $\beta$ -catenin in U87MG and LN382 cells, while no detectable changes were observed in the total levels of GSK-3 $\beta$  and  $\beta$ -catenin. However, the expression level of phosphorylated YAP-1 (p-YAP-1) in the signal pathway did not change. Furthermore, Wnt/ $\beta$ -catenin signaling pathway inhibitor significantly impaired lncRNA SLC8A1-AS1-induced glioma development. These results suggested that lncRNA SLC8A1-AS1 could facilitate the progression of glioma by regulating the Wnt/ $\beta$ -catenin pathway activity.

Although we analysed only two cell lines (U87MG and LN382) in these studies, our nude mouse tumorigenicity assay results provide powerful evidence; therefore, we speculate that other glioma cell lines will show similar results.

## Conclusions

Taken together, we identified for the first time that lncRNA SLC8A1-AS1 is up-regulated in glioma tissues and cell lines. Our findings demonstrated that knockdown of SLC8A1-AS1 inhibits proliferation, migration and invasion of glioma cells. These effects were dependent on Wnt/ $\beta$  catenin activation status, highlighting the potential of lncRNA SLC8A1-AS1 as a candidate therapeutic target in glioma.



# Abbreviations

LncRNAs: Long non-coding RNAs; EMT: Epithelial to Mesenchymal Transition; TCGA: The Cancer Genome Atlas; WHO: World Health Organization; ATCC: American Type Culture Collection; DMEM: Dulbecco's-modified Eagle medium; FBS: Fetal bovine serum; PI: Propidium iodide; ROC: Receiver Operating Characteristic; AUC: Area under the curve; RTCA: Real time cellular analysis; p-GSK3 $\beta$ : phosphorylated GSK-3 $\beta$ ; p- $\beta$ -catenin: phosphorylated  $\beta$ -catenin

# Declarations

## Authors' contributions

Ling He performed the research and wrote the manuscript. Hui Yang collected clinical samples and corresponding clinical data. Xiao-long Zhu performed cell culture and the assessment of cell functions *in vitro*. Yan Zhang and Kun Lv revised the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

The dataset supporting the conclusions of this article was retrieved by using the TCGA <http://cancergenome.nih.gov>

## Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of the The First Affiliated Hospital of Wannan Medical College (Wuhu, China) and performed in accordance with the relevant guidelines and regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Wannan Medical College (Wuhu, China)

## Acknowledgements

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

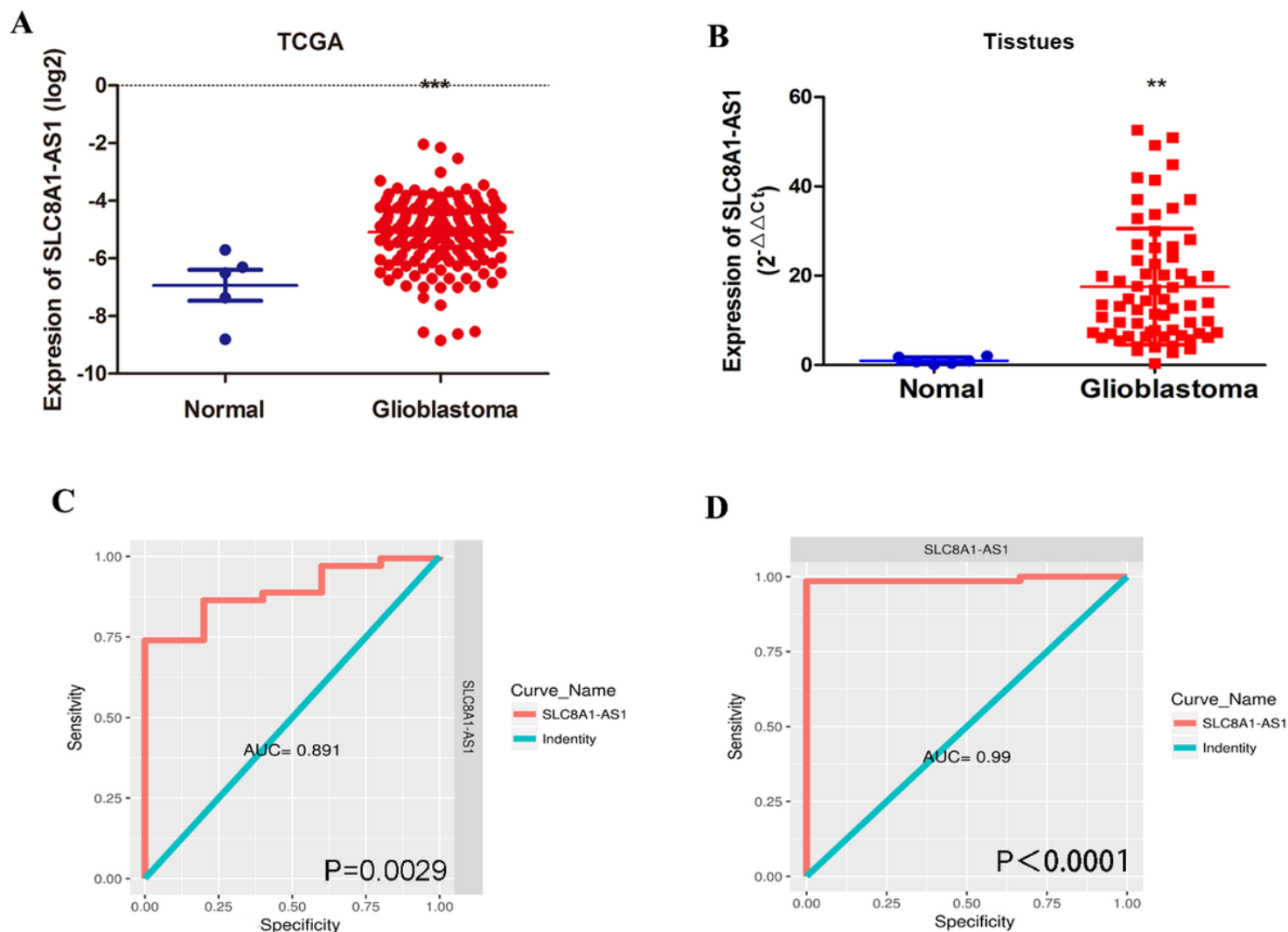
The authors declare no conflict of interest.

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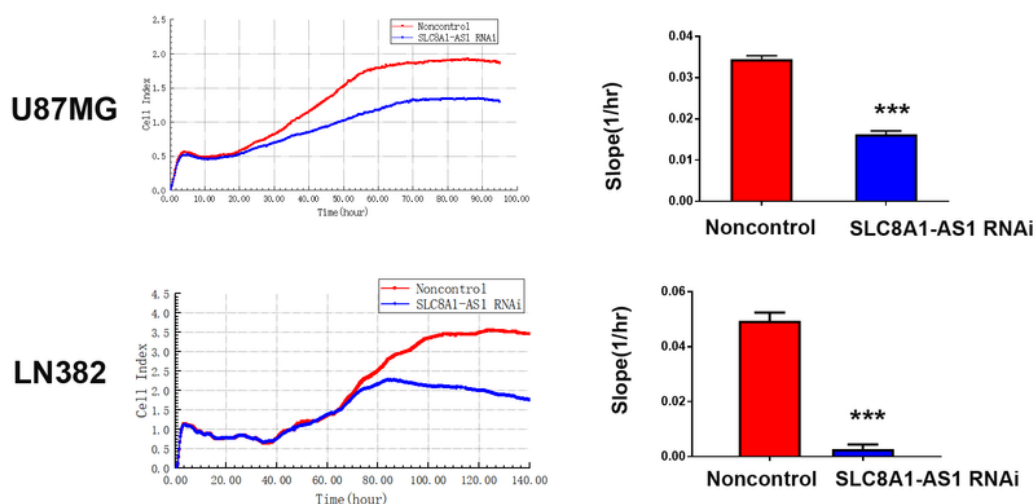
## Figures



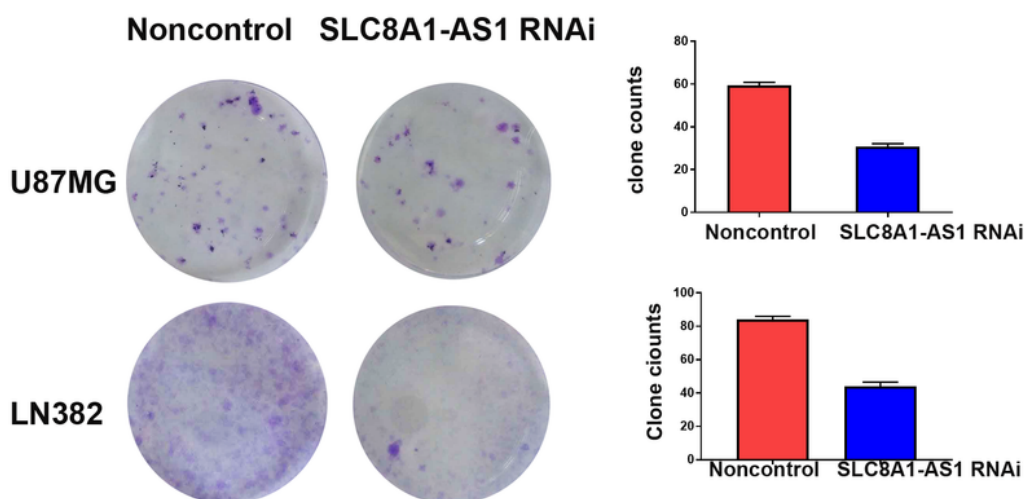
**Figure 1**

LncRNA SLC8A1-AS1 is up-regulated in glioma. A. Relative expression levels of SLC8A1-AS1 in TCGA database. B. Relative expression of SLC8A1-AS1 in the tumor tissues (n=67) compared to normal tissues (n=7). SLC8A1-AS1 expression was evaluated by qRT-PCR and normalized to GAPDH expression. C. ROC curve for prediction of glioblastoma based on SLC8A1-AS1 expression, using corresponding adjacent normal tissues as a control. D. ROC curve for prediction of glioma based on SLC8A1-AS1 expression, using corresponding normal tissues as a control. \*\*\*P < 0.001

A.



B.



C.

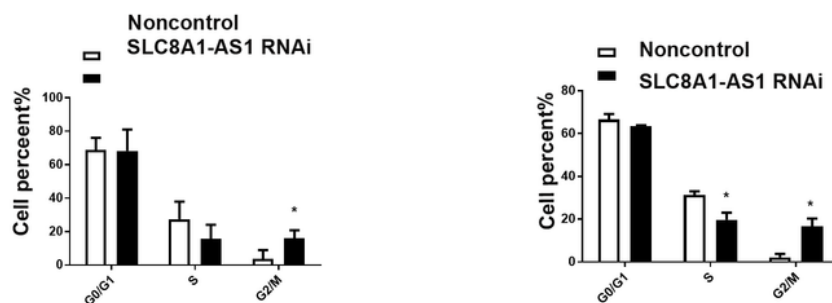
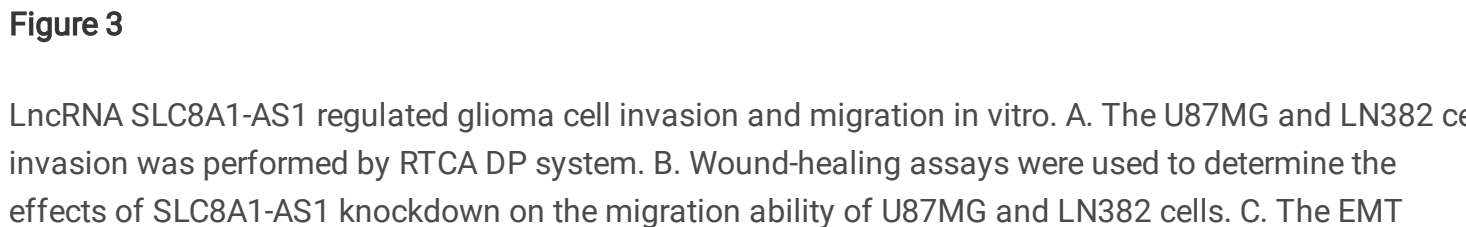


Figure 2

Knockdown of lncRNA SLC8A1-AS1 inhibited glioma cell proliferation in vitro. A. RTCA system were performed to determine the proliferation of SLC8A1-AS1 siRNA transfected U87MG and LN382 cells. B. Colony-forming assays were conducted to determine the proliferation of SLC8A1-AS1 siRNA transfected U87MG and LN382 cells. C. Flow cytometric analysis were conducted to determine the cell cycle of

**A.** **B.**





pathway markers was assessed by western-blot. The experiments were performed in triplicate, and the data are expressed as the mean  $\pm$  S.D. \*,  $P \leq 0.05$ , \*\*,  $P \leq 0.01$  and \*\*\*,  $P \leq 0.001$ .

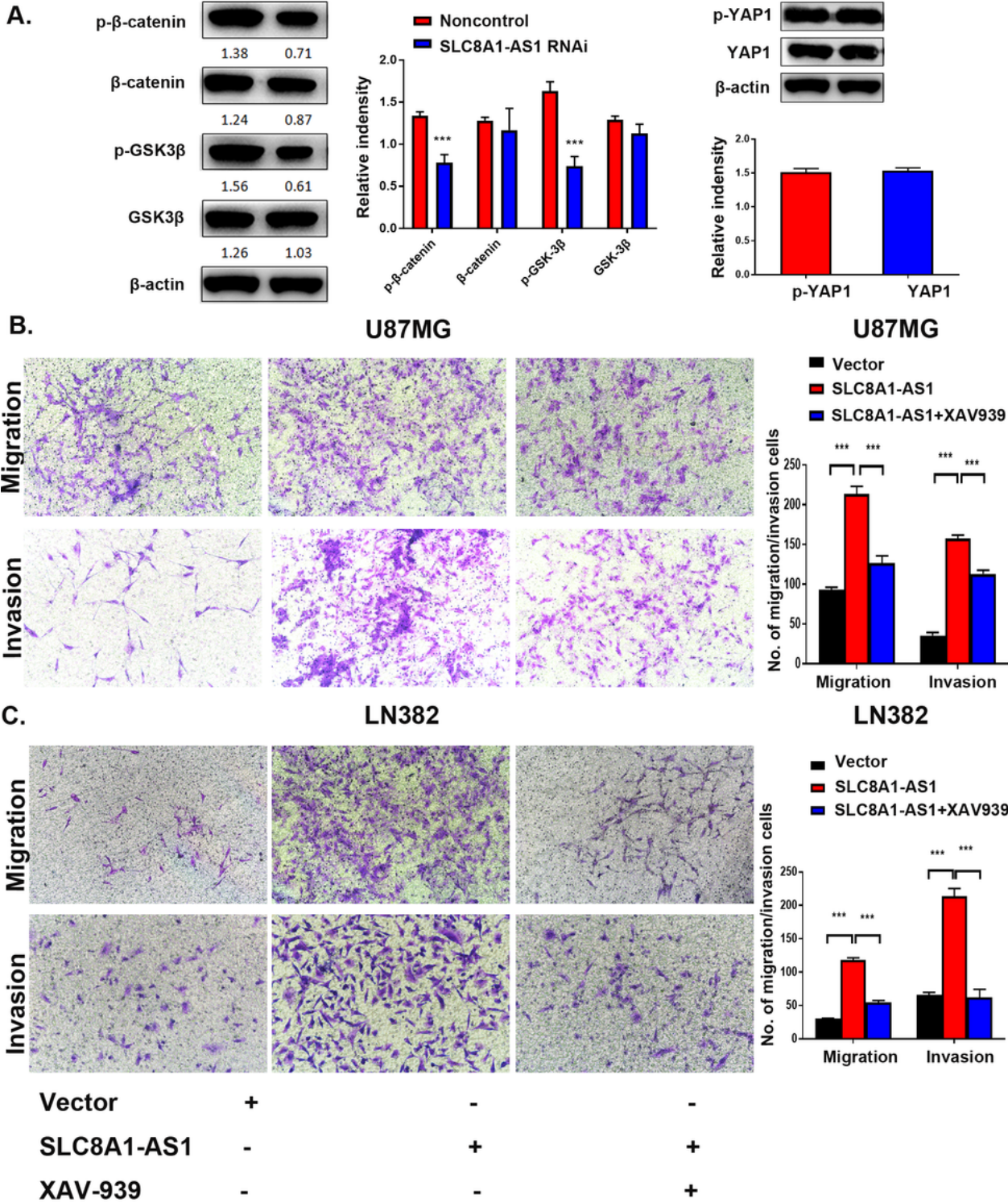
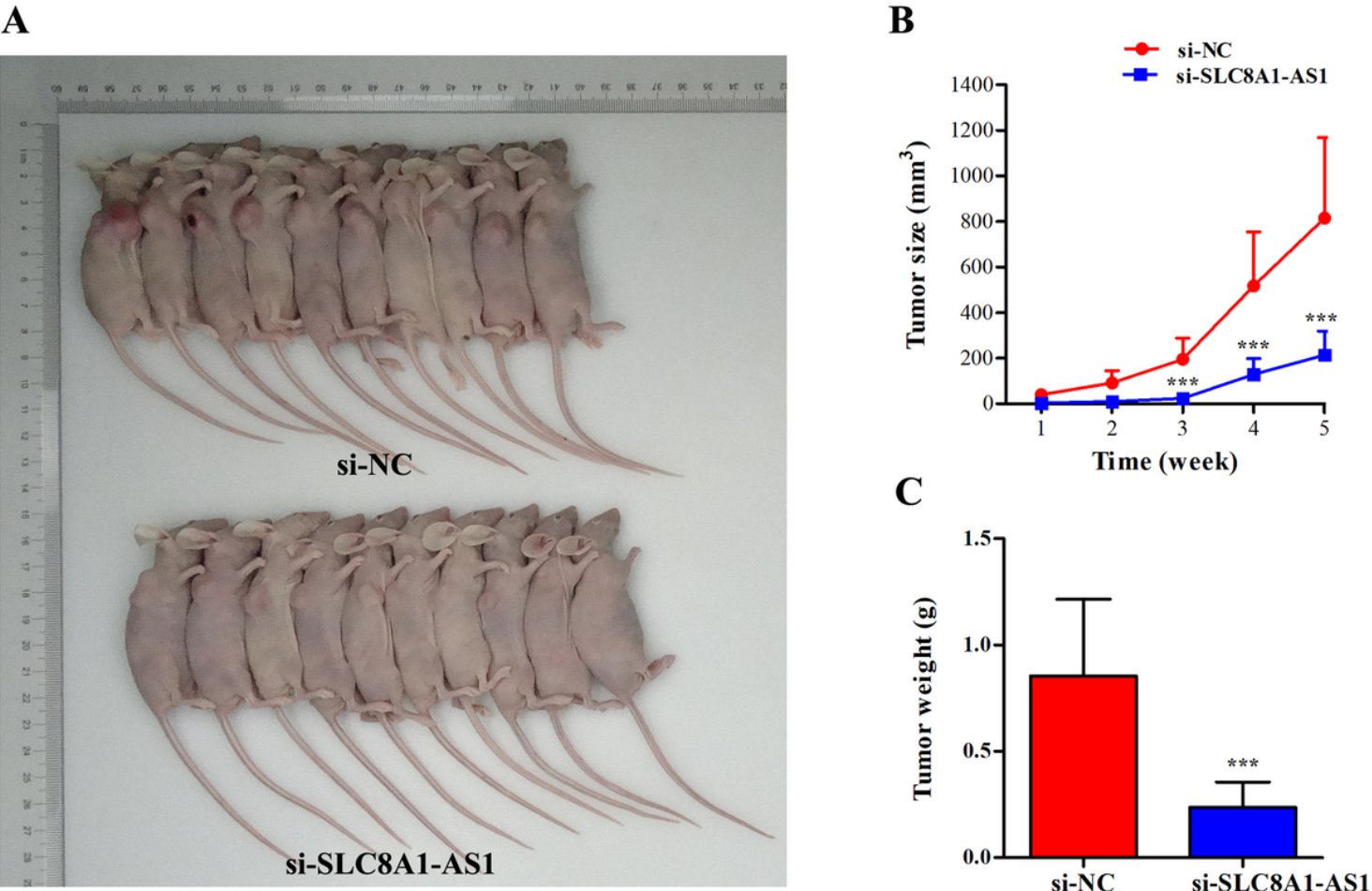


Figure 4

Down-regulation of lncRNA SLC8A1-AS1 suppressed glioma cell Wnt/β-catenin pathway. A. Effect of SLC8A1-AS1 knockdown on Wnt/β-catenin and Hippo pathway. B. Representative images of transwell assays showed that XAV939 reversed the effects of SLC8A1-AS1-overexpressing glioma cells. Scale bars:

100  $\mu$ m. p-values were calculated by using two-tailed t-tests. The error bars represent standard deviations of three independent experiments. \*p < 0.05, \*\*p < 0.01, \*\*\*, P $\leq$ 0.001.



**Figure 5**

SLC8A1-AS1 suppressed U87MG and LN382 cell proliferation in vivo. A. U87MG cells with stable SLC8A1-AS1 knockdown and control cells were inoculated into nude mice. These graphs show the tumor xenografts 6 weeks after ectopic-subcutaneous implantation in nude mice. B. U87MG cells with downregulated SLC8A1-AS1 expression exhibited attenuated tumor growth in nude mice. The effect of SLC8A1-AS1 on glioma growth was evaluated based on tumor volume in the two groups.