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RESEARCH ARTICLE

LncRNA GAS5-AS1 inhibits glioma proliferation, migration, and invasion via miR-106b-5p/TUSC2 axis

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Abstract

Glioma is one of the most common malignant tumors and shows a high metastasis rate and poor prognosis. Abnormal expression of long non-coding RNAs (lncRNAs) contributes to various human tumors including gliomas. This study aimed to investigate the regulatory role of the antisense RNA of growth arrest special 5 (GAS5-AS1), a novel lncRNA, in gliomas. Expression of GAS5-AS1 and microRNA-106b-5p (miR-106b-5p) in glioma tissues and cells was detected by quantitative reverse transcription PCR, northern blotting, or fluorescent in situ hybridization. Cell proliferation, migration, and invasion were analyzed by CCK-8 and Transwell assays. BALB/c nude mice were used to establish a glioma xenograft animal model by subcutaneous injection of U251 cells transfected with small interfering RNA targeting GAS5-AS1. A dual-luciferase reporter assay was conducted to confirm the targeting relationship between GAS5-AS1 and miR-106b-5p. GAS5-AS1 expression was downregulated in glioma tissues and cells, and upregulation of GAS5-AS1 expression inhibited glioma cell proliferation, migration, and invasion. GAS5-AS1 expression was correlated with the glioma tumor grade. In nude mice, upregulation of GAS5-AS1 markedly suppressed glioma tumor growth. GAS5-AS1 overexpression significantly increased the miR-106b-5p level in glioma cells, and GAS5-AS1 expression was negatively related to miR-106b-5p expression in glioma tissues. Overexpression of miR-106b-5p reversed the inhibitory effects of GAS5-AS1 upregulation on glioma cell proliferation and metastasis, while restoration of TUSC2 rescued the proliferation and invasion of glioma cells transfected with miR-106b-5p mimics. In summary, lncRNA GAS5-AS1 inhibited glioma proliferation, migration, and invasion by sponging miR-106b-5p and regulating the expression of TUSC2. Our results suggest GAS5-AS1 as a novel target for the treatment and prognosis prediction of gliomas.

Keywords GAS5-AS1 · miR-106b-5p · TUSC2 · Glioma · Long non-coding RNA · Biomarker

Introduction

Gliomas are characterized by rapid progression and early metastasis and are among the most malignant tumors occurring in the central nervous system, accounting for more than 50% of intracranial neoplasms [1, 2]. Gliomas can be graded as I–IV based on pathologic growth and diffusion velocity features according to the World Health Organization [3]. Surgical resection followed by radiotherapy and chemotherapy is the most common clinical treatment for gliomas; however, mortality and recurrence rates are high, and patients generally have a poor prognosis, with a median survival time of less than 1 year [2]. Therefore, it is important to understand the molecular mechanism of glioma development and identify biomarkers for early diagnosis.

Long non-coding RNAs (lncRNAs) are transcripts of more than 200 nucleotides [4, 5], and are closely related to

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various diseases, including cancer [6]. Abnormal lncRNA expression has been observed in various cancer types [7]. Acting as tumor facilitators or suppressors, lncRNAs are considered as potential biomarkers for cancer diagnosis [8, 9]. GAS5-AS1, mapped on chromosome 1q25.1, is the antisense RNA of growth arrest special 5 (GAS5), which is a tumor suppressor [10]. Recent studies have shown that GAS5-AS1 contributes to the development and progression of several types of cancer, such as non-small cell lung cancer (NSCLC) [11] and hepatocellular carcinoma [12]. However, a role of GAS5-AS1 in gliomas has not been reported.

MicroRNAs (miRNAs) are small non-coding RNAs of 18–24 nucleotides [13]. They regulate gene expression at the transcription level by binding to the 3′-untranslated regions of their target genes [14]. MiRNAs regulate a range of biological processes, including proliferation, invasion, and migration of cancer cells [15]. MiR-106b-5p, a member of the miR-106b-25 cluster, is abnormally expressed in various cancer tissues, showing an increase in non-NSCLC and hepatocellular carcinoma [16] and a decrease in colorectal cancer tissues [17]. These results indicate that miR-106b-5p has a dual role as a tumor suppressor or oncogene in oncogenesis. Recent studies have shown that miR-106b-5p can inhibit or promote tumor growth by binding to lncRNAs. In colorectal cancer, miR-106b-5p directly matched with the 3′-untranslated region of MALAT1 [18], thereby promoting invasion and metastasis. Additionally, miR-106b-5p inhibited the invasion and metastasis of renal cell carcinoma by targeting XIST [18]. Our previous results found that miR-106b-5p was significantly upregulated in glioma [19]. Whereas, there is little information elaborating the interaction between lncRNA and miR-106b-5p in glioma. In this study, we aimed to elucidate the role of GAS5-AS1 in glioma cell proliferation, and the underlying mechanism. Bioinformatic analysis was employed to confirm the relationship found between miR-106b-5p and GAS5-AS1. In addition, we investigated the interaction between GAS5-AS1 and miR-106b-5p in regulating glioma cell growth.

Materials and methods

Clinical sample collection and cell culture

A total of fifty paired glioma tissues and corresponding adjacent normal tissues were used in this study. Pathological examination revealed that the adjacent tissues were normal brain tissues. All samples were collected from patients who underwent glioma resection in Nanjing Medical University Affiliated Changzhou No. 2 People's Hospital and had not been administered radiotherapy and/or chemotherapy. All patients were clinically staged before operation and signed an informed consent form. This study was approved by the ethics committee of Nanjing Medical University Affiliated Changzhou No. 2 People's Hospital.

Glioma cell lines (U251, U87MG, U373, and LN229) and the human astrocyte cell line NHA were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco, Grand Island, NY, USA) at 37 °C in the presence of 5% CO_2.

Quantitative reverse transcription (qRT-) PCR

Total RNA was extracted from tissues or cell lines using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Five micrograms of total RNA was reverse transcribed into cDNA using a PrimeScript™ RT-PCR Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Expression of GAS5-AS1 and miR-106b-5p was measured by qPCR using a SYBR Premix Ex Taq kit (Thermo Fisher Scientific). U6 was used as an internal control. Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. All PCRs were run in triplicate. Primer sequences were as follows: GAS5-AS1 5′-TGTGCCCTTTATACCCAC TTT -3′, U6: 5′-CTCGCTTCGGCAGCACA-3′, GAPDH: 5′-AATCCCATCACCATCTTCCAG-3′, Stem-loop primers for miR-106b-5pwere bought from ABI.

Northern blot analysis

Northern blotting to measure GAS5-AS1 expression was conducted using an Ambion Northern Max-Gly Kit (Austin, TX, USA). Total RNA was electrophoresed on a 1.2% agarose gel containing 0.4 M formaldehyde, transferred to a nylon membrane, and then fixed by UV crosslinking. Then, the membrane was prehybridized with UltraHyb-Oligo Hybridization Buffer(Thermo Fisher Scientific) and hybridized with GAS5-AS1-specific oligonucleotide probes (GCCCAACTAGTGATAGGCATTA) labeled with digoxigenin-ddUTP in roller bottles. The quantitative data were analyzed by Gel-Pro Analyzer 4.0 software (Media Cybernet- ics, MD).

Fluorescence in situ hybridization (FISH)

LncRNA-GAS5-AS1 labeled with Cy3 and U6 probes labeled with DAPI were purchased from RiboBio (Guangzhou, China). RNA-FISH assays were carried out using a FISH kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions.

Cell transfection

Small interfering RNA (siRNA) plasmid of GAS5-AS1 and its control, lenti virus(LV) overexpressing GAS5-AS1 and its control, plasmid of TUSC2 and its blank vector were packed from Obio technology (Shanghai, China). When designing the small interfering RNA (siRNA) plasmid of GAS5-AS1, we constructed more than three siRNA and we choose the most effective one in the following experiment. MiR-106b-5p mimic, miR-106b-5p inhibitor, and negative controls (50 nM) were synthesized by RiboBio (RiboBio Co. Ltd, Guangzhou, China). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Cell proliferation assay

Cell proliferation was measured by CCK-8 assay. Cells in the exponential growth phase were resuspended and inoculated into a 96-well plate at 4000 cells/well. After the addition of 10 μL of CCK-8 solution (Sigma), the cells were incubated at 37 °C for 4 h. Absorbance at 450 nm was measured with a spectrophotometer (SmartSpec 3000; Bio-Rad, CA, USA). The experiment was repeated at least 3 times.

Flow cytometry

The cell cycle was analyzed by flow cytometry after the nuclei were stained with propidium iodide (PI). After transfection, U251 and U87MG cells were resuspended in PBS and then fixed with cold 70% ethanol at 4 °C overnight. The next day, the cells were washed with PBS and stained with 50 mg/mL PI and 20 mg/mL RNase A (Sigma) in the dark for 30 min. The cell cycle was analyzed by fluorescence-activated cell sorting (FACS) in an LSR II (BD Biosciences, Franklin Lakes, NJ, USA).

Colony formation assay

Cells in the exponential growth phase were resuspended and inoculated in a petri dish for 6 days. Then, the cells were washed with PBS, fixed with methanol, stained with crystal violet, and observed under a microscope. The colonies were counted in 6 photomicrographs from each well.

Migration and invasion assays

To evaluate cell invasion, at 24 h after transfection, cells were transferred into serum-free medium for 12 h. Upper Transwell chambers were coated with 50 μL of Matrigel (1:8, BD Biosciences) and incubated at 37 °C for 30 min for gel formation. Medium containing 10% fetal bovine serum was added to the lower chamber and the cell suspension was added to the upper chamber. After 24 h of incubation at 37 °C, the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Cells were counted in 8 random visual fields. To evaluate migration, no Matrigel was used, whereas the other assay steps were the same.

Mouse tumor model

Four-week-old male BALB/c nude mice were raised under specific pathogen-free conditions. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University (Permit Number: NJMU 18–0232) and performed in an SPF animal facility. All procedures were conducted in accordance with the Care and Use of Laboratory Animals issued by the Chinese Association for Laboratory Animal Care. The mice were randomly divided into two groups ($n = 12$ per group): one group was subcutaneously injected in the left flank with U251 cells transfected with LV-GAS5-AS1, the other with U251 cells transfected with LV-NC. Tumor volumes were measured every 7 days and were calculated as follows: tumor volume (mm^3) = length × width^2 × 0.5. The mice were sacrificed after 4 weeks, and tumors were resected and weighed.

Hematoxylin and eosin (H&E) and immunohistochemical staining

After resection, tumor tissues were immersed in 10% formalin and embedded with paraffin. Paraffin-embedded sections were cut at 5 μm and stained with H&E to observe histological changes. For Ki-67 staining, the sections were deparaffinized and rehydrated. The slides were incubated with anti-Ki-67 antibody (1: 50, Abcam, Cambridge, UK) after blocking, followed by counterstaining with hematoxylin.

Luciferase reporter assays

The GAS5-AS1 sequence, containing a putative miR-106b-5p-binding site, was cloned and inserted into the pmirGlO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to prepare GAS5-AS1-WT. The GAS5-AS1 sequence with a mutated binding site was similarly used to prepare GAS5-AS1-Mut. U251 cells were cotransfected with the GAS5-AS1 or control vector, miR-106b-5p mimic, or miR-106b-5p inhibitor using Lipofectamine 2000. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega), and firefly luciferase activity was normalized against Renilla luciferase activity.

Western blot analysis

Total protein was extracted and its concentration was analyzed through the BCA protein Assays Kit (Thermo Fisher Scientific; Shanghai, China). Fifteen micrograms of each protein samples were separated on SDS-PAGE gel and transferred onto the polyvinylidene fluoride (PVDF) membranes. Next, the PVDF membranes were incubated with 10% BSA, followed by primary (ab246970, Abcam Cambridge, MA) and secondary antibody incubations. Finally, the protein bands were visualized by GeneSnap using SynGene system.

Statistical analysis

Data were analyzed with SPSS20.0 software (SPSS, Inc., Chicago, IL, USA) and are reported as the mean ± SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc tests, or and unpaired *t* test. $p < 0.05$ was considered statistically significant.

Results

GAS5-AS1 expression is decreased in glioma tissues and cell lines

First, we investigated GAS5-AS1 expression in normal brain and glioma tissues by northern blotting. Northern blotting results revealed that GAS5-AS1 was significantly downregulated in glioma compared to adjacent tissues (Fig. 1a, b). FISH analysis showed that GAS5-AS1 stain intensity was significantly decreased in glioma tissues, and the expression level decreased with increasing tumor grade (Fig. 1c, d). RT-PCR results further confirmed that the GAS5-AS1 level was correlated with tumour grade and in gliomas (Fig. 1e),. GAS5-AS1 expression was also significantly decreased in glioma cell lines (U251, U87MG, U373, and LN229) compared to the human astrocyte cell line NHA (Fig. 1f). As downregulation of GAS5-AS1 was more evident in U251 and U87MG cells, these cell lines were selected for subsequent experiments. To evaluate the effect of GAS5-AS1 in glioma in vitro, we transfected LV-NC and LV-GAS5-AS1 into U251 and U87MG cells. Relative GAS5-AS1 expression was markedly increased upon LV-GAS5-AS1 transfection (Fig. 1g). These results suggested that the lncRNA GAS5-AS1 is downregulated in glioma tissues and cell lines.

GAS5-AS1 overexpression inhibits cell proliferation, induces G0/G1 arrest, and suppresses glioma cell migration and invasion

To analyze the effect of GAS5-AS1 overexpression on cell proliferation, we conducted CCK8 assays using U251 and U87MG cells transfected with LV-GAS5-AS1. As shown in Fig. 2a, overexpression of GAS5-AS1 significantly suppressed cell proliferation at 72 h after transfection. Similarly, LV-GAS5-AS1 markedly decreased colony formation by U251 and U87MG cells (Fig. 2b). FACS analysis showed that GAS5-AS1 overexpression decreased the entry into the S phase of the cell cycle (Fig. 2c, d). Transwell assays were conducted to determine the effects of GAS5-AS1 on tumor migration and invasion; cell migration and invasion abilities were dramatically suppressed after LV-GAS5-AS1 transfection compared to LV-NC transfection in both U251 and U87MG cells (Fig. 2e, f). These data suggested that overexpression of GAS5-AS1 inhibited cell proliferation, migration, and invasion of glioma cells.

Upregulation of GAS5-AS1 inhibits tumor growth in vivo

To determine the effect of GAS5-AS1 on tumor growth suppression in vivo, LV-NC- or LV-GAS5-AS1-transfected U251 cells were injected into nude mice to establish xenograft models. Compared to the negative control group, GAS5-AS1 overexpression markedly decreased tumor volume and weight, as shown in Fig. 3a–c. In addition, immunohistochemical staining of Ki-67, a marker of cell proliferation ability, was lower in tumor issues derived from mice injected with LV-GAS5-AS1-transfected U251 cells than in those derived from control mice (Fig. 3d), whereas H&E staining revealed no significant changes upon GAS5-AS1 overexpression (Fig. 3d). These results indicated that upregulation of GAS5-AS1 can inhibit tumor growth in vivo.

MiR-106b-5p is targeted by GAS5-AS1

LncRNAs exert their regulatory functions by competing with endogenous miRNAs. According to miRcode (https://www.mircode.org/mircode/) results, we predicted miR-106b-5p as a potential target of GAS5-AS1 (Fig. 4b). In addition, an inverse correlation was found between the expression of miR-106b-5p and GAS5-AS1 in glioma tissues (Fig. 4a). To confirm the direct interaction between miR-106b-5p and GAS5-AS1, we employed a dual-luciferase reporter assay. MiR-106b-5p mimic significantly inhibited luciferase activity, whereas miR-106b-5p inhibitor increased luciferase activity in U251 cells transfected with the GAS5-AS1-WT reporter plasmid; there was no difference in cells transfected with GAS5-AS1-Mut (Fig. 4c). Furthermore, qRT-PCR results showed that upregulation of GAS5-AS1 resulted in a significant decrease expression of miR-106b-5p, while downregulation of GAS5-AS1 resulted in a significant increase expression of miR-106b-5p in U251 and U87MG cells (Fig. 4d, e). These data suggested a direct and negative correlation between GAS5-AS1 and miR-106b-5p.

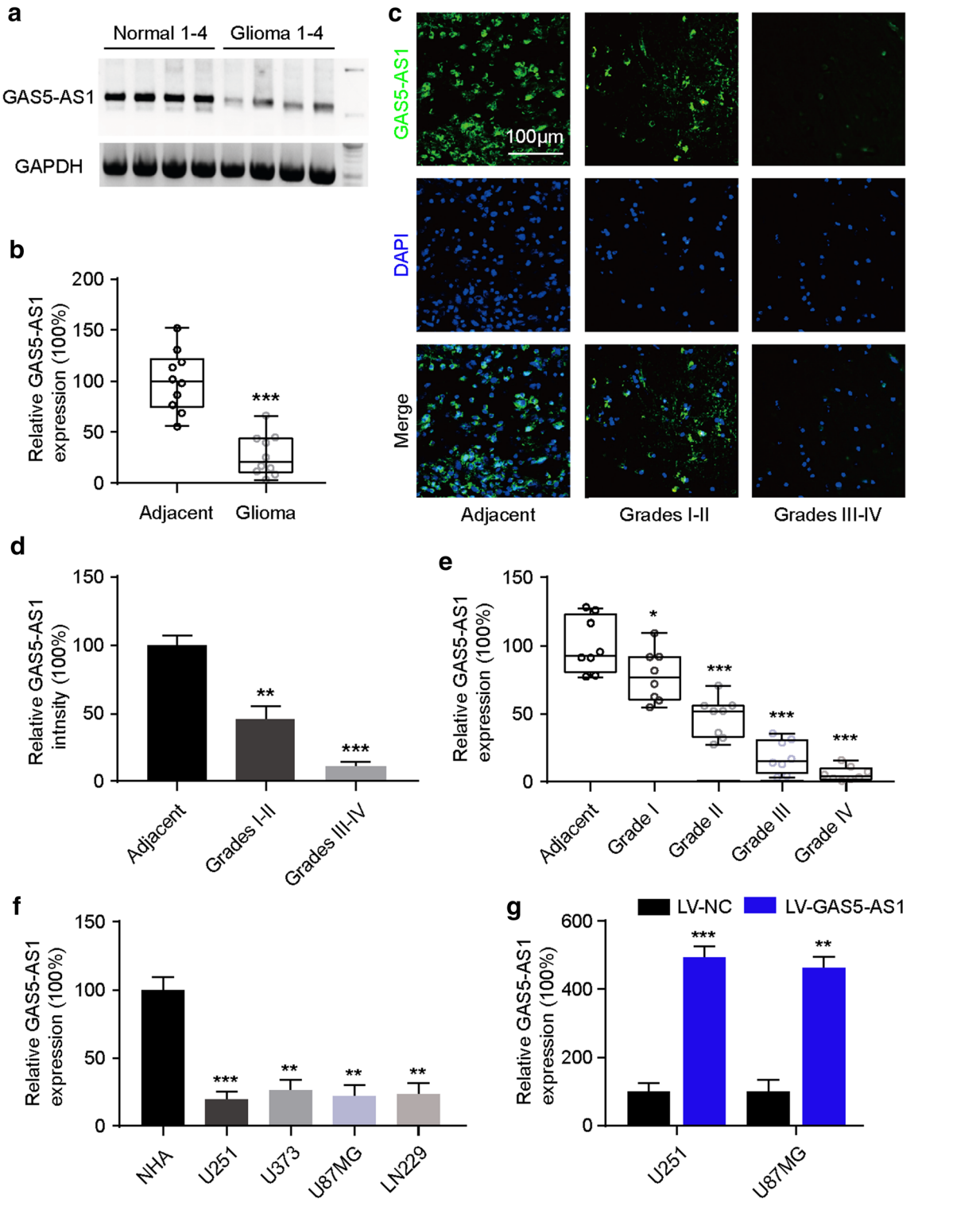


Fig. 1 Relative GAS5-AS1 expression levels in glioma tissues and cell lines. **a**, **b** Northern blot analysis of GAS5-AS1 in paired glioma tissues and adjacent normal tissues. $n = 10$. **c**, **d** Expression level of GAS5-AS1 in glioma tissues graded as I–II or III–IV and in adjacent normal tissues was analyzed by FISH. $n = 6$–8. Scale bar = 100 μm. **e** GAS5-AS1 expression in different tumor grades of glioma were analyzed by real-time PCR. $n = 10$. **f** GAS5-AS1 expression was decreased in U251, U87MG, U373, and LN229 cells. $n = 6$–8. **g** GAS5-AS1 expression was increased in U251 and U87MG cells after transfection with LV-GAS5-AS1. $n = 6$–8. One-way ANOVA was used in **d**–**f** and t tests in **b** and **g**. Data are shown as the mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. adjacent or NHA cells

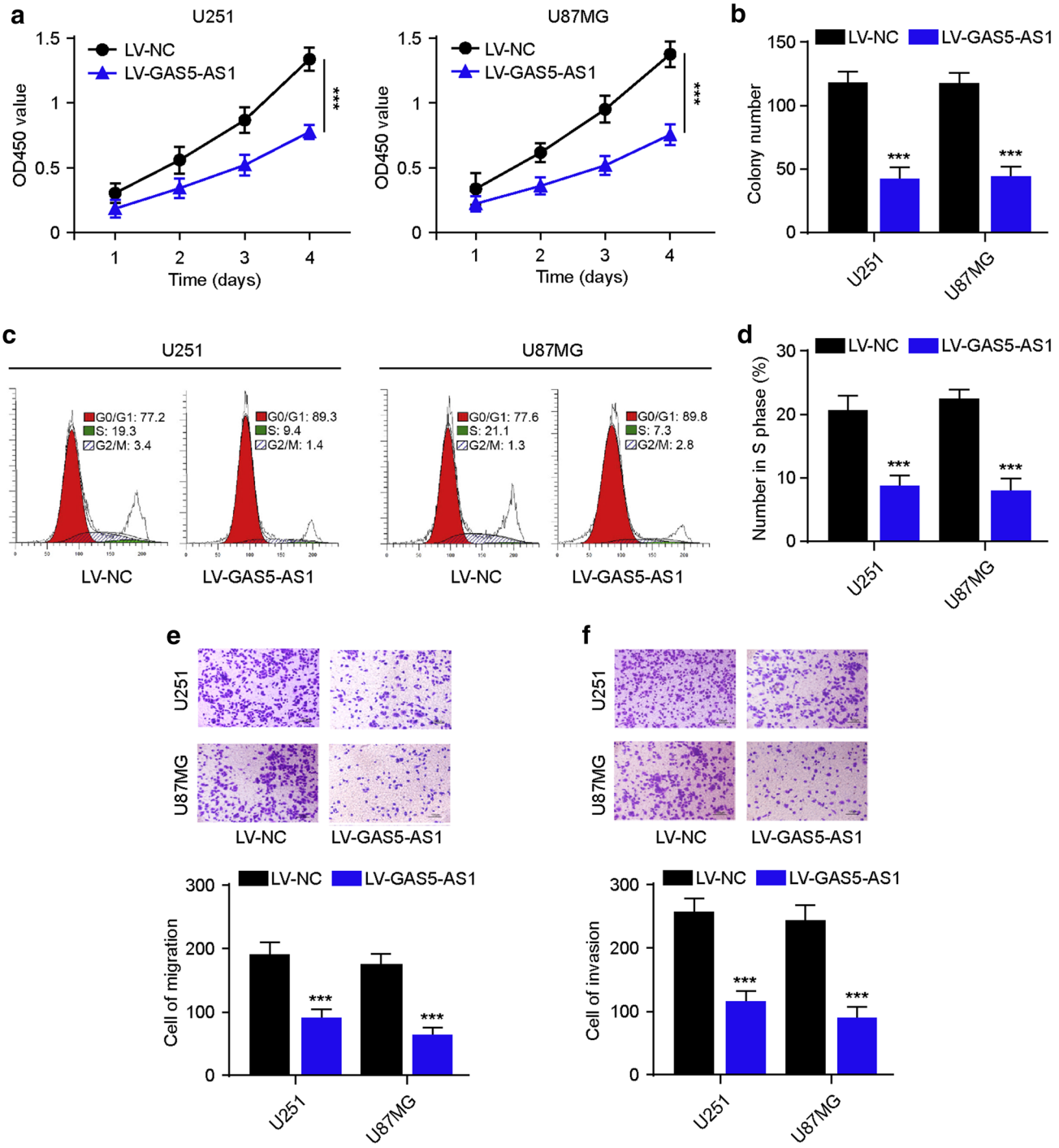


Fig. 2 Effects of GAS5-AS1 on cell growth, cell cycle, migration, and invasion. **a** CCK-8 assays were employed to assess the viability of U251 and U87MG cells after transfection with LV-NC or LV-GAS5-AS1. $n=6$–7. **b** Colony numbers of U251 and U87MG were decreased after LV-GAS5-AS1 transfection. $n=7$–9. **c**, **d** Cell cycle distribution was evaluated in U251 and U87MG cells by FACS, which showed that GAS5-AS1 overexpression decreased the number of cells in S phase. $n=7$–8. **e**, **f** Transwell assay showed that LV-GAS5-AS1 inhibited the migration and invasion of U251 and U87MG cells (×200). $n=8$–10. One-way ANOVA was used in **a**, and *t* tests in the left. Representative data of three independent experiments are shown. Data are shown as the mean ± SD. ***$p<0.001$ vs. LV-NC

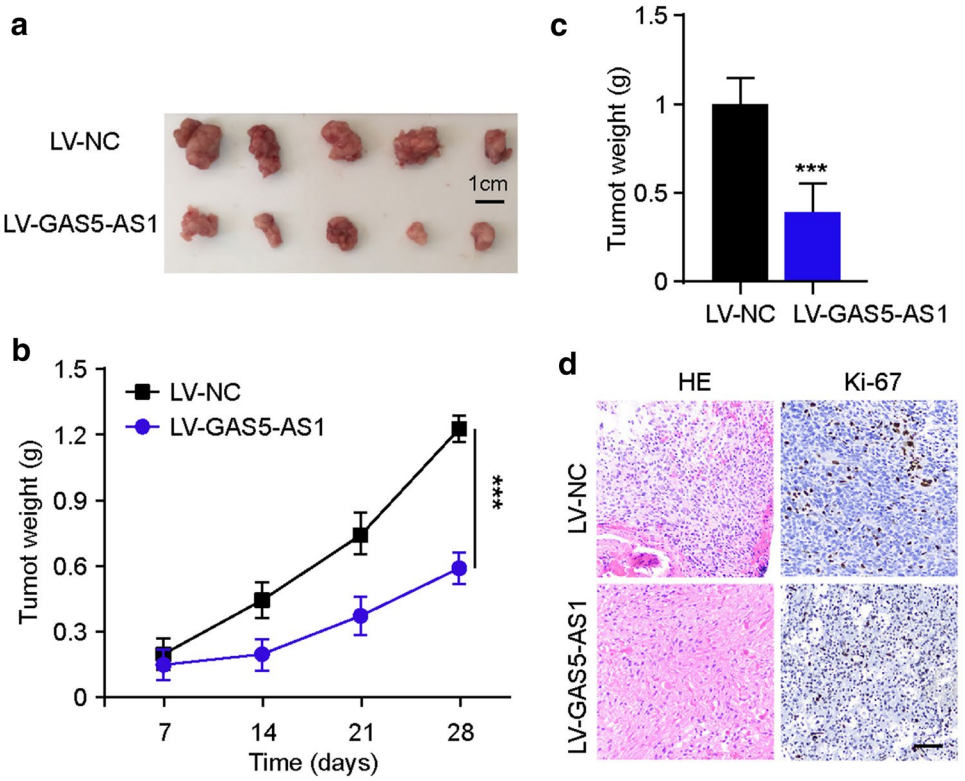


Fig. 3 GAS5-AS1 inhibits tumor growth. U251 cells transfected with LV-NC or LV-GAS5-AS1 were injected into nude mice ($n = 12$ per group), and tumor weight (**b**) and tumor volume (**c**) were measured. Images of dissected tumors (**a**) are shown, tumor volumes were measured every other 7 days, and tumor weights were measured at the end of the experiments. Scale bar = 1 cm. **d** Representative images of H&E staining and immunohistochemical Ki-67 staining are shown. Scale bar = 100 μm. *t* test was used for in **a** and one-way ANOVA was used in **b**. Data are presented as the mean ± SD, ***$p < 0.001$ vs. LV-NC

MiR-106b-5p mimic reverses the inhibitory effect of LV-GAS5-AS1 on colony formation, migration, and invasion in glioma cell lines

To investigate whether GAS5-AS1 suppresses glioma cell proliferation, migration, and invasion by targeting miR-106b-5p, we simultaneously overexpressed GAS5-AS1 and transfected miR-106b-5p mimic into U251 and U87MG cells. The number of colonies formed was decreased in U251 and U87MG cells with upregulated GAS5-AS1 and miR-106b-5p (Fig. 5a). Transwell assays showed that the migration and invasion abilities of U251 and U87MG cells were significantly inhibited by GAS5-AS1 overexpression, and abrogated by miR-106b-5p mimic (Fig. 5b, c). These results indicated that GAS5-AS1 suppresses glioma cell proliferation, migration, and invasion by targeting miR-106b-5p.

MiR-106b-5p promotes the colony formation, migration, and invasion in glioma cell lines by downregulating the expression of TUSC2

The online TargetScan (www.targetscan.org) indicated that TUSC2 might be a novel target gene of miR-106b-5p (Fig. 6a). Results of dual-luciferase reporter assay demonstrated that miR-106b -5p directly bound to the 3′-UTR of TUSC2 (Fig. 6b). Overexpression of miR-106b-5p suppressed the expression of TUSC2 in U251 cells (Fig. 6b). In addition, we examined the expression level of TUSC2 in glioma tissue and cell lines and found that TUSC2 expression was significantly decreased (Fig. 6c, d). In addition, RT-PCR validated that miR-106b-5p-mimic trasnsfection significantly reduced the mRNA level of TUSC2 in U251 cells. Furthermore, we observed that restoration of TUSC2 rescued miR-106b-5p-mimics effects on U251 cell proliferation, migration, and invasion (Fig. 6f–h). These findings suggested that miR-106b-5p exerted its roles by targeting TUSC2. Overall, the above data indicated that lncRNA GAS5-AS1 positively regulated TUSC2 through sponging miR-106b-5p, constituting the lncRNA GAS5-AS1/miR-106b-5p/TUSC2 pathway.

Discussion

Gliomas are the most common and malignant tumors occurring in the central nervous system [20]. Despite the availability of advanced surgery with radio-/chemotherapy, the 5-year survival rate of patients with advanced stage gliomas is below 6% [21]. Thus, new strategies for treating gliomas are urgently needed. Previous studies have reported that the lncRNA GAS5-AS1 plays a critical role in tumorigenesis and metastasis, Wu et al. found that the GAS5-AS1 expression was reduced in NSCLC tumors, and reduced GAS5-AS1 expression was significantly correlated with tumor size, tumor grade, and lymph node metastasis in NSCLC

patients [11]. However, its expression in gliomas tissues and the regulatory mechanism in glioma cells remained unclear.

In the present study, we found that the GAS5-AS1 expression was decreased in human glioma tissues and cell lines. Upregulation of GAS5-AS1 suppressed glioma cell proliferation and metastasis in vitro and in vivo. Our data revealed a regulatory relationship between lncRNA GAS5-AS1 and miR-106b-5p; downregulation of GAS5-AS1 was related to upregulation of miR-106b-5p in glioma tissues. Furthermore, upregulation of miR-106b-5p reversed the suppressive effects of upregulated GAS5-AS1 on cell proliferation, migration, and invasion in glioma cell lines. Our findings indicate that GAS5-AS1 might serve as a biomarker and therapeutic target in glioma treatment.

LncRNAs reportedly have an oncogenic or a tumor-suppressive role in glioma pathogenesis [22]. TUSC7 and HOTTIP, which are abnormally downregulated in glioma tissues, suppress the growth and promote the apoptosis of glioma cells [23]. GAS5-AS1 is reportedly downregulated in hepatocellular carcinoma tissues [24], and knockdown of GAS5-AS1 expression increased the migration and invasion of SPC-A1 cells [11]. However, few studies have demonstrated the regulatory functions of GAS5-AS1

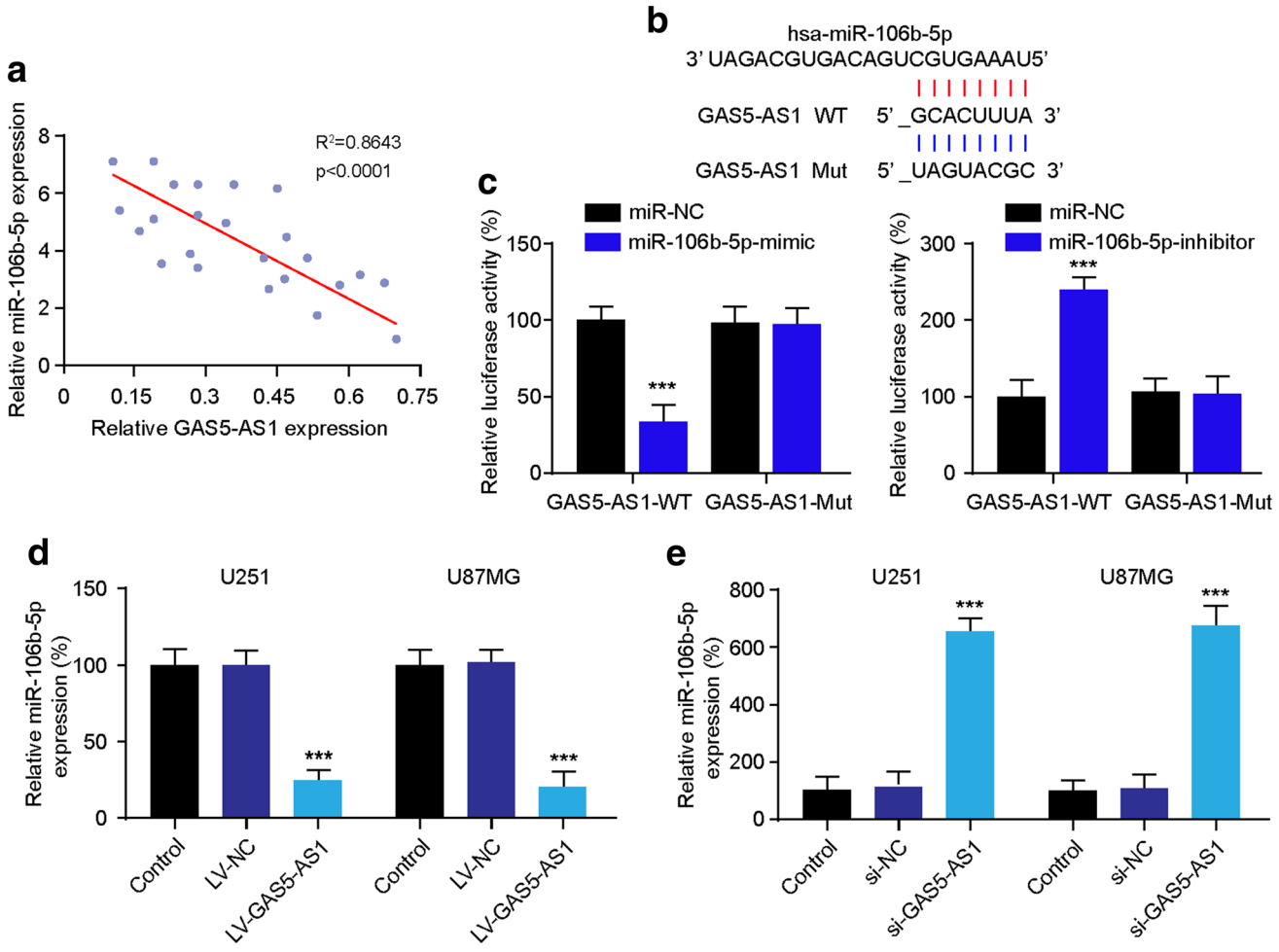
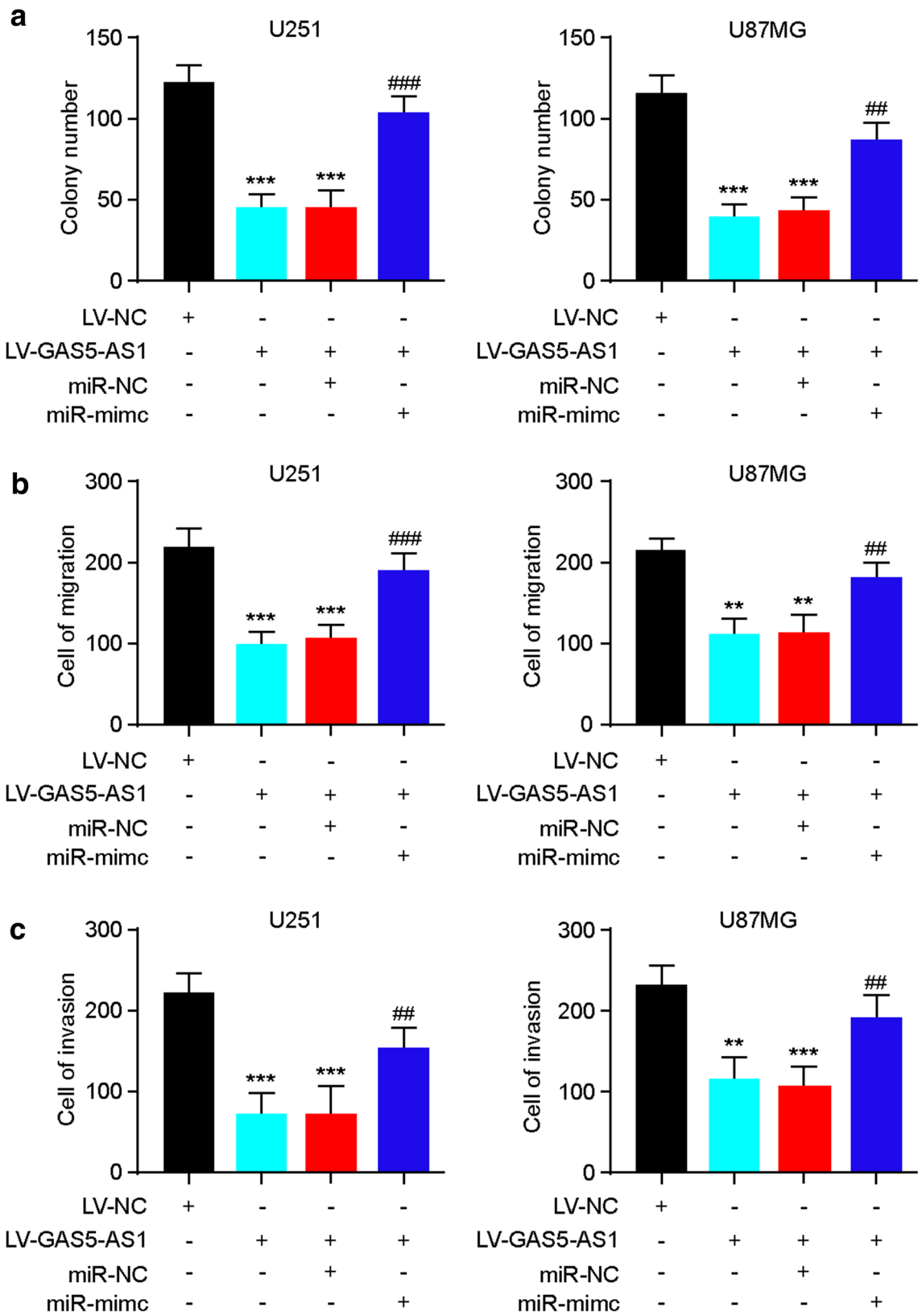


Fig. 4 miR-106b-5p directly interacts with GAS5-AS1. **a** Negative association between miR-106b-5p expression and GAS5-AS1 measured in 24 glioma patient. **b** miR-106b-5p-binding sites in GAS5-AS1 were predicted by bioinformatics analysis(LncBase Predicted v.2). **c** Luciferase reporter assays were conducted by co-transfecting miR-106b-5p mimic or inhibitor and GAS5-AS1-WT or GAS5-AS1-Mut reporter plasmid into U251 cells. $n=7$–9. MiR-106b-5p was significantly decreased in si-GAS5-AS1-transfected U251 and U87MG cells (**d**), while significantly increased in si-GAS5-AS1-transfected U251 and U87MG cells (**e**). $n=8$–10. Regression analysis was used in **a,** and *t* test was used in the following. Data are shown as the mean ± SD. ***$p<0.001$ vs. GAS5-AS1-WT or LV-NC

Fig. 5 GAS5-AS1 inhibits glioma colony formation, migration, and invasion by targeting miR-106b-5p. GAS5-AS1 and miR-106b-5p overexpression increased colony formation of U251 and U87MG cells (**a**). Transwell assays showed that decreased migration and invasion in U251 and U87MG cells caused by LV-GAS5-AS1 was reversed by miR-106b-5p mimic (**b**, **c**). One-way ANOVA was used for statistical analysis. $n=7$–10. Data are shown as the mean ± SD. **$p<0.01$, ***$p<0.001$ *vs.* LV-NC, ##$p<0.01$ vs. LV-GAS5-AS1 and miR-106b-5p inhibitor


a
U251
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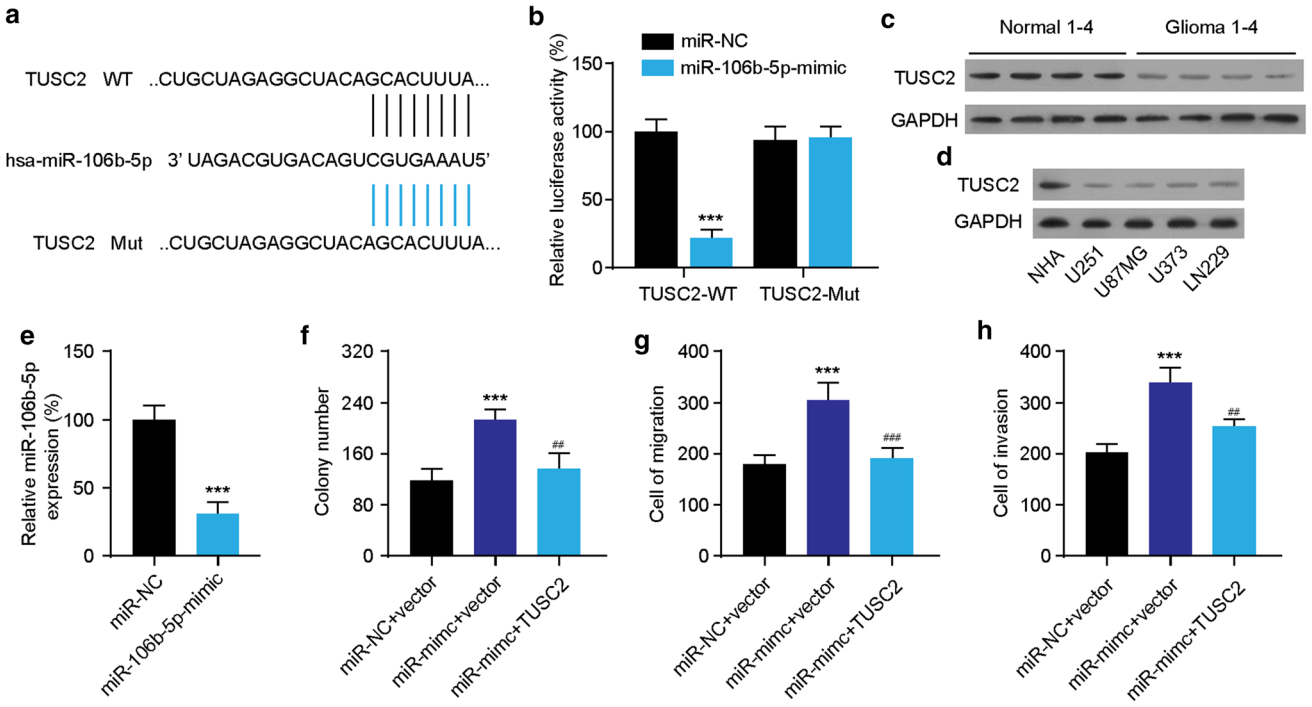

Fig. 6 TUSC2 might be the key target of GAS5-AS1/miR-106b-5p axis in glioma. **a** Predicted target sequence of miR-206-5p from TargetScan and its binding sites. **b** The binding relationship of TUSC2 to miR-106b-5p validated by dual-luciferase reporter gene assay. $n=7$–8. **c** Protein data showing the relative expression of TUSC2 in glioma tissues. **d** Protein data showing the relative expression of TUSC2 in glioma cell lines. **e** RT-qPCR data showing the relative expression of TUSC2 mRNA in miR-106n-5p upregulated U251 cells. $n=8$. **f**–**g** Restoration of TUSC2 block the effect of increased colony formation, migration, and invasion in U251 cells caused by miR-106b-5p overexpression. $n=8$–10. *t* tests was used in **b** and **e**, and one-way ANOVA was used in **f**–**h**. Data are shown as the mean ± SD. $^{**}p<0.01$, $^{***}p<0.001$ vs. miR-NC or miR-NC + vector. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. miR-mimic + vector

in gliomas. We found that GAS5-AS1 is significantly downregulated in human glioma tissues and cell lines. To explore the biological function of GAS5-AS1 in glioma progression, we conducted in vitro and in vivo assays. Our data showed that GAS5-AS1 overexpression led to significant inhibition of cell proliferation and migration and induced cell cycle arrest at the G0/G1 phase in glioma cells. Furthermore, GAS5-AS1 upregulation suppressed xenograft tumor growth in nude mice. These data indicate that high GAS5-AS1 expression inhibits the progression of glioma and that GAS5-AS1 may play a tumor-suppressor role in human glioma.

LncRNAs exhibit sequence complementarity with miRNAs to suppress or activate them [25]. GAS5, the sense RNA of GAS5-AS1, reportedly is significantly downregulated in glioma tissues, and overexpression of GAS5 by sponging miR-222-inhibited cell growth, apoptosis, and metastasis in U87 and U251 glioma cells [26]. In our study, bioinformatic analyses and luciferase reporter assays revealed that miR-106b-5p is a potential target of GAS5-AS1 in glioma cells. In addition, we observed that downregulation of GAS5-AS1 was closely related with upregulation of miR-106b-5p in glioma tissues. In this study, we confirm that miR-106b-5p is a direct target of GAS5-AS1.

Tumor suppressor candidate-2 (*TUSC2*), also known as *Fus-1*, is a novel tumour suppressor gene and its dysfunction is implicated in the molecular pathogenesis of cancer. Loss of TUSC2 expression has been observed in lung carcinoma due to either a structural destruction or post-transcriptional repression by microRNAs. TUSC2 had been reported as candidate targets for tumor metastasis by binding multiple microRNAs in breast cancer [27], ovarian cancer [28], and glioma [29]. Our laboratory previously showed that miR-106b-5p was able to downregulate retinoblastoma-like 1 (RBL1) and RBL2 translation by direct targeting [19]. In this study, our data further validate that TUSC2 is a novel target which bind to miR-106b-5p. Restoration of TUSC2 neutralized the promotion of growth and migration in miR-106b-5p transfected glioma cells.

There were some limitations to this study. Marker proteins of cell cycle arrest (cyclins A, D, E) and apoptosis (Bax, Bcl-2) were not measured in each cell cycle phase, and the percentage of cells undergoing apoptosis was not determined. Furthermore, the downstream regulator of GAS5-AS1/miR-106b-5p in glioma cells was not identified.

In conclusion, lncRNA GAS5-AS1 was suppressed, whereas miR-106b-5p was upregulated in glioma tissues and cells. Our results demonstrated that lncRNA GAS5-AS1 can bind miR-106b-5p through a sponge effect, then promoting the expression of its target gene, TUSC2, and inhibits glioma proliferation and metastasis, which may provide a promising therapeutic target for glioma.

Author contributions FL conceived and designed research; WH and YS performed experiments; BH analyzed data; QW interpreted results of experiments; BZ prepared figures; CQ drafted, edited, and revised manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of Nanjing University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of Changzhou No. 2 People's Hospital, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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