LncRNA SNHG5 Promotes Proliferation of Glioma by Regulating miR-205-5p/ZEB2 Axis

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Xiangrui Meng^{1,*}
Yanyao Deng^{2,*}
Zhicheng Lv³
Chao Liu²
Ziqing Guo²
Yuan Li²
Hua Liu²
Bing Xie²
Ziqi Jin²
Fangbo Lin²
Hongwei Zhu¹

¹Department of Gastroenterology, The Third Xiangya Hospital of Central South University, Changsha, Hunan, People's Republic of China; ²Department of Neurology, The First Hospital of Changsha, Changsha, Hunan, People's Republic of China; ³Department of Neurosurgery, Chenzhou First People's Hospital, Chenzhou, Hunan, People's Republic of China

*These authors contributed equally to this work

Background: Glioma is a common primary brain tumor with extremely poor prognosis outcomes. Increasing evidences have proved the relation between lncRNAs and glioma onset and progression. LncRNA SNHG5 involves in the biological activities of tumor cells, such as proliferation, migration and metastasis. Nevertheless, it is still necessary to explain the molecular mechanism and biofunction of SNHG5 in glioma.

Materials and methods: Quantitative real-time PCR (qRT-PCR) was performed to analyze expressions of SNHG5, miR-205-5p and ZEB2 in tumor tissues and cell lines. The cell counting kit-8 (CCK-8) assay, plate and soft agar colony formation assays were performed to evaluate cell proliferation ability. RNA immunoprecipitation assay and dual-luciferase reporter assay were used to confirm the interaction among SNHG5, miR-205-5p and ZEB2. The protein level of ZEB2 was measured by Western blot.

Results: Based on our findings, compared with normal tissues, the elevated expression of SNHG5 and decreased expression of miR-205-5p were observed in glioma tissues. The downregulation of SNHG5 exerted an obvious inhibitory effect on glioma cells in terms of their proliferation. With regard to the underlying mechanism, SNHG5 presented a direct inhibitory influence on miR-205-5p which targeted to the 3'-UTR region of zinc finger E-box binding homeobox 2 (ZEB2) mRNA. As a competing endogenous RNA (ceRNA), SNHG5 sponged miR-205-5p, regulating the expression of ZEB2 thereby.

Conclusion: These discoveries indicate that SNHG5 promotes proliferation of glioma by regulating miR-205-5p/ZEB2 axis.

Keywords: long non-coding RNA, SNHG5, miR-205-5p, ZEB2, glioma

Introduction

Glioma is a common primary intracranial tumor, thought to derive from the neuroglial stem or progenitor cells.¹ Although the relevant reports are moderately rare, it may cause significant morbidity and mortality. Glioblastoma in particular is the most malignant type of glioma. Patients with glioblastoma have a 5-year survival rate of less than 5%.² Despite advances in treatment modalities, it is still largely incurable.³ As an extensively infiltrative tumor of the central nervous system, patients with glioma own a median survival time of less than 18 months for glioma cells can rapidly migrate from primary lesions to nearby normal brain tissue and distant sites.⁴ Malignant progression, including proliferation, migration and invasion, can critically lead to poor clinical prognosis and usually is the important cause of the failure in realizing total resection using surgery.⁵

Despite of a large number of studies on the molecular mechanisms of glioma malignant progression, scholars have not yet completely explained the interaction

Correspondence: Hongwei Zhu
Department of Gastroenterology, The Third Xiangya Hospital of Central South University, No. 138 Tongzipo Road, Yuelu District, Changsha, Hunan Province, People's Republic of China
Tel +86-13657351235
Fax +86-15526491006
Email zhw_xysy@163.com

of non-coding RNAs (ncRNAs) and mRNAs as a bridge in a potential network. ncRNAs exhibit a close relation to tumor growth, development, migration and invasion, as well as drug resistance.^{6,7} Biofunctions of ncRNAs involved in glioma have been characterized by many studies, and solicitous attention has been paid to microRNAs (miRNAs) together with long non-coding RNAs (lncRNAs).^{8,9} lncRNAs, characterized as ncRNAs with the length of over 200 nucleotides, are now known to have numerous capacities, acting as decoys that bind to proteins or miRNAs and as scaffolds or guides that regulate the interactions between proteins and genes.¹⁰ The transcription of lncRNAs may either positively or negatively affect the downstream gene expressions by directly interfering with the promoters or adjusting the structure of chromatin.¹¹ Recent investigations demonstrate that interactions between lncRNAs and miRNAs in glioma are able to inter-regulate their own expressions, in this way forming a complex regulatory system that greatly affects cell pathophysiological processes.^{12,13}

The abnormal expression of lncRNA SNHG5 has been uncovered in various tumors. An obvious up-regulation of SNHG5 has been revealed in colorectal carcinoma, with a positive association with tumor proliferation and poor survival.¹⁴ In addition, this ceRNA in the osteosarcoma can promote the T tumorigenesis, further modulating miR-26a expression.¹⁵ For all this, the expression and the possible function of SNHG5 in glioma remain unknown. In this study, SNHG5 has been identified and characterized as a steady cytoplasmic lncRNA which is up-regulated in glioma, and its role in glioma has also been investigated.

MiRNAs as small ncRNAs bind to 3'-UTR regions of mRNAs, regulating expressions of genes thereby.¹⁶ It is reported that miRNAs are involved in many human carcinogenesis including glioma.¹⁷ MiR-205-5p, a gene located on 1q32.2 locus of the human genome with multiple biofunctions, has been illuminated to participate in decreasing tumor chemoresistance by cooperatively repressing E2F1.¹⁸ Moreover, miR-205-5p is recognized as a prognostic biomarker of pancreatic cancer, prostate cancer and bladder cancer.^{19,20} We have predicted that miR-205-5p has putative binding sites of lncRNA SNHG5, based on our search in lncRNASNP2 database (<http://bioinfo.life.hust.edu.cn/lncRNASNP2>). Nevertheless, data about whether miR-205-5p can influence biological processes of glioma are limited.

ZEB2 belongs to the ZEB family of the 2-handed zinc finger/homeodomain proteins and shows a close association with epithelial-mesenchymal transition (EMT) to promote tumor initiation and development.^{21,22} We speculate that ZEB2 perhaps is an assumed target of miR-205-5p based on the miRNA target prediction software TargetScan (<http://www.targetscan.org/>). According to previous studies, ZEB2 promotes tumor cell migration through EMT in triple-negative breast cancer.²³ However, we are ambiguous about how ZEB2 affects glioma.

In this study, we aimed at investigating the expression of SNHG5, miR-205-5p and ZEB2 in glioma. We also explored the roles in regulating the glioma malignant behavior and the potential molecular pathways among them. These findings will shed a new light on the precise treatment for glioma.

Materials and Methods

Clinical Samples

Twenty glioma samples were obtained from patients who experienced surgical resection and 14 normal brain tissues were obtained from individuals who died in traffic accidents from January 2016 to December 2018 in the Department of Neurosurgery at the Chenzhou First People's Hospital. Glioma specimens had been pathologically diagnosed and were divided into low grade group (stage I–II, *n*=9) and high grade group (stage III–IV, *n*=11) according to the WHO classification system by at least 2 experienced clinical pathologists. None of these patients underwent chemotherapy or radiotherapy before surgery. The study was conducted in accordance with the Declaration of Helsinki. All patients and traffic accident victims provided written informed consent for the use of their specimens and disease information. The project was approved by the Chenzhou First People's Hospital. All samples were stored in the liquid nitrogen following resection until they were used.

Cell Culture

The human glioma cell lines LN229, A172, U251 and U87, as well as normal human astrocytes (NHA) were obtained from ATCC. We maintained and cultured all cells in Dulbecco's Modified Eagle Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone) without antibiotics at 37°C in a humidified atmosphere of 5% CO₂.

Cell Transfection

All constructs used to knock down SNHG5 were purchased from RiboBio (Guangzhou, China). The siRNA sequences were as follows: si-SNHG5-1: 5'-GCUCUGAAGAUGCAAAGAUUU-3'; si-SNHG5-2: 5'-CCTCTGGTCTCATCTGCATATTGACTTA-3'. MiRNA mimics and miRNA inhibitors were synthesized by Genepharma Company (Shanghai, China). The miR-205-5p mimics sequences were as follows: sense: 5'-UCCUUCAUUCCACCGGAGUCUC-3' and antisense: 5'-GACUCCGGUGGAAUGAAGGAUU-3'. The mimics control sequences were as follows: sense: 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense: 5'-ACGUGACACGUUCGGAGAATT-3'. The sequence of the miR-205-5p inhibitor was: 5'-CAGACUCCGGUGGAAUGAAGGA-3'. The control sequence of the inhibitor was: 5'-CAGUACUUUUGUGUAGUACAA-3'. LipofectamineTM3000 transfection reagent (Invitrogen) was applied to the construction of cell transfection according to the manufacturer's instructions.

Quantitative Real-Time PCR

Trizol (Invitrogen) and TRIzolTM LS Reagent (Invitrogen) were used to extract total RNAs from cells and plasma, respectively. RNA was extracted based on the manufacturer's instructions. For lncRNA and mRNA, cDNA was generated with the help of a PrimeScriptTM RT reagent Kit (TaKaRa); real-time PCR was implemented relying on the TB GreenTMPremix Ex TaqTM II (TaKaRa); GAPDH was applied as the internal control. For miRNA, cDNA was generated with the help of a miRcute Plus miRNA First-Strand cDNA Kit (TIANGEN); real-time PCR was performed using a miRcute Plus miRNA qPCR Kit (SYBR Green); U6 small nuclear RNA (snRNA) was adopted as the internal control. The real-time PCR was carried out by the ABI 7500 real-time PCR system (Applied Biosystems). RNA relative expression was calculated with the $2^{-\Delta\Delta C_t}$ method. All primers used in this study were synthesized by Sangon Biotech (Shanghai, China). Primer sequences were as follows: SNHG5, forward: 5'-CGCTTGGTTAAACCTGACACT-3' and reverse: 5'-CCAAGACAATCTGGCCTCTATC-3'; ZEB2, forward: 5'-AATGCACAGAGTGTGGCAAGGC-3' and reverse: 5'-CTGCTGATGTGCGAACTGTAGG-3'; GAPDH, forward: 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse: 5'-ACCACCCTGTTGCTGTAGCAA-3'. miR-205-5p forward: 5'-UCCUUCAUUCCACCGGAGUCUG-3'; U6 snRNA forward: 5'-CTCGCTTCG

GCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'. The miRcute Plus miRNA qPCR Kit (SYBR Green) supplied the reverse primer of miR-205-5p.

RNA Isolation from Nucleus and Cytoplasmic Fractions

Nucleus and cytoplasmic fractions were isolated by the PARISTM Kit (Invitrogen) based on the manufacturer's protocol. In brief, experimenters collected cells, lysed the cells using the cell fractionation buffer, and separated nucleus and cytoplasmic fractions by centrifugation. Experimenters collected supernatant which contained cytoplasmic fractions and then transferred them to a fresh tube without RNase. The nucleus pellet was lysed using a cell disruption buffer. The nucleus lysate and cytoplasmic fraction were mixed with the 2× lysis/binding solution, followed by adding 100% ethanol. A filter cartridge helped draw the sample mixture and washing solution wash it. RNAs of nucleus and cytoplasmic fractions were eluted by the elution solution. U6 snRNA served as the positive control for the nucleus fraction and GAPDH served as the positive control for the cytoplasmic fraction.

Cell Proliferation Assay

The cell proliferation was detected by the CCK-8 (Dojindo) assay. Cells were seeded in 96-well plates (5×10^3 cells/well) and treated under various transfection conditions. We added 20 μ L of CCK-8 to each well at 24 h, 48 h, and 72 h after transcription. The Elx800 (BioTek) was used to measure each sample regarding the absorbance at 490 nm. The viability of cells was calculated based on absorbance values.

Western Blot

Total proteins were extracted from glioma cells using a BCA protein assay kit (Sigma) and protein concentrations were detected. Proteins were fractionated using 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE; Sigma). The separated protein was transferred to the PVDF membrane (Bio-Rad), which were incubated with ZEB2 antibody (Cat No. 14026-1-AP; proteintech) and GAPDH (Cat No. 25778; Santa Cruz Biotechnology) at 4°C overnight. Then, HRP-conjugated secondary antibody was used to incubate blotted membranes for 2 h at room temperature. Signals were visualized using ECL substrates (Millipore).

Luciferase Reporter Assay

We predicted the putative binding sites of miR-205-5p in the 3'-UTR of SNHG5/ZEB2. The pMIR-REPORTTM (RiboBio) which contained wild type (WT) or mutant (MT) 3'-UTR sequences of SNHG5/ZEB2 was used to operate the dual-luciferase reporter assay. Transient co-transfection in cells (1×10^5) with the miR-205-5p mimic or the negative control accompanied by WT or MT SNHG5/ZEB2 3'-UTR vector. After the 48h transfection, we harvested the cells and used the Luc-PairTM Duo-Luciferase Assay Kit (Yeasen) to determine the luciferase results.

RNA Immunoprecipitation Assay

A Magna RNA-Binding Protein Immunoprecipitation Kit (Millipore) was adopted to implement the RNA immunoprecipitation. Briefly, a RIP buffer which contained magnetic beads was used to incubate whole-cell lysates, which were conjugated with normal mouse IgG (Millipore) or human anti-Ago2 antibody (1:50, Millipore), used as a negative control. All samples were incubated by the proteinase K buffer. Immunoprecipitated RNAs were extracted and expressions of SNHG5 and miR-205-5p were analyzed by qRT-PCR.

Soft Agar

The transfected cells were seeded in 6-well plates at 1000–2000 cells per well and incubated at 37°C for 2–4 weeks. Suspension cells was cultured in an upper layer of 0.35% agarose (Lonza Rockland) in DMEM supplemented with 10% FBS. The cells suspension was overlaid with 0.5% basal agar and 10% FBS in a 6-well plate and placed at room temperature until the agarose solidified. Agars were destained with phosphate-buffered saline prior to photo-capturing. Only cell mass with more than 50 cells was counted as a colony and representative photos of the assay were shown in the result section.

Colony Formation Assay

The transfected cells were seeded in 6-well plates with the culture medium containing 10% FBS. They were cultured overnight. After 14-day incubation, cells were fixed with methanol and stained with 0.1% crystal violet. Colonies were manually counted under a light microscope.

Statistical Analysis

All statistical analyses were performed in the SPSS 21.0 software. Each experiment was independently performed 3

times, with the results expressed as the Mean $\pm$ SD. The two-tailed Student's *t*-test or one-way ANOVA was adopted to estimate differences between the aforesaid two groups. Correlations were analyzed by Spearman rank correlation. A *P* value of < 0.05 was considered as statistical significant.

Results

LncRNA SNHG5 Was Up-Regulated in Glioma and Promoted Glioma Cells Proliferation

For identifying deregulated lncRNA SNHG5, the expression was profiled in glioma from a cohort of 20 glioma tissues and 14 adjacent normal tissues, as well as 4 glioma cell lines. As a result, we found that SNHG5 in glioma cells, including A172 ($p < 0.05$), U87 ($p < 0.05$), especially LN229 ($p < 0.01$), and U251 ($p < 0.01$), exhibited obviously higher expression levels compared with NHA (Figure 1A). In clinical samples, SNHG5 exhibited a remarkable increase in glioma tissues than that in normal tissues and the expression level positively correlated with tumor grades (Figure 1B). The expression level of SNHG5 was remarkably decreased due to SNHG5 knockdown with the specific SNHG5 siRNA. We found that SNHG5 was successfully knocked down in LN229 and U251 cell lines after transfecting si-SNHG5-2 rather than si-SNHG5-1 by qRT-PCR. Therefore, we chose si-SNHG5-2 for the following assays (Figure 1C). Effects of SNHG5 on glioma cell proliferation would be further determined. According to results of the proliferation ability by CCK-8 assay, the downregulation of SNHG5 remarkably weakened the proliferation ability of both LN229 and U251 cells, compared with the si-NC group (Figure 1D). These data suggested that the up-regulation of SNHG5 in glioma was accompanied by an inhibitory effect on glioma cell proliferation.

LncRNA SNHG5 Directly Interacted with miR-205-5p

Experiments were then implemented for further investigating the latent mechanism of SNHG5 in glioma cells. We observed the cytoplasmic enrichment of SNHG5 (Figure 2A), and found that SNHG5 could reduce target mRNA repression as molecular sponges for miRNAs. Based on experimental results, the binding sequence of miR-205-5p was complementarily shared by that of the 3'-UTR of SNHG5 (Figure 2B). Furthermore, the dual-luciferase reporter assay revealed the direct targeting of miR-205-5p to SNHG5-WT, rather than SNHG5-MT (Figure 2C). The expression of miR-205-5p reduced in glioma

Figure 1 LncRNA SNHG5 was up-regulated in glioma and promoted proliferation in LN229 and U251 cells. **(A)** qRT-PCR analysis of lncRNA SNHG5 in NHAs, A172, LN229, U87 and U251 cell lines. **(B)** qRT-PCR analysis of SNHG5 in 9 low grade (stage I-II) glioma tissues, 11 high grade (stage III-IV) glioma tissues and 14 normal tissues. **(C)** LN229 and U251 cells were transfected with SNHG5 siRNA (si-SNHG5) or control siRNA (si-NC), and SNHG5 expression was detected by qRT-PCR. **(D)** CCK-8 assay was performed to evaluate cell proliferation. * $P < 0.05$, ** $P < 0.01$.

Abbreviations: qRT-PCR, quantitative reverse transcription PCR; NHAs, normal human astrocytes; CCK-8, Cell Counting Kit-8.

tissues compared with normal tissues, and the expression levels negatively correlated with tumor grades (Figure 2D). The correlation between SNHG5 and miR-205-5p was then analyzed. Pearson correlation analysis revealed the negative association between SNHG5 and miR-205-5p in glioma cells (Figure 2E). In addition, miR-205-5p expression increased in SNHG5 knockdown glioma cells, which was further proved by transfecting with the miR-205-5p inhibitor (Figure 2F). Whether the RNA-induced silencing complex (RISC) where SNHG5 lied was the same as that of miR-205-5p was determined by an RNA immunoprecipitation (RIP) assay. Results showed that levels of SNHG5 and miR-205 in anti-Ago2 group were higher than those in the anti-normal IgG group (Figure 2G). These findings suggested that SNHG5 directly interacted with miR-205-5p.

SNHG5 Exerted Its Functions via Negatively Regulating miR-205-5p Expression

To expound whether the role of SNHG5 in glioma was dependent on miR-205-5p, glioma cells were transfected with si-SNHG5 or co-transfected with si-SNHG5 and

the miR-205-5p inhibitor. As shown in Figure 3A–C, the miR-205-5p inhibitor attenuated the si-SNHG5-induced proliferation of glioma cells. This indicated that SNHG5 negatively modulated miR-205-5p, facilitating glioma cells proliferation thereby.

SNHG5 Acted as a ceRNA for miR-205-5p to Regulate ZEB2 Expression

The downstream target of miR-205-5p was further identified using a bioinformatics method and ZEB2 was screened out (Figure 4A). It was revealed that miR-205-5p remarkably repressed the activity of ZEB2-WT reporter using a luciferase reporter assay (Figure 4B). In clinical samples, we found that ZEB2 mRNA expression was elevated in glioma tissues compared with normal tissues, and the expression levels positively correlated with tumor grades (Figure 4C). Then we analyzed the correlation between ZEB2 and miR-205-5p by Pearson correlation analysis, and there was a negative correlation between them (Figure 4D). Furthermore, the involvement of miR-205-5p in the regulatory effect of SNHG5 on ZEB2 was investigated. It was found that the expression of ZEB2 was remarkably reduced due to SNHG5 downregulation. However, the miR-205-5p inhibitor revised this effect (Figure 4E–F).

Figure 2 LncRNA SNHG5 targeted miR-205-5p. (A) qRT-PCR analysis of SNHG5 RNA levels percentage of nuclear and cytoplasmic. (B) Potential sites targeted by miR-205-5p in lncRNA SNHG5 sequence. (C) Luciferase activity in LN229 and U251 co-transfected with miR-205-5p mimics and luciferase reporters containing SNHG5-WT or SNHG5-MT transcript. ** $P < 0.01$ vs miR-NC group. (D) qRT-PCR was used to analyze the expression of miR-205-5p in 9 low grade (stage I-II) glioma tissues, 11 high grade (stage III-IV) glioma tissues and 14 normal tissues. (E) Pearson's correlation scatter plot of SNHG5 and miR-205-5p in glioma tissues. (F) LN229 and U251 were transfected with si-SNHG5 or co-transfected with si-SNHG5 and miR-205-5p inhibitor. Relative expression of miR-205-5p was detected via qRT-PCR assay. (G) RNA immunoprecipitation analysis for the enrichment of SNHG5 and miR-205-5p in anti-Ago2 group and anti-IgG as control. * $P < 0.05$, ** $P < 0.01$. **Abbreviation:** qRT-PCR, quantitative reverse transcription PCR.

These results suggested that SNHG5 promoted ZEB2 expression by sponging miR-205-5p in glioma.

Discussion

This study demonstrated the roles of lncRNA SNHG5 in glioma. It was found that lncRNA SNHG5 was overexpressed in glioma tissues and SNHG5 knockdown inhibited glioma development with repressed tumor cell proliferation. Furthermore, SNHG5-induced upregulation

of ZEB2 expression was initiated by the direct binding of SNHG5 to miR-205-5p, facilitating glioma progression thereby. Our findings expound the significant role of SNHG5/miR-205-5p/ZEB2 pathway in glioma.

Glioma is an aggressive and common primary brain tumor in adults, accounting for about 70% of all primary malignant brain tumors.^{24,25} Despite the development of new treatments, the survival rate of glioma especially glioblastomas still remains low with a median survival of

Figure 3 SNHG5 exerted its functions via negatively regulating miR-205-5p expression. (A–C) U251 and LN229 were transfected with si-SNHG5 or co-transfected with si-SNHG5 and miR-205-5p inhibitor. Cell proliferation ability was analyzed by CCK-8 assay, plate and soft agar colony formation assays. * $P < 0.05$.

Abbreviation: CCK-8, Cell Counting Kit-8.

15 months following the standard treatment.²⁶ Thus, more new therapeutic targets become a hotspot for glioma treatment.²⁷ Growing evidences indicate the important biofunction of lncRNAs in tumor onset and progression.⁹ However, the first imperative is still to explore the possible molecular mechanism of lncRNAs in glioma before achieving therapeutic effects on this malady.

The data in the study have revealed the positive correlation between lncRNA SNHG5 and human glioma progression, and SNHG5 knockdown can inhibit the proliferation of the tumor cells. The same effect has been reported in other researches. Baoming He has reported that SNHG5, acting as a ceRNA for miR-205-5p, is up-regulated in the isolated peripheral blood

cells of CML patients in comparison with the healthy controls, increasing the imatinib resistance in CML thereby.²⁸ In a previous study, the overexpression of SNHG5 in colorectal cancer (CRC) can promote the proliferation, invasiveness and metastasis of CRC cells by regulating the miR-132-3p/CERB5 axis.²⁹ Our findings indicated the important role of SNHG5 in promoting glioma malignancy.

LncRNAs finely modulated expressions of genes via intricate ways, such as translation and splicing, transcriptional regulation, post-transcriptional regulation, as well as epigenetic modulation.³⁰ LncRNAs as ceRNAs can bind to their common miRNAs and crosstalk with mRNAs. For detecting the possible mechanism of

The downstream targets of miR-205-5p have been predicted using the bioinformatics screening, and ZEB2 has been screened out. Data from the luciferase reporter assay has revealed the post-transcriptional regulation of ZEB2 by miR-205-5p through a direct binding. Glioma cells are developmentally derived from the neuroepithelial lineage. Thus, they do not undergo the traditional EMT like the classical epithelial cells. Nonetheless, glioma adopts a phenotype that could be considered mesenchymal and therefore the term “EMT-like” process has been proposed.³⁴ As an EMT-inducing transcription factor, ZEB2 is also related with EMT-like in GBM.³⁵ Previous research has found that ZEB2 was associated with tumorigenicity in glioma and knockdown of ZEB2 expression inhibited cell proliferation, migration, and invasion of glioma cells.^{36,37} In our study, ZEB2 was overexpressed in glioma tissues. SNHG5 knockdown can suppress the expression of ZEB2, and the inhibition of miR-205-5p can partially reverse the effect, suggesting that SNHG5 can bind to miR-205-5p and regulate ZEB2 expression thereby.

Conclusion

Our research has elucidated the function of SNHG5 and its molecular mechanism in malignant glioma progression. Briefly, the SNHG5 knockdown-induced overexpression of miR-205-5p down-regulates ZEB2, resulting in inhibited malignant biological behavior in glioma. This study, for the first time, stresses the importance of the interaction among SNHG5, miR-205-5p and ZEB2. In addition, SNHG5/miR-205-5p/ZEB2 axis as a promising therapeutic target will inspire more effective treatments for human glioma.

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Disclosure

The authors report no conflicts of interest in this work.

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