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LncRNA SNHG1 facilitates the epithelial-mesenchymal transition and invasiveness in glioblastoma multiforme via regulation of the miR-128- 3p/RRAS2 axis

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Abstract

Purpose The present study aimed to identify the role of long non-coding RNA (lncRNA) small nucleolar RNA host gene 1 (SNHG1) in the progression of glioblastoma multiforme (GBM) and the regulatory mechanism of the SNHG1/miR-128-3p/RRAS2 network.

Methods The TargetScan method was used to analyze the target mRNAs and associated miRNAs of SNHG1. The levels of SNHG1, miRNAs, and mRNAs were determined in glioma and normal brain tissues based on The Cancer Genome Atlas. Pathway analysis was used to identify the significant pathways of the predicted target genes of SNHG1 according to the Kyoto Encyclopedia of Genes and Genomes. The expressions of SNHG1 in GBM cells were determined by real-time quantitative polymerase chain reaction (qRT-PCR). A scratch wound-healing and Transwell assays were used to determine GBM cell migration and invasion. The dual-luciferase reporter assay was used to validate the miR-128-3p target relationships with lncRNA SNHG1. Western blotting was performed to determine whether SNHG1 and RRAS2 regulated the epithelial-mesenchymal transition (EMT) process of GBM cells.

Results SNHG1 was upregulated in glioma tissues and cells. Knockdown of SNHG1 inhibited migration and invasion of GBM cells. A competing endogenous RNA interaction network of SNHG1-6miRNAs-17mRNAs was constructed and revealed the functional role of the SNHG1/miR-128-3p/RRAS2 feed-forward loop. MiR-128-3p expression was downregulated in glioma tissues, whereas RRAS2 expression was elevated in glioma tissues. QRT-PCR and dual luciferase reporter analyses of the results indicated that SNHG1 bound to miR-128-3p, while SNHG1 expression was negatively regulated by miR-128-3p. Additionally, miR-128-3p regulated the expression of RRAS2 mRNA. Moreover, knockdown of RRAS2 inhibited GBM cell migration and invasion. Collectively, SNHG1 promoted GBM progression by sponging miR-128-3p and regulating RRAS2 expression. In addition, down-regulations of SNHG1 and RRAS2 significantly reduced the expressions of mesenchymal markers, N-cadherin and vimentin, while it increased the protein level of E-cadherin epithelial marker, showing that silencing SNHG1 reversed the EMT phenotype.

Conclusion Our findings confirmed that lncRNA SNHG1 promoted the malignant progression of GBM by the SNHG1/miR-128-3p/RRAS2 axis, which activated the EMT process. The SNHG1/miR-128-3p/RRAS2 axis therefore may provide a novel therapeutic treatment for GBM.

Introduction

Glioma is a common primary intracranial malignancy of neuroepithelial origin, notorious for its high recurrence and high patient mortality (Moon et al. 2020; Hervey-Jumper and Berger. 2019). Glioblastoma multiforme (GBM) is the most malignant and most common type of brain tumor, contributing to approximately half of all gliomas (Protopapa et al. 2018). In recent decades, treatments of GBM have made great advances in surgical resection, radiotherapy, and chemotherapy. However, due to its strong invasion and metastasis, the prognoses of GBM patients remain dismal (Ma etal. 2021; Rius-Rocabert et

al. 2020). Therefore, there is an urgent need to identify the molecular mechanisms responsible for the development of GBM, in order to discover new therapeutic targets.

Long non-coding RNA (lncRNA) is a type of functional long RNA, that cannot encode for proteins, with a length less than 200 bases (Ali and Grote 2020; Liang et al. 2020). LncRNAs usually regulate gene expression at the post-transcriptional level and play an important role in regulating tumorigenesis. Among many lncRNA studies, we found that the small nucleolar RNA host gene 1 (SNHG1), located on chromosome 11q12.3, was aberrantly highly expressed in many tumors, and it has become an indicator of prognoses in many types of cancers (Li et al. 2020; Tan et al. 2021). MicroRNAs (miRNAs) are small endogenous RNA molecules of approximately 20 nucleotides in length that do not encode proteins. They post-transcriptionally regulate gene expression and play a key role in influencing cellular processes and promoting cancer progression (Tsuji et al. 2020; Lu and Rothenberg. 2018; Ferragut et al. 2020). Recently, it was reported that some lncRNAs acted as competitive endogenous RNAs (ceRNAs) that bound to miRNA sequences through specific mRNA sequences, and played important regulatory roles in tumor development (Wang et al. 2020; Yang et al. 2020). LncRNAs can also be involved in targeted miRNA degradation, shaping miRNA pools, and in translation (Ang et al. 2020).

Studies found that highly expressed SNHG1 was a ceRNA, binding to miRNAs to promote malignant progression of gliomas. For example, Li et al (Li et al. 2019) found that SNHG1 regulated the expression of FOXP2 and KDM5B by binding to miR-154-5p and miR-376b-3p, which in turn regulated the malignant behavior of glioma cells. In addition, Mi et al (Mi et al. 2020) found that SNHG1, which was highly expressed in gliomas, was positively correlated with FtMt and competitively bound miR-9-5p to promote glioma development and angiogenesis. Based on these results, we constructed ceRNA networks with SNHG1, and differentially expressed miRNAs and downstream target genes using bioinformatics methods. Among these SNHG1-associated miRNAs and target genes, we identified binding sites for SNHG1 to miR-128-3p and miR-128-3p to RRAS2. MiR-128-3p is one of the most abundant miRNAs in the human brain (Shao et al. 2010). In addition, it has been shown that miR-128-3p was upregulated in vascular smooth muscle expression and affected vascular smooth muscle cell (VSMC)proliferation, migration, differentiation, and contraction (Farina et al. 2020). However, it has been reported that miR-128-3p expression inhibited the malignant progression of gliomas, but LncRNA PVT1 inhibited miR-128-3p expression, thereby decreasing the inhibitory effect of miR-128-3p on GREM1 expression, and promoting the proliferation and metastatic potential of glioma cells (Fu et al. 2018). RRAS2, also known as recombinant human related RAS virus (R-Ras) oncogene homolog 2, is a GTP-binding protein, which together with RRAS1 and RRAS3, forms part of the R-Ras GTPase subfamily (Ehrhardt et al. 2002). RRAS2 has been implicated in the pathogenesis of human cancers. One study confirmed that RRAS2 played an important role in the development and late metastasis of breast cancer (Larive et al. 2014). Another study found that overexpression of RRAS2 in human central nervous system tumors promoted the malignant progression of nervous system tumors (Gutierrez-Erlandsson et al. 2013). More importantly, we found that RRAS2 may be involved in and contribute to the epithelial-mesenchymal transition (EMT). The EMT is a typical program of developmental phenotypic plasticity controlled by the transformation of the epithelial cell phenotype to mesenchymal cells (Das et al. 2019). It has been

reported that the EMT enhanced invasiveness, spread to distant sites, and was involved in metastatic colonization, cancer stemness, and chemoresistance (Williams et al. 2019). By exploiting the EMT process, tumor cells enhance their plasticity and metastatic potential, thus acquiring favorable characteristics for their survival and reproduction (Kröger et al. 2019).

In the present study, we verified the endogenous expression of lncRNA-SNHG1 in GBM cells and investigated its important role in the biological behavior in these same cells. By constructing ceRNA networks through bioinformatics analyses, we identified the SNHG1/miR-128-3p/RRAS2 axis pathway, and confirmed that SNHG1 promoted the EMT process through the SNHG1/miR-128-3p/RRAS2 axis, which in turn facilitated malignant progression of GBM. To our knowledge, this is the first report that SNHG1 was found to promote the EMT process through regulating RRAS2 expression in GBM, which revealed a novel potential mechanism for regulating the biological behavior of GBM cells, as well as providing new ideas for targeted treatment of human GBM.

Materials And Methods

Construction and functional analysis of a CeRNA network

Data for miRNA-LncRNA SNHG1 and LncRNA SNHG1-mRNA interactions were obtained from StarBase, version 2.0. Before analyzing the data downloaded from StarBase, version 2.0, duplicates were filtered out. Then, differential miRNAs and mRNAs were filtered out by analyzing 156 GBM tissues and five normal brain tissues in The Cancer Genome Atlas (TCGA) database. Next, correlations between differential miRNAs and mRNAs were obtained from StarBase, version 2.0. Differential mRNAs with down-regulated expressions were then analyzed for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichments. The analysis revealed that some mRNAs were involved in up-regulated pathways. Next, miRNAs negatively correlated with these mRNAs were selected and multiple SNHG1-miRNA-mRNA interaction networks were constructed.

Cell culture

The human GBM A172 and U251 cell lines, and glioma H4 and HS683 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and normal human astrocytes (HA) were obtained from ScienCell Research Laboratories (San Diego, CA, USA). A172 and U251 cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS, Gibco). H4 and HS63 cells were grown in high glucose Dulbecco's Modified Eagle Medium (Gibco) containing 10% fetal bovine serum (Gibco). HA cells were grown in astrocyte medium (Sciencell Research Laboratories) supplemented with 10% fetal bovine serum (Sciencell Research Laboratories), and all cell lines were cultured at 37°C in an environment containing 5% CO_2.

Reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of the cell lines was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). The cDNA was reverse transcribed from the extracted RNA after removal of genomic DNA using a PrimeScript™ RT kit (Takara Bio, Shiga, Japan). The qRT-PCR was performed using an Applied Biosystems 7500 Real Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using a SYBR Green PCR Master Mix (Takara) under the following conditions. The initial activation step was performed for 30 s at 95°C for 40 cycles, denaturation at 94°C for 5 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. GAPDH was used as an internal control. MiRNA was reverse transcribed using the miDETECT A Track™ miRNA RT kit (Ribobio, Guangzhou, China) based on the polyA tailing method. Quantitative RT-PCR was performed using the Applied Biosystems 7500 Real Time PCR System (Thermo Fisher Scientific), using the miDETECT A Track™ miRNA 2X SYBR Green PCR Master Mix (Ribobio) under the following conditions: an initial activation step of 95°C for 30 s, 40 cycles, denaturation at 94°C for 5 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. U6 was used as an internal control. The qPCR was repeated three times. Data were analyzed using the $2^{-\Delta\Delta CT}$ method. The primers for amplification of each gene and its internal reference gene are shown in Table 1.

Table 1
Primer sequences used for quantitative RT-PCR

Primers	Sequence (5'-3')
SNHG1	F*: CCCAGACTTTGCTGCCTTTCTTACA
	R#: AGAGGAACAGACACGAAGTGGAGTT
RRAS2	F: TAAAGGATCGTGATGAGTTCCCA
	R: AATTTCCTGATAACCCGGACAAG
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: TGGTGAAGACGCCAGTGGA
miR-128-3p	F: GTTGGATTCGGGGCCGTAG
	R: AAGCAGCTGAAAAAGAGACCG
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCG

*F = forward; #R = reverse

Western blot analysis

Cells were lysed on ice with RIPA buffer containing 1% protease inhibitor, followed by extraction of total protein. The concentration of total protein was determined using a BCA kit (Solarbio, Beijing, China) and adjusted to an acceptable concentration. Total proteins were then separated by 10% (SDS-PAGE) (Solarbio) and then transferred to polyvinylidene difluoride membranes. Membranes were incubated with 5% skim milk (Solarbio) for 2 h, and then N-cadherin (1:1,000), E-cadherin (1:1,000), vimentin (1:1,000),

and β-Actin primary antibodies (1:1,000) were added and incubated at 4°C overnight. The membranes were then washed to remove the primary antibody, and then horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:2,000) was added and incubated for 1 h at 25°C. Antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Chemiluminescence was detected using an electrochemiluminescence kit (Calan Biotechnology, Shanghai, China) and a Bio-Rad ChemiDoc MP imaging system (Yihui Biotechnology, Shanghai, China). Grayscale values were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Vector construction and cell transfection

All transfections were performed in A172 and U251 cells. Three targeted inhibited SNHG1 RNAs, three targeted inhibited RRAS2 RNAs, and negative control RNA were purchased from GenePharma (Shanghai, China). MiR-128-3p mimics and miR-NC were synthesized by RiboBio. A172 and U251 cells were inoculated into 6-well plates the day before transfection, ensuring a cell density of 30%–50% for the next day of transfection. Transfections were performed according to the Lipofectamine 2000 (Invitrogen) manufacturer's instructions. Then, glioblastoma cells were cultured in DMEM medium containing 10% FBS for 24 – 48 h. Finally, the cells were collected and transfection efficiency was determined by the quantitative real-time polymerase chain reaction (qRT PCR) or western blot analysis.

Transwell migration and invasion assays

To assess the migration and invasion potential of cells, we used 8 μm pore size Transwell chambers (Corning, Corning, NY, USA). The Transwell chambers were covered or uncovered with 50 μL Matrigel (1:10 dilution; BD Biosciences, San Jose, CA, USA). The transfected cells were harvested and dissociated into single cell suspensions. Next, approximately 50,000 cells in 100 μL of serum-free medium were added to the upper chamber, and 500 μL of medium containing 20% FBS was added to the lower chamber. After 24 h of incubation, the cells above the membrane were removed, and the cells that penetrated the membrane were fixed with methanol plus acetone and stained with 1% Crystal Violet. Cells that underwent migration or invasion were counted using at least three randomly selected microscope fields.

Luciferase reporter assay

The SNHG1 fragment containing the miR-128-3p binding site was cloned into the downstream site of the pmiR-RB-ReportTM dual luciferase miRNA target gene and the 3′-UTR plasmid vector Renilla luciferase (*hRlc*) gene to construct the SNHG1 wild type reporter vector (SNHG1 Wt:UUGUGUGUGAUAGCACUGUGG). Similarly, the corresponding mutant with the miR-128-3p binding site was synthesized to construct the SNHG1 mutantreporter vector (SNHG1 Mut: UUGUGUGUGAUAGGUGACACG). The dual luciferase reporter plasmids of WT-SNHG1 or MUT-SNHG1 and miR-NC or miR-128-3p mimics were then co-transfected into A172 and U251 cells using Lipofectamine 2000 (Invitrogen). At a total of 24 h after transfection, the relative luciferase activity was measured using a dual luciferase reporter kit (Promega, Milwaukee, WI, USA). Relative luciferase activity was expressed as the ratio of Renilla luciferase activity to firefly luciferase activity.

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) was used to analyze the data and for graphing. Data are shown as the mean ± standard deviation (SD) of at least three independent experiments, and were analyzed using a two-tailed, unpaired Student's *t*-test. A value of $p < 0.05$ was considered a significant difference. The values of $p < 0.05$, $p < 0.01$, and $p < 0.001$ are indicated by *, **, and ***, respectively, in the figures.

Results

LncRNA SNHG1 was overexpressed in glioblastoma tissues and cells

To determine the expression levels of SNHG1 in GBM, we examined the expression levels of SNHG1 in tumor tissues from 156 GBM patients and five normal brain tissues in TCGA. The results showed that SNHG1 was significantly up-regulated in GBM tissues compared with that in normal brain tissues (Fig. 1a). Next, we screened a cohort of glioma cell lines for their SNHG1 expressions and showed that the majority of the glioma cells had elevated expressions, when compared to human astrocytes (HAs), especially in the A172 and U251 GBM cells (Fig. 1b). Together, the results showed that SNHG1 was highly expressed in GBM tissues and cells, and that it may be related to the genesis of GBM.

LncRNA SNHG1 enhanced GBM cells invasion and induced the EMT response

GBM cells show highly aggressive character. To investigate the effects of reduced expression of SNHG1 on cell migration and invasion, wound healing and Transwell assays were performed for two GBM cell lines. The SNHG1 expression was repressed by RNA interference. We measured SNHG1 levels in A171 and U251 cells after treatment with siSNHG1-1, siSNHG1-2, or siSNHG1-3. The results indicated that siSNHG1-1, -2, and − 3 caused visible reductions in SNHG1 levels, with a greater inhibitory effect of siSNHG1-3, when compared to siSNHG1-1 and siSNHG1-2 in the two cell lines (Fig. 2a). The wound healing and Transwell migration assays indicated that SNHG1 knockdown significantly inhibited the migration capacity of GBM cells, when compared with the control group (Fig. 2b), and showed that the cell invasion ability was decreased in SNHG1-inhibiting GBM cell lines (Fig. 2c). Taken together, these results indicated an important promoting role for SNHG1 in GBM migration and invasion.

Because the invasive ability of tumor cells is related to the EMT, we used western blot assays to assess the expression of EMT markers. Knockdown of SNHG1 was associated with increased production of the E-cadherin epithelial marker and reduced production of the vimentin and N-cadherin mesenchymal markers (Fig. 2d), showing that SNHG1 promoted GBM cells during the EMT.

Construction of the lncRNA SNHG1-miRNA-mRNA ceRNA network

It has been previously shown that some lncRNAs acted as molecular sponges in ceRNA networks to bind to miRNAs and regulate their expressions (Peng et al. 2018; Zhang et al. 2018). Based on these results, we hypothesized that SNHG1 may act as a ceRNA and bind to miRNAs, which may also bind to downstream target genes to regulate the progression of glioma. First, 120 miRNAs targeting SNHG1 and 161 mRNAs associated with SNHG1 were found through bioinformatics analyses in the ENCORI website (https://starbase.sysu.edu.cn/) (Supplemental Tables 1, 2). Second, we analyzed the expression levels of 120 miRNAs and 161 mRNAs in 156 GBM tissues and five normal brain tissues based on TCGA database. A total of 80 mRNAs were identified that had differential expression in the GMB group, when compared with the normal brain group (Fig. 3a, Supplemental Table 3, $p < 0.05$). The five miRNAs were up-regulated (FC > 1.2) and 26 miRNAs were down-regulated (FC < 0.9) in the GBM group (Supplemental Table 4). Of the 80 mRNAs, 17 up-regulated mRNAs (with a FC > 2, Fig. 3b, Supplemental Table 5) were selected for further function analyses. KEGG pathway analysis was used to analyze the significant pathway of 17 mRNAs. The results indicated that CXCL3, CASP7, and RRAS2 mRNAs participated in 10 up-regulated pathways, which involved diverse biological processes, including environmental information processing (signal transduction), human diseases (cancer), and cellular processes (cell motility and cell growth and death) (Fig. 3c). RRAS2 played a key role in tumor progression, and was involved in six pathways, including MAPK, cAMP, ras, proteoglycans in cancer, cellular senescence, and regulation of the actin cytoskeleton signaling pathway (Fig. 3d). It was found that 17 up-regulated mRNAs were associated with six miRNAs by the ENCORI website (Table 2). The miR-181band miR-128-3p were then selected, whose expression levels were negatively correlated with SNHG1, CXCL3, CASP7, and RRAS2 mRNAs to obtain four SNHG1/miRNA/mRNA interaction networks (Fig. 3e). The roles of the SNHG1/miR-128-3p/RRAS2 regulation network in GBM were further detected.

Table 2
17mRNAs corresponding to six miRNAs

mRNAs	miRNAs		
LMNB1	hsa-miR-1323	hsa-miR-128-3p	hsa-miR-7-5p
DEPDC1B	hsa-miR-1323	hsa-miR-128-3p	hsa-miR-181b
TSPAN12	hsa-miR-1323	hsa-miR-128-3p	hsa-miR-421
WDR76	hsa-miR-1323	hsa-miR-182	hsa-miR-421
FNDC3B	hsa-miR-1323	hsa-miR-181b	hsa-miR-181b
CXCL3	hsa-miR-128-3p	hsa-miR-182	hsa-miR-7-5p
PHLDA1	hsa-miR-181b	hsa-miR-181b	hsa-miR-421
RBMS1	hsa-miR-1323	hsa-miR-7-5p	hsa-miR-181b
MSI1	hsa-miR-128-3p	hsa-miR-128-3p	hsa-miR-421
VANGL1	hsa-miR-1323	hsa-miR-182	hsa-miR-181b
TRIM4	hsa-miR-1323	hsa-miR-7-5p	hsa-miR-421
TMPO	hsa-miR-181b	hsa-miR-182	hsa-miR-128-3p
NRP2	hsa-miR-1323	hsa-miR-7-5p	hsa-miR-7-5p
CASP7	hsa-miR-128-3p	hsa-miR-182	
RRAS2	hsa-miR-1323	hsa-miR-421	
MEX3C	hsa-miR-128-3p	hsa-miR-128-3p	
CRYBG1	hsa-miR-1323	hsa-miR-7-5p	
	hsa-miR-1323	hsa-miR-181b	
	hsa-miR-181b		
	hsa-miR-1323		
	hsa-miR-181b		
	hsa-miR-128-3p		
	hsa-miR-1323		
	hsa-miR-7-5p		

LncRNA SNHG1 functioned as a sponge of miR-128-3P and miR-128-3p expression and was down-regulated in GBM tumors

We used the online software, RNAhybrid, to predict the target sequence of SNHG1 for miR-128-3p. SNHG1 was found to contain complementary binding sites to miR-128-3p seed regions (Fig. 4a). To determine whether SNHG1 directly interacted with miR-128-3p, luciferase reporter plasmids containing the wild-type or mutated miR-128-3p binding sites in SNHG1 were constructed, as shown in Fig. 4b, and co-transfected with miR-control or miR-128-3p mimics into A172 and U251 cells. The transfection efficiencies of miR-128-3p mimics were estimated by qRT-PCR (Fig. 4c). The luciferase reporter assay showed that overexpression of miR-128-3p significantly reduced the luciferase activity of SNHG1-WT, but not that of SNHG1-MUT (Fig. 4d). Taken together, these results indicated that SNHG1 directly interacted with miR-128-3p. The effects of miR-128-3p on expression of SNHG1 were detected using qRT-PCR. Compared with the control group, SNHG1 expression in the miR-128-3p mimics group was significantly decreased in both A172 and U251 cells (Fig. 4e). Together, these results showed miR-128-3p targeted and regulated SNHG1 expression.

We also determined miR-128-3p expressions in the WHO II, III, and IV grade tissues from glioma patients from the Chinese Glioma Genome Atlas (CGGA) database. The expression of miR-128-3p in GBM tissues (WHO IV) was lower than that in low grade glioma tissues (WHO II and III) (Fig. 4f), suggesting the involvement of miR-128-3p in the progression of GBM.

SNHG1 regulated RRAS2 expression

As a first step in determining whether SNHG1 regulated the expression of RRAS2, we determined RRAS2 expressions in large cohorts of GBM patients available from TCGA database. The results showed that RRAS2 expression levels were significantly increased in GBM tissues (from 528 patients) compared with those in 10 normal brain tissues (Fig. 5a). Further statistical analyses showed a significant and positive correlation between *SNHG1* and *RRAS2* gene expressions (Fig. 5b). Thus, we concluded that increased expression of RRAS2 may play an important role in GBM progression and development. Next, we investigated changes in RRAS2 mRNA after silencing of SNHG1 by RT-qPCR. Knockdown of SNHG1 resulted in a decrease in RRAS2 mRNA (Fig. 5c), indicating that SNHG1 regulated RRAS2 expression.

MiR-128-3p targeted and regulated RRAS2, and knockdown of RRAS2 suppressed GBM cell migration, invasion, and affected the EMT

We identified the binding site of miR-128-3p on the RRAS2 3′-UTR using RNAhybrid analysis (Fig. 6a). To examine whether miR-128-3p was able to directly regulate RRAS2 expression, A172 and U251 cells were transfected with miR-128-3p mimics. The level of RRAS2 mRNA was determined using qRT-PCR. Compared with the NC group, RRAS2 expression was downregulated after overexpression of miR-128-3p (Fig. 6b).

Our previous data revealed a higher level of RRAS2 in GBM tumors. We therefore examined the migration and invasion ability and EMT markers of GBM cells after RRAS2 down-regulation. First, the knockdown efficiency of siRRAS2 was estimated by qRT-PCR. The siRRAS2-2 showed a greater inhibitory effect of RRAS2, when compared with siRRAS2-1 and siRRAS2-3 in A172 and U251 cell lines (Fig. 6c). Next,

Transwell migration and invasion arrays were performed to investigate whether RRAS2 affected GBM cell migration and invasion. The results showed that RRAS2 knockdown significantly inhibited the migration and invasion of GBM cells, when compared with the control group (Fig. 6d). Finally, the effects of RRAS2 on protein expression of vimentin, N-cadherin, and E-cadherin were determined using western blotting. Compared with the control group, vimentin and N-cadherin protein expression in the siRRAS2 group significantly decreased, whereas E-cadherin protein expression significantly increased (Fig. 6e). Taken together, the results showed that reduction of RRAS2 inhibited the induction of the EMT through decreased expression of E-cadherin and increased expression of vimentin in GBM.

Discussion

In the present study, lncRNA SNHG1 was highly expressed in human glioma tissues and cell lines. There existed a regulatory relationship between lncRNA SNHG1 and miR-128-3p, as well as between miR-128-3p and RRAS2. Compared with normal brain tissues, miR-128-3p was down-regulated, whereas RRAS2 was up-regulated in glioma tissues. Overexpression of RRAS2 promoted the proliferation and metastatic potential of glioblastoma cells, whereas the miR-128-3p mimic targeted and inhibited the expression of RRAS2. Furthermore, both SNHG1 and RRAS2 facilitated the EMTprocess in GBM cells. Finally, we showed that lncRNA SNHG1 acted as a sponge of miR-128-3p and promoted the EMT process in GBM by regulating RRAS2, which in turn enhanced the malignant progression of GBM.

In recent years, increasing evidence has shown that lncRNAs play a critical role in the development of different tumors, as well as being prognostic markers for tumor patients (Shuai et al. 2020; Marín-Béjar et al. 2017). Small nucleolar RNA host genes (SNHGs) are newly recognized lncRNAs, which have become the focus of current oncology studies. For example, lncRNA SNHG6 is overexpressed in primary high grade ductal breast cancers, and it may promote the proliferation, migration and EMT of breast cancer cells (Jafari-Oliayi and Asadi 2019). Moreover, lncRNA SNHG1 promotes sorafenib resistance in hepatocellular carcinoma (HCC) by activating the Akt pathway, its nuclear expression is promoted by miR-21, and its nuclear translocation is induced by sorafenib, suggesting that SNHG1 may be a valuable target for overcoming sorafenib resistance to HCC (Li et al. 2019). In addition, Wang et al. reported that SNHG1 was upregulated in gliomas, which predicted poor prognosis (Wang et al. 2017). However, the role and mechanism of SNHG1 in glioma still needs to be further characterized. In the present study, we found that SNHG1 expression was upregulated in both glioma tissues and cell lines, which is consistent with the Wang findings, which indicated that SNHG1 may play an important role in glioma genesis and development.

GBM is the most common and serious malignancy among intracranial primary gliomas, which is incurable owing to its highly invasive and metastatic potentials (Shergalis et al. 2018). There is therefore an urgent need to find ways to control the invasive and metastatic ability of GBM. In this study, we performed functional experiments in two GBM cell lines, A172 and U251. Our results showed that the proliferation, migration, and invasive abilities of GBM cells were effectively inhibited after SNHG1 knockdown, suggesting that SNHG1 functioned as an oncogene in GBM.

MiR-128-3p is also known as an miR-128 and its precursor is miR-128-1 (Hu et al. 2014). MiR-128-3p plays an important role in tumor development (Chen et al. 2019). For example, Zhao et al (Zhao et al. 2019) found that overexpression of miR-128-3p suppressed the expression of LIMK1, thereby inhibiting breast cancer progression. Simultaneously, they also found that patients with high expression levels of miR-128-3p may have a better prognosis. In addition, Shi et al (Shi et al. 2012) found that overexpression miR-128 suppressed the expression of p70S6K1, thereby attenuating glioma development and progression. This result was similar to the findings of our present study, in which miR-128-3p overexpression suppressed SNHG1 expression and also regulated RRAS2 expression, thereby affecting GBM development and progression.

Previous studies had shown that many lncRNAs bound and interacted with miRNAs as ceRNAs, which in turn regulated downstream target genes (Wang et al. 2021; Liu et al. 2019). For example, SNHG1 activated the oncogene HOXA1 in breast cancer cells by repressing the expression of miR-193a-5p, thereby promoting malignant progression of breast cancer (Li et al. 2020). Based on these results, we conducted an in-depth mining of the regulatory mechanism of SNHG1 as ceRNA in gliomas. Bioinformatics prediction helped us to identify six down-regulated miRNAs and 17 up-regulated mRNAs associated with SNHG1. KEGG pathway analysis was used to analyze the significant pathway of 17 mRNAs. From this analysis, we found that RRAS2 played a key role in tumor progression. RRAS2 is a member of the RAS superfamily of GTPases (Drivas et al. 1990). RRAS2 controls a variety of cellular processes, including proliferation, survival, and migration, and its dysfunction had been shown to contribute to tumorigenesis (Capri et al. 2019). For example, RRAS2 partially attenuates the effect of the anticancer drug JQ1 in NMC tumor cells by maintaining ERK pathway function during oncogene BRD4 inhibition, thereby promoting the progression of midline cancers in the NUT (Liao et al. 2018). In our research, we confirmed that the expression of RRAS2 was also decreased after SNHG1 knockdown in GBM cells. This suggested the potential regulatory effect of SNHG1 on RRAS2. Furthermore, knockdown of RRAS2 also effectively inhibited the proliferation, migration, and invasion ability of GBM cells, indicating that RRAS2 may function as an oncogenic gene in GBM.

EMT is a transformation process that causes cells to lose their epithelial properties and acquire mesenchymal cell characteristics, which plays an important role in primary tumor formation and metastasis (Bakir et al. 2020). E-cadherin, N-cadherin, and vimentin proteins play key roles in the EMT. E-cadherin is a tumor suppressor protein whose major effect in normal cells involves inhibiting cancer cell metastasis, and the loss of E-cadherin expression has been correlated with the EMT in the progression of cancer metastasis (Na et al. 2020). The expression of N-cadherin is increased, which promotes tumor cell shedding, leading to tumor cell invasion and metastasis (Cao et al. 2019). Cancer cells expressing the intermediate filament protein, vimentin, are more motile and invasive *in vitro*, and also more metastatic *in vivo* (Thompson et al. 1992). Furthermore, it was found that EMT-related induction factors played important roles in the malignant progression of glioma (Tao et al. 2020). In the present study, the expression of the EMT marker proteins E-cadherin, N-cadherin, and vimentin were lowered after SNHG1 and RRAS2 knockdown in GBM cells, suggesting that SNHG1 and RRAS2 regulated the EMT. To our knowledge, this is the first report that SNHG1 regulated the EMT process in GBM.

Nevertheless, there were several limitations of this study. To better explain the regulatory mechanism of lncRNA SNHG1 in glioblastomas, *in vitro* experiments in nude mice need to be further investigated. In addition, the regulatory mechanism of the SNHG1/miR-128-3p/RRAS2 network in other biological functions of glioblastoma cells remains to be further investigated.

In the present study, we found miR-128-3p up-regulation after SNHG1 knockdown in GBM. Both SNHG1 and RRAS2 expressions were down-regulated after overexpression of miR-128-3p in GBM cells. These results suggested that SNHG1 and miR-128-3p had antagonistic effects in the pathological process of GBM, and that miR-128-3p also had a dampening effect on the expression of its downstream target RRAS2. In addition, luciferase reporter gene experiments further confirmed the close relationship between SNHG1 and miR-128-3p. Taken together, our results suggested that SNHG1 regulated the EMT process through the SNHG1/miR-128-3p/RRAS2 axis, which in turn promoted the malignant progression of GBM.

Conclusions

In summary, this study showed that SNHG1 was upregulated in glioma tissues and cells. This study demonstrated for the first time that SNHG1 knockdown inhibited the EMT in GBM cells by regulating the miR-128-3p/RRAS2 axis. Importantly, the regulatory mechanism of SNHG1 promoted the malignant progression of GBMthrough activation of the EMT process by the SNHG1/miR-128-3p/RRAS2 axis, providing a basis for the mechanism of GBM development and targeted therapy for human GBM.

Declarations

Funding This study was supported by the grant from the Natural Science Foundation of Jilin Province (No. 20200201491JC).

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions XYH, YQW and QL contributed to the idea, drafting and editing of the manuscript. XCY and WDZ completed statistical analysis. RPZ, ZJS and HTW contributed to literature search and data analysis. XYH and QL revised the manuscript with critical reviews and comments and all the authors approved the final version.

Data availability statement The raw data used and analyzed in the current study are available from the corresponding author upon a reasonable request.

Disclosure TCGA belong to public databases. The patients involved in the database have obtained ethical approval. Users can download relevant data for free for research and publish relevant articles. Our study is based on open source data, so there are no ethical issues and other conflicts of interest.

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Figures

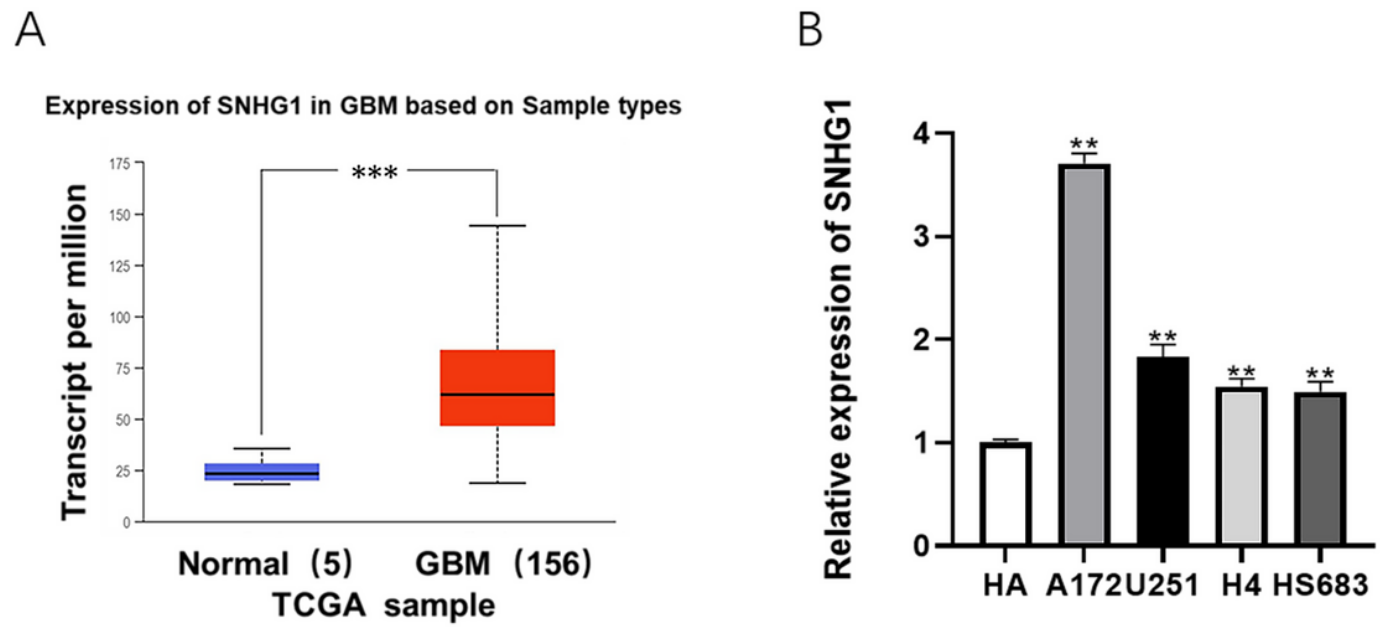

Figure 1

The expression level of lncRNA SNHG1 in glioblastoma tissues and glioma cells. **a** The expression level of SNHG1 in GBM tissues and normal tissues from TCGA. **b** SNHG1 levels in human astrocytes HA and glioblastoma cells (A172 and U251) and low grade glioma cells (H4 and HS683) ($^{**}p < 0.01$, $^{***}p<0.001$)

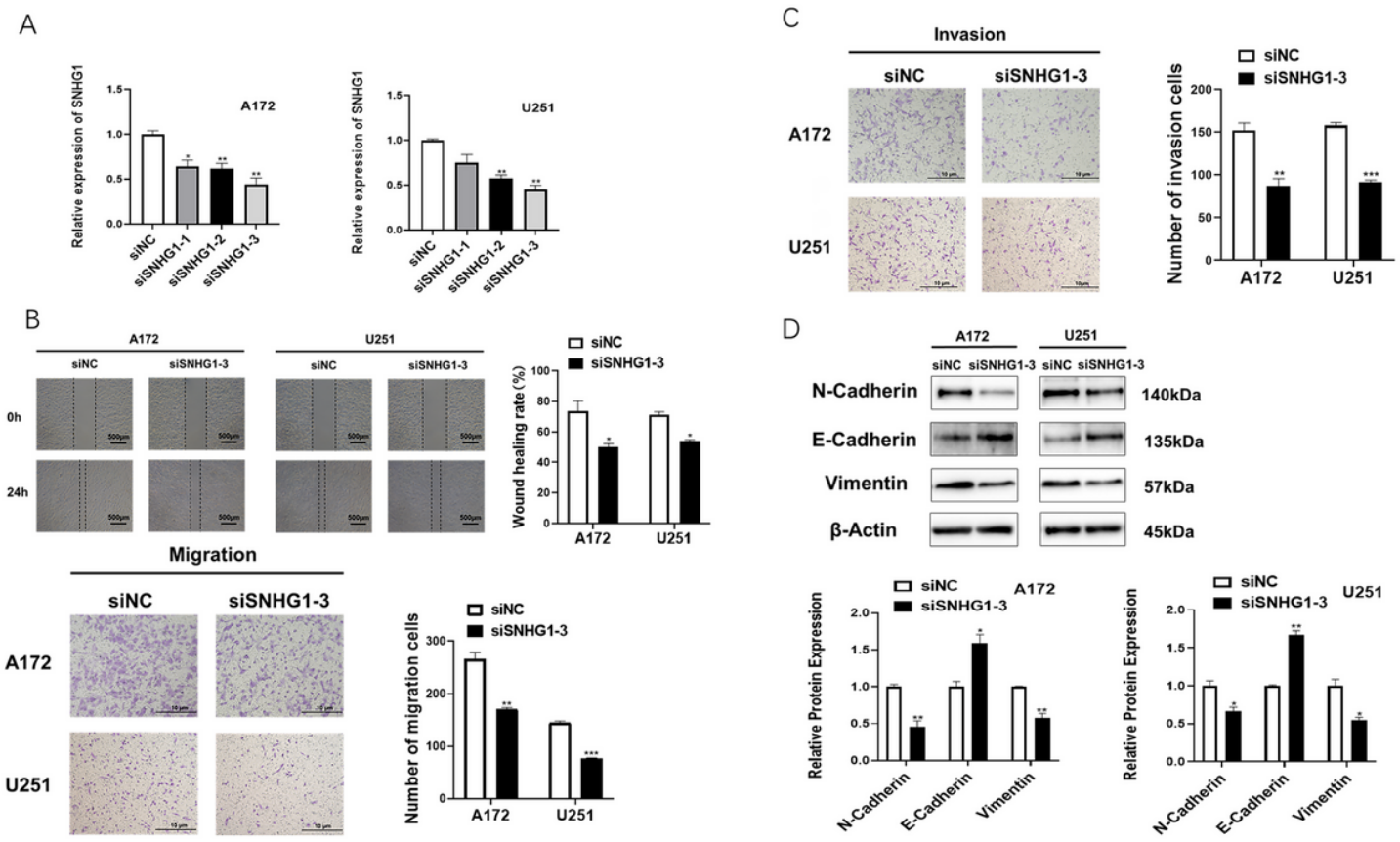

Figure 2

Inhibition of lncRNA SNHG1 suppressed GBM cells invasion, migration affect EMT process. **a** The SNHG1 knockdown efficiencies by three siRNAs were detected by qRT-PCR. **b** Representative images from wound healing assays (b, upper left, magnification: 4×, scale bar: 500 μm), Graphic representation of the quantification of the wound healing rate after 24 hours (b, upper right). Representative images from transwell migration assays (b, lower left, magnification: 10×, scale bar: 10μm) for GBM cells infected by NC and siRNA-SNHG1-3, Graphic representation of the quantification of the transwell assays after 24 hours (b, lower right). **c** Representative images from transwell invasion assays (c,left magnification: 10×, scale bar: 10μm) for GBM cells infected by NC and siRNA-SNHG1-3, Graphic representation of the quantification of the transwell assays after 24 hours (c, right). **d** Western blot for protein levels of three EMT markers, N-cadherin, E-cadherin and Vimentin from A172 and U251 cells infected by siRNA-NC and siRNA-SNHG1-3. β-Actin was used as a loading control (d, upper). Graphic representation of the quantification of the three EMT markers (d, lower) ($^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.001$)

Figure 3

Construction of LncRNA-miRNA-mRNA ceRNA network from TCGA and KEGG analysis. **a** Expression levels of 161 mRNAs and 120 miRNAs in 156 GBM tissues and 5 normal brain tissues from TCGA. **b** Expression levels of 17 mRNAs in 156 GBM tissues and 5 normal brain tissues (FC>2, P<0.05). **c**

Enrichment and variability of signaling pathways involving 17mRNA were derived from KEGG(P<0.05). **d** Signaling pathways involved in RRAS2 from KEGG(P<0.05). **e** The ceRNA network constructed by SNHG1 with miR-7-5p, miR-128-3p and CXCL3, CASP7, RRAS2

Figure 4

LncRNA SNHG1 functioned as a sponge of miR-128-3P and miR-128-3p expression were down-regulated in GBM. **a** Alignment prediction showing three possible target sites of miR-128-3p on the 3'UTR of the SNHG1 gene. **b** The predicted miR-128-3p binding sites on SNHG1 by StarBase v.2.0 and schematic of wild and mutant SNHG1 constructs. **c** Expression of miR-128-3p in A172 and U251 cells transfected by miR-128-3p mimic. **d** Relative Luciferase Rluc/Luc ratio transfected by miR-128-3p mimics and SNHG1-WT in A172 and U251 cells. **e** Expression of SNHG1 in A172 and U251 cells transfected by miR-128-3p mimic. **f** Expression of miR-128-3p in gliomas of different grades in CGGA ($^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.001$)

Figure 5

LncRNA SNHG1 regulates RRAS2 expression. **a** The expression level of RRAS2 in 528 GBM tissues and 10 normal brain tissues analyzed by TCGA. **b** Spearman's correlation analysis demonstrated that the expression of SNHG1 and RRAS2 were positively correlated in 156 GBM tissues. **c** RRAS2 expression in A172 and U251 cells transfected with si-SNHG1-3 or si-NC was detected by qRT-PCR ($^{**}p<0.01$)

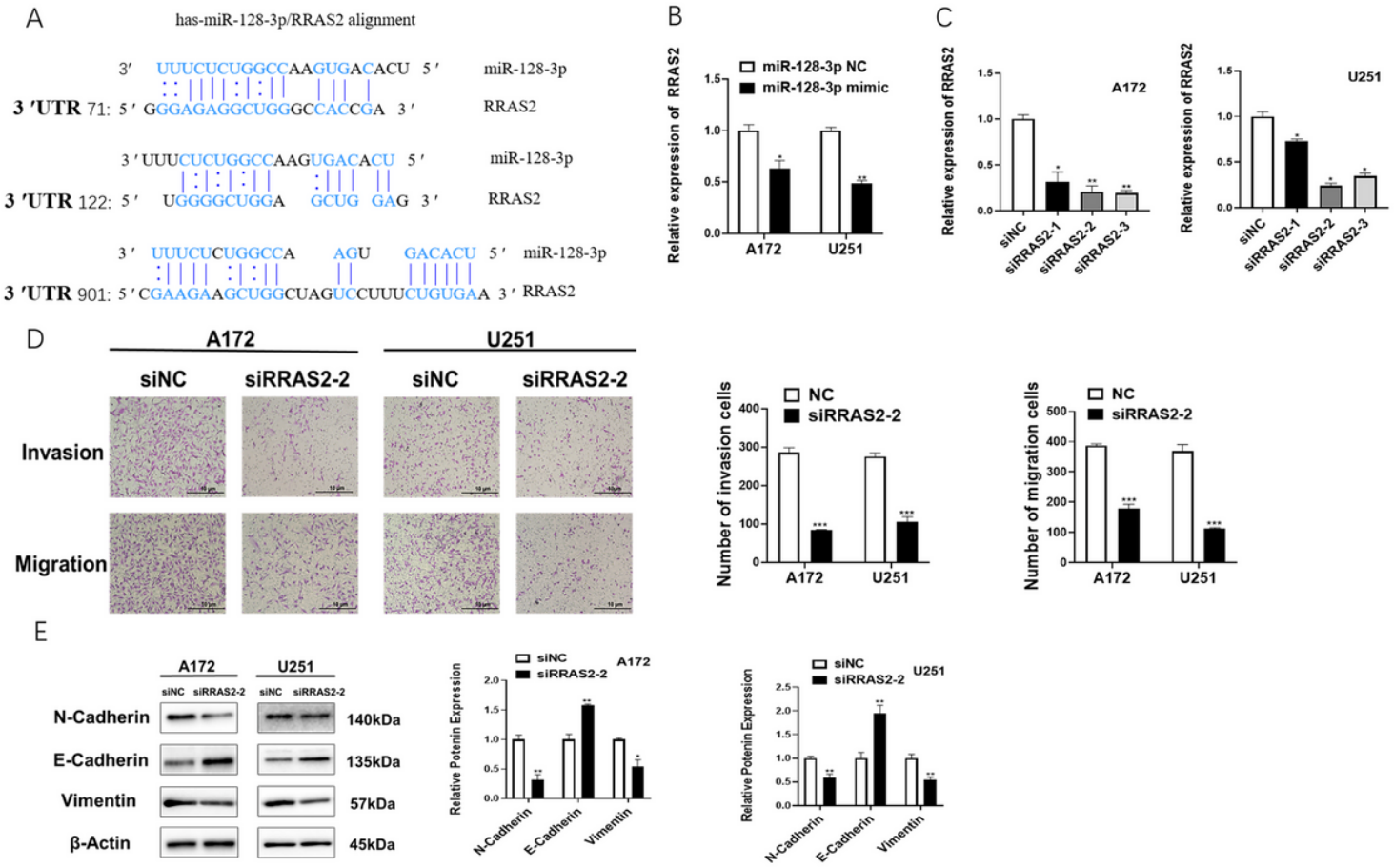

Figure 6

MiR-128-3p targeted and regulated RRAS2 and knockdown of RRAS2 suppressed GBM cells migration, invasion and affect EMT process. **a** Alignment prediction showing three possible target sites of miR-128-3p on the 3'UTR of the RRAS2 gene. **b** Expression of RRAS2 in A172 and U251 cells transfected by miR-128-3p mimic. **c** The RRAS2 knockdown efficiencies by three siRNAs were detected by qRT-PCR. **d** Representative images from transwell assays (d, left magnification: 10×, scale bar: 10μm) for glioma cells infected by NC and siRNA-RRAS2-2, Graphic representation of the quantification of the transwell assays after 24 hours (d, right). **e** Western blot for protein levels of three EMT markers, N-cadherin, E-cadherin and Vimentin from A172 and U251 cells infected by siRNA-NC and siRNA-RRAS2-2. β-Actin was used as a loading control (e, left). Graphic representation of the quantification of the three EMT markers (e, right) ($^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.001$)

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