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ORIGINAL RESEARCH

CELSR1 Acts as an Oncogene Regulated by miR-199a-5p in Glioma

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Purpose: This study aimed to elucidate the biological function and upstream regulatory mechanism of CELSR1 in glioma.

Materials and Methods: We evaluated the expression of CELSR1 in glioma by TCGA_GEPIA tool, RT-qPCR, and Western blot assays. CCK-8, wound healing, and transwell invasion assays were, respectively, performed to detect the effect of CELSR1 on cell proliferation, migration, and invasion. The upstream regulatory miRNAs of CELSR1 were predicted by TargetScan and validated by luciferase activity reporter assay.

Results: CELSR1 is overexpressed in glioma ($P<0.05$). CELSR1 promoted glioma cell proliferation, migration and invasion ($P<0.01$). CELSR1 was a direct target of miR-199a-5p. miR199a-5p mimics significantly inhibited CELSR1 mRNA and protein expression ($P<0.01$). miR199a-5p mimics reversed the effects of CELSR1 on glioma cell behaviors ($P<0.01$).

Conclusion: CELSR1 acts as an oncogene promoting glioma cell proliferation, migration, and invasion, which is regulated by miR199a-5p.

Keywords: CELSR1, miR-199a-5p, glioma, proliferation, migration, invasion

Introduction

Glioma, accounting for about 40% of all brain tumors, is characterized by high morbidity and poor survival.[1–4] High-grade gliomas are obviously characterized by high invasiveness, high proliferation rate and poor differentiation.[5] Previous research achievements have greatly increased life expectancy and quality of life. However, the molecular mechanisms underlying its pathology remain unclear.

CELSR1 is a member of the flamingo subfamily, which is a part of the cadherin superfamily. CELSR1 is likewise a member of planar cell polarity signaling pathway.[6] Mutations of CELSR1 are linked to the severe neural tube defect craniorachischisis.[7] Besides, aberrant CELSR1 expression is reported in a variety of tumors (including lung cancer,[8] breast cancer,[9] hepatocellular carcinoma,[10] ductal carcinoma in situ,[11] oral squamous cell carcinoma,[12] lymphoid leukemia,[13,14] and glioma[15]). However, until now, the biological function of CELSR1 has not been identified in cancers including glioma.

MicroRNAs (miRNAs), a type of non-coding RNA, are single-stranded RNAs of 18~24 nucleotides in length. miRNAs regulate gene expression at a post-transcriptional level by degrading or repressing target mRNAs, resulting in translational repression or mRNA degradation.[16,17] Our previous study reported that miR199a-5p suppresses glioma cell proliferation, migration, and invasion by

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inhibiting MAGT1.[18] However, the correlation between CELSR1 and miR-199a-5p has not been reported.

The purpose of our study was to investigate the biological function of CELSR1 and the upstream regulatory mechanism in glioma. Specifically, we evaluated the expression of CELSR1 in glioma by TCGA_GEPIA tool, RT-qPCR, and Western blot assays. Dual-luciferase reporter assay was utilized to determinate the mechanism upstream of CELSR1.

Given that the glioma biomarkers are scarce for clinical, our study on CELSR1 has significant implications in the diagnosis and treatment of glioma.

Methods and Materials

Cell Lines

The human glioma cell lines (U251, U118, U87-MG, and A172) and normal human astrocyte cell line (NHA) were obtained from the Type Cell Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific Inc, Rockford, USA) containing 10% fetal bovine serum (FBS), penicillin (100U/mL) and streptomycin (100μg/mL) at 37°C under 5% CO_2.

Transfection

GenePharm (Shanghai, China) was responsible for synthesizing miR-199a-5p mimics and inhibitors. CELSR1 overexpression vector was synthesized by Sangon Biotech (Shanghai, China) and cloned into pCDNA3.1 (+) plasmid. Cell transfection was performed by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

Bioinformatics Analysis

The function of CELSR1 in cancer development was explored in Cancer Hallmarks Analytics Tool (CHAT) (http://chat.lionproject.net/).[19] GEPIA[20] (http://gepia.cancer-pku.cn/index.html) online analysis tool was used to analyze the expression of CELSR1 in glioma. The targets of miR-199a-5p were analyzed by targetscan[21] database.

Western Blot

To determine the effect of miR199a-5p on CELSR1, we detected the protein expression levels of CELSR1 according to the following procedures. Lysis Buffer (CST, Danvers, USA) was used to lyse cells on ice for 30 min. Then, the protein samples were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant protein was collected and normalized, and equal protein samples were electrophoresed in SDS-PAGE gels and transferred onto the polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% fat-free milk at room temperature for 1 hour and then incubated with primary antibodies (CELSR1: ab225889, GAPDH: ab181602; both from Abcam, Cambridge, USA) at 4°C overnight. Upon being washed three times with washing buffer (Tris buffered saline Tween, TBST), the membranes were incubated with secondary antibody (ab6721, Abcam) diluted in blocking buffer for 60 min at room temperature. The membranes were also washed in TBST. Proteins were then visualized by ECL reagents (KeyGen, Nanjing, China). Image J 1.42 software was used to analyze the relative expression levels.

Quantitative Real-Time PCR

Total RNAs were extracted from cells and specimens using Trizol reagent (Invitrogen, Rockville, USA) following the provided instructions. Complementary DNA (cDNA) was reverse transcribed using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). The mRNA expression level was detected using TB Green Fast qPCR Mix (Takara, Dalian, China). The mature miR-199a-5p expression was detected by the premix EX Taq (Takara, Dalian, China). The primers are: miR-199a-5p, 5′-TCAAGAG CAATAACGAAAAATGT-3′ (forward), 5′-GCTGTCAA CGATACGCTACGT-3′ (reverse); U6, 5′-ATTGGAACGA TACAGAGAAGATT-3′ (forward), 5′-GTCCTTGGTGCC CGAGTG-3′ (reverse); CELSR1, 5′-GGCGTTGTTTGAGA ACGAACC-3′ (forward), 5′-AGAGTCGATTCGGAAGTA GCC-3′ (reverse); GAPDH, 5′-GGAGCGAGATCCCTCCA AAAT-3′ (forward), 5′-GGCTGTTGTCATACTTCTCAT GG-3′ (reverse); ROCK1, 5′-AACATGCTGCTGGATAAA TCTGG-3′ (forward), 5′-TGTATCACATCGTACCATGCC T-3′ (reverse); Galectin1, 5′-GGACATCCTCCTGGA CTCA-3′ (forward), 5′-GTTGAAGCGAGGGTTGAAGT -3′ (reverse); MMP2, 5′-CCCACTGCGGTTTTCTCGAAT -3′ (forward), 5′-CAAAGGGGTATCCATCGCCAT-3′ (reverse); MMP9, 5′-AGACCTGGGCAGATTCCAAAC-3′ (forward), 5′-CGGCAAGTCTTCCGAGTAGT-3′ (reverse); Snail, 5′-TACAAAAACCCACGCAGACA-3′ (forward), 5′-ACCCCACATCCTTCTCACTG-3′ (reverse); GAPDH and U6 were used as the standard for normalization. The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

CCK-8 Assay

Cell viability was assessed by Cell Counting Kit-8 (DOJINDO, Japan) assay. U251 and U118 cells (5×10^3/well) were plated in 96-well plates and incubated 24 h, 48 h, or 72 h. Then, old medium was replaced with 10μL CCK-8 reagent and 100μL fresh medium. The optical density (OD) values were measured at a wavelength of 450 nm after 1 h at 37°C.

Wound Healing Assay

Wound healing assay was performed to evaluate cell migration activity. To make sure the same visual field in the photograph, a mark was left on the back of the 12-well plate. U251 and U118 cells were seeded into plate. When cells approximately 100% covered the plate bottom, a sterilized tip was used to create a wound at the cell surface. Then, we washed cells with phosphate-buffered saline for three times and add medium. Cells were photographed at a fixed location at 0 h and 24 h.

Transwell Invasion Assay

To evaluate the effect of CELSR1 on glioma cell invasion, a matrix (BD Biosciences, San Jose, USA) precoated transwell chamber was used. In brief, U251 and U118 cells were serum starved for 24 h and then were resuspended in 1% FBS.1×10^4 cells were seeded on the upper chamber, whereas the lower chamber was supplemented with 20% FBS. After incubation for 48 h, the upper non-invasive cells were gently wiped. The invasion cells were fixed with methanol and stained with 0.5% crystal violet. Cell numbers were averaged under five random fields from triplicate experiments.

Dual-Luciferase Reporter Assay

To determine the binding site between miR-199a-5p and CELSR1, wild-type (wt) or mutated (mut) CELSR1 sequences were cloned into the psiCHECK2 vector (Promega, Madison, USA). Cells were co-transfected with the wt or mut CELSR1 3′-UTR, and the miR-199a-5p mimics/inhibitors/NC. Luciferase activity was measured through Dual-Luciferase Reporter Assay Kit (Promega, Madison, USA) after 48 h.

Statistical Analysis

SPSS 21.0 software (SPSS, Chicago, USA) was used for statistical analysis. Data is presented as mean± standard deviation (SD) of three independent experiments. Differences in measurement data were analyzed by the Student's *t*-test and one-way ANOVA. *P* values <0.05 were considered statistically significant.

Results

CELSR1 is Overexpressed in Glioma

Using Cancer Hallmarks Analytics Tool (CHAT), we found that CELSR1 is associated with genome instability and mutation, evading growth suppressors, inducing angiogenesis, invasion and metastasis, and sustaining proliferative signaling (Figure 1A). To determine the expression levels of CELSR1 in glioma, we analyzed the TCGA datasets by GEPIA tools and detected the protein and mRNA expression levels of CELSR1 in glioma cells. TCGA datasets show that CELSR1 is significantly overexpressed in glioma patients (Figure 1B; $P<0.05$). Besides, Western blot and RT-qPCR assays, respectively, revealed that the protein and mRNA expression levels of CELSR1 are increased in glioma cells (Figure 1C and D; $P<0.001$).

CELSR1 Promoted Glioma Cell Proliferation, Migration and Invasion

To investigate the biological function of CELSR1 in glioma, we significantly knocked down and overexpressed CELSR1 expression in U251 and U118 cells (Figure 2A and B; $P<0.01$). CCK-8 assays revealed that CELSR1 significantly increased cell viability, and blockade of CELSR1 significantly inhibited cell viability in U251 and U118 cells (Figure 2C and D; $P<0.01$). Besides, wound healing assay found that CELSR1 significantly promoted cell migration (Figure 2E; $P<0.01$). Transwell invasion assay revealed that CELSR1 significantly promoted cell invasion (Figure 2F; $P<0.01$).

CELSR1 Was a Direct Target of miR-199a-5p

To determinate the upstream regulation mechanism of CELSR1, we predicted the correlated miRNA using targetscan database. We found that CELSR1 probably is a direct target of miR199a-5p (Figure 3A). To further investigate the correlation between miR199a-5p and CELSR1, we transfected miR199a-5p mimics and inhibitors in U251 and U118 cells (Figure 3B; $P<0.01$) and detected the CELSR1 expression levels in different groups. RT-qPCR assays found that miR199a-5p mimics significantly inhibited CELSR1 mRNA expression

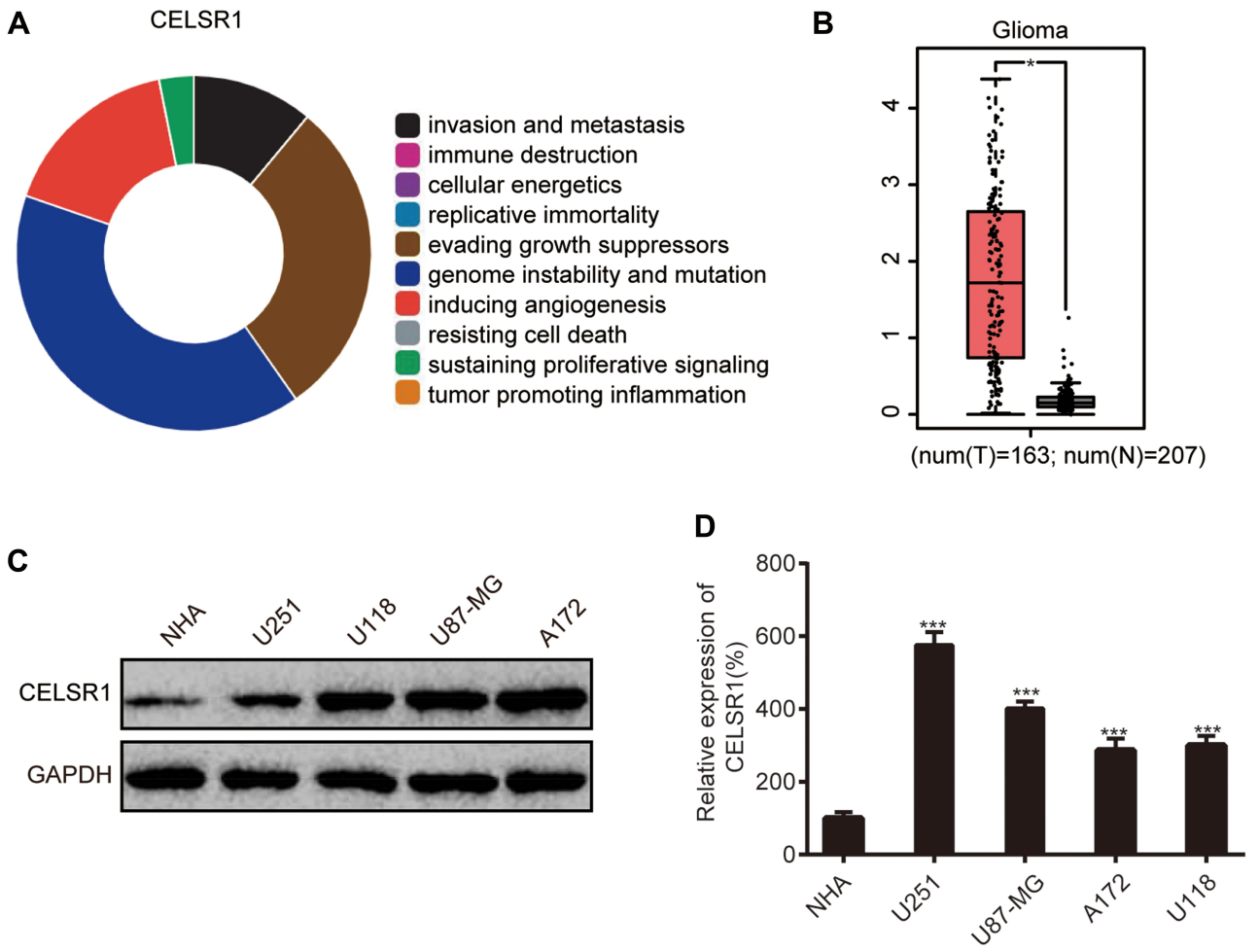


Figure 1 CELSR1 expression levels in glioma. (**A**) Association between CELSR1 and hallmarks of cancer from Cancer Hallmarks Analytics Tool (CHAT). (**B**) CELSR1 expression levels in TCGA-glioma datasets. CELSR1 protein (**C**) and mRNA (**D**) were upregulated in glioma cell lines (U251, U118, U87-MG, A172) compared with normal human astrocyte cell line (NHA). *P<0.05; ***P<0.001. Each experiment was conducted in triplicates.

(Figure 3C; P<0.01) and miR199a-5p inhibitors significantly promoted CELSR1 mRNA expression (Figure 3D; P<0.01). Western blot assays revealed that miR199a-5p mimics significantly inhibited CELSR1 protein expression and miR199a-5p inhibitors significantly promoted CELSR1 protein expression (Figure 3E; P<0.05). Hence, to further verified the binding sites between miR199a-5p and CELSR1, we detect the relative luciferase activity after co-transfection assays. The dual-luciferase report system revealed that miR-199a-5p mimics significantly inhibit the luciferase activity of CELSR1-wt 3′UTR, and miR-199a-5p inhibitors significantly increase the luciferase activity of CELSR1-wt 3′UTR among U251 and U118 cells, whereas miR-199a-5p mimics or inhibitors do not regulate the luciferase activity of CELSR1-mut 3′UTR among U251 and U118 cells (Figure 3F and G; P<0.05).

CELSR1 Was an Effector for the Role of miR-199a-5p

To further illuminate the regulatory process of miR199a-5p/CELSR1 axis on cell biological function, we co-transfected CELSR1 and miR199a-5p mimics (Figure 4A; P<0.05) and detected cell proliferation, migration and invasion in U251 and U118 cells. CCK-8 assays revealed that miR-199a-5p mimics significantly reversed the effect of CELSR1 promoting cell proliferation (Figure 4B; P<0.01). Wound healing assay found that miR-199a-5p mimics significantly reversed the effect of CELSR1 promoting cell migration (Figure 4C; P<0.01). Transwell invasion assay significantly reversed the effect of CELSR1 promoting cell invasion (Figure 4D; P<0.01). Besides, we found that CELSR1 significantly reversed the effect of miR-199a-5p inhibiting the mRNA expression of ROCK1, Galectin1, and Snail.

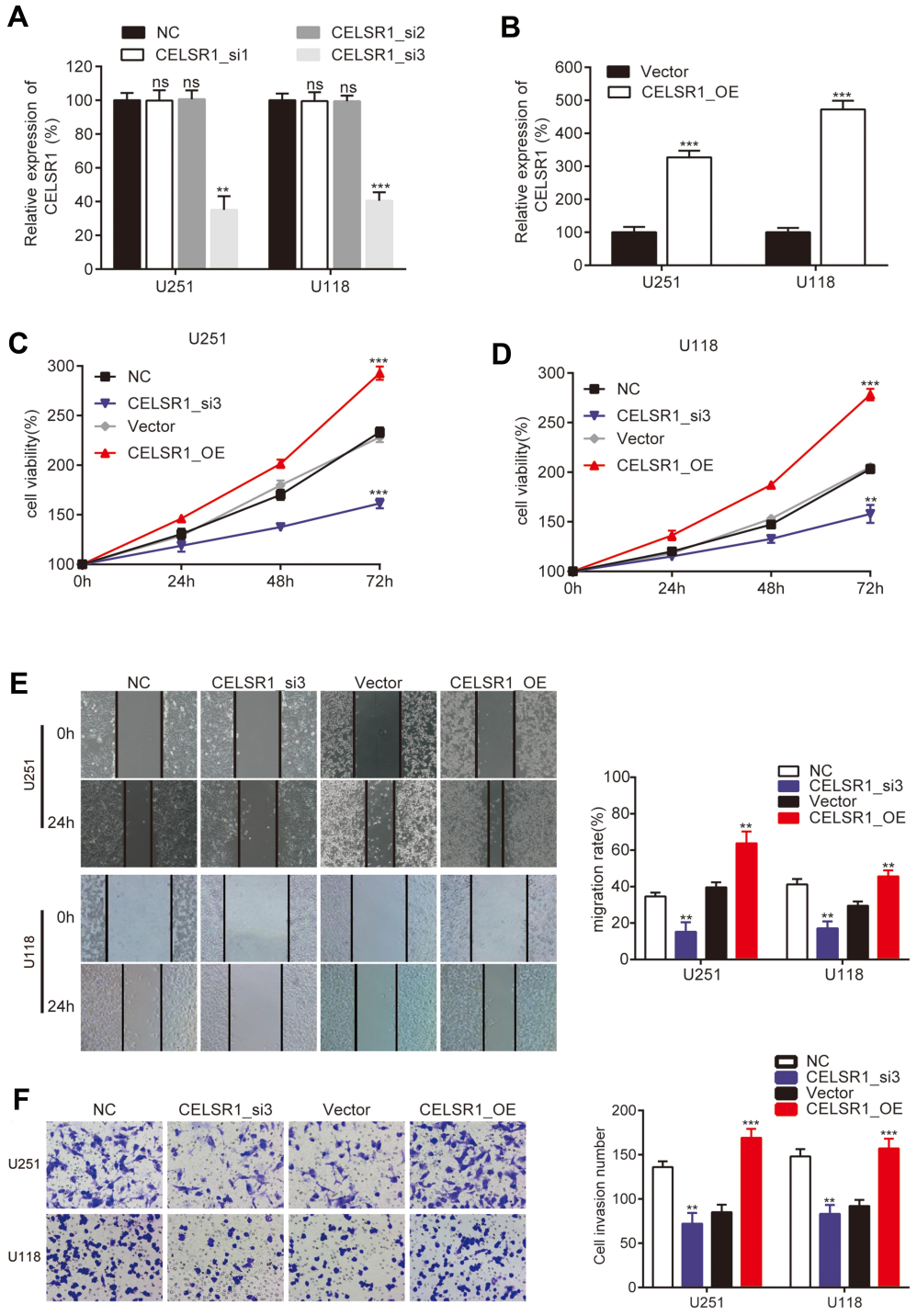


Figure 2 CELSR1 promoted glioma cell proliferation, migration, and invasion. RT-qPCR detected the efficiency of CELSR1 siRNA (**A**) and CELSR1 overexpressed plasmids (**B**). CCK-8 (**C, D**), wound healing (**E**) and transwell invasion assay (**F**) were respectively performed to detect the effect of CELSR1 on cell proliferation, migration, and invasion. ns, not significant; ***P*<0.01; ****P*<0.001. Each experiment was conducted in triplicates.

Discussion

In this study, we found that CELSR1 acts as an oncogene by regulating cell proliferation, cell migration, and cell invasion in glioma, which was negatively regulated by miR199a-5p. The result of Figure 1A indicated that CELSR1 is associated with these cancer hallmarks (such as genome instability and mutation, evading growth suppressors, invasion and metastasis), which provided the research direction. Subsequently, in light of TCGA_GEPIA expression analysis, RT-qPCR and Western blot results, we confirmed that CELSR1 is

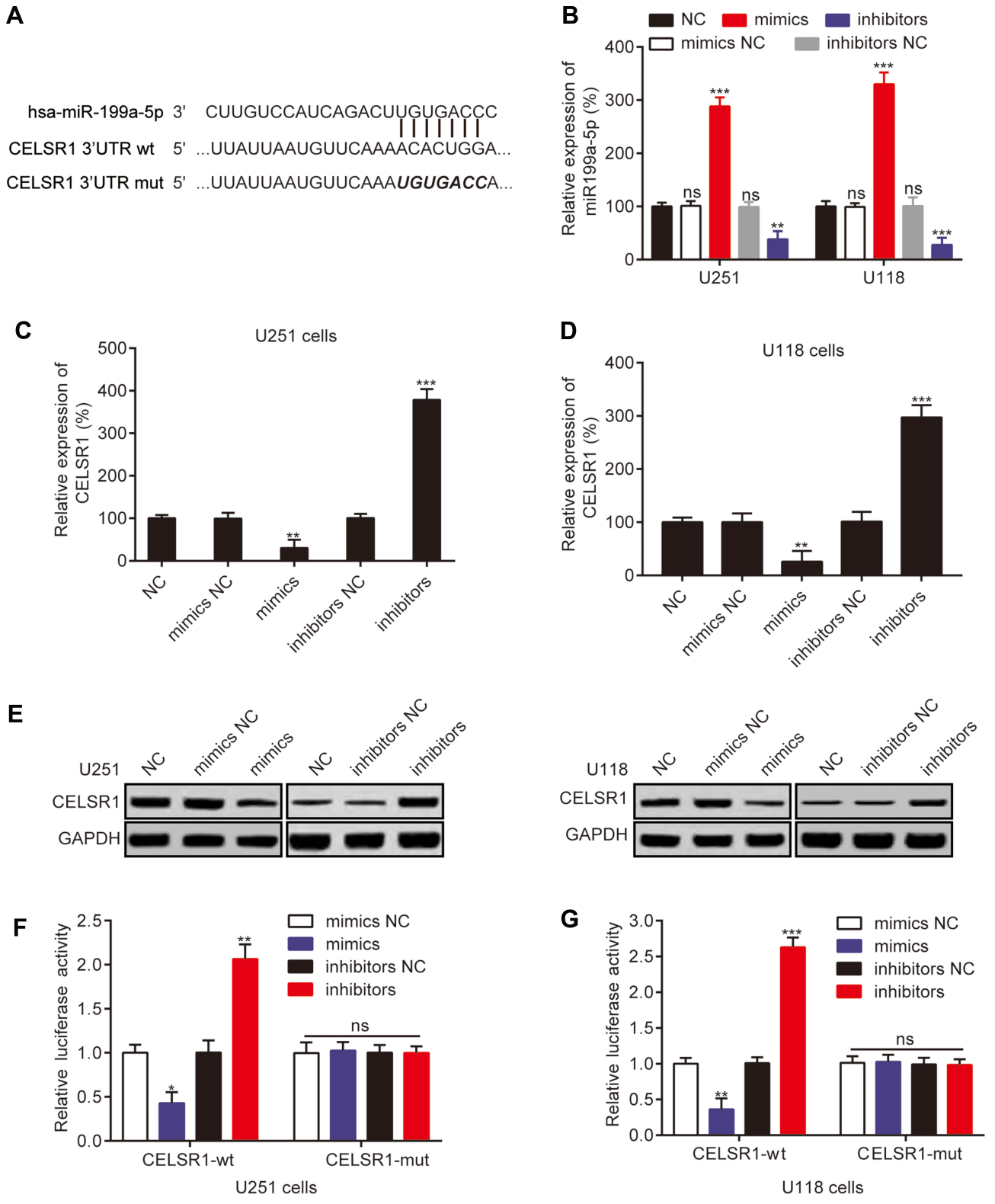


Figure 3 CELSR1 was a direct target of miR-199a-5p. (**A**) Sequence alignment of miR-199a-5p and the CELSR1 3′-UTR. (**B**) Relative miR199a-5p expression after transfection. (**C, D**) miR-199a-5p negatively regulated CELSR1 mRNA expression. (**E**) miR-199a-5p negatively regulated CELSR1 protein expression. (**F, G**) The dual-luciferase report system was used to detect the effect of miR-199a-5p on CELSR1 in U251 and U118 cells. **P*<0.05; ***P*<0.01; ****P*<0.001; ns, not significant; Each experiment was performed in triplicates.

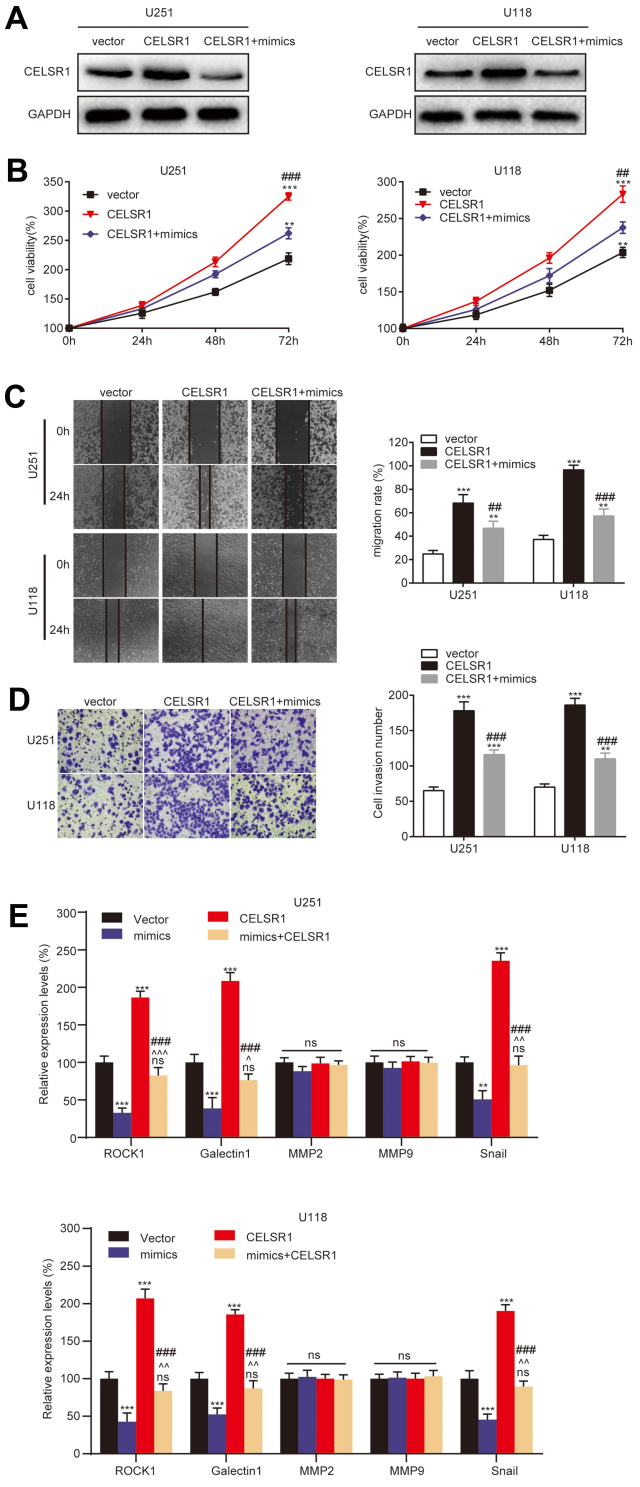


Figure 4 CELSR1 was an effector for the role of miR-199a-5p. (**A**) CELSR1 protein expression, (**B**) cell proliferation, (**C**) cell migration, and (**D**) cell invasion in U251 and U118 cells after vector, CELSR1, or CELSR1/miR199a-5p mimics transfection. (**E**) miR199a-5p regulated the mRNA expression levels of ROCK1, Galectin1, and Snail by CELSR1. $^{**}P<0.01$ or $^{***}P<0.001$ vs vector group; $^{\#\#}P<0.01$ or $^{\#\#\#}P<0.001$ vs CELSR1 group; $^{\wedge}P<0.05$ or $^{\wedge\wedge}P<0.01$ or $^{\wedge\wedge\wedge}P<0.001$ vs mimics group, ns, not significant; Each experiment was conducted in triplicates.

overexpressed in glioma (Figure 1B–D). These results lead us to detect the role of CELSR1 in tumor cell proliferation, migration and invasion. Our findings revealed that CELSR1 significantly promoted cell proliferation, migration, and invasion (Figure 2C–F). Next, we further explored the upstream regulatory mechanism. We confirmed that miR199a-5p regulates CELSR1 expression at a post-transcriptional level by degrading CELSR1 mRNA (Figure 3A), which indicated that CELSR1 is a direct target of miR-199a-5p.

miR199a-5p regulates cell biological function by multiple target genes. Previous study reported that miR-199a-5p significantly regulated cell proliferation, migration, and invasion.[18,22,23] In glioma, Zhang et al reported that miR-199a inhibits tumor growth and attenuates chemoresistance by targeting K-RAS via ATK and ERK signaling.[24] Our previous study also found that miR199a-5p suppresses glioma cell proliferation, migration, and invasion by inhibiting MAGT1. Then, we hypothesized that miR199a-5p regulates glioma progression via targeting CELSR1. Our results revealed that miR-199a-5p mimics significantly reversed the effect of CELSR1 promoting cell proliferation, migration, and invasion (Figure 4B–D). Besides, we detected the mRNA expression of several known motility and adhesion regulators including ROCK1, Galectin1, MMP2, MMP9, and Snail.[25] Our results found that CELSR1 significantly reversed the effect of miR-199a-5p inhibiting the mRNA expression of ROCK1, Galectin1, and Snail (Figure 4E), which indicated that CELSR1 is an effector for the role of miR-199a-5p.

Based on the above results, CELSR1 is an oncogene in glioma, which regulated cell proliferation, migration, and invasion. Besides, we preliminarily demonstrated CELSR1 upstream regulatory mechanism that miR199a-5p inhibits CELSR1 expression at a post-transcriptional level by degrading CELSR1 mRNA. However, there are some limitations to this study. On the one hand, in vivo assays should be performed to verify these results. On the other hand, more experiments should be performed to explore probable mechanisms downstream of CELSR1 regulating glioma progression (such as cell apoptosis, cell cycle). Hence, the next step is to explore the detailed regulatory mechanism of CELSR1 in animal models and patients with glioma.

Above all, our findings validated that CELSR1 promotes cell migration and invasion regulated by miR-199a-5p in glioma, which will provide strong evidence supporting the function of CELSR1 and miR199a-5p in glioma occurrence and development.

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Disclosure

The authors report no conflicts of interest in this work.

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