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LncRNA HANR aggravates the malignant progression of glioma *via* targeting miRNA-335

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Abstract. – OBJECTIVE: The aim of this study was to uncover the role of lncRNA HANR in the progression of glioma and the underlying mechanism.

PATIENTS AND METHODS: HANR expression level in 36 matched glioma tissues and adjacent non-tumoral tissues was determined by qRT-PCR. The relationship between HANR expression and pathological indexes of the glioma patients was analyzed. The Kaplan-Meier method was introduced to investigate the survival of glioma patients. After the knockdown of HANR, the proliferative, migratory, and invasive changes of U251 and SHG44 cells were determined. Bioinformatics and Dual-Luciferase Reporter Gene Assay were applied to predict and verify the downstream target of HANR, respectively. Furthermore, the rescue experiments were conducted to clarify the role of HANR/miRNA-335 regulatory loop in the progression of glioma.

RESULTS: HANR was significantly upregulated in glioma tissues and cell lines. Glioma patients with a high expression level of HANR presented remarkably higher rates of lymphatic metastasis and distant metastasis, as well as worse prognosis. The silence of HANR remarkably attenuated the proliferative, migratory, and invasive capacities of U251 and SHG44 cells. MiRNA-335 was the direct target of HANR and was significantly downregulated in glioma tissues. Meanwhile, the miRNA-335 level was negatively regulated by HANR. In addition, the knockdown of miRNA-335 partially reversed the regulatory effects of HANR on cellular behaviors of glioma.

CONCLUSIONS: LncRNA HANR is upregulated in glioma, which is closely correlated with metastasis and poor prognosis of glioma patients. In addition, HANR aggravates the progression of glioma by negatively regulating miRNA-335.

Key Words:
LncRNA HANR, MiRNA-335, Glioma, Metastasis.

Introduction

Glioma is the most common primary malignant tumor in the adult central nervous system. Meanwhile, it accounts for more than 40% of all primary intracranial tumors[1-3]. Currently, significant advances have been achieved in traditional treatments for glioma, including surgical resection, radiation therapy, and chemotherapy. However, the overall prognosis of glioma patients is still far from satisfactory[4-6]. The occurrence and development of glioma are complex processes involving multiple genes and pathways[7]. The oncogenes and tumor-suppressor genes have been proved to be involved in the tumorigenesis of glioma[8,9]. Therefore, it is necessary to uncover the molecular mechanism of glioma, thus providing new therapeutic and prognostic markers[10].

Long non-coding RNAs (lncRNAs) are a type of RNAs with over than 200 nucleotides in length. Due to the reason that they cannot encode proteins[11,12], they were initially considered as transcriptional noises. Several reports[13,14] have confirmed their regulatory effects on human diseases. Recently, it has been speculated that lncRNAs may be utilized as new targets for tumor treatment[15]. Functionally, lncRNAs are able to regulate the gene expression at transcriptional, post-transcriptional, and epigenetic levels[16,17]. Mao et al[17] have also proposed a new regulatory loop "lncRNA-miRNA", which is interacted with each other to further regulate the downstream genes.

LncRNA HANR has been found abnormally expressed in multiple diseases by relevant microarray[18,19]. In this experiment, we mainly investigated the relationship between HANR expression level and the pathological indexes of the

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glioma patients. Furthermore, the potential role of HANR in influencing the progression of glioma was explored.

Patients and Methods

Patients and Glioma Samples

Glioma tissues and adjacent non-tumoral tissues were surgically resected from 36 glioma patients (32-79 years). The tumor staging of glioma was assessed based on the criteria proposed by UICC. The informed consent was obtained from patients and their families. This study was approved by the Ethics Committee of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University. We followed the Declaration of Helsinki.

Cell Culture

Normal glial cell line (HEB) and glioma cell lines (A172, T98-G, U87, U251, and SHG44) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator.

Cell Transfection

The transfection plasmids were provided by GenePharma (Shanghai, China). The cells were first seeded into 6-well plates. The cell transfection was performed according to the instructions of Lipofectamine 2000 at 50-70% of confluence. At 48 h, the transfected cells were harvested for verification of the transfection efficacy and subsequent experiments.

Cell Counting Kit (CCK-8) Assay

The transfected cells were first seeded into 96-well plates at a density of 2×10^3 cells per well. At 24, 48, 72 h, and 96 h, respectively, the absorbance value at 450 nm was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan). Finally, the viability curve was plotted.

Wound Healing Assay

The transfected cells were seeded into 24-well plates with 5.0×10^5 cells/well. After cell adherence (0 h), an artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. The wound closure images were taken using an inverted microscope at 0 and 24 h, respectively. Finally, the percentage of wound closure was calculated.

Transwell Assay

The concentration of the cells was first adjusted to 3.0×10^5/mL. 200 μL cell suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA) and inserted in a 24-well plate. Meanwhile, 700 μL of complete medium containing 10% FBS was added to the lower side. After 48 h of incubation, the cells penetrated to the bottom side were fixed with methanol for 15 min and dyed with crystal violet for 20 min. The invasive and migratory cells were observed under a microscope, and the numbers of the invasive and migratory cells were counted. 5 fields of view were randomly selected for each sample (magnification 200×).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified by the DNase I treatment. Subsequently, the extracted RNA was reverse transcribed into cDNA using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). β-actin and U6 were used as internal references for mRNAs and miRNAs, respectively. Each sample was performed in triplicate. The relative expression level of the gene was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer 5.0 was used for designing the qRT-PCR primers. The primers used in this study were as follows: LncRNA HANR: forward: 5'-AAGTACCAGGCAGTGACAGC-3', reverse: 5'-TTCTCCACGTTCTTCTCGGC-3'; U6: forward: 5'-CTCGCTTCGGCAGCACATATACTA-3', reverse: 5'-ACGAATTTGCGTGTCATCCTTGCG-3'; MiRNA-335: forward: 5'-AACGTCTTCTCCCTTCTCTCTCTGTCA-3', reverse: 5'-CCACAGCAGCAGAAACT-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'.

Dual-Luciferase Reporter Gene Assay

The Wild-type and mutant-type HANR Luciferase vectors were constructed based on the binding sites in the promoter regions of HANR and miRNA-335, respectively. Subsequently, the cells were co-transfected with HANR-WT/

HANR-MUT and miRNA-335 mimics/NC. After 48 h, the co-transfected cells were lysed, and the Luciferase activity was determined.

Statistical Analysis

The Statistical Product and Service Solution (SPSS 22.0, IBM Corp., Armonk, NY, USA) was used for all statistical analyses. The experimental data were expressed as mean ± standard deviation (SD). The intergroup differences were analyzed by the *t*-test. The Kaplan-Meier method was introduced for survival analysis. The Chi-square test was performed to evaluate the correlation between the two genes. $p < 0.05$ was considered statistically significant.

Results

HANR Was Highly Expressed in Glioma Tissues and Cell Lines

Compared with adjacent non-tumoral tissues, HANR was markedly upregulated in glioma tissues (Figures 1A, 1B). Similarly, HANR was highly expressed in glioma cell lines compared with normal glial cells (Figure 1C). Among the five selected glioma cell lines, U251 and SHG44 cells expressed the highest levels of HANR. Therefore, these two cell lines were selected for the following experiments.

HANR Expression Was Correlated with Lymphatic and Distant Metastasis in Glioma Patients

The relationship between HANR level and pathological indexes of glioma patients was analyzed. The results indicated that the HANR expression was positively correlated with lymphatic metastasis and distant metastasis of glioma patients, rather than age, gender, and tumor staging (Table I). Moreover, the survival analysis identified that a significantly worse prognosis was observed in glioma patients with the higher expression level of HANR (Figure 1D).

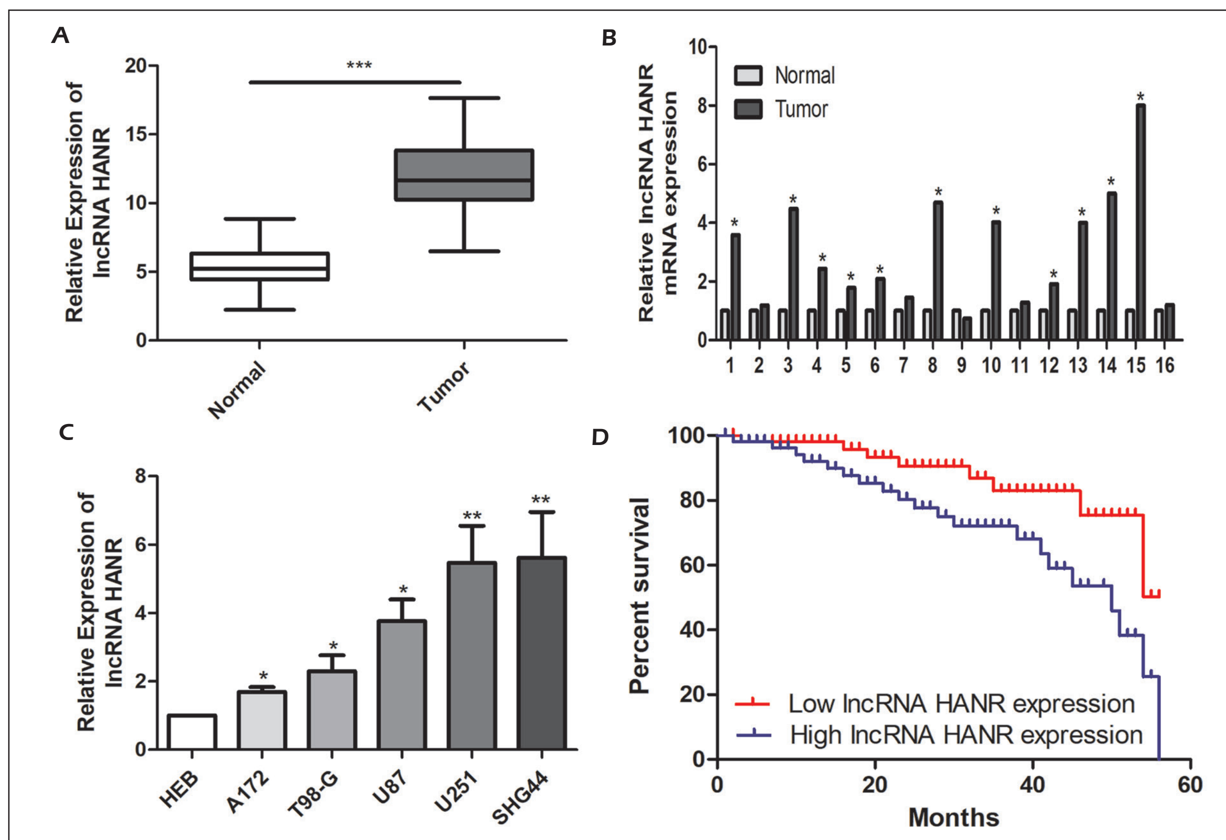

Figure 1. HANR was highly expressed in glioma tissues and cell lines. **A,** HANR level in glioma tissues and normal tissues. **B,** HANR level in 16 matched glioma tissues and adjacent non-tumoral tissues. **C,** HANR level in normal glial cell line (HEB) and glioma cell lines (A172, T98-G, U87, U251, and SHG44). **D,** Overall survival in glioma patients with high and low level of HANR.

Table I. Association of lncRNA HANR expression with clinicopathologic characteristics of glioma.

Parameters	Number of cases	lncRNA HANR expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.516
< 60	19	11	8	
≥ 60	17	8	9	
Gender				0.516
Male	17	8	9	
Female	19	11	8	
T stage				0.923
T1-T2	23	12	11	
T3-T4	13	7	6	
Lymph node metastasis				**0.042**
No	25	16	9	
Yes	11	3	8	
Distance metastasis				**0.018**
No	24	16	8	
Yes	12	3	9	

Knockdown of HANR Suppressed Proliferation, Migration and Invasion of Glioma Cells

To uncover the biological function of HANR, we constructed three HANR shRNAs, namely sh-HANR-1, sh-HANR-2, and sh-HANR-3. The transfection efficacy in U251 and SHG44 cells was verified by qRT-PCR (Figure 2A). Finally, sh-HANR-1 was chosen for *in vitro* experiments due to its best transfection efficacy. The transfection of sh-HANR-1 markedly reduced the viability of U251 and SHG44 cells (Figure 2B). Meanwhile, the downregulation of HANR significantly suppressed the migratory and invasive capacities of the glioma cells (Figure 2C). Besides, the percentage of wound closure remarkably decreased after transfection of sh-HANR-1, further confirming the inhibited proliferative ability (Figure 2D).

Interaction of HANR and MiRNA-335

By searching TargetScan, miRDB, and Starbase, miRNA-335 was finally screened out as the target gene of HANR (Figure 3A). After transfection of sh-HANR-1, the expression of miRNA-335 significantly increased (Figure 3B). Based on the binding sites in the promoter regions of HANR and miRNA-335, HANR-WT and HANR-MUT Luciferase vectors were constructed (Figure 3C). Luciferase activity was significantly reduced after the co-transfection of HANR-WT and miRNA-335 mimics. These results verified the binding relationship between HANR and miRNA-335 (Figure 3D). In glioma tissues, miRNA-335 was markedly downregulated compared to that of the adjacent non-tumoral tissues (Figure 3E). Moreover, a negative correlation was observed between the expression levels of HANR and miRNA-335 in glioma (Figure 3F).

HANR Influenced Glioma Progression Through Targeting MiRNA-335

Subsequently, the involvement of miRNA-335 in the progression of glioma was specifically discussed. The miRNA-335 inhibitor was constructed, and its transfection efficacy in glioma cells was verified by qRT-PCR (Figure 4A). The transfection of miRNA-335 inhibitor significantly accelerated the proliferation, migration, and invasion of glioma cells. Notably, the attenuated viability, migration, invasion, and wound closure in glioma cells with HANR knockdown could be partially reversed by the co-transfection of miRNA-335 inhibitor (Figures 4B-D).

Discussion

In recent years, tumor research has been greatly advanced due to the great progress in microarray analyses and high-throughput sequencing. A plenty number of non-coding RNAs have been discovered in tumors. Meanwhile, they are of great significance in the occurrence and progression of tumors[11,12]. These molecules, including miRNAs and lncRNAs, can mediate the differentiation, stemness, metastasis, and other aspects of

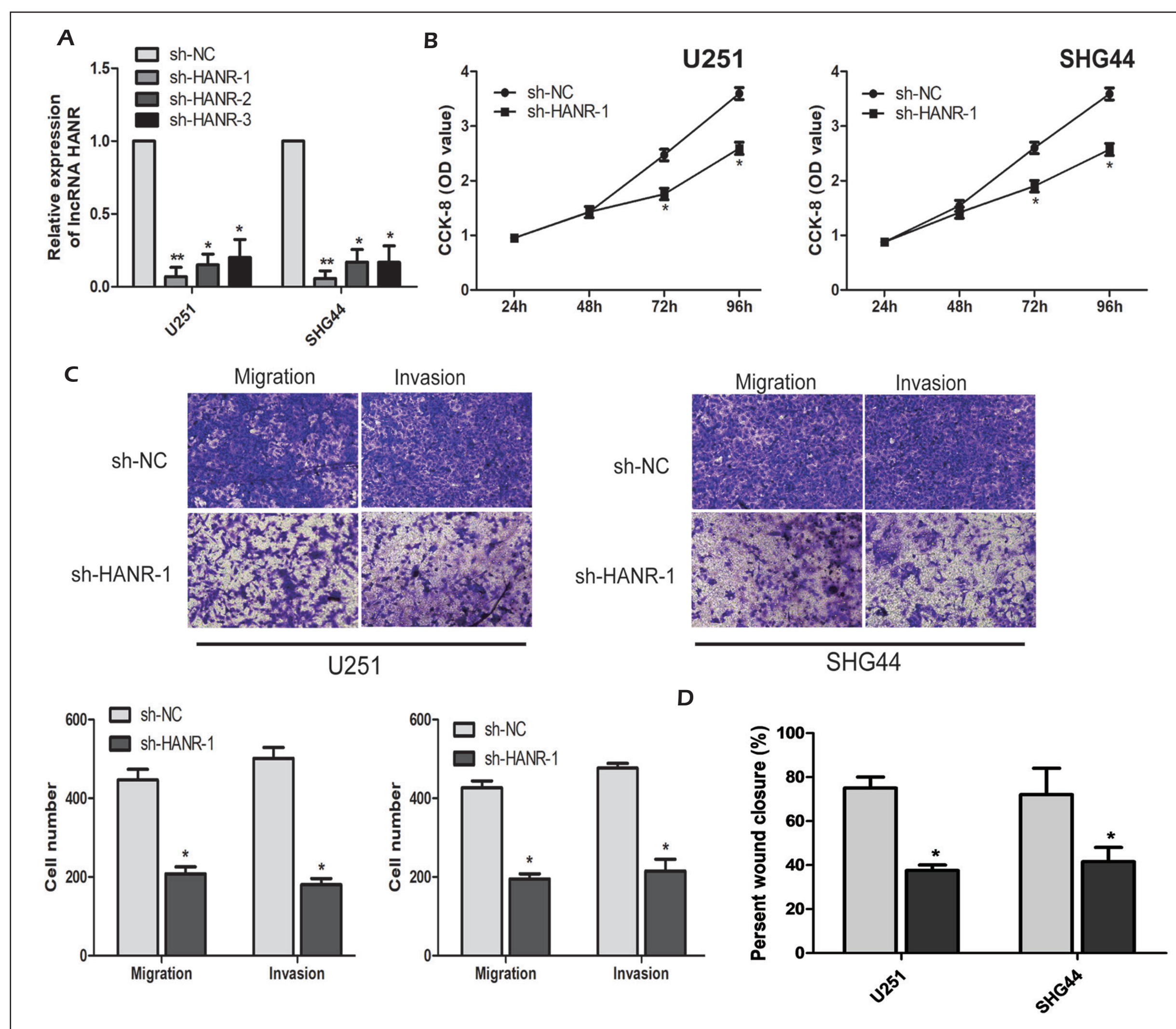

Figure 2. Knockdown of HANR suppressed the proliferation, migration, and invasion of glioma cells. **A,** Transfection efficacy of sh-HANR-1, sh-HANR-2, and sh-HANR-3 in U251 and SHG44 cells. **B,** Viability of U251 and SHG44 cells transfected with sh-NC or sh-HANR-1. **C,** Migration and invasion of U251 and SHG44 cells transfected with sh-NC or sh-HANR-1 (magnification: 100x). **D,** Wound closure in U251 and SHG44 cells transfected with sh-NC or sh-HANR-1.

tumor cells. Moreover, their expression levels are correlated with clinical indexes and prognosis of tumor patients. These findings indicate that these molecules provide a possibility to be developed as therapeutic targets[13-15]. Some researchers[10,15] have mainly focused on searching for effective and sensitive non-coding RNAs that are differentially expressed in tumor/non-tumoral tissues.

Currently, the pathogenesis of glioma remains unclear. Surgical resection combined with postoperative chemotherapy or radiotherapy is still the major treatment for glioma[4-6]. The therapeutic efficacy of the targeted molecular drugs for glioma treatment, including temozolomide and bevacizumab, is only about 10-15%[2,4-7]. Therefore, it is urgent to develop more sensitive drugs for glioma. Differentially expressed lncRNAs in glioma have been searched by analyzing microarrays[10-12]. These lncRNAs have been confirmed to exert regulatory effects on glioma progression by targeting the key genes or pathways[20,21].

In this paper, lncRNA HANR was found significantly upregulated in glioma tissues and cell lines. The expression level of HANR was positively correlated with metastatic rates of glioma. This indicated its carcinogenic role in the progression of glioma. *In vitro* experiments demonstrated that the knockdown of HANR attenuated the proliferation, migration, and invasion of glioma cells.

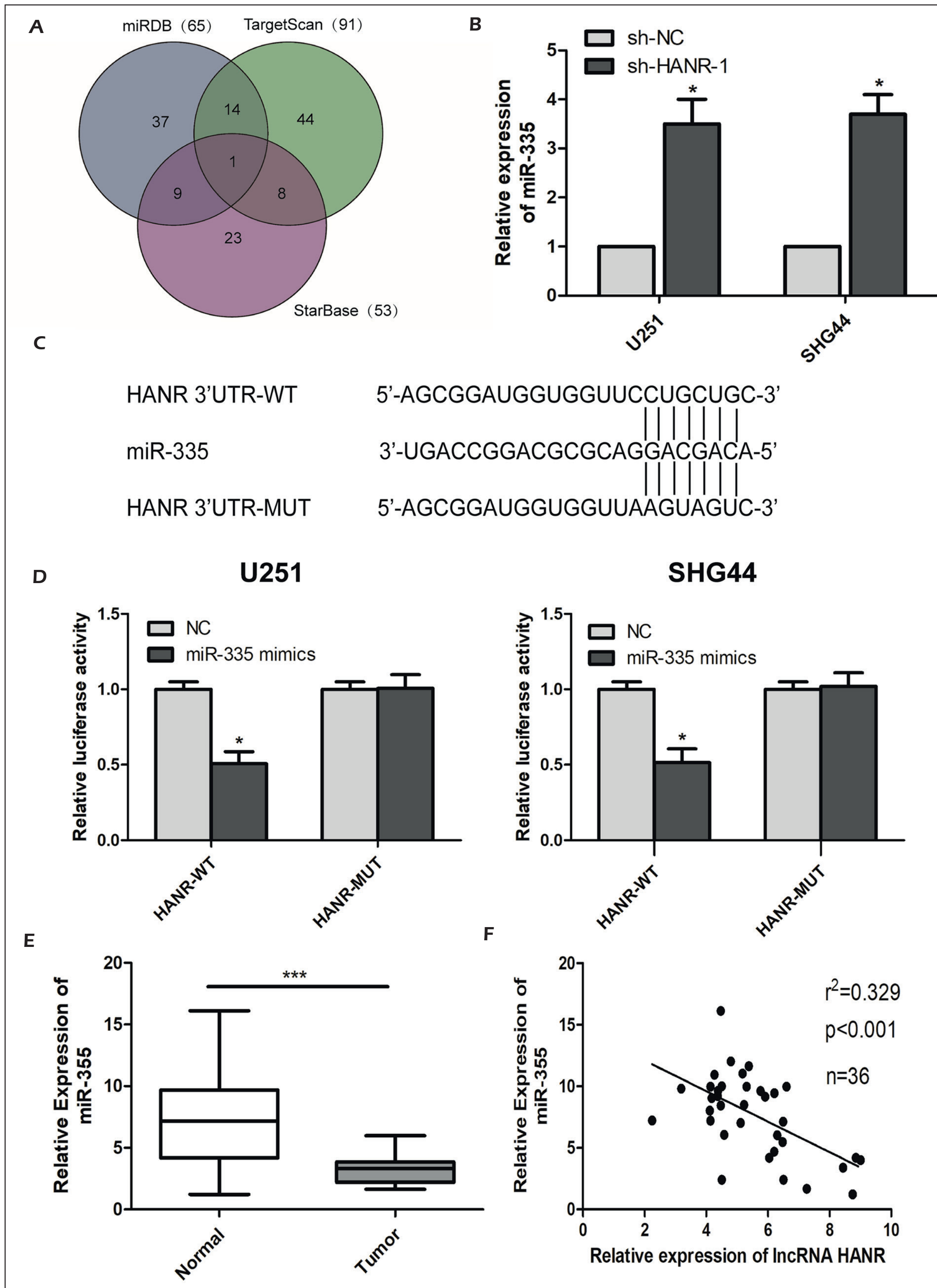

Figure 3. Interaction of HANR and miR-335. **A,** The potential targets of HANR predicted by miRDB, TargetScan, and StarBase. **B,** MiR-335 level in U251 and SHG44 cells transfected with sh-NC or sh-HANR-1. **C,** The binding sites in the promoter regions of miR-335 and HANR. **D,** Luciferase activity in U251 and SHG44 cells co-transfected with HANR-WT/HANR-MUT and miR-335 mimics/NC, respectively. **E,** MiR-335 level in glioma tissues and normal tissues. **F,** A negative correlation was observed between the expression levels of HANR and miR-335 in glioma tissues.

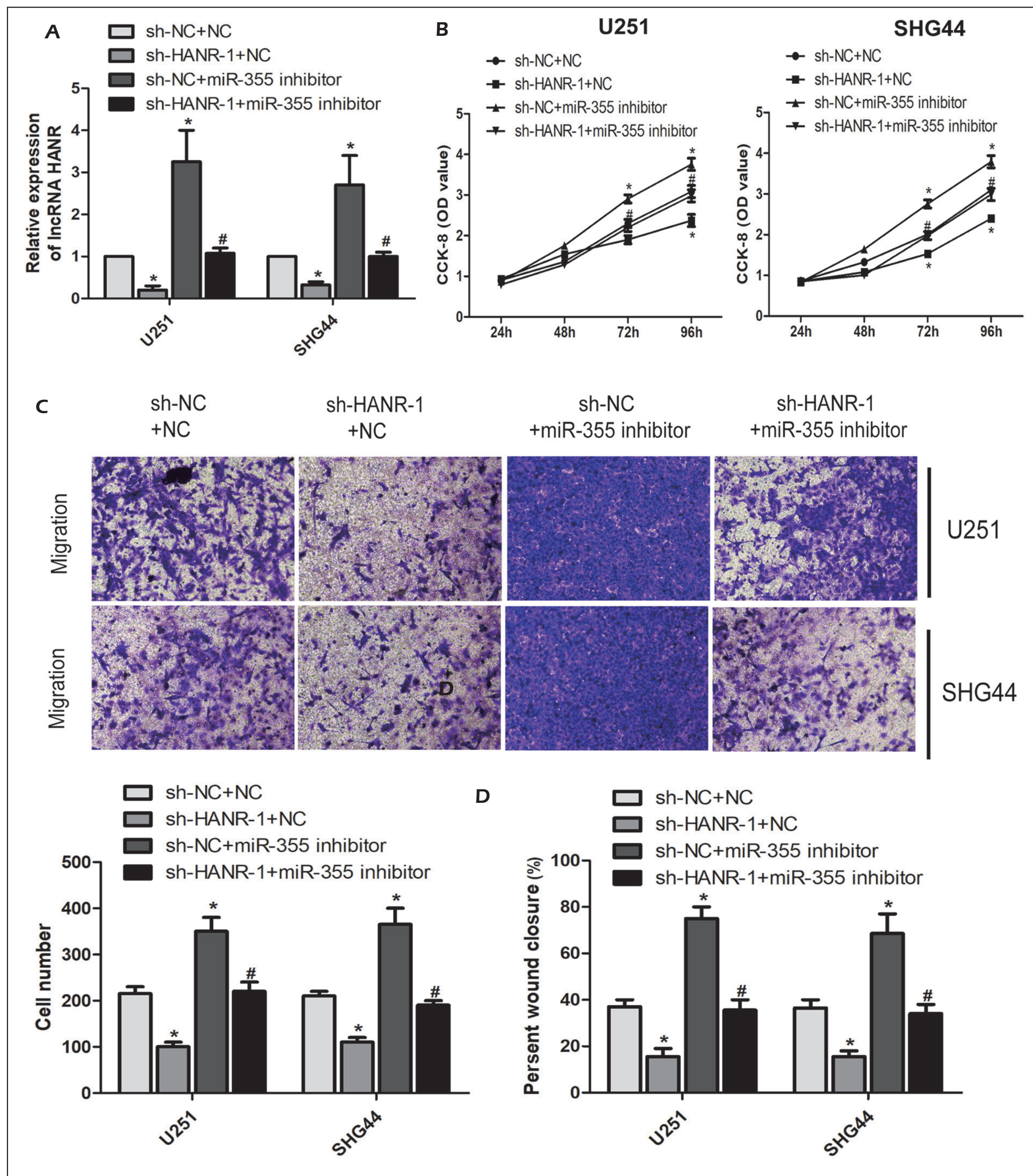

Figure 4. HANR influenced glioma progression by targeting miR-335. U251 and SHG44 cells were transfected with sh-NC + NC, sh-HANR-1 + NC, sh-NC + miR-335 inhibitor, or sh-HANR-1 + miR-335 inhibitor. **A,** HANR level. **B,** Viability. **C,** Migration and invasion (magnification: 100x). **D,** Wound closure.

The competing endogenous RNA (ceRNA) hypothesis proposes that transcripts, such as mRNAs, pseudogenes, and lncRNAs, can competitively bind to target miRNA-responsive elements. This may eventually affect the target gene expression and their functions[16,17]. Here, miRDB, TargetScan, and StarBase were used to search for potential targets of HANR. Finally, miRNA-335 was selected and verified to be the downstream gene of HANR. QRT-PCR results illustrated that

miRNA-335 expression was negatively regulated by HANR. Notably, the knockdown of miRNA-335 partially reversed the regulatory effects of HANR on cellular behaviors of glioma. All our findings suggested that HANR aggravated the progression of glioma by targeting miRNA-335.

Conclusions

We first demonstrated that lncRNA HANR is significantly upregulated in glioma and is closely correlated with metastasis and poor prognosis of glioma patients. In addition, HANR aggravates the progression of glioma through negatively regulating miRNA-335.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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