European Review for Medical and Pharmacological Sciences 2018; 22: 7798-7806

MiR-638 serves as a tumor suppressor by targeting HOXA9 in glioma

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Abstract. – OBJECTIVE: MiR-638 is constantly downregulated and serves as a tumor suppressor in various cancers. Its role in gliomas remains unclear. This study is designed to investigate the clinical significance and the pathogenic role of miR-638 in human gliomas.

PATIENTS AND METHODS: Quantitative Real-time PCR was performed to analyze the expression of miR-638 in the tumor and adjacent tissues of 24 glioma patients. The association between the expression of miR-638 and clinical features were examined. Survival of patients was studied by Kaplan-Meier curves. The impact of miR-638 on cell growth and apoptosis was determined by CCK-8 assay, colony formation assay, cell cycle analysis and Annexin V-FITC-PI apoptosis assay. The effect of miR-638 on HOXA9 was determined by luciferase assay and Western blot. The effect of miR-638 and HOXA9 on expression of oncogenes, Cyclin D1 and C-MYC was determined by Western blot.

RESULTS: MiR-638 expression was constantly downregulated in glioma tumor tissue, which is negatively correlated with the WHO grade. MiR-638 expression was associated with clinical features such as tumor size, KPS score and WHO grade. Patients with low miR-638 had a worse overall survival than those with high expression. Experimentally, miR-638 directly targeted HOXA9 to suppress its expression, leading to attenuations of cell proliferation, colony formation and cell cycle progression and enhanced basal apoptosis level. MiR-638/HOXA9 axis also suppressed the expression of Wnt/beta-catenin-regulated oncogenes, Cyclin D1 and C-MYC.

CONCLUSIONS: MiR-638 is a constantly downregulated microRNA in gliomas and is associated with its prognosis. MiR-638 regulates cellular malignancy of gliomas through targeting HOXA9. Thus, miR-638/HOXA9 signaling axis may have therapeutic potential in gliomas.

Key Words

microRNA, HOXA9, Cell proliferation, Cell cycle, Glioma.

Introduction

Gliomas are the most prevalent brain tumors and have been recognized as malignancies that are the most difficult to treat[1]. High-grade gliomas have been characterized by its high invasiveness, high proliferation rate and poor differentiation[1-3]. Although numbers of studies have described the molecular changes in the pathogenesis of gliomas, much more efforts should be made regarding on how to improve patient survival. Thus, finding the aberrantly expressed genes and investigating their roles may help to uncover the mechanisms and develop new effective treatment for gliomas. MicroRNAs are a class of non-coding RNAs that are usually about 22 nt in length and processed by multiple enzymes in a programed biological process[4]. Since their discovery, their gene silencing function has been recognized as one of the most important balancing forces to keep gene expression in check[4-6]. Downregulation and upregulation of microRNAs have been common phenomena in tumor tissues, which possibly contribute to repression of oncogenes and suppression of tumor suppressive genes. It has been reported[7-9] by accumulating evidence that aberrant expression of microRNAs have a tremendous role in the pathogenesis of gliomas. Therefore, a set of microRNAs has shown great potentials in diagnosis, prognosis and treatment of gliomas[7,9,10]. Human miR-638 is located in chromosome 19, and it has been implicated in the pathogenesis of many diseases such as lupus nephritis, gastric cancer, hepatocellular carcinoma and breast cancer[11-15]. However, the expression and function of miR-638 have not been experimentally tested. Our current study aimed to fully uncover the expression and biological role of miR-638 in human gliomas. Our data show that miR-638 is downregulated in glioma tissues and associated with some of the clinical features. MiR-638 has a tumor suppressive role

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by restricting glioma cell proliferation, cell cycle progression and Wnt signaling and promoting apoptosis. We observed that these functions may be achieved by targeting HOXA9, an oncogenic transcriptional factor. Thus, miR-638 might be a novel candidate for the therapy of gliomas.

Patients and Methods

Patients

24 patients were enrolled in our study; these patients had surgeries to resect glioma tumors in Shunde Hospital, Southern Medical University from September 2011 to December 2013. The baseline characteristics have been summarized in Table I. The tumor specimens were snap frozen in liquid nitrogen and rapidly transferred to -80°C. The histological examination was conducted by two independent experienced pathologists. Follow-up studies on patient's survival were updated every month according to the medical record. The study was approved by the Ethics Committee of Shunde Hospital, Southern Medical University. Informed consents were obtained from all patients.

Cell Lines

The two-glioma cell lines, U87 and U251 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Both cell lines were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium that was supplemented with 10% fetal bovine serum (FBS, Gibco, Carsbad, CA, USA). Cells were cultured in a humidified atmosphere with 5% CO_2 in 37°C. The cells were subcultured every 2 days, and medium was changed. Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used to deliver the miR-638 (RiboBiotech., Guangzhou, China), negative control (miR-NC, RiboBiotech., Guangzhou, China) and HOXA9 plasmids (Origene, Beijing, China) into these cells. The dosages were 150 nM for microRNAs and 2 μg/ml for plasmids, and we followed a standard instruction provided by the manufacturer to complete the transfection process.

Quantitative Real-Time PCR

The total RNA samples were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The expression of miR-638 was detected using a Bulge-Loop primer set purchased from RiboBiotech. (Guangzhou, China). A SYBR green-based amplification method (TaKaRa, Dalian, China) was conducted on an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA, USA). U6 was used as a normalizing control; relative expression was processed using $2^{-\Delta\Delta CT}$ method.

Cell Viability Assay and Colony Forming Assay

Cells were seeded in 96-well plates with an amount of 200 cells per well. Cells transfected with microRNAs or plasmids were assessed by

Table I. The association between miR-638 expression and clinical features.

Clinical feature	No. of patients	miR-638 expression		p
		Low	High	
Age (years)				
≥ 50	15	7	8	1.0000
< 50	9	5	4	
Gender				
Male	13	7	6	1.0000
Female	11	5	6	
Tumor Size				
≥ 5 cm	10	8	2	0.0361
< 5 cm	14	4	10	
KPS score				
≥ 70	15	4	11	0.0094
< 70	9	8	1	
WHO grade				
I+II	5	0	5	0.0373
III+IV	19	12	7	

the Cell Counting Kit- 8 assay (CCK-8, Beyotime, Shanghai, China) according to the specification provided by the manufacturer. Colony forming assay was also performed according to the standard procedure, after transfection, 1000 cells were cultured in 6-well plates. Two weeks later, cells were fixed with 95% alcohol and stained with crystal violet solution (Beyotime, Shanghai, China), the colony number in each group was then counted.

Apoptosis and Cell Cycle Assay

Apoptosis was determined using Annexin V-FITC-PI Apoptosis Detection Kit (Vazyme, Nanjing, China). 200,000 cells were collected for each test, and resuspended in binding buffer. The cells were incubated with Annexin V-FITC for 20 min., followed by a 30 min PI incubation process. Cell cycle was determined by PI staining. A FACSCalibur Flow cytometry system (BD Biosciences, Franklin Lakes, NJ, USA) was used for detection and analysis.

Western Blot

The expression level of proteins was determined by Western blot assay. Cells were harvested using RIPA lysis buffer (Beyotime, Shanghai, China). The protein concentration was determined using BCA kit (Beyotime, Shanghai, China). 60 μg protein were used for electrophoresis in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring onto polyvinylidene difluoride (PVDF) membrane (Thermo Fisher, Waltham, MA, USA). After being blocked with 5% non-fat milk at 4°C for 1 h, the membranes were incubated with primary antibodies targeting HOXA9 (Abcam, Cambridge, MA, USA), Cyclin D1 (Cell Signaling Technology, Danvers, MA, USA), C-myc (Abcam, Cambridge, MA, USA) and β-actin (ZSGB, Beijing, China) at 4°C overnight. On the next day, the membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies. Protein bands were developed using ECLplus kit (Beyotime, Shanghai, China). Image J software was used for measuring the density. The expression of protein was normalized to that of β-actin.

Luciferase Assay

The position 1-360 of 3'UTR of HOXA9 mRNA was amplified and inserted into the multiple cloning site of pmiRGLO construct (Promega, Madison, WI, USA). The seed sequence was mutated using site-direct mutagenesis kit (TaKaRa, Dalian, China). The wild type or mutant luciferase reporter was transfected into U87 and U251 cells along with miR-NC or miR-638. Cells were incubated for 24 h, and luciferase activity was detected using the Dual-Luciferase Detection System (Promega, Madison, WI, USA).

Statistical Analysis

Data were expressed as means± SEMs. MiR-638 expression was compared with Student's *t*-test. Overall survival for miR-638-high and miR-638-low groups was compared by log-rank test. Associations between miR-638 expression and clinical features were assessed by Fisher's exact test. One-way ANOVA followed by Bonferroni post-hoc test was used for comparison of multiple groups. A two-tailed *p*-value less than 0.05 was considered statistically significant.

Results

MiR-638 is Underexpressed in Glioma Tissues, and Low mir-638 Expression is Associated with Worse Prognosis

The expression and function of miR-638 in human glioma tissues have not reported. To understand the role of miR-638 in the development and progression of gliomas, we examined its expression in both tumor tissues and adjacent normal tissue using quantitative Real-time PCR. As shown in Figure 1A, a lower level of miR-638 expression was detected in tumor tissues. Moreover, when we analyze its potential differential expression according to the WHO grade, we found that high-grade glioma tissues have a significantly lower expression of miR-638 compared with that of low-grade glioma tissues (Figure 1B). Based on the median expression of miR-638 in the study subjects, we classified study subjects into miR-638-low expression group and miR-638-high expression group. Our data have shown that miR-638 expression was associated with important clinical features such as tumor size, KPS score and WHO grade (Table I). To investigate whether miR-638 is correlated with the survival of patients, a follow-up study was conducted and a Kaplan-Meier survival curve was constructed. As shown in Figure 1C, we found that patients with high miR-638 survived relatively longer than patients with low miR-638 expression. These data suggest that miR-638 may be a potential prognosis marker for glioma patients and that low expression of miR-638 in may confer the tumorigenicity of glioma cells.

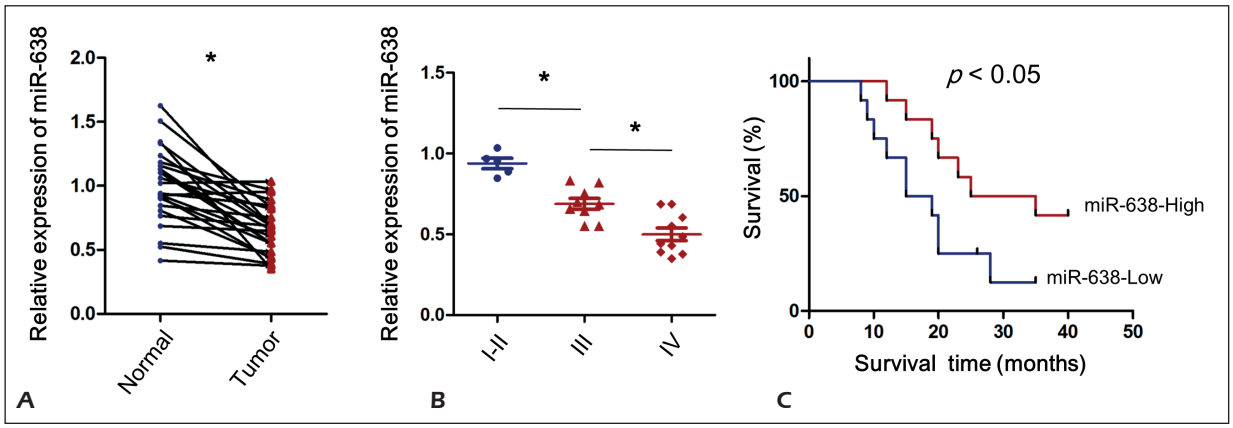

Figure 1. MiR-638 is underexpressed in glioma tissues, and low miR-638 expression is associated with worse prognosis. **A**, The expression of miR-638 in glioma tissues and their paired adjacent normal tissues. **B**, The expression of miR-638 in glioma tissues of different grades. **C**, The survival of patients with high and low miR-638 expression. *p<0.05.

Overexpression of miR-638 Suppresses Cell Proliferation, Induces Cell Cycle Arrest and Promotes Apoptosis in Glioma Cells

We next utilized *in vitro* experiments to explore the molecular mechanism of miR-638 in the pathogenesis of gliomas. Glioma cell lines U87 and U251 were used in our study. MiR-638 was overexpressed in these cells by transfecting miR-638 mimics. To our expectation, miR-638 was able to attenuate the proliferation and colony formation of glioma cells (Figure 2A-C). Flow cytometry analysis showed that miR-638 transfection induced cell cycle arrest at G1 phase (Figure 2D). Apoptosis was also analyzed by flow cytometry, miR-638 transfected U87 and U251 cells have a significantly higher level of basal apoptosis (Figure 2E). These results indicated that the miR-638 mediates a tumor suppressive effect in glioma cells by inducing cell cycle arrest and promoting apoptosis.

MiR-638 Directly Targets HOXA9 to Exert its Tumor Suppressive Function

To further understand the molecular basis of the tumor suppressive action of miR-638, we searched the Targetscan database (www.targetscan.org) to find its potential target gene. We found that the mRNA of HOXA9, which encodes a previously established oncogenic transcriptional factor in glioma[16], contains a potential binding site in its 3'UTR (Figure 3A). We constructed a luciferase reporter having the HOXA9 3'UTR inserted in pmiRGLO luciferase reporter backbone. Site-directed mutagenesis induced mutation of the binding sites was conducted to construct a mutant luciferase reporter (Figure 3A). Our data showed that in U87 and U251 cells, miR-638 effectively suppressed the luciferase activity of wild type (WT) reporter, but showed no effect on the activity of mutant luciferase reporter (Figure 3B), which indicated that miR-638 directly targets HOXA9. Western blot analysis confirmed that miR-638 suppressed the expression of HOXA9 (Figure 3C). To further ascertain the functional role of HOXA9 in miR-638-mediated cellular effect, we overexpressed HOXA9 in miR-638 transfected cells to test whether there was a reversal of the inhibitory effect on cell growth. Indeed, HOXA9 abolished the inhibitory effects of miR-638 on many aspects of cell growth such as cell proliferation, colony formation and cell cycle progression in both cell lines (Figure 3D-F). These data suggest that HOXA9 is a functional target in glioma cells.

MiR-638/HOXA9 Signaling Controls the Expression of Wnt/beta-Catenin-Regulated Oncogenes

A recent study has shown that Wnt/beta-catenin signaling mediates the oncogenic effect of HOXA9[17], we, therefore, tested whether this is also true in gliomas. We analyzed the protein level of Cyclin D1 and C-MYC, two well-established Wnt/beta-catenin-regulated oncogenes. We found that miR-638 inhibited the expression of Cyclin D1 and C-MYC in U87 and U251 cells, which was partially abrogated by HOXA9 (Figure 4A and B). These data suggest that Wnt/beta-catenin signaling may act downstream of miR-638/HOXA9 axis to regulate cellular malignancy in gliomas.

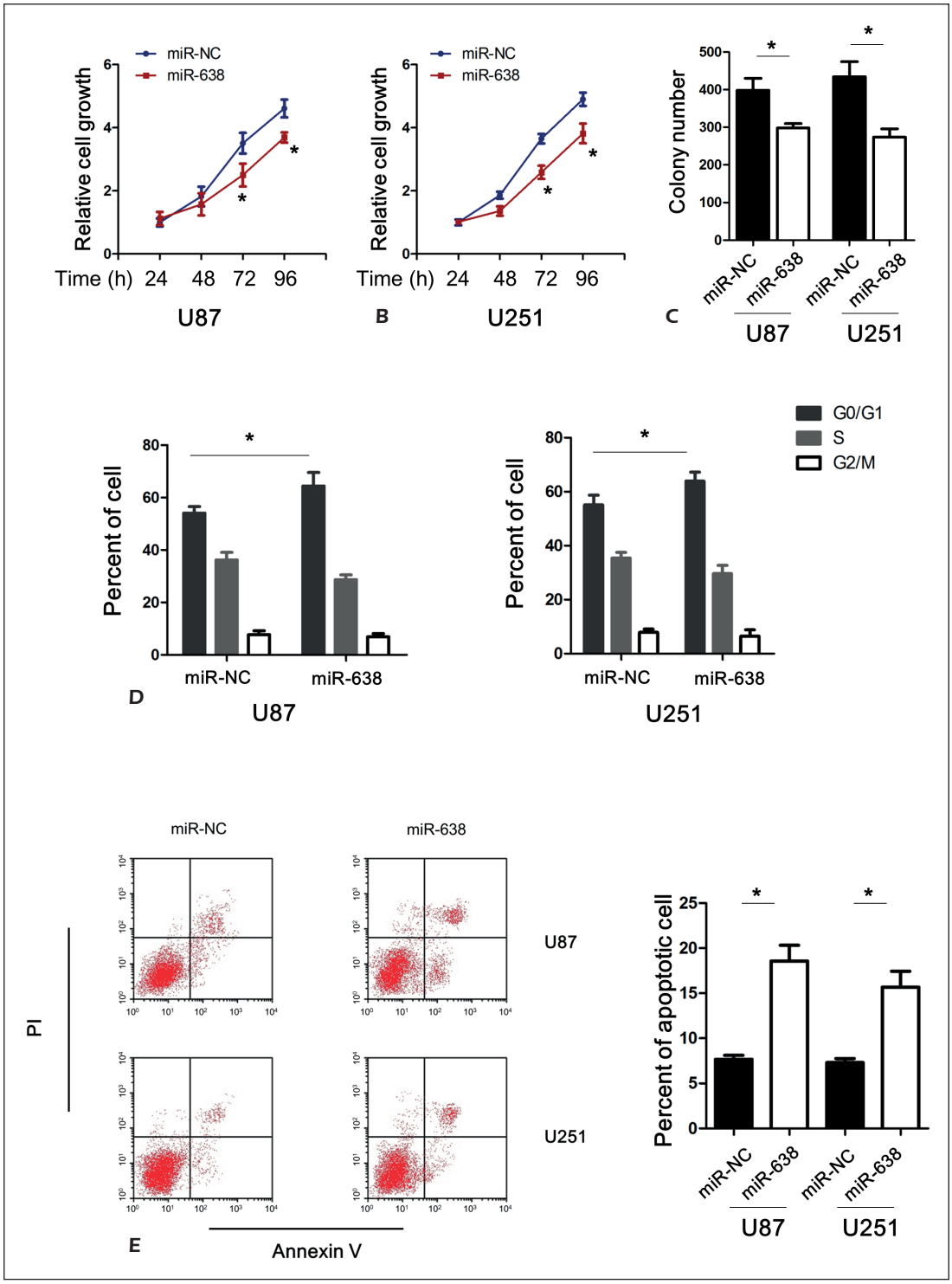

Figure 2. Overexpression of miR-638 suppresses cell proliferation, induces cell cycle arrest and promotes apoptosis in glioma cells. (***A*** and ***B***) Cell viability assay conducted by CCK-8 kit in U87 and U251 cells transfected with miR-NC and miR-638. ***C***, Colony formation of U87 and U251 cells transfected with miR-NC and miR-638. ***D***, Cell cycle distribution of U87 and U251 cells transfected with miR-NC and miR-638. ***E***, Apoptosis of U87 and U251 cells transfected with miR-NC and miR-638. *$p<0.05$ vs. miR-NC, n=3.

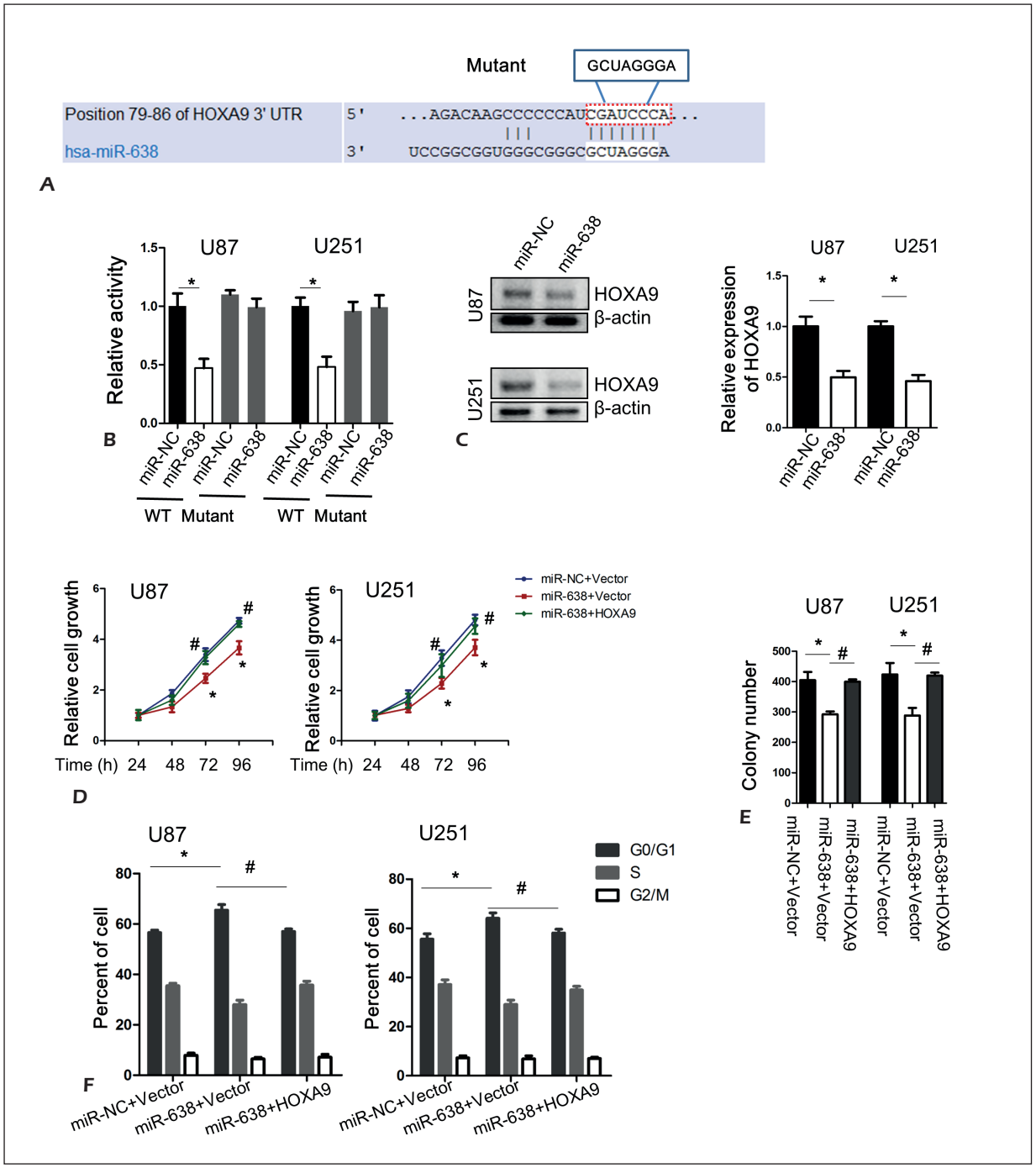

Figure 3. MiR-638 directly targets HOXA9 to exert its tumor suppressive function. **A**, HOXA9 is a predicted target of miR-638 in Targetscan database. **B**, The wild type (WT) and mutant luciferase reporter activity in U87 and U251 cells transfected with miR-NC and miR-638. *p<0.05 vs. miR-NC, n=3. **C**, The expression of HOXA9 in U87 and U251 cells transfected with miR-NC and miR-638. *p<0.05 vs. miR-NC, n=3. **D**, The combinatory effect of miR-638 and HOXA9 on cell proliferation in U87 and U251 cells. *p<0.05 vs. miR-NC+ Vector, #p<0.05 vs. miR-638+ Vector, n=3. **E**, The combinatory effect of miR-638 and HOXA9 on colony formation in U87 and U251 cells. *p<0.05 vs. miR-NC+ Vector, #p<0.05 vs. miR-638+ Vector, n=3. **F**, The combinatory effect of miR-638 and HOXA9 on cell cycle distribution in U87 and U251 cells. *p<0.05 vs. miR-NC+ Vector, # p<0.05 vs. miR-638+ Vector, n=3.

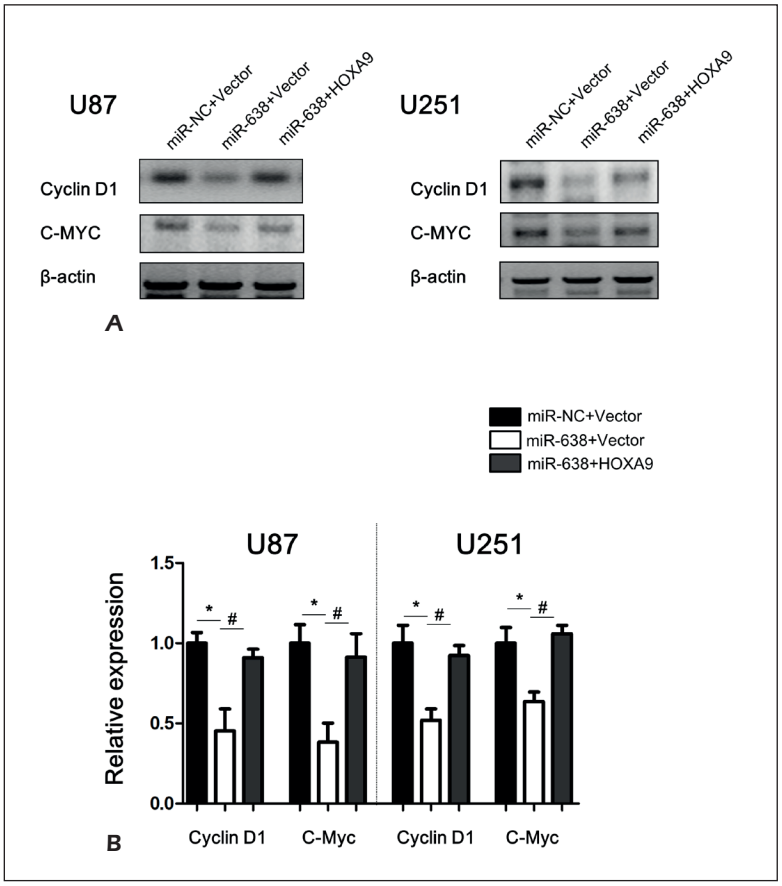

Figure 4. MiR-638/HOXA9 signaling controls the expression of Wnt/beta-catenin-regulated oncogenes. **A**, The expression of miR-638 and HOXA9 on Cyclin D1 and C-MYC protein expression in U87 and U251 cells. **B**, The quantification of Cyclin D1 and C-MYC proteins. *$p<0.05$ vs. miR-NC+ Vector, $^{\#}p<0.05$ vs. miR-638+ Vector, n=3.

Discussion

In this study, we demonstrate for the first time that miR-638 serves as a novel tumor suppressor that is under-expressed in glioma tissues. Importantly, miR-638 expression is significantly associated with some of the important clinicopathological features of glioma such as tumor size, KPS score and tumor grade. Patients with high miR-638 expression are more likely to survive longer that those with low miR-638 expression. We uncovered that miR-638 have multiple roles to regulate the carcinogenicity of glioma cells. Overexpression of miR-638 inhibited cell cycle progression and increased the basal apoptosis. Moreover, these effects were likely to be connected with the expression of HOXA9. HOXA9 was confirmed as a direct target of miR-638 in both glioma cell lines (U87 and U251). Overexpression of HOXA9 can partially abolish the effects of miR-638. Finally, we show that cyclin D1 and C-myc, two Wnt signaling effectors that are important for cancer cell growth[18], are affected by miR-638 and HOXA9, corroborating the previous study showing that HOXA9 act upstream of Wnt signaling[17]. Collectively, we show that miR-638 may be both a prognostic marker and a therapeutic biological agent. The misregulations of microRNAs in gliomas have been reported repeatedly. Based on the expression data of microRNAs, a recent work has shown that the glioblastoma patients can be classified into two groups, long term survival and extremely short term survival, which is a comprehensive understanding of the microRNAs with prognostic function[19]. For example, the expression of miR-497 and miR-125b can be applied to distinguish the grade of glioma[20], which is a crucial prognostic factor for glioma; and miR-125 is correlated with overall survival in glioblastoma multiform patients[19]. In this study, we provide evidence that miR-638 is significantly lower in high-grade gliomas. MiR-638 expression is associated with multiple prognostic factors such as tumor size, KPS score and WHO grade and is indeed closely associated with patient survival in our follow-up study. Intriguingly, previous microRNA-based consensus clustering study using the Cancer Genome Atlas

(TCGA) datasets showed that subclass with miR-638 signature has longer survival[21]; our data, which show that patients with high miR-638 expression have a relatively longer survival time, are consistent with this TCGA study. Although this conclusion should be warranted with a multicenter study with big sample size, our data shed light on the novel function of miR-638 in glioma prognosis and treatment. Our *in vitro* researches have unraveled a potential underlying mechanism of the clinical outcome of miR-638 low expression in gliomas. Overexpression of miR-638 in glioma cells significantly caused G1/S cell cycle arrest and promoted apoptosis, which significantly attenuated cell proliferation activity. Previously, the function of miR-638 has not been reported in glioma. However, researches have shown that it plays a favorable role in gastric cancer, cervical cancer and breast cancer[12,22-24]. Our study, together with these investigations, demonstrates that despite distinct molecular targets in different cancers, miR-638 generally functions as a tumor suppressor. We proposed that miR-638 exerts its tumor suppressive function through HOXA9, a transcriptional factor that has been shown to promote glioma cell growth. Costa et al[16] have shown that HOXA genes are aberrantly activated within confined chromosomal domains in some glioblastoma, and that HOXA9 expression is associated with poorer survival and controlled by PI3K mediated transcriptional process. In osteosarcoma, HOXA9 can activate downstream Wnt/beta-catenin signaling and promote cell proliferation[17]. This is also confirmed by our research, since two crucial Wnt target genes, cyclin D1 and C-myc, were downregulated by miR-638, and HOXA9 expression restored their expression. Goncalves et al[25] showed that HOXA9 expression in glioma cells is relevant to biological processes known as cancer hallmarks such as cell cycle, DNA repair, stem cell phenotype and cell adhesion, the current study suggests that miR-638 may be an important modulator of these biological processes. More precise experimental approaches can be made to fully characterize the role of miR-638 in gliomas.

Conclusions

We showed that miR-638 was under-expressed in glioma tissues and has a very important prognostic significance. MiR-638 plays as a potential negative regulator of cell growth by targeting HOXA9. Thus, we showed that miR-638 represents a potential molecule for the effective treatment of gliomas.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

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