MiR-330-3p functions as a tumor suppressor that regulates glioma cell proliferation and migration by targeting *CELF1*

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Abstract

Introduction: Glioma is a common type of neoplasm that occurs in the central nervous system. miRNAs have been demonstrated to act as critical regulators of carcinogenesis and tumor progression in multiple cancers, but the molecular mechanism of miR-330-3p in glioma remained unclear. The purpose of the study was to explore the role of miR-330-3p in glioma cell reproduction and migration.
Material and methods: The expression levels of miR-330-3p and CELF1 in 27 glioma tissue specimens and human glioma cell lines were examined by qRT-PCR and western blot. The TargetScan database was used to predict the relationship between miR-330-3p and CELF1. Then the target relationship was verified using dual-luciferase reporter assay. The effects of miR-330-3p/CELF1 on glioma cell proliferation were evaluated by MTT and colony formation assay. Wound healing assay was employed to measure the migration ability of glioma cells.
Results: MiR-330-3p was found lowly expressed in glioma tissues and cells compared with adjacent tissues and normal astrocytes, while CELF1 expression was relatively high in the glioma tissues and cells. Dual-luciferase reporter assay confirmed that miR-330-3p could directly target CELF1. Furthermore, miR-330-3p could down-regulate the expression of CELF1, therefore suppressing glioma cell reproduction and migration.
Conclusions: MiR-330-3p inhibited the propagation and migration of glioma cells by repressing CELF1 expression.

Key words: glioma, miR-330-3p, *CELF1*, proliferation, migration.

Introduction

Glioma is a kind of common neoplasm in the central nervous system, with a high morbidity and mortality rate worldwide [1–3]. Currently, the combined treatment of tumor resection with radiation and chemotherapy has been considered as a primary therapeutic method and widely applied to the treatment of neoplasms. Despite tremendous progress in the treatment of glioma, the average 5-year survival rate remains at a low level among glioma patients [4–6].

MicroRNAs (miRNAs) are highly conserved, non-coding small RNA molecules composed of approximately 20 nucleotides, which are involved

in the process of tumor initiation and progression [7–9]. Numerous previous studies have found a variety of miRNAs aberrantly expressed in glioma tissues and cells, contributing to tumorigenesis or carcinogenesis [10–13]. miRNAs have been found to function as tumor suppressors or facilitators through binding to 3'UTRs of target genes in multiple diseases [14–17]. They regulate glioma cell proliferation and metastasis by regulating corresponding target genes [18, 19]. For instance, miR-330-3p functions in different types of cancers and has been extensively studied [20, 21]. Mesci *et al.* reported that miR-330-3p enhances breast cancer cell invasion and metastasis abilities by targeting *CCBE1* [20]. Meng *et al.* verified that miR-330-3p acted as an oncogenic mRNA in esophageal squamous cell carcinoma by repressing *PDCD4* expression [22]. However, the function and the specific molecular mechanism of miR-330-3p in malignant gliomagenesis remain unknown, and to understand the effects of miR-330-3p on the biological functions of glioma cells is critical for the development of novel therapeutic strategies of glioma.

CELF1, also known as CUGBP1, is an RNA binding protein that belongs to the CUGBP embryonic lethal abnormal vision-like family. *CELF1* can control the post-transcriptional regulation of genes involved in cell survival [23]. Recent studies have shown that *CELF1* was abnormally expressed in malignant T cells and laryngeal cancer cells [24, 25]. Its aberrant expression may suggest its possible oncogenic role in various cancers; for instance, Xia *et al.* found that overexpression of *CELF1* promoted glioma cell growth [26]. In this study, we also found the overexpression of *CELF1* in glioma cells, which facilitated glioma cell propagation and migration. The specific mechanism of action underlying how *CELF1* affects gliomagenesis needs to be determined.

Based on the findings of previous studies, we attempted to explore the role of miR-330-3p and *CELF1* in glioma. We hypothesized that *CELF1* might be a potential target of miR-330-3p and miR-330-3p might influence glioma cell reproduction and migration via regulation of *CELF1*.

Material and methods

Tissue specimens

Twenty-seven pairs of glioma tissues and adjacent normal tissues were obtained from patients with glioma undergoing surgical resection in the Affiliated Hospital of Hebei University of Engineering between August 2014 and August 2016 (Table I). All glioma tissue specimens were confirmed based on histopathological evaluation. We froze all tissues in liquid nitrogen and conserved them at −80°C for the following assay. The study

obtained permission from the Research Ethics Committee of the Affiliated Hospital of Hebei University of Engineering and informed consent from all participants.

Cell culture, transfection and grouping

Glioma cell lines (U87, U251, and C6) and the normal astrocyte cell line HA (ATCC, Manassas, VA) were cultured in DMEM medium (GIBCO, Gaithersburg, MD) plus 10% FBS, 100 U/ml penicillin, and 100 ng/ml streptomycin, followed by incubation at 37°C. MiR-330-3p mimics, negative control (NC) and *CELF1*-cDNA were purchased from Shanghai GenePharma Ltd. (Shanghai, China). Briefly, 1×10^5 U251 cells were inoculated into a 6-well plate and cultured at 37°C, 5% CO_2 overnight, followed by transfection with miR-330-3p mimics and *CELF1* cDNA using Lipofectamine 2000 reagents (Invitrogen). The grouping of cells in the experiment was as follows: (1) the control group

Table I. Association between miR-330-3p expression and clinical characteristics

Clinical characteristics	N	miR-330-3p expression		P-value
		Low	High	
Sex:				0.8826
Male	14	9	5	
Female	13	8	5	
Age (years):				0.8068
≤ 60	17	11	6	
> 60	10	6	4	
Tumor location:				0.7781
Parenchyma	17	11	6	
Ventricle	10	7	3	
Tumor size:				0.8826
≤ 3 cm	14	9	5	
> 3 cm	13	8	5	
WHO grade*:				0.0341
I + II	13	5	8	
III + IV	14	11	3	
Karnofsky score*:				< 0.0001
≤ 80	16	15	1	
> 80	11	2	9	
Resection range:				0.7215
Total resection	15	9	6	
Local resection	12	8	4	

p < 0.05, performed by χ² test.

(without transfection); (2) the NC group (transfection with antisense sequence); (3) the miR-330-3p group (transfection with miR-330-3p mimics); (4) *CELF1* group (transfection with *CELF1* cDNA) and (5) miR-330-3p + *CELF1* group (co-transfection with miR-330-3p mimics and *CELF1* cDNA).

qRT-PCR

Trizol reagent and NanoDrop 1000 (Thermo Fisher Scientific) were respectively used for the extraction and quantification of total RNA. Total RNA was subjected to reverse transcription by using the PrimeScript RT reagent kit (Takara, Japan). Expression levels of miR-330-3p were measured using a TaqMan microRNA assay (Applied Biosystems, San Diego, USA). U6 (RUN6B) was assessed as an endogenous control. *CELF1* mRNA levels were quantified by a SYBR Green PCR kit (TaKaRa, Dalian, China). GAPDH was used as an endogenous control to normalize the amount of total mRNA in each sample. The primer sequences used were as follows: for CELF1 5′-TCCTGC-CGTTTGTTCATCGTT-3′ (forward) and 5′-TTTC-CCCTTCAGCAGTCGTTC-3′ (reverse); for GAPDH: 5′-TATTGTTGCCATCAATGACCC-3′ (forward) and 5′-ACTCCACGACGTACTCAGC-3′ (reverse). MiR-330-3p and *CELF1* expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

Western blot

SDS-PAGE and bicinchoninic acid (BCA) Kit (Sigma-Aldrich, St. Louis, Missouri, USA) were used to separate cell lysate and quantify protein concentration. Proteins were transferred from SDS-PAGE gel to PVDF membranes, which were then soaked in 5% nonfat milk and incubated with antibodies against CELF1 and GAPDH (CELF1, GAPDH, 1 : 1000, 1 : 2000, Abcam, Cambridge, UK) at 4°C for 24 h. Afterwards, the membranes were rinsed with TBST three times and incubated with HRP-labeled mouse anti-rabbit secondary antibody (1 : 2000) for 1.5 h at 37°C. After washing with TBST for 15 min, the immunoreactive proteins were visualized and analyzed by enhanced chemiluminescence (ECL) western blotting detection reagent (GE Healthcare, Milwaukee, WI, USA).

Dual-luciferase reporter gene assay

To validate the relationship between miR-330-3p and *CELF1*, we first predicted the binding site of hsa-miR-330-3p on 3′UTR of *CELF1* by means of the TargetScan database (http://www.targetscan.Org/). Meanwhile, dual-luciferase reporter gene assay was also used to further validate the correlation between miR-330-3p and *CELF1*. The wild type (wt) or mutated (mut) 3′-UTR of *CELF1* and the predicted binding site of miR-330-3p were amplified and cloned into the pmirGLO vectors (Thermo Fisher Scientific). Then, we co-transfected pmirGLO recombinant vector and miR-330-3p mimics or negative control mimics into glioma cells and the relative Luciferase activity was detected after 48-hour transfection.

MTT assay

The cell proliferation assay was performed with an MTT kit (Sigma, St. Louis, Mo, USA). We first inoculated glioma cells with different transfection into a 96-well plate (1×10^3 cells/well). Subsequently 20 μl of MTT (15 mg/ml Sigma) was added to each well and kept for 3 h. After the supernatants were discarded, 150 μl of dimethyl sulfoxide (Sigma, St. Louis, Mo, USA) was added to each well. Lastly, the absorbance value of each well was assessed at 490 nm. The assay was carried out three times.

Colony formation assay

Glioma cells were put into 60 mm culture dishes and incubation was performed in a standard environment for 7 days. Each group had six reduplicative wells. 4% paraformaldehyde and 0.1% crystal violet were added to the cells for fixation and staining. The colonies were observed through a microscope. All reactions were conducted three times.

Wound healing assay

We seeded glioma cells into 6-well plates (4×10^5 cells/well) 24 h post-transfection. Following adhesion, the medium was removed and the monolayer of cells was scraped with a 1000 μl pipette tip. After rinsing with PBS twice and incubation for 24 h, the scratch healing area was photographed by an inverted microscope and cell migration rates were measured at 0 h and 24 h respectively.

Xenograft tumor assay

Ten immunodeficient female nude mice (15–20 g, Beijing Laboratory Animal Center, Beijing, China) were used to test the effects of miR-330-3p on glioma *in vivo*. These nude mice were randomly divided into two groups (5 mice per group). Approximately 2×10^6 logarithmically growing glioma cells stably expressing NC or miR-330-3p mimics were subcutaneously injected into the nude mice. After 3 weeks, the subcutaneous tumors were stripped and weighed. Tumor volume was calculated according to the formula: V (mm³) = $(a \times b^2)/2$ (*a* represents the longest axis and *b* the shortest axis). The ethics committee of the Animal Experimental Center of Affiliated Hospital of Hebei University approved this study.

Statistical analysis

Statistical analysis was conducted using SPSS 21.0 software (IBM, Chicago, Illinois, USA). All results were displayed as mean ± standard deviation (SD). Normally distributed measurement data were analyzed by Student's *t*-test or one-way ANOVA, otherwise by the Mann-Whitney rank sum test. *P*-value < 0.01 or 0.05 denoted statistical significance.

Results

MiR-330-3p was lowly expressed in glioma tissues and cells, while *CELF1* was highly expressed in glioma tissues and cell lines.

We analyzed miR-330-3p and *CELF1* expressions in tissues specimens using qRT-PCR and western blotting assay. As shown in Figures 1 A, B, glioma tissues presented a significantly lower expression
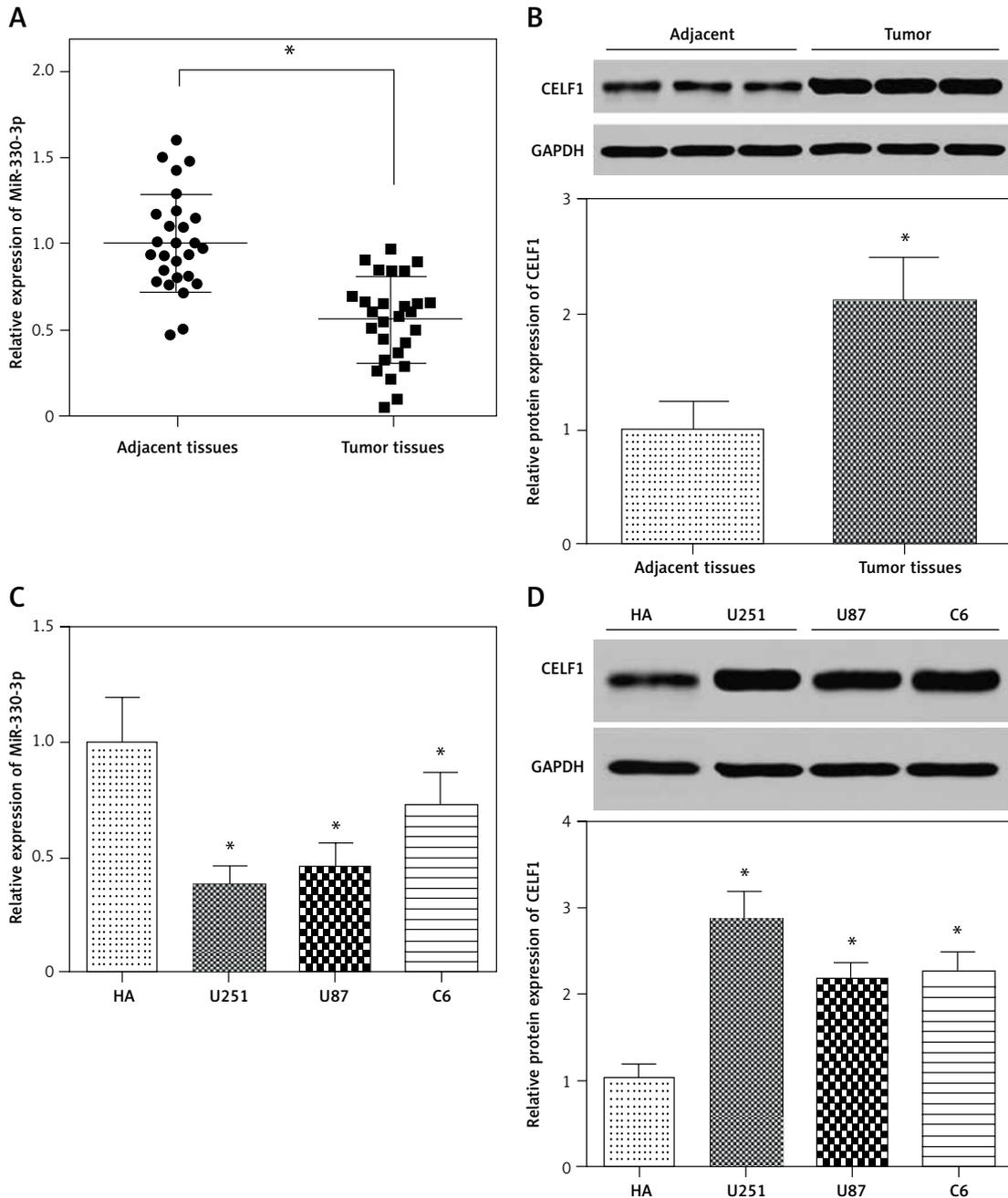

Figure 1. MiR-330-3p was lowly expressed in glioma tissues and cell lines, while *CELF1* was highly expressed in glioma tissues and cell lines. **A** – The expression level of miR-330-3p mRNA in glioma tissues was significantly lower than that in adjacent normal tissues detected by qRT-PCR. **B** – The expression level of CELF1 protein was remarkably higher in glioma tissues in comparison with that of normal tissues detected by western blot. **C** – The expression level of miR-330-3p mRNA was found to be significantly lower in glioma cell lines (U87, U251 and C6) compared with that of normal cell line (HA cells) using qRT-PCR. **D** – The protein expression level of CELF1 was higher in glioma cell lines than in normal cell line detected by western blot. **P* < 0.05, compared with adjacent tissues and normal cell line (HA)

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level of miR-330-3p compared with normal tissues ($^*p < 0.05$), whereas *CELF1* expression was remarkably higher in in glioma tissues ($^*p < 0.05$). Likewise, the expression levels of miR-330-3p in glioma cell lines (U87, U251 and C6) were significantly lower compared with that in HA cells ($^*p < 0.05$) (Figure 1 C). Moreover, results from western blot indicated that *CELF1* protein expression in glioma cell lines was remarkably higher in comparison with normal cells, especially in the U251 cell line ($^*p < 0.05$, Figure 1 D). Overall, compared with U87 and C6, U251 had higher expression of miR-330-3p and lower expression of *CELF1*, which made it a proper subject to adapt the change of miR-330-3p and *CELF1* in our further study. Therefore, the U251 cell line was chosen for the subsequent experiments.

MiR-330-3p directly modulated *CELF1* by targeting its 3′UTR

We performed prediction analysis for binding sites of miR-330-3p on 3′UTR *CELF1* using Target-Scan. As shown in Figure 2 A, *CELF1* was one of the potential targets of miR-330-3p. Meanwhile, results of dual-luciferase reporter gene assay indicated that the luciferase activities of U251 cells transfected with *CELF1* 3′UTR-WT vectors were significantly weaker in the miR-330-3p mimic group than in the NC group. A difference of luciferase activities of U251 cells transfected with *CELF1* 3′UTR-MUT vectors was not found between the NC group and the miR-330-3p group (Figure 2 B).

MiR-330-3p suppressed glioma cell growth and migration by targeting *CELF1*

To evaluate the functions of miR-330-3p and *CELF1* in glioma cell growth and migration, we examined the expression of miR-330-3p in cells. Results from qRT-PCR revealed that the expression of miR-330-3p mRNA in the miR-330-3p group and miR-330-3p + *CELF1* group was significantly up-regulated in comparison with the control group after transfection ($^*p < 0.05$, Figure 3 A). Furthermore, western blot showed that CELF1 protein expression significantly decreased in the miR-330-3p mimic group, while CELF1 expression in cells transfected with *CELF1* cDNA dramatically increased. MiR-330-3p could down-regulate the expression level of *CELF1* through targeting *CELF1* ($^*p < 0.05$, Figure 3 B). In addition, according to the results of MTT assay, overexpression of miR-330-3p significantly inhibited glioma cell viability or growth, while overexpression of *CELF1* significantly promoted glioma cell viability or growth in comparison with the control group ($^*p < 0.05$, Figure 3 C). Similarly, the colony formation assay demonstrated that miR-330-3p overexpression significantly repressed the proliferation ability of glioma cells, while *CELF1* overexpression facilitated glioma cell proliferation. MiR-330-3p could suppress glioma cell proliferation via downregulation of *CELF* ($^*p < 0.05$, Figure 3 D). Meanwhile, the wound healing assay indicated that the migration rate of cells in the miR-330-3p overexpression group was significantly lower compared with the NC group, suggesting that miR-330-3p could suppress glioma cancer cell migration. On the other hand, the wound healing area in the *CELF1* group was remarkably larger in comparison with the control group, which indicated that *CELF1* could promote glioma cancer cell migration ($^*p < 0.05$, Figure 3 E). After U251 cells were co-transfected with miR-330-3p and *CELF1*, no significant difference of cell migration was found between the miR-330-3p +
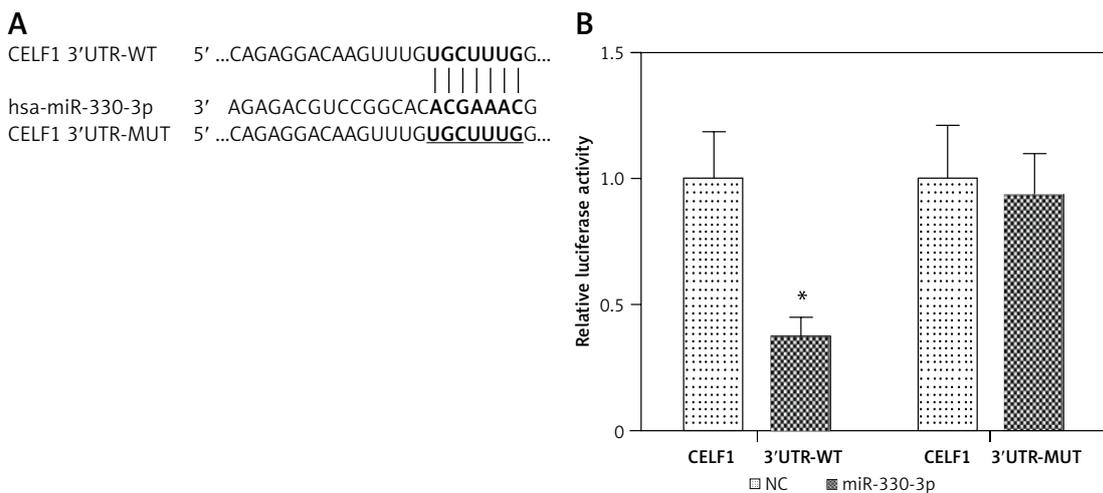
A

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CELF1 3′UTR-WT    5′ ...CAGAGGACAAGUUUGUGCUUUGG...
                                    | | | | | | |
hsa-miR-330-3p    3′  AGAGACGUCCGGCACACGAAACG
CELF1 3′UTR-MUT   5′ ...CAGAGGACAAGUUUGUGCUUUGG...
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B

Figure 2. MiR-330-3p directly modulated *CELF1* by targeting its 3′UTR. **A** – Alignment of the miR-330-3p putative binding site and corresponding mutated site on *CELF1* 3′UTR. **B** – The luciferase activity of U251 cells transfected with *CELF1* 3′UTR-WT vectors was significantly weaker in the miR-330-3p mimic group than in the NC group. Difference of luciferase activities of U251 cells transfected with *CELF1* 3′UTR-MUT vectors was not found between NC group and miR-330-3p group. *$P < 0.05$, compared with NC group
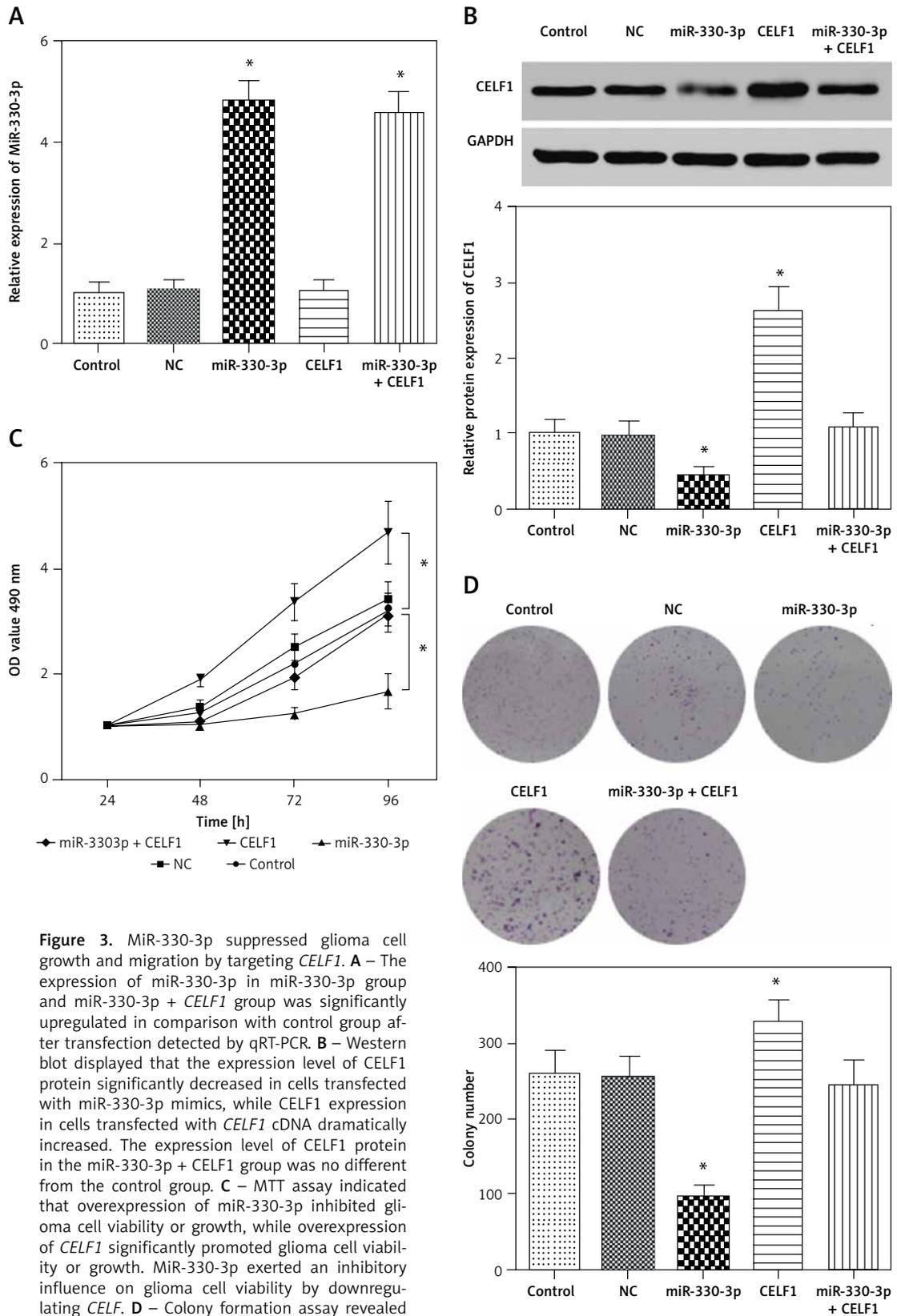
Figure 3. MiR-330-3p suppressed glioma cell growth and migration by targeting *CELF1*. **A** – The expression of miR-330-3p in miR-330-3p group and miR-330-3p + *CELF1* group was significantly upregulated in comparison with control group after transfection detected by qRT-PCR. **B** – Western blot displayed that the expression level of CELF1 protein significantly decreased in cells transfected with miR-330-3p mimics, while CELF1 expression in cells transfected with *CELF1* cDNA dramatically increased. The expression level of CELF1 protein in the miR-330-3p + CELF1 group was no different from the control group. **C** – MTT assay indicated that overexpression of miR-330-3p inhibited glioma cell viability or growth, while overexpression of *CELF1* significantly promoted glioma cell viability or growth. MiR-330-3p exerted an inhibitory influence on glioma cell viability by downregulating *CELF*. **D** – Colony formation assay revealed that overexpression of miR-330-3p significantly repressed the proliferation ability of glioma cells, while *CELF1* overexpression facilitated glioma cell proliferation. MiR-330-3p could suppress glioma cell proliferation via downregulation of *CELF*. *P < 0.05, compared with the control group
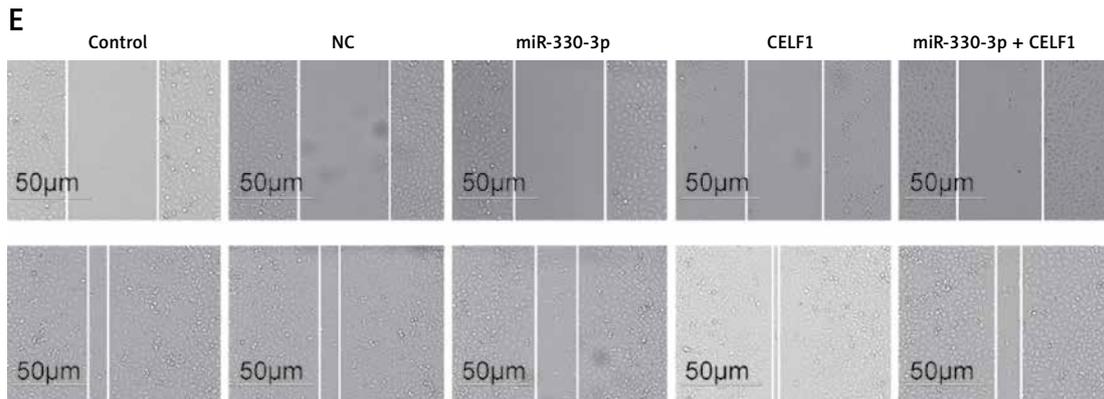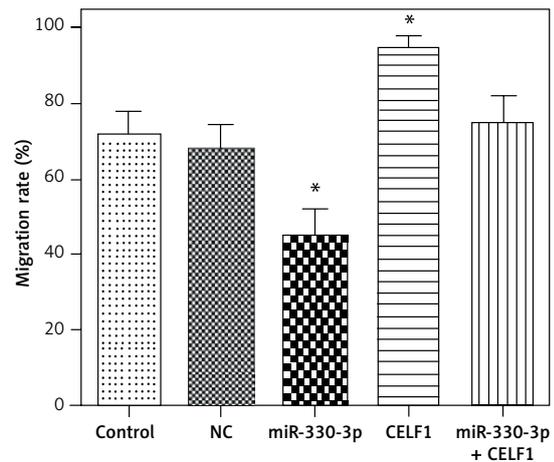
E

| Control | NC | miR-330-3p | CELF1 | miR-330-3p + CELF1 |

Figure 3. Cont. **E** – Wound healing assay showed that miR-330-3p overexpression significantly inhibited glioma cell migration, whereas *CELF1* overexpression drastically enhanced the migration ability of glioma cell. MiR-330-3p could suppress glioma cell migration through downregulating *CELF*. *$P < 0.05$, compared with the control group

CELF1 group and the control group (*$p > 0.05$). All the results above demonstrated that miR-330-3p might suppress glioma cell propagation and migration via down-regulating *CELF1*.

Up-regulation of miR-330-3p inhibited tumor growth *in vivo*

Xenograft tumor assay was used to test the effects of miR-330-3p on glioma *in vivo*. The tumor progression of glioma *in vivo* could be inhibited by up-regulation of miR-330-3p. Over-expression of miR-330-3p showed better performance in suppressing the volume of glioma tumor than the NC group, and simultaneously up-regulating miR-330-3p minimized the tumor growth in nude mice ($p < 0.01$, Figures 4 A–C).

Discussion

In the current study, we detected lower expression of miR-330-3p and higher expression of *CELF1* in glioma tissues and cell lines. Furthermore, we validated the target relationship between miR-330-3p and *CELF1* and demonstrated that miR-330-3p inhibited glioma cell reproduction and migration via down-regulation of *CELF1* expression. These findings identified a new target for the therapeutic strategy of glioma and laid

a foundation for extensive study on the molecular mechanism of miR-330-3p in glioma cells.

There is considerable evidence suggesting that dysregulation of miRNAs could incur the aberrant expression of genes and tumorigenesis [10, 11, 27]. For instance, miR-637 was found significantly decreased in glioma tissues, which could suppress glioma cell growth and metastasis [28]. MiR-330 promoted the malignant behavior of glioblastoma stem cells and significantly activated both the ERK and PI3K/AKT signaling pathways [29]. Down-regulation of miR-145 expression suppressed metastasis of glioma stem cells by targeting *ABCG2* in glioblastoma multiforme tumors [30]. However, miRNA can also act as a tumor facilitator in multiple cancer cells. Chen *et al.* found that miR-106 could induce the proliferation of glioma cells through regulating the JNK/MAPK pathway [31]. Zhou *et al.* revealed that miRNA-155 acted as a facilitator of glioma cell proliferation via regulation of *MXI1* [32]. Palumbo *et al.* studied different glioma cell lines and found the activity and the possibility to generate neurospheres [33]. Inspired by it, we used three glioma cells to find a proper subject to adapt the change of miR-330-3p and *CELF1* and for the subsequent experiments. In our study, miR-330-3p was significantly lowly expressed in glioma specimens and cells. Further-
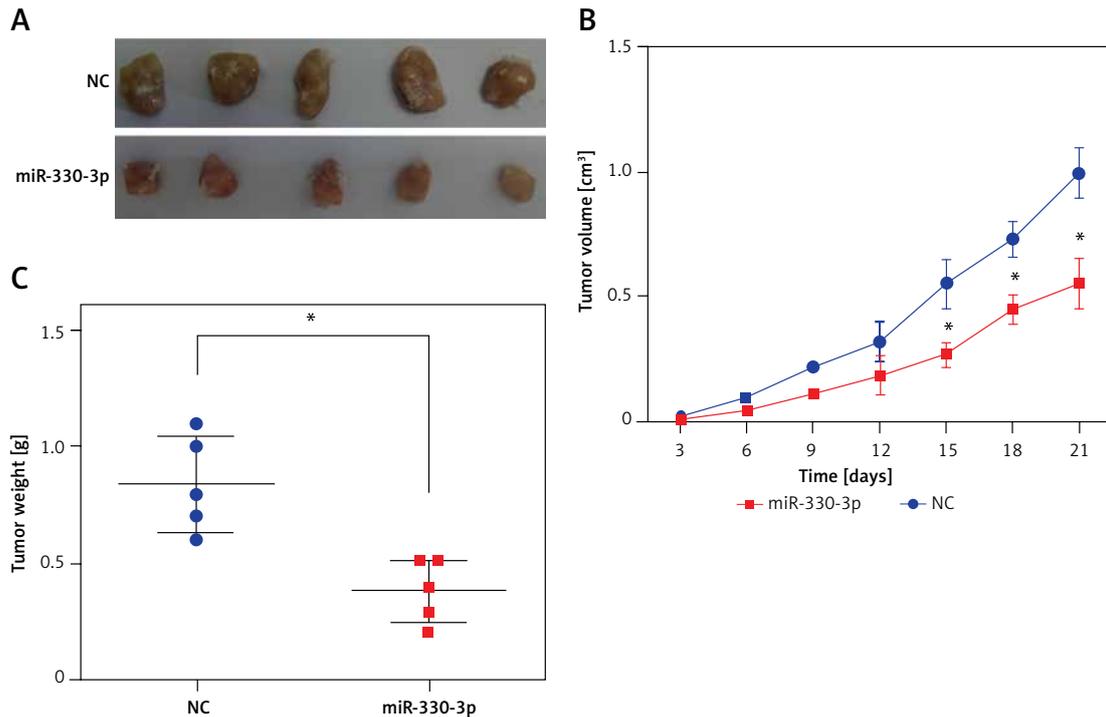
Figure 4. Up-regulation of miR-330-3p inhibited tumor growth *in vivo*. **A** – The xenograft model of nude mice transplanted with glioma cells showed that transfection of miR-330-3p mimics inhibited tumor growth in nude mice. **B**, **C** – The tumor volume and tumor weight in the miR-330-3p group were smaller than those in the NC group. *$P < 0.05$, compared with the NC group

more, miR-330-3p overexpression could drastically repress the proliferation and migration of glioma cells. Combined with *in vivo* experiments, it was verified in our study that overexpression of miR-330-3p could suppress the tumor growth of glioma. Similar studies also indicated that miR-330-3p can modulate gene expression in various cancers. For instance, miR-330-3p could promote metastasis in human breast cancer by targeting *CCBE1* [20]. Nonetheless, there are few studies on the effects of miR-330-3p on glioma progression. In accordance with bioinformatic analysis and the dual-luciferase reporter assay, we further identified *CELF1* as a functional target of miR-330-3p. Moreover, *CELF1* expression in glioma tissues and cells could be repressed by ectopic expression of miR-330-3p, which was also consistent with a previous study [26].

Intriguingly, *CELF1*, as a broadly expressed member of the CUGBP ELAV-like family of RNA-binding proteins, regulates post-transcriptional gene expression by facilitating alternative splicing, translation as well as mRNA degradation, controlling cell growth, motility, and apoptosis [34–36]. House *et al*. reported that RNA-binding protein *CELF1* could promote tumor growth and change gene expression in oral squamous cell carcinoma [37]. Rizzoli *et al*. demonstrated that *CELF1* promoted glioma cell propagation via suppression of *CDKN1B* [38]. Talwar *et al*. found that overex-

pression of *CELF1* inhibited oral cancer cell apoptosis [39]. Gao *et al*. revealed that downregulation of *CELF1* promoted apoptosis and suppressed cell proliferation of non-small cell lung cancer [40]. The previous study presented an 11-component genetic pathway, and showed that CELF1 protein functions as a central node controlling translational activation of genes driving EMT and ultimately tumor progression [41]. In our study, *CELF1* was overexpressed in glioma tissues and cells. Zhong *et al*. found that the enhanced expression of syntenin and phosphorylated FAK may correlate with the increase of the malignancy of human gliomas [42]. In addition, the finding reported by Cui *et al*. identified miR-503 as both a novel regulator of *CELF1* expression and a modulator of intestinal epithelial homoeostasis [43]. Furthermore, miR-214-3p was found to act as a tumor suppressor and that its downregulation contributed to chemoresistance in esophageal cancer cells by targeting *CELF1* [44]. We demonstrated that overexpressed miR-330-3p inhibited glioma cell migration by targeting *CELF1*. Furthermore, we also substantiated that the interplay between miR-330-3p and *CELF1* played a crucial role in glioma development. MiR-330-3p might inhibit glioma cell reproduction and migration by targeting *CELF1*.

Nevertheless, there are several limitations in the study. Firstly, the number of patients was not big enough. Secondly, miR-330-3p is not the only

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miRNA which regulates *CELF1*. As for this point, on line sequence target prediction sites are taken into consideration and bioinformatics analysis will be used in our further study. The tumorigenesis cell pathway miR-330-3p/*CELF1* involved needs to be further studied.

In conclusion, miR-330-3p directly targeted *CELF1* and might inhibit glioma cell reproduction and migration by down-regulating the expression of *CELF1*. The study might provide a new tumor marker for glioma diagnosis and prognosis.

Conflict of interest

The authors declare no conflict of interest.

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