

MiR-137 targets and regulates E2F7 to suppress progression of glioma cells

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Abstract

Introduction: The paper aimed to explore the mechanism of miR-137 in modulating glioma.

Material and methods: qRT-PCR detected miR-137 and E2F7 mRNA expression in cells. The protein expression of E2F7 was measured using Western blot assay. Cell proliferation, scratch healing, transwell and programmed cell death assays were conducted to examine the influences of the genes on the biological function of glioma cells. The dual-luciferase assay verified the interaction between miR-137 and E2F7.

Results: MiR-137 was lowly expressed in glioma cells, and E2F7 was highly expressed. MiR-137 suppressed progression and promoted programmed cell death of glioma cells. MiR-137 could target and negatively regulate E2F7 expression to further accelerate programmed cell death of glioma cells.

Conclusions: It was found that miR-137 could target E2F7 to restrain cell progression and accelerate programmed cell death of glioma cells, which is helpful to search for new molecular therapeutic targets for glioma.

Key words: miR-137, E2F7, glioma, malignant progression.

Introduction

With an annual incidence of 3-8 per 100,000 people, glioma is a general term for tumours derived from the neuroepithelium [17,20]. Glioma can be divided into 4 grades: grade I – pilocytic cell astrocytoma, grade II – diffuse astrocytoma, grade III – anaplastic astrocytoma, and grade IV – glioblastoma. Current treatments for glioma include surgery, radiotherapy and chemotherapy, but the prognosis is still not optimistic. The median survival time is only 12-15 months and the 5-year survival rate is less than 10% [15,21,22]. Hence, it is of paramount significance to understand the specific mechanism of progression of glioma and

to unveil molecular therapeutic targets for glioma to improve the survival of patients with glioma.

MicroRNAs (miRNAs) can inhibit the translation or degrade the target mRNA by combining with the 3'UTR of the target gene [2]. Current research has shown that some miRNAs are important regulators in glioma. For instance, miR-139-3p restrains progression of glioma via targeting MDA-9/syntenin [24]. MiR-424 targets KIF23 to inhibit glioma cell migration, invasion, and epithelial-mesenchymal transition (EMT) [34]. In this study, attention was focused on miR-137, a miRNA that has been extensively studied in cancers. For instance, miR-137 inhibits transforming growth factor β (TGF- β)/Smad pathway through combining with GREM1, thereby inhibiting invasion, migration and EMT in cer-

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vical cancer cells [16]. MiR-137 represses cell migration and invasion *via* targeting EZH2-STAT3 signalling in hepatocellular carcinoma [8]. Based on the fact that miR-137 is down-regulated in various cancers and is used for tumour suppression, this paper aimed to investigate whether miR-137 could modulate progression of glioma.

E2F transcription factors are important drivers in tumour growth, and are involved in diverse biological activities, including cell cycle, differentiation, and programmed cell death [18,28]. E2F7 is an atypical E2F factor that also regulates the progression of various tumours like lung cancer [31], prostate cancer [26] and breast cancer [11]. There are also several studies on E2F7 in gliomas. For example, high E2F7 expression is associated with poor prognosis in glioma patients [19,29]. E2F7 participates in regulating autophagy in gliomas, thereby promoting tumour progression [5]. High E2F7 expression indicates that patients with high-grade gliomas respond poorly to radiotherapy, while silenced E2F7 reveals a higher cell killing effect [30]. Previous studies proved the research value of E2F7 in gliomas, but not yet enough. Accordingly, we further explored the regulatory role and the molecular mechanism of E2F7 in glioma.

Herein, miR-137 expression in glioma cells was tested by reverse transcription quantitative polymerase chain reaction (qRT-PCR), and influences of miR-137 on progression of glioma cells were explored as well. Collectively, miR-137 was revealed to inhibit progression of glioma cells and promote programmed cell death of glioma cells, which indicates its potential as a candidate biomarker for targeted therapy for glioma.

Material and methods

Microarray analysis

Expression data of miRNAs and mRNAs and clinical data of TCGA-GBMLGG were downloaded. Then, the expression data of the two types of RNA were analysed by edgeR ($|\log_{2}FC| > 2$, $p_{adj} < 0.01$), and survival analysis was performed combined with the clinical information in the dataset. After the differential expression miRNAs (DEmiRNAs) for subsequent studies was selected, downstream target genes were predicted through miRDB, TargetScan, miRTarBase and starBase databases. E2F7 regulated by the differential miRNA was finally obtained by taking an intersection of the differential expression mRNAs (DEmRNAs) in TCGA and the predicted downstream target genes.

Cell incubation

Human glioma cell lines U251 (TCHu 58) and LN299 (HS-C335708) were procured from the Cell Bank of the Chinese Academy of Sciences. Human glioma cell line

HS683 (BNCC339564) and normal human astrocytes (NHA) (BNCC347734) were procured from the Cell Bank of BeNa Culture Collection. The cells were nurtured in Dulbecco's modified Eagle medium (DMEM) (BNCC342239) (added with 10% foetal bovine serum, FBS) at 37°C with 5% CO₂.

Cell transfection

MiR-137-mimic, miR-137-inhibitor, overexpression-E2F7 (oe-E2F7, obtained after the cDNA fragment of E2F7 was cloned into pcDNA3.1 plasmid) and related negative control (NC) were acquired from GenePharma. The sequence of miR-137-mimic was: 5'-UUAUUGCUUAAGAAUACGCGUAG-3', and the sequence of miR-137 inhibitor was: 5'-CUACGCGUAUUCUUAAGCAAUA-3'. Lipofectamine[®] 3000 kit (cat. L3000075, Invitrogen, Carlsbad, USA) was utilized to transfect human glioma U251 cells with the above prepared sequences and plasmids. Transfection efficiency was measured within 48 h.

qRT-PCR

Total RNA of human glioma cells was separated by TRIzol (cat. 15596018, Invitrogen) reagent. Complementary DNAs (cDNAs) of mRNA and miRNA were synthesized under the DNA Reverse Transcription Kit (cat. 4374967, Applied Biosystems) and the One Step miRNA cDNA Synthesis Kit (cat. D1801, HaiGene, Harbin, China) respectively. Afterwards, these samples were loaded on the SYBR Premix Ex Taq kit (cat. RR820A, TaKaRa, Dalian, China) for qRT-PCR on the Applied Biosystems 7500 sequence detection system. U6 and GAPDH were adopted for internal reference of miR-137 and E2F7, respectively. Primer sequences were listed in Table I.

Western blot assay

The pre-treated cells were rinsed with pre-cooled phosphate buffered saline (PBS) (cat. 003002 Thermo Fisher Scientific, MA, USA), and then added with an appropriate amount of radioimmunoprecipitation assay lysis buffer involving protease inhibitor (cat. 89900 Thermo Fisher Scientific, MA, USA) for ice lysis for 30 min. Next, the supernatant containing protein samples was obtained by centrifugation at 4°C. BCA kit (cat. 23225 Thermo Fisher Scientific, MA, USA) was implemented to quantify the obtained protein samples. Proteins were transferred to a nitrocellulose membrane following SDS-PAGE by means of wet transfer. The nitrocellulose membrane was sealed with a blocking solution (5% BSA/TBST) for 60 min firstly, and then added with primary antibodies for incubation in a refrigerator at 4°C overnight. After that, goat anti-rabbit IgG labelled with horseradish peroxidase (ab205718, Abcam, Cambridge, UK) was added into the membrane for 120 min.

Table I. qRT-PCR primer sequences

Gene	Sequence
<i>miR-137</i>	Forward: 5'-CGGGCTTATTGCTTAAGAATA-3'
	Reverse: 5'-GCAGGGTCCGAGGTATTC-3'
<i>U6</i>	Forward: 5'-CTCGCTTCGGCAGCACA-3'
	Reverse: 5'-AACGCTTCACGAATTTGCGT-3'
<i>E2F7</i>	Forward: 5'-CACCAAGAGTTCGCATCTGA-3'
	Reverse: 5'-AGTTTGCTGTTGCCTTTCCT-3'
<i>GAPDH</i>	Forward: 5'-TATGATGATATCAAGAGGGT AGT-3'
	Reverse: 5'-AGGAGACAACCTGGTCCTCA-3'

Finally, ECL kit (Solarbio, Beijing, China) was used for chemiluminescence, and photos were taken. The assay was repeated 3 times. Primary antibodies used in the assay were: E2F7 (PA5-114731, Invitrogen, USA), and GAPDH (ab128915, Abcam, Cambridge, UK).

Cell proliferation

Proliferative capacity of glioma cells was tested by cell counting kit-8 (CCK-8) reagent (HY-K0301, MedChem-Express, USA). After transfection and other operations, the digested cells were transferred to 96-well plates at a concentration of 2×10^4 cells/ml, with 100 μ l for each well, and 3 multiple wells were set in each group. After inoculation for 1, 2, 3 and 4 d, 10 μ l CCK-8 reagent was put in and cells were placed in an incubator for 2 h. After incubation, the plates were placed on a shaking table for 10 min. The optical density (OD) value of per well was detected by a microplate reader (Multiskan MK3, Thermo) at 450 nm, and the assay was repeated 3 times.

Scratch healing assay

Cell suspension was moved to a 6-well plate (cat. CLS3506, Corning, USA) at a concentration of 4×10^5 cells/well, and a 200 μ l sterile pipette was adopted to scratch a wound when cell density reached about 90%. After the scraped cells were washed, the left cells were cultivated in FBS-free medium at 37°C with 5% CO₂. The cells were observed and photographed after culture for 0 h and 48 h, respectively. The cell migratory rate was analysed on ImageJ and calculated as (0 h scratch width – 48 h scratch width)/0 h scratch width \times 100%. The assay was repeated 3 times.

Transwell assay

The upper chamber (cat. CLS3428, Corning, USA) of a 24-well plate (cat. CLS3527, Corning, USA) was covered with 50 μ l Matrigel matrix (cat. 354234, BD, USA) and placed at 4°C. Then, 200 μ l cell suspension at a concentration of 1×10^5 cells/ml was introduced to the upper chamber, while 650 μ l fresh medium containing 10% FBS was added to the lower chamber as

a chemokine. After 24 h of cultivation in an incubator at 37°C with 5% CO₂, cells not invading to the lower chamber were wiped away. The lower surface of the transwell insert was treated with 4% paraformaldehyde for 15 min, and then stained with 0.1% crystal violet for 10 min. Finally, 5 fields were casually chosen by an inverted microscope for photographing and cell counting. The assay was repeated 3 times.

Flow cytometry

The cells at the logarithmic growth stage were dissolved, and prepared into cell suspension with a concentration of 1×10^5 cells/ml. 2 ml cell suspension was taken and inoculated in 6-well plates for culture in an incubator for 48 h. According to the BD Annexin V FITC Apoptosis Kit (cat. 556547, BD, USA), corresponding reagents were added in turn and then, the programmed cell death rate was detected by flow cytometry.

Dual-luciferase assay

The predicted sequences of wild-type (WT) E2F7 3'UTR and mutant (MUT) were cloned into the downstream polyclonal sites of pGL3 (cat. E1751 Promega, WI, USA). The pGL3 E2F7-WT or E2F7-MUT vectors and miR-137-mimic or NC-mimic were co-transfected into 293T cells utilizing Lipofectamine[®] 3000 kit. Luciferase activities of firefly and renilla were detected using the luciferase reporter analysis system (E1910, Promega, USA). The assay was repeated 3 times.

Statistical analysis

All data analysis were performed using the Graph-Pad Prism 7 Software (GraphPad Software, Inc., La Jolla, CA). Measurement data were reflected as mean \pm standard deviation (M \pm SD). For the significance test, the analysis of variance (*F* test) was adopted to check whether the data followed the normal distribution, and the homogeneity of variance was also adopted. Thereafter, *t*-test was applied to analyse the significance of differences between two groups.

Results

MiR-137 is lowly expressed in glioma

Various studies demonstrated that miR-137 is a vital factor in progression of cancers, like liver cancer, renal cell cancer, endometrial cancer, and colon cancer [3,4,25,33]. Here, TCGA database was applied to analyse the differential expression of miR-137 in glioma, and it was unveiled that miR-137 showed a low expression in the tumour group (Fig. 1A). As qRT-PCR results indicated, compared with the NHA, miR-137 in glioma cells U251, LN299 and HS683 was remarkably-down-regulated, and downregulation of miR-137 was

the most significant in U251 (Fig. 1B). Then, U251 was selected for relevant studies in the subsequent experiments. Based on the above results, it was determined that miR-137 was constrained in glioma.

MiR-137 inhibits progression and promotes programmed cell death of glioma cells

Above, we proved that miR-137 was repressed in glioma. In order to further observe whether the low expression of miR-137 was relevant to the biological function of glioma cells, glioma cells were transfected with miR-137-inhibitor or miR-137-mimic. qRT-PCR result revealed great transfection efficiency (Fig. 2A). As indicated in cell proliferation assay results, inhibition of miR-137 expression would promote the proliferation of glioma cells, whereas overexpressed miR-137 would constrain the proliferation of glioma cells (Fig. 2B). Conclusions of scratch healing and transwell assays unveiled that downregulation of miR-137 expression would improve the migration and invasion of glioma cells, whereas overexpressed miR-137 would decrease those capacities of glioma cells (Fig. 2C, D). In the cell programmed cell death assay, inhibition of miR-137 would lead to a decrease in the programmed cell death rate of glioma cells, while overexpressed miR-137 would increase the programmed cell death rate of glioma cells (Fig. 2E). These experimental findings implied that miR-137 could facilitate programmed cell death and repressed progression of glioma cells.

E2F7 is a target gene of miR-137

With the aim of further studying the regulatory mechanism of miR-137 in glioma, bioinformatics methods were used. Firstly, edgeR was used for differential

analysis, and 1,318 differential mRNAs were obtained (Fig. 3A). Next, miRDB, TargetScan, miRTarBase and starBase databases were used for predicting the downstream target genes of miR-137. Then, predicted results were intersected with the differential mRNAs to get a differential mRNA (E2F7) with a targeted binding site of miR-137 (Fig. 3B). TCGA data implied that E2F7 was boosted in glioma (Fig. 3C), and patients with a high expression of E2F7 had a bad prognosis (Fig. 3D). In an effort to verify the validity of bioinformatics analysis, the E2F7 level in glioma cells U251, LN299, HS683 and NHA was detected by qRT-PCR firstly. Results unveiled that E2F7 was significantly facilitated in glioma cells (Fig. 3E). Next, E2F7 was detected in glioma cells with inhibited or overexpressed miR-137. qRT-PCR results suggested that inhibition of miR-137 promoted E2F7 expression in glioma cells, while overexpressed miR-137 inhibited E2F7 (Fig. 3F). Finally, the dual-luciferase assay was to determine whether E2F7 was directly targeted by miR-137. After transfection with miR-137-mimic, it was observed that the luciferase activity of WT E2F7 3'-UTR was repressed, while the luciferase activity of MUT E2F7 3'-UTR did not change significantly (Fig. 3G). These results strongly suggested that E2F7 was directly modulated by miR-137.

MiR-137 targets E2F7 modulate progression of glioma cells

To determine whether miR-137 targeting E2F7 can regulate progression and programmed cell death of glioma cells, both miR-137 and E2F7 were overexpressed in glioma cells, and thus the experiment was conducted with 4 groups: NC-mimic + oe-NC, NC-mimic + oe-E2F7, miR-137-mimic + oe-NC, and miR-137-mimic + oe-E2F7. Firstly, qRT-PCR and western blot unveiled that boosted miR-137 inhibited expressions of E2F7

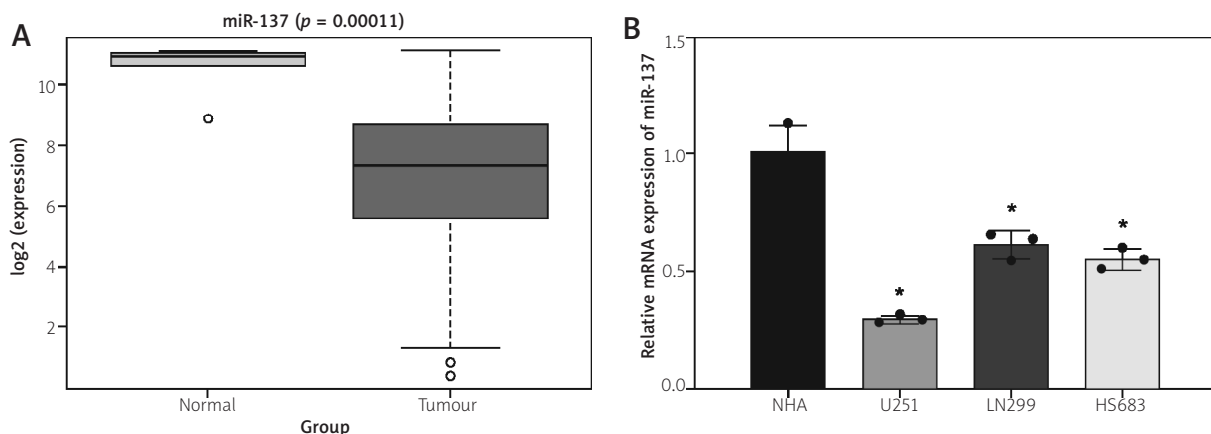


Fig. 1. The expression level of miR-137 in glioma is down-regulated. **A)** MiR-137 expression in two groups (normal and tumour); **B)** The expression of miR-137 in glioma cells U251, LN299, HS683 and normal human astrocytes (NHA); * $p < 0.05$.

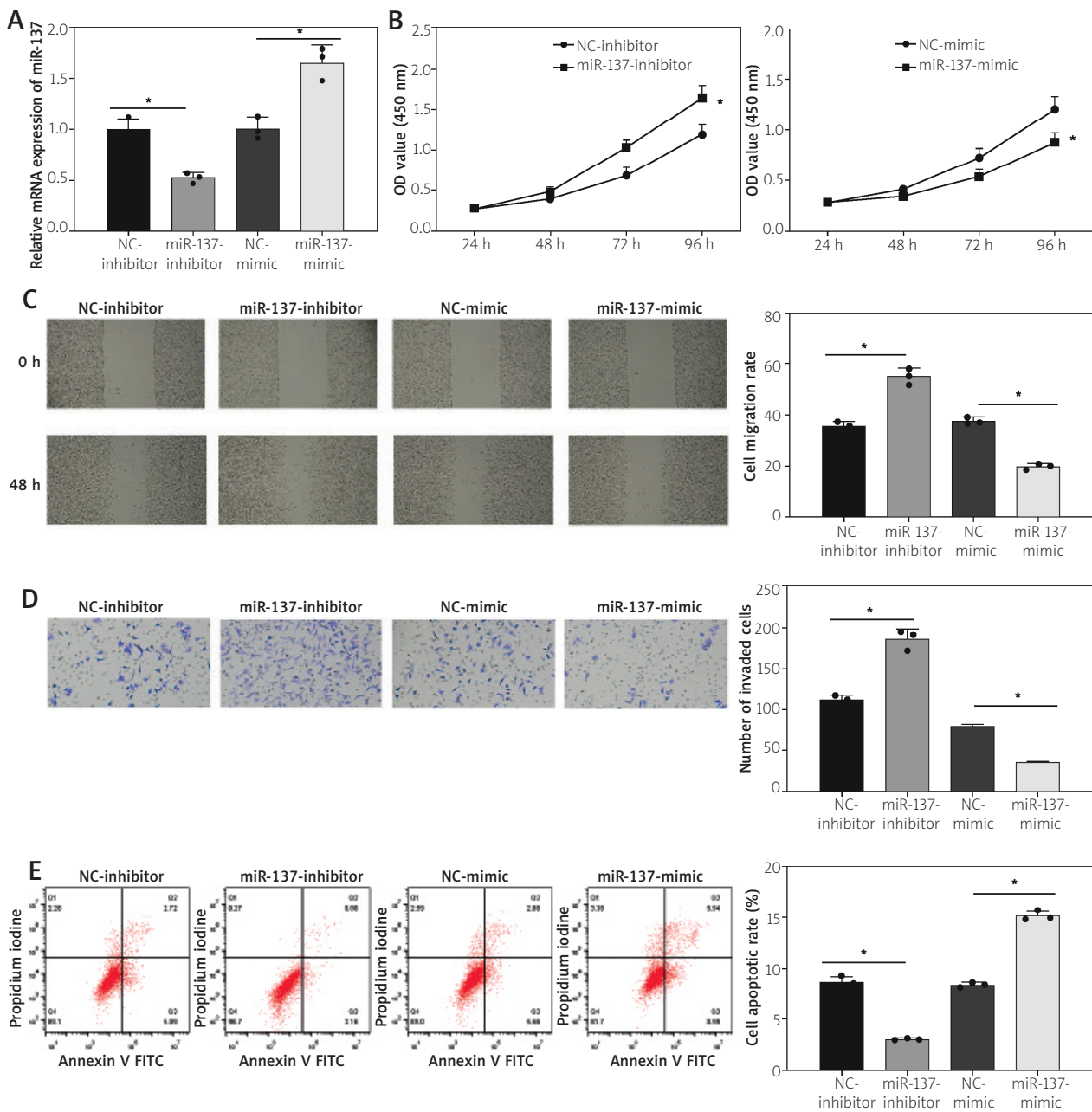


Fig. 2. Upregulated miR-137 in glioma cells represses glioma cell progression. **A)** The efficiency of inhibiting and overexpressing miR-137 in glioma cells in groups NC-inhibitor, miR-137-inhibitor, NC-mimic, miR-137-mimic; **B)** Changes in the proliferation of glioma cells; **C)** Changes in the migratory capacity of glioma cells; **D)** Changes in the invasive ability of glioma cells; **E)** The changes in the programmed cell death rate of glioma cells; * $p < 0.05$.

mRNA and protein, while the overexpression of both E2F7 and miR-137 reversed this phenomenon (Fig. 4A, B). Then, cell proliferation, scratch healing, transwell and programmed cell death assays were carried out to examine the biological function of the transfected glioma cells. Cell proliferation assay results unveiled that

E2F7 eliminated the suppressive effects of miR-137 on glioma cell proliferation (Fig. 4C). Scratch healing and transwell assays showed that E2F7 reversed suppressive effects of miR-137 on migration and invasion of glioma cells (Fig. 4D, E). Similarly, E2F7 also reversed the way miR-137 affected the programmed cell death

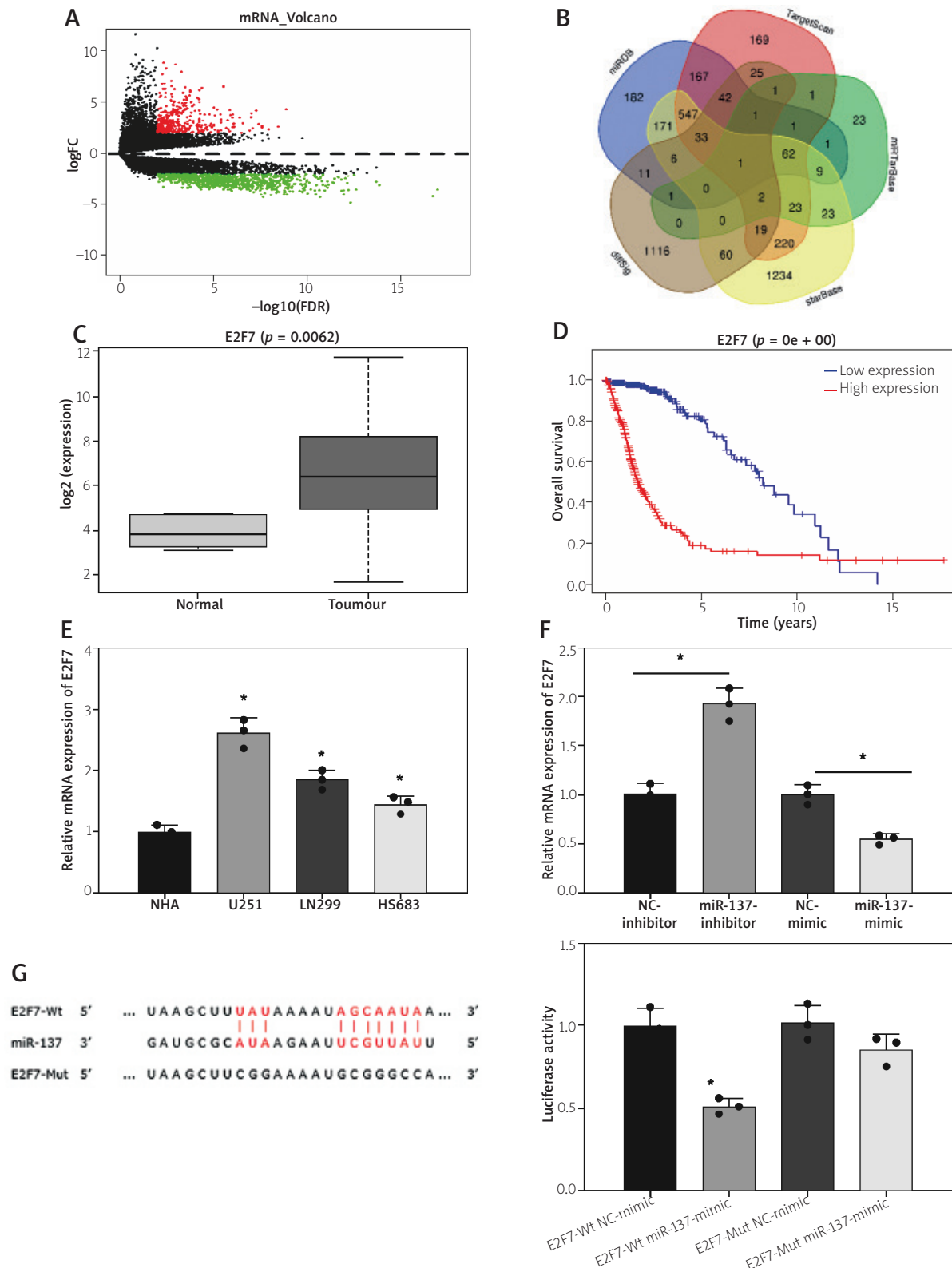


Fig. 3. MiR-137 directly binds to E2F7. **A)** A volcano plot of differential mRNAs in TCGA-GBMLGG; **B)** A Venn diagram displaying differential mRNAs and predicted target genes; **C)** A different expression of E2F7 between normal and tumour groups; **D)** A survival analysis of the E2F7 gene; red: patients with a high E2F7 expression; blue: patients with a low E2F7 expression; **E)** E2F7 expression in glioma cells U251, LN299, HS683 and normal human astrocytes (NHA); **F)** E2F7 expression in glioma cells with inhibited or overexpressed miR-137; **G)** The interaction between miR-137 and E2F7; * $p < 0.05$.

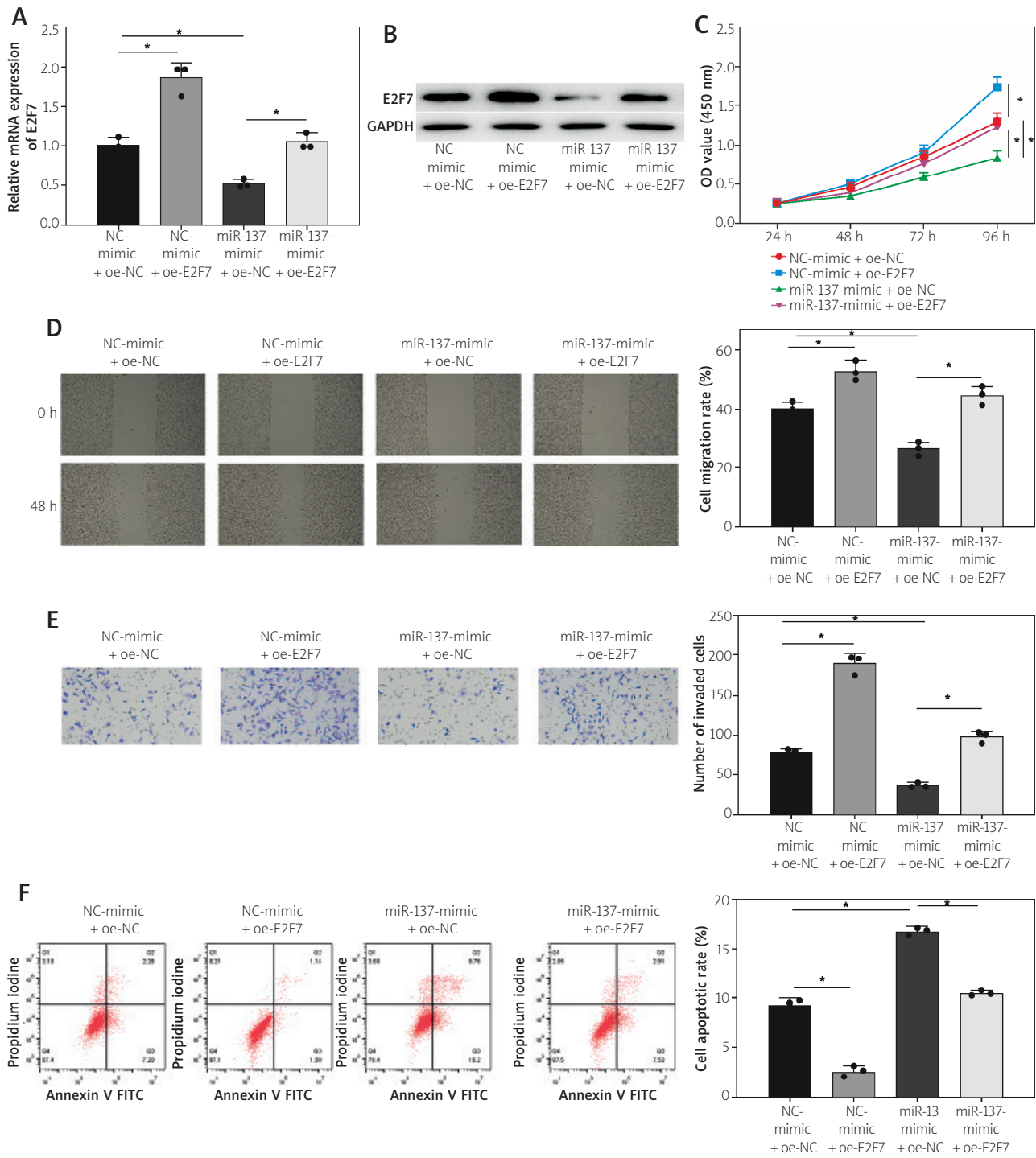


Fig. 4. MiR-137 targets E2F7 to attenuate the ability of glioma cells to proliferate, migrate and invade, and increase the rate of apoptosis of glioma cells. **A)** mRNA level of E2F7 in glioma cells; **B)** Protein expression of E2F7 in glioma cells transfected with miR-137 mimic and oe-E2F7; **C)** Changes in proliferation of glioma cells; **D)** Changes in migratory ability of glioma cells; **E)** Changes in the invasive ability of glioma cells; **F)** Changes of apoptosis rate of glioma cells; * $p < 0.05$.

of glioma cells in the programmed cell death assay (Fig. 4F). In summary, miR-137 constrained progression of glioma cells through modulating E2F7.

Discussion

There is increasing evidence that miRNA is often dysregulated in tumours, and aberrant miRNA may regulate multiple biological and pathological activities, like tumour cell proliferation, metastasis, and programmed cell death [1,12,14,35]. These findings suggest that miRNA may have functions similar to oncogenes or tumour suppressor genes, and connect to cancer development [9,27,32]. MiR-137 is proven to be suppressed in liver cancer, renal cell cancer, endometrial cancer, and colon cancer [3,4,25,33], and it regulates the occurrence and progression of these cancers. Based on the above literature and the predicted results, we speculated that miR-137 was a modulator in glioma. Subsequent *in vitro* functional experiments in this study confirmed our supposition. Cell proliferation, scratch healing, transwell and programmed cell death assays were conducted and it was determined that miR-137 repressed progression and promoted programmed cell death of glioma cells. All of the above confirmed that miR-137 had a role of tumour suppressor in glioma. Later, it was predicted that the target gene had a binding site with miR-137 and was differentially up-regulated in glioma through the bioinformatics database. In addition, the dual-luciferase assay also confirmed that miR-137 could bind to the 3'-UTR of E2F7, which led to degradation of E2F7 mRNA.

E2F7 is a significant modulator in a number of cancers. For example, miR-30a can directly target E2F7 to repress progression of thyroid papillary cancer cells [6]. MiR-302a/d can target E2F7/AKT/ β -catenin/CCND1 signalling axis to constrain proliferation of liver cancer stem cells [13]. These findings were consistent with our study, where we verified the inhibitory effect of miR-137/E2F7 axis on glioma progression. Besides, the results of the rescue assay fully demonstrated that E2F7 could promote glioma progression, and the inhibitory effect of miR-137 on glioma malignant progression could be attenuated by overexpressing E2F7. This was first demonstrated by our study. In addition to some common cellular phenotypes, such as proliferation, migration, and invasion that were investigated in this study, previous studies have revealed that E2F7 can be regulated by various miRNAs to affect other biological functions of tumours. For example, E2F7 can be regulated by miR-378c which in turn promotes cancer cell resistance to 5-FU in colorectal cancer [10]. LncRNA MYLK-AS1 promotes tumour progression and angiogenesis by targeting the miR-424-5p/E2F7 axis and activating the VEGFR-2 signalling pathway in hepato-

cellular carcinoma [23]. We will also carry out further studies for these cellular functions in the future. E2F7 can function as a transcription factor in addition to being regulated by some miRNAs. For example, E2F7 transcriptionally regulates miR-199b expression, which in turn promotes USP47 expression, thereby enhancing the stem cell activity and facilitating colon cancer progression [7]. Similar studies have also been conducted in glioma where Yang *et al.* reported that E2F7-EZH2 axis regulates PTEN/AKT/mTOR signalling pathway and glioblastoma progression [28]. In these studies, E2F7 works as a transcription factor, which may also be one of the directions for our next in-depth study to further refine the regulatory network of this topic.

Taken together, findings of this study clarified that miR-137 can significantly inhibit progression and promote the programmed cell death of glioma cells, and E2F7 is the direct target of miR-137 in glioma. This study fully elucidates how miR-137 works in regulating progression of glioma. The miR-137/E2F7 signalling axis may be an underlying therapeutic target for glioma.

Disclosure

The authors report no conflict of interest.

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