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MiR-138-5p Targets Nir1 to Regulate Glioma Cell Invasion

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Abstract

Objective: To investigate the relationship between miR-138-5p and PYK2 N-terminal domain-interacting receptor 1 (Nir1) and determine whether miR-138-5p affects glioma invasion through binding with Nir1. **Methods:** The expression level of miR-138-5p in different glioma cell lines and its effect on Nir1 protein in glioma cells U251 and H4 were compared. The invasion ability of glioma cells after transfection, and whether miR-138-5p could target Nir1 were detected. **Results:** The level of miR-138-5p in low-invasive human glioma cells H4 was significantly higher than that in highly invasive human glioma cells U251, LN229 and U87 ($p < 0.05$). In U251 and H4 cells, the expression of Nir1 protein was lowered in the miR-138-5p overexpression group compared to the miR-negative control (miR-NC) group, but increased in miR-138-5p inhibitor group relative to inhibitor NC group. However, Nir1 mRNA level in miR-138-5p overexpression group had no significant changes compared with the miR-NC group ($p > 0.05$). The number of U251 and H4 cells in the miR-138-5p overexpression group passing through the small hole in the basement membrane was significantly less than that in the miR-NC group ($p < 0.05$). MiR-138-5p overexpression evidently reduced luciferase activity of Nir1-3'-UTR. **Conclusion:** MiR-138-5p binding to Nir1 in glioma cells can inhibit the invasion of glioma cells by reducing the translation of Nir1 and the expression of Nir1 protein.

1 Introduction

Gliomas are common malignant primary central nervous system tumors with an invasive growth pattern, poor surgical outcomes, and suboptimal prognosis. Therefore, studying the molecular mechanisms of glioma invasion is of significant importance for the treatment of gliomas [1-4]. PYK2 N-terminal domain-interacting receptor 1 (Nir1), as a functional receptor of C-C motif chemokine ligand 18 (CCL18), can mediate phosphorylation of protein kinase B (Akt) to impact filamentous actin (F-actin) accumulation in glioma cells, and regulate Snail family transcriptional repressor 2 (Slug) to induce mesenchymal transition (MT) of glioma cells, thereby promoting the invasion and migration of glioma cells [5-11]. On this basis, blocking or inhibiting Nir1 has become a new method and idea for treating gliomas; however, there is no clinical report relevant to effective blockage and inhibition of Nir1. The prediction results of online bioinformatics software TargetScan found Nir1 as a potential target gene of miR-138-5p. Therefore, this study aims to verify the scientific hypothesis that miR-138-5p can inhibit the invasive and infiltrative capacity of gliomas by targeting and regulating Nir1. The study focuses on exploring the relationship between miR-138-5p and Nir1, as well as the effectiveness of miR-138-5p in targeting and regulating Nir1.

2 Materials and methods

2.1 Materials

Glioma cell lines H4, LN-229, and U251 were purchased from American Type Culture Collection (ATCC). Nir1 Plasmid Midi Preparation Kit and TaqMan mRNA reverse transcription kits were obtained from Beyotime (China). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were procured from Hyolone (USA). Nir1 monoclonal and Anti-β-actin polyclonal antibodies were ordered from Cell Signaling Technology (USA) and Santa Cruz Biotechnology (USA), respectively. Transwell chambers and PVDF membranes were purchased from Corning (USA). Matrigel, Lipofectamine 2000 transfection reagents and the dual-luciferase reporter assay kit was provided by BD Biosciences (USA), Thermo Fisher Scientific (USA) and GenePharma (China).

2.2 Histochemistry

62 cases from January 2014 to June 2021 were selected, including 41 males and 21 females aged 8-72 years. According to the 2007 WHO glioma classification, low-grade gliomas were graded I-II, and high-grade gliomas were graded III-IV [1]. The 5-year survival rate was 13 out of 26 in the low-grade group and 11 out of 36 in the high-grade group, with 2 cases lost to follow-up in the high-grade group. Comparative histochemical data and morphology (20 ×) are presented in Table 1 and Figure 1.

2.3 Methods

2.3.1 Cell culture

Glioma cell lines (H4, LN-229, U251, U87) were cultured in DMEM with 10% FBS and 100 μg/mL penicillin-streptomycin at 37 °C with 5% CO_2. Cells in logarithmic growth phase after the third passage were cultured for 24 h before transfection.

2.3.2 Detection of miR-138-5p expression levels

Total ribonucleic acid (RNA) of glioma cell lines (H4, LN-229, U251, U87) was extracted using Trizol reagent kit. Reverse transcription was performed according to the instructions of the complementary deoxyribonucleic acid (cDNA) reverse transcription kit (the reaction system for reverse transcription was 20 μL). Primers were designed using Primer 6.0 software, and the reverse transcription quantitative polymerase chain reaction (RT-qPCR) primers (RiboBio) were diluted according to the instructions for PCR reaction. Pre-denaturation was performed at 95 ℃ for 3 min, followed by 35 cycles of denaturation at 95 ℃ for 30 s,

annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 1 min. U6 snRNA was selected as the internal reference, and the relative expression of miRNA was precisely calculated using the Ct value.

Table 1 Comparison of histochemical data between the two groups.

Clinical parameters	n	Nir1		χ^2	p
		+	−		
Age/(years)				0.136	0.713
⩽ 55	12	10	2		
> 55	50	46	4		
Tumor size/(cm)				6.998	0.008
⩽ 2	29	20	9		
> 2	33	32	1		
Histological grade				40.112	< 0.001
Ⅰ～Ⅱ	26	5	21		
Ⅲ～Ⅳ	36	35	1		

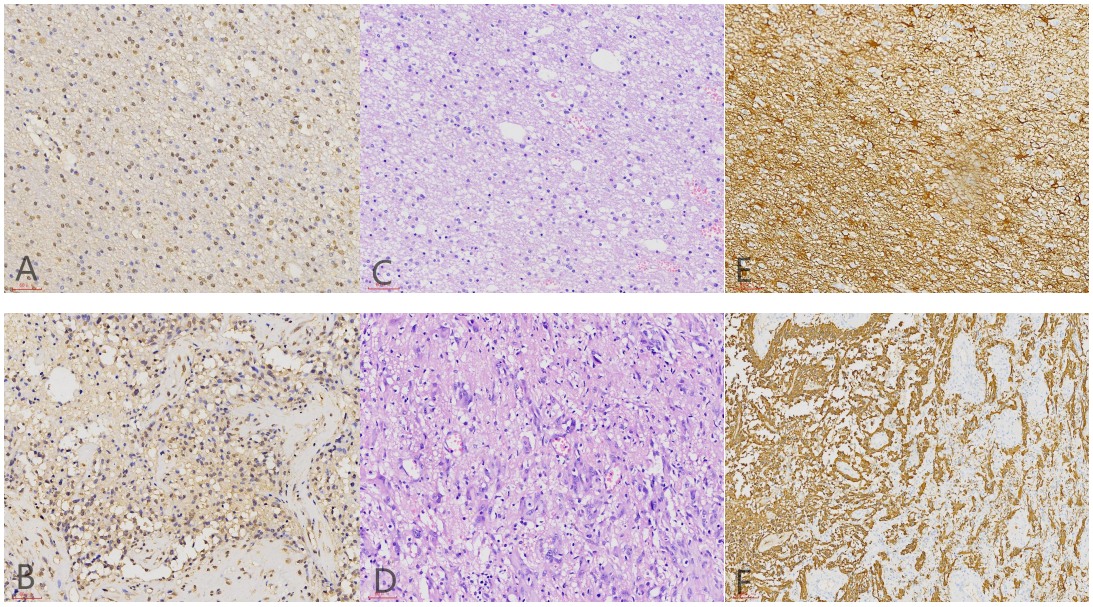

Figure 1 The morphology of low and high grade gliomas (20 ×). (A) Nir1 in low-grade glioma. (B) Nir1 in high-grade gliomas. (C) Low-grade glioma (HE). (D) High-grade gliomas (HE). (E) Low-grade glioma (GFAP). (F) High-grade gliomas (GFAP).

2.3.3 Construction of miR-138-5p plasmid and cell transfection

The miR-138-5p plasmid, miR-138-5p inhibitor, miR-negative control (miR-NC), and miR-138-5p inhibitor NC were all purchased from Invitrogen (USA), and transfected into cells according to the instructions of the transfection reagent. U251 or H4 cells were divided into four groups: (1) Overexpression control group (miR-negative control, miR-NC): cells were transiently transfected with a plasmid containing a scrambled sequence (scramble1). (2) Overexpression miR-138-5p group (miR-138-5p): cells were transiently transfected with a plasmid containing the target fragment 5'-AGCUGGUGCCUUGUGAAUCAGGCCG-3'. (3)

MiR-138-5p inhibitor group (miR-138-5p inhibitor): cells were transiently transfected with a plasmid containing the antisense sequence of miR-138-5p. (4) Inhibitor control group (inhibitor negative control (inhibitor NC)): cells were transiently transfected with a plasmid containing a scrambled sequence (scramble2).

2.3.4 Western blot

The proteins were from the transfected cells, then electrophoresed with SDS-PAGE, transferred to membrane, and blocked with 5% skimmed milk. The membrane was incubated with primary antibody at 4 ℃ overnight and secondary antibody for 1 h, followed by development in a darkroom. The internal reference was β-actin. The relative expression level of the target protein was calculated by the ratio of the gray value of the target band to that of the internal reference band.

2.3.5 RT-PCR

Total RNA extracted from the cells was reversely transcribed into cDNA according to the instructions of the MMLV reagent. ARK5 and β-actin primers were diluted with double distilled water as per the instructions of SYBR Premix (PerfectReal Time) kit (Beyotime, Beijing, China), followed by RT-PCR amplification (two-step method), pre-denaturation at 95 ℃ for 20 s, and 40 cycles of reaction at 95 ℃ for 5 s and 60 ℃ for 30 s reaction. Anti-β-actin was used as the internal reference, and the relative expression of Nir1 in each group of cells was calculated using the Ct value.

2.3.6 Luciferase activity assay

The target site of Nir1 regulated by miR-138-5p was predicted using TargetScan software. A non-conserved site 5'-UUGGCUUUCCAAAGGCACCAGCU-3' was identified in the 3'-untranslated region (3'-UTR) of Nir1. The eukaryotic expression vector pmiRGLO target gene reporter plasmid and the 3'-UTR mutant reporter plasmid were constructed by GenePharma (Shanghai, China). pRL-TK was selected as the internal control. According to the instructions of Lipofectamine 2000 and the experimental grouping, U251 and H4 cells were co-transfected with the luciferase reporter plasmid and miR-138-5p or the control scramble1 sequence. After 4 h of transfection, the medium was changed and the cells were cultured for 48 h at 37 ℃ with 5% CO_2. Luciferase activity was measured as per the instructions of the Dual-Luciferase Reporter Assay Kit (GenePharma, Shanghai, China).

2.3.7 Transwell invasion assay

The Transwell chamber (with an 8 μ m pore membrane) was pre-coated with Matrigel. A cell suspension (5×10^5/mL) of transfected cells was added to the upper chamber, and DMEM culture medium containing 10% FBS was added to the lower chamber. The cells were cultured in a 5% CO_2 incubator at 37 ℃ for 24 h. The cells were then fixed with 4% paraformaldehyde for 20 min, washed with PBS, stained with Giemsa for 20 min, rinsed with PBS, air-dried and counted under a microscope.

2.4 Statistical analysis

Data were analyzed using SPSS 23.0 software. Measurement data were expressed as mean ± standard deviation. The independent samples *t*-test was used to compare differences between two groups. A p-value of less than 0.05 was considered statistically significant.

3 Results

3.1 Expressions of miR-138-5p in different glioma cell lines

RT-PCR was performed to measure the miR-138-5p expression in H4, U251, LN-229, and U87 glioma cells. The results revealed that significantly higher miR-138-5p expression in low-invasive H4 cells

compared to high-invasive U251, LN229, and U87 cells ($p < 0.05$, Figure 2).

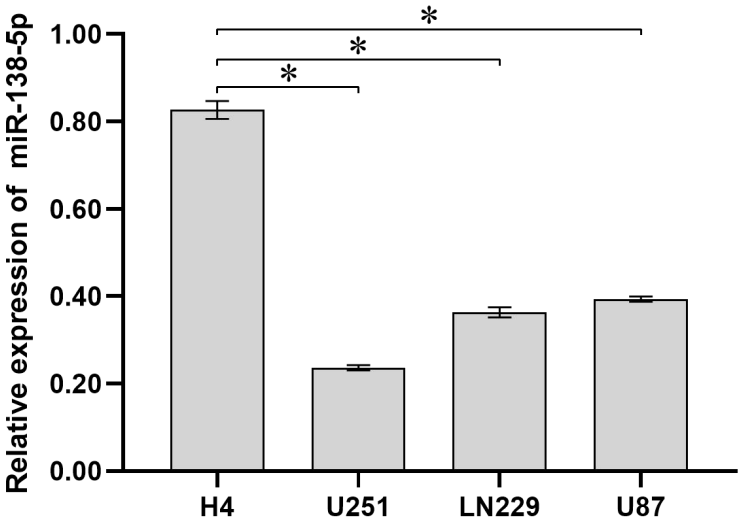

Figure 2 The expression of miR-138-5p in H4, U251, LN229 and U87cells. Note: * $p < 0.05$.

3.2 Impact of miR-138-5p level on Nir1 protein expression

Cultivated U251 and H4 cells were transfected in overexpression control (miR-NC) group, inhibition (miR-138-5p inhibitor) group, inhibition control (inhibitor NC) group, and overexpression (miR-138-5p) group, respectively. After successful transfection, Western blot experiment was performed to detect the expression level of Nir1 protein in each group of cells. The results showed that the expression level of Nir1 protein in U251 and H4 cells of miR-138-5p group was lower than that of miR-NC group. The expression level of Nir1 protein in U251 and H4 cells of miR-138-5p inhibitor group was higher than that in inhibitor NC group ($p < 0.05$), as shown in Figure 3.

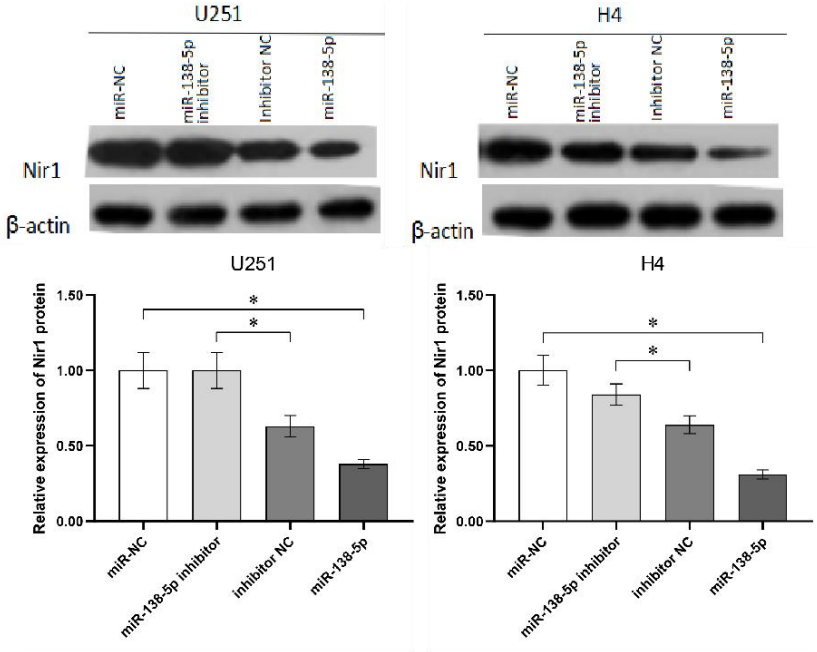

Figure 3 Impacts of miR-138-5p expression on Nir1 in U251 and H4 cells. Note: * $p < 0.05$.

3.3 Impacts of miR-138-5p on Nir1 mRNA level

In order to investigate whether miR-138-5p inhibits the expression of Nir1 protein by regulating the degradation of Nir1 mRNA or by translation inhibition, RT-qPCR experiments were conducted to detect changes in the mRNA levels of Nir1 in U251 and H4 cells of miR-138-5p and miR-NC groups. The data showed that the mRNA levels of Nir1 in U251 and H4 cells of miR-138-5p group were not significantly different from those of miR-NC group (p > 0.05, Figure 4).

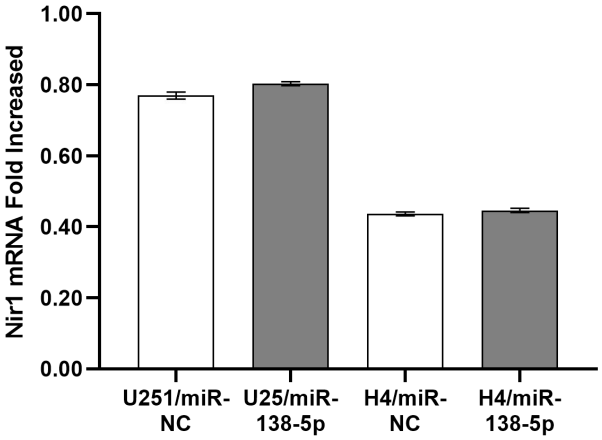

Figure 4 RT-qPCR was applied to detect Nir1 mRNA after overexpression of miR-138-5p.

3.4 Effects of miR-138-5p on glioma cell invasion

Transwell invasion assay was conducted to examine the effect of miR-138-5p on glioma cell invasion. The data demonstrated that the number of U251 and H4 cells passing through the basement membrane pores in the miR-138-5p group was significantly lower than that in the miR-NC group (p < 0.05, Figure 5).

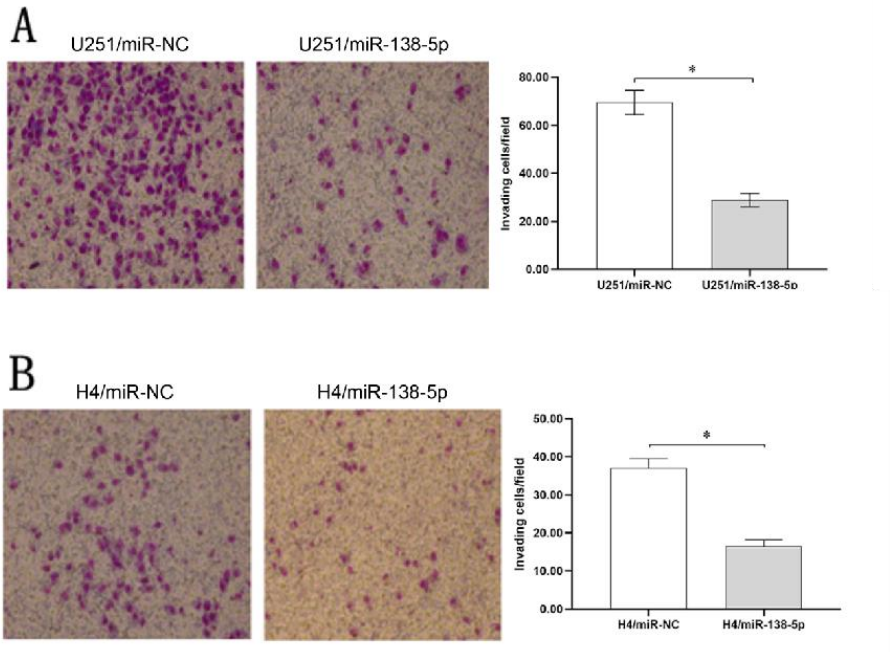

Figure 5 The effects of miR-138-5p on the invasion of gliomas cells (200×). (A) miR-138-5p overexpression promoted the invasion of U251 cells and the invasion ability was statistically analyzed (* p < 0.05). (B) miR-138-5p overexpression boosted the invasion of H4 cells and the invasion ability was statistically analyzed (*

$p < 0.05$).

3.5 Validation of Nir1 as a miR-138-5p's target gene

To search for the target gene of miR-138-5p, TargetScan target gene prediction software was used and found that miR-138-5p complementarily bound to the 3'UTR region of Nir1 (Figure 6A). The results of luciferase reporter gene detection showed that the luciferase activity of pGL3-Nir1-3'-UTR in U251 and H4 cells overexpressing miR-138-5p was apparently reduced compared to that in the corresponding control group ($p < 0.05$, Figure 6B).

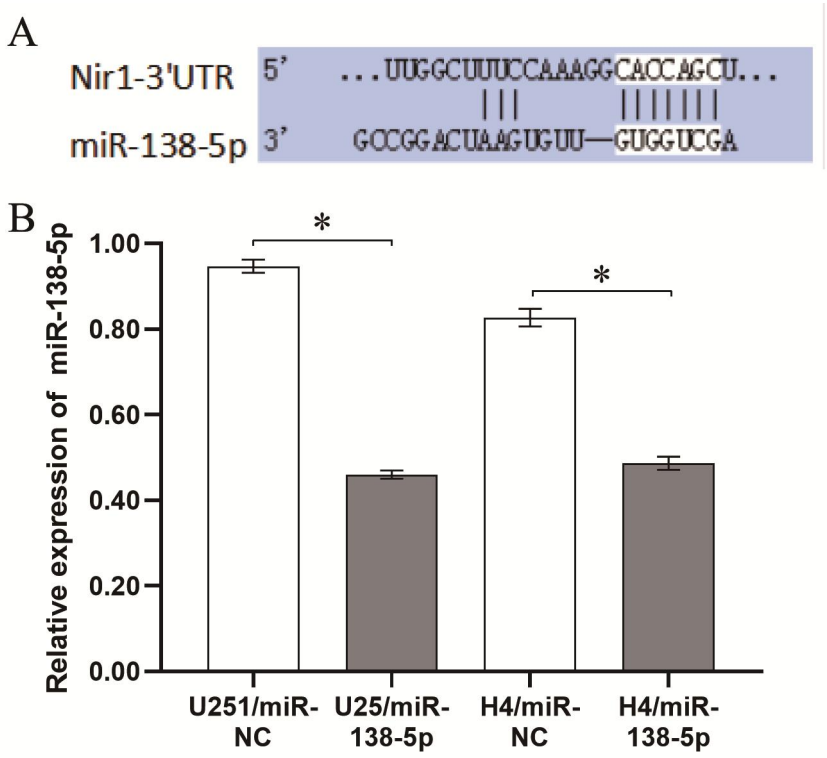

Figure 6 Luciferase reporter gene assay data verified that miR-138-5p targeted Nir1 in glioma cells. (A) Targetscan data predicted that Nir1 might be a target gene of miR-138-5p. (B) Relative activities of the reporter plasmids in different groups of glioma cells (* $p < 0.05$).

4 Discussion

Many studies [5,12,13] have confirmed that Nir1 is a target for inhibiting malignant tumor invasion and metastasis, and its expression is closely related to the infiltration, metastasis, and prognosis of tumors such as breast and lung cancer. Our team previously explored Nir1 expression in glioma cells and its correlation with glioma cell invasion and metastasis [7]. The results indicated that reduced Nir1 expression resulted in attenuated Akt phosphorylation and F-actin polymerization in glioma cells, which is an important reason for the weakened motility and invasion abilities of glioma cells. The detection results of epithelial-to-mesenchymal transition (EMT) related proteins showed that the Nir1 protein induced the transformation of glioma H4 cells to MT by regulating the transcription factor Snail. However, whether Nir1-driven glioma cell invasion is regulated by specific miRNAs remains unstudied. Some studies [14-18] have suggested that miR-138-5p overexpression can inhibit the invasion and metastasis of pancreatic and bladder cancers, and reduce glioma cell vascularization, invasion and metastasis abilities. Consequently, we employed the TargetScan target prediction software and identified complementary binding relation between miR-138-5p and Nir1's

3'UTR.

Herein, the RT-PCR experiment results showed that the level of miR-138-5p in low-invasive human glioma H4 cells was significantly higher than that in high-invasive glioma U251, LN229, and U87 cells. After transfection of miR-138-5p, miR-138-5p inhibitor, and corresponding negative controls into U251 and H4 cells, Western blot analysis data unveiled that miR-138-5p overexpression in U251 and H4 cells resulted in downregulation of Nir1 protein, indicating that miR-138-5p complementarily bound to the 3'UTR region of Nir1, which can inhibit glioma cell invasion. Zhang et al. [19] reported downregulated miR-138 in clinical glioma tissues, and miR-138 upregulation signally suppressed glioma cell proliferation and invasion and promoted apoptosis. Jiang et al. [20] found Nir1 expression was higher in cancer than normal tissues, and Nir1 plays an oncogenic role in malignant tumors. Our findings aligned with these studies, further confirming miR-138-5p exerted inhibitory effects on glioma cell invasion through complementarily binding to Nir1's 3'UTR.

MiRNAs binding specifically to the 3'-UTR of target gene mRNA can directly cause target mRNA degradation or translation inhibition, reducing post-transcriptional target protein expression to regulate target genes [21,22]. To verify this mechanism, RT-PCR assay was carried out to detect changes in Nir1 mRNA levels in miR-138-5p-overexpressing glioma cells and revealed no changes. Cell invasion assays showed reduced invasive capacity, and luciferase reporter assays revealed lower luciferase activity of pGL3-Nir1-3'-UTR in miR-138-5p-overexpressing glioma cells. Thus, miR-138-5p inhibited Nir1 protein expression through translation inhibition rather than mRNA degradation. Besides, miR-138-5p significantly suppressed glioma cell invasion and Nir1 acted as as a target gene of miR-138-5p. Jun Wu et al. [23] demonstrated that miR-22-3p directly binds to NLRP3-3'UTR to inhibit carcinogenesis, which is similar to our results, further confirming the complementary binding relation of miR-138-5p and Nir1's 3'UTR.

In conclusion, miR-138-5p binds to Nir1 in glioma cells, which can inhibit the invasive ability of glioma cells by reducing the translation of Nir1 and the expression of Nir1 protein. MiR-138-5p is expected to become a new target for inhibiting glioma invasion, but the molecular mechanism by which miR-138-5p represses Nir1 protein translation needs further investigation.

Acknowledgements

Not applicable.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Investigation, P.C.; Data collection, Y.P. and Y.Z.; Validation, D.M.; Supervision, H.G.; Writing—original draft, F.L., B.S. and L.W.; Writing—review and editing, H.L. and S.S. All authors have read and agreed to the published version of the manuscript.

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Availability of Data and Materials

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Supplementary Materials

Not applicable.

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