MiR-129-5p inhibits glioma cell progression in vitro and in vivo by targeting TGIF2

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Abstract
This study purposed to explore the correlation between miR-129-5p and TGIF2 and their impacts on glioma cell progression. Differentially expressed miRNA was screened through microarray analysis. MiR-129-5p expression levels in glioma tissues and cells were measured by qRT-PCR. CCK-8 assay, flow cytometer, transwell assay and wound-healing assay were employed to detect cell proliferation, apoptosis and cycle, invasiveness and migration, respectively. Dual-luciferase reporting assay was performed to confirm the targeted relationship between miR-129-5p and TGIF2. The effects of TGIF2 expression on cell biological functions were also investigated using the indicated methods. Tumour xenograft was applied to explore the impact of miR-129-5p on tumorigenesis in vivo. MiR-129-5p expression was down-regulated in both glioma tissues and glioma cells, while TGIF2 expression was aberrantly higher than normal level. Dual-luciferase reporter assay validated the targeting relation between miR-129-5p and TGIF2. Overexpression of miR-129-5p or down-regulation of TGIF2 inhibited the proliferation, invasion and migration capacity of glioma cells U87 and U251, and meanwhile blocked the cell cycle as well as induced cell apoptosis. MiR-129-5p overexpression repressed the tumour development in vivo. MiR-129-5p and TGIF2 had opposite biological functions in glioma cells. MiR-129-5p could inhibit glioma cell progression by targeting TGIF2, shining light for the development of target treatment for glioma.

KEYWORDS
glioma, miR-129-5p, TGIF2

1 INTRODUCTION
Glioma is the most untreatable tumour of the central nervous system with significant mortality and poor survival rate.1 According to the latest statistics, the incidence of glioma increased from 5.9 of 100 000 people in 1973 to 6.61 of 100 000 people in 2016 after the application of improved radiological diagnosis.2 Recent years, tremendous progress have been achieved in tumour diagnosis and treatment including radiotherapy, neurosurgery and chemotherapy, while the prognosis of glioma remains unfavourable.1 The 5-year relative survival rate in the United States merely improved from 22% in 1977 to 35% in 2011.3 Therefore, it is necessary to explore novel therapeutic strategy for this intractable disease.

MicroRNAs (miRNAs) are a varied collection of non-protein-coding tiny (19-22 nucleotides) RNAs with regulatory impacts on gene expression at post-transcriptional level.4 By targeting or silencing target genes, miRNAs play a crucial role in controlling the progression of tumour cells. For example, Zhou et al reported that miR-224 could drive colorectal cancer cell proliferation by targeting SMAD4.5 MiR-203 was reported to suppress tumour growth and metastasis of...
non-small cell lung cancer by down-regulating RGS17.6 Previous studies have also provided some novel perspectives for the therapy of human glioma based on the modulating mechanism of miRNAs. For instance, miR-145 could induce glioma cell apoptosis by targeting BNP3 and Notch signalling,7 while miR-543 could suppress glioma in vitro and in vivo.8 MiR-129-5p is an essential member of miR-129 family.9 Dysregulation of miR-129 family members has been investigated in various cancers such as human prostate carcinoma,10 breast cancer,11 lung cancer,12 gastric cancer.13 Some researchers have also explored the mechanisms of miR-129 family members in affecting the glioma cell processes. For example, Koukkan et al reported that miR-129-1 acted as a suppressor in glioblastoma cells through targeting IGFBP3 and MAPK1.14 and miR-129-2 targeting HMGB1 was reported by Yang et al.15 Xu et al also reported that miR-129-5p inhibited glioblastoma cell viability and metastasis by targeting FNDC3B.9 Despite the antecedent studies, researches on the specific mechanism of miR-129-5p regulation in glioma cells are still insufficient.

Transforming growth factor-beta-induced 2 (TGIF2), whose overexpression was first identified in ovarian cancer, had been reported in studies concerning various malignancies.16 While few study investigated the mechanism underlying TGIF2 and glioma. According to the reports of Jin et al, TGIF2 mRNA was detected at high level at E12.5 and E15.5 in the mice nervous system, which might participate in the regulation of neural stem cell.17 And there is a similarity between neural stem cell and glioma stem cell. Therefore, this study analysed the TGIF2 effects on glioma cells activity. On the other hand, accumulating evidence showed that the regulatory mechanisms of some mRNAs in certain cancers were associated with TGIF2, such as miR-541-3p in non-small cell lung cancer,16 miR-148a in skin cancer,18 and miR-34a in gastric cancer.19 Generally, TGIF2 was found up-regulated in these tumours and acted as an antagonist of relative tumour-suppressing miRNAs.16,18,19 But the correlation between TGIF2 and miR-129-5p still remains unknown. Based on the importance of TGIF2 and previous researches, we employed experiments regarding the molecular network of TGIF2 and miR-129-5p in glioma.

In this study, we purposed to explore the mechanism of miR-129-5p on glioma cell processes. We measured the expression levels of miR-129-5p in both cells and tissues and demonstrated its association with glioma cell progression. In addition, we investigated the relationship between miR-129-5p and TGIF2 and explored their impacts on glioma cell progression.

2 MATERIALS AND METHODS

2.1 Tissue samples

Forty-nine glioma tissue samples and 19 non-tumorous brain tissues were provided by patients receiving surgery in the First Affiliated Hospital of Xinxiang Medical University from January 2012 to January 2016. Written consents were obtained from patients. All tissues were directly preserved in liquid nitrogen and stored at −80°C. The study was carried out under the approval of the ethic committee of the First Affiliated Hospital of Xinxiang Medical University.

2.2 Cell culture

Human astrocytes (HA) and human brain glioma cell lines A127, U251, U87, U373 and SHG44 were procured from BeNa Culture Collection (BNCC, Beijing, China). Cell lines were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% foetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) in 5% CO2 at 37°C.

2.3 Microarray analysis

The three pairs of tissue samples were randomly analysed. Total extracted RNA was analysed through Affymetrix Multispecies miRNA-4 Array (Affymetrix, Santa Clara, CA, USA) and quantified by Spectrophotometry and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). R program and Bayesian test were used for the screening of differentially expressed genes based on the criteria of over twofold difference and P-value < .05.

2.4 QRT-PCR

Total RNA was extracted from the samples with TRIzol® reagent (Invitrogen) and then quantified by NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The reverse transcription of 200 ng total RNA was utilizing ReverTra Ace qPCR RT Kit (Toyobo, Japan) and quantitative real-time PCR with THUNDERBIRD SYBR® qPCR Mix (Toyobo, Japan). The PCR set was at the initial denaturation of 2 minutes at 94°C, following with 30 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C in a total of 30 cycles, and another 10 minutes at 72°C in the end. All experiments were carried out in triplicate. The internal control was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and relative expression of mRNA was calculated using 2^(-ΔΔCT) method. The primers were listed in Table 1.

2.5 Cell transfection

U87 and U251 cells were seeded in 6-well plates and cultured in 5% CO2 at 37°C for 18-24 hours till 80%-90% confluence. MiR-129-5p
mimics (miRNA-129-5p group), miR-129-5p inhibitor (anti-miR-129-5p group) and negative control oligonucleotide (mock group) were purchased from Sangon Biotech, Shanghai, China. Lipofectamine 2000 (Invitrogen) was used for cell transfection. The eukaryotic expression vector pcDNA 3.1-TGIF2 containing complete sequence of TGIF2 cDNA and pcDNA3.1-TGIF2 shRNA was constructed by Sangon Biotech, Shanghai, China. With Lipofectamine 2000, the vector pcDNA 3.1-TGIF2 and pcDNA3.1-shTGIF2 were transfected into glioma cells according to the indicated protocol. Cells were transferred into complete medium 6 hours post-transfection.

2.6 CCK-8 assay

At 12 hours post-transfection, the cells (U251 or U87) were transfected into 96-well plates, and 10 μL CCK-8 solution (Beyotime, Shanghai, China) was added to each well after cultured for 24, 48 and 72 hours. After incubation for another 4 hours at 37°C, the absorbance value was measured at 450 nm.

2.7 Flow cytometric analysis

Collected cells were fixed with 75% ethanol at 4°C for 1 hour and washed with phosphate-buffered saline (PBS) three times before adding 1 mL PBS containing 40 μg propidium iodide (PI) and 100 μg RNase A. A flow cytometer FACS Calibur (Becton Dickinson, San Jose, CA, USA) was employed for the detection of cell cycle distribution and cell apoptosis with FITC Annexin V Apoptosis Detection Kits (Becton Dickinson). The data were analysed by FACS Diva (Becton Dickinson). All experiments were carried out in triplicate.

2.8 Wound-healing assay

At 24 hours post-transfection, cells were seeded in six-well plates and cultured until 90% confluence. Then cell layers were scratched with a 200-μL sterile pipette tip. After removing cell culture medium and suspension cells and cell debris, each well was added with serum-free medium and stored in incubator for 24 hours. Cell migration was then viewed and photographed after incubation for 24 hours.

2.9 Transwell assay

The Matrigel (BD, USA) melted at 2°C to 8°C overnight and was mixed with triple-volume serum-free medium and added into 24-well transwell chambers (50 μL per well). After incubation for 30 minutes, 250 mL of 10% FBS DMEM was added to the lower chambers, and 4 × 10³ collected cells in total were planted in the upper ones and cultivated in the incubator for another 24 hours. Cotton swabs were utilized to slightly clean the cells on the upper surface of the membrane, and cells penetrated across the polycarbonate membrane were fixed with methanol and stained with 0.1% crystal violet. Five random fields were selected, and invaded cells were counted under a microscope. The experiments were carried out in triplicate.

2.10 Tumour xenograft

Twelve male BALB/c nude mice at an average age of 6 weeks, weighing 16-20 g, were purchased from Department of Laboratory Animal Science of China Medical University (Shenyang, China) and equally and randomly divided into two groups: mice injected with U87-Mock cells as control group or U87 cells transfected with Agomir-129-5p (Riboio, Guangzhou, China). Animal experiments were strictly conducted in accordance with the protocols of Animal Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University. Mice of the two groups were each subcutaneously injected with 100 μL (1 × 10⁶ cells) of U87-Mock or U87-miR-129-5p cell sap in the right axillary. After 7 days post-injection, a vernier calliper was used to measure the subcutaneous tumours over different time (every 3 days). The volume size was calculated based on the formula: Volume = Long Diameter × Short Diameter. All the mice were sacrificed on the 28th day after injection. Tumours were taken out for further measurement.

2.11 Dual-luciferase reporter assay

As shown in TargetScan (http://www.targetscan.org/), TGIF2 is one of miR-129-5p’s targets. TGIF2-3’UTR was mutated using multisitedirected mutagenesis. 3’UTRs (wild and mutated type) were introduced into pmirGLO vector (Promega, Madison, WI, USA). MiR-129-5p mimics or mocks and TGIF-2-wt or TGIF-2-mut 3’UTRs were co-transfected into cells. Relative Luciferase activity was detected 48 hours after transfection.

2.12 Western blot

The concentration of extracted protein was quantified with Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, USA). After segregated by SDS-PAGE, proteins were transferred to PVDF membrane (Invitrogen) and blocked in Tris-buffered saline-Tween (TBST) with 5% skim milk at room temperature for 1 hour. Subsequently, the proteins were first cultured with primary antibodies (Rabbit Anti-Human TGIF2, BOSTER Biological Technology Co., Ltd., Wuhan, China, 1:500) at 4°C overnight, and then the secondary antibodies ( Peroxidase AffiniPure Goat Anti-Rabbit IgG, 1:2000) at room temperature for 1.5 hours. Proteins were visualized by ECL (enhanced chemiluminescence) Plus (Life Technology), and the IOD (integrate optical density) was determined and analysed by software Lab Works 4.5. GAPDH protein was detected as the internal control.

2.13 Statistical analysis

Statistics were analysed by SPSS 21.0 (SPSS Inc) and presented as the mean ± SD. Student’s t-test method was utilized for comparison between two groups while one-way ANOVA and LSD-t test were used for data comparison in multiple groups repeated-measures.
ANOVA for comparison of nude mice tumour volumes. $P < .05$ was considered as statistically significant.

3 | RESULTS

3.1 | MiR-129-5p expression was suppressed in glioma tissues and cells

Three paired glioma samples were analysed through microarray analysis to screen out differentially expressed miRNAs (Figure 1A-B). MicroRNAs with $\log_2(\text{FoldChange}) > 2$ and adjusted $P$ value $< .01$ were identified and plotted as heat map. Statistical analysis showed that miR-129-5p was down-regulated in the glioma samples. According to qRT-PCR results, compared with the normal brain tissues, miR-129-5p expression level in glioma tissues was considerably lower ($P < .05$, Figure 1C). MiR-129-5p expression level was also remarkably lower in glioma cells in comparison with normal glial cells ($P < .05$, Figure 1D). Results of qRT-PCR validated the down-regulation of miR-129-5p in glioma tissues and cells.

3.2 | MiR-129-5p overexpression impeded the multiplication and metastasis of glioma cell lines U87 and U251

MiR-129-5p overexpression or knockdown in glioma cells U87 or U251 were established by transfection ($P < .05$, Figure 2A). Results of CCK-8 assay showed that cell viability in anti-miR-129-5p group was significantly stronger, while that in miR-129-5p overexpression group was much weaker compared with the mock group ($P < .05$, Figure 2B-C), indicating that overexpression of miR-129-5p impeded the proliferation of U87 and U251 cells. Results of the wound-healing assay in both cell lines demonstrated the remarkably lower migration rate in miR-129-5p overexpression group as well as the significantly higher migration rate in anti-miR-129-5p group in comparison with the mock group ($P < .05$, Figure 2D-F). Results of transwell assay showed that more cells penetrated across the membrane in the anti-miR-129-5p group and much less invaded cells in miR-129-5p overexpression group compared with the mock group ($P < .05$, Figure 2G-H). The results mentioned above indicated that

![Figure 1](image-url)
miR-129-5p inhibited the proliferation, migration and invasive ability of glioma cells.

### 3.3 MiR-129-5p induced the apoptosis and blocked the cell cycle of U87 and U251

Flow cytometric analysis showed that cell apoptosis rate was notably higher in miR-129-5p overexpression group while was lower in anti-miR-129-5p group compared with the mock group, indicating that overexpression of miR-129-5p induced the apoptosis of glioma cells U87 and U251 (P < .05, Figure 3A). In addition, the results of cell cycle distribution indicated that the cell cycle progression of cells in miR-129-5p group was arrested in G0/G1 phase (P < .05, Figure 3B). The above results showed that miR-129-5p promoted glioma cell apoptosis and arrested cell cycle at G0/G1 phase.

### 3.4 MiR-129-5p suppressed glioma cell U87 development in vivo

After manipulating miR-129-5p expression in U87 cells with Ago-miR-129-5p, cells were subcutaneously injected into the nude mice while mice injected with untreated U87 cells as mock group. After 7 days post-injection, a vernier caliper was used for the measurement of the subcutaneous tumours in twelve nude mice every 3 days. The nude mice were sacrificed on the 28st day, and the tumours were isolated. Tumour development in U87-miR-129-5p
group was obviously attenuated than in the mock group \( (P < .05, \text{Figure 4A-C}). \)

### 3.5 Targeting relationship between miR-129-5p and TGIF2

To further investigate the underlying mechanism of miR-129-5p’s function, TargetScan Database (http://www.targetscan.org/) was employed to predict the potential targets. Combining the literature search and the expression measurement, TGIF2 was selected, and the wild-type and mutated 3’UTR were subcloned into pmirGLO vector (Figure 5A). The targeting relation between miR-129-5p and TGIF2 was validated by dual-luciferase reporter assay \( (P < .05, \text{Figure 5B}). \)

According to the qRT-PCR results, TGIF2 was overexpressed in glioma tissues \( (P < .05, \text{Figure 5C}), \text{and its expression in glioma cells U87, U251, A172, U373 and SHG44 was dramatically higher than that in normal cell HA (P < .05, Figure 5D). Western blot assay also indicated that TGIF2 protein expression levels were much higher in the five glioma cell lines than that in normal cell HA (P < .05, Figure 5E). Compared with the mock group, dramatically lower protein expression levels of TGIF2 were displayed in the miR-129-5p overexpression cells while higher protein expression levels were displayed in miR-129-5p knockdown cells \( (P < .05, \text{Figure 5F}), \text{suggesting that miR-129-5p could regulate TGIF2 expression in U87 and U251.}\)

### 3.6 TGIF2 stimulated glioma cell multiplication and metastasis

The results of qRT-PCR indicated that TGIF2 mRNA expression was promoted by TGIF2 cDNA, while impeded by TGIF2 shRNA...
Co-transfection of anti-miR-129-5p rescued the expression level of TGIF2. CCK-8 results demonstrated that the viability of glioma cell was remarkably stronger in TGIF2 overexpression group while was notably weaker in TGIF2 knockdown group in comparison with the mock group ($P < .05$, Figure 6B,C). And knocking down of miR-129-5p impaired the inhibitory effect of TGIF2 shRNA on cell viability. Results of wound-healing assay demonstrated that cell migration rate in TGIF2 overexpression group was remarkably higher.

**FIGURE 4** MiR-129-5p suppressed tumour formation of glioma cell U87 in nude mice. (A-B) Tumours in nude mice were remarkably larger in miR-129-5p overexpression group compared with the mock group. (C) Tumours in nude mice were remarkably lower in miR-129-5p overexpression group. *$P < .05$, compared with mock group.

**FIGURE 5** Targeting relationship between MiR-129-5p and TGIF2 in U87 and U251. (A) The predicted base pairing in miR-129-5p and TGIF2 from TargetScan. *$P < .05$, compared with mock group. (B) Validation of targeting relation between miR-129-5p and TGIF2 through dual-luciferase reporter assay. (C) TGIF2 expression was up-regulated in glioma tissues. *$P < .05$, compared with normal tissues. (D) The expression of TGIF2 was higher in five glioma cell lines (U87, U251, A172, U373 and SHG44) than that in normal cell line HA. *$P < .05$, compared with HA cell lines. (E) TGIF2 protein expression was higher in five glioma cell lines (U87, U251, A172, U373 and SHG44) than that in normal cell line HA. *$P < .05$, compared with HA cell lines. (F) In cell lines U87 and U251, the protein expression of TGIF2 was significantly higher in anti-miR-129-5p group while was lower in miR-129-5p overexpression group. *$P < .05$, compared with mock group.
whereas that in TGIF2 knockdown group was much lower compared with the mock group ($P < .05$, Figure 6D-F). Meanwhile, there was no significant difference between TGIF2 shRNA + anti-miR-129-5p group and mock group. In transwell assay, more invaded cells were seen in TGIF2 overexpression group while much less cells penetrated across the membrane in TGIF2 knockdown group compared with the mock group ($P < .05$, Figure 6G,H). In addition, the inhibitory effect of TGIF2 shRNA on cell invasion was impaired by anti-miR-129-5p. These results denoted that TGIF2 promoted U87 and U251 cell migration and invasion, and anti-miR-129-5p could reverse the negative effect of TGIF2 shRNA on cell metastasis. All the results above demonstrated a negative correlation between TGIF2 and miR-129-5p with regard to their impacts on glioma cell functions.

3.7 | TGIF2 inhibited U87 and U251 cells apoptosis and promoted cell cycle

For further investigation into the impact of TGIF2 on cell functions, the cell apoptosis and cell cycle distribution were also detected in the TGIF2 overexpression and knockdown cells. The results demonstrated that overexpression of TGIF2 decreased cell apoptosis rate, while the inhibition of TGIF2 accelerated the apoptosis of glioma cells, which was impeded by anti-miR-129-5p ($P < .05$, Figure 7A). Meanwhile, down-regulation of TGIF2 arrested cell cycle at G0/G1 phase while anti-miR-129-5p reversed the impacts of TGIF2 shRNA on cell cycle ($P < .05$, Figure 7B). The above results showed that TGIF2 induced the development of tumours, which was exactly
contrary to the biological functions of its targeting regulator miR-129-5p.

4 | DISCUSSION

According to the experiment results in our study, the expression of miR-129-5p was suppressed in glioma tissues and cell lines. Overexpression of miR-129-5p not only impeded glioma progression, induced tumour cell apoptosis and blocked cell cycle in vitro, but also suppressed tumour growth in vivo. TGIF2 was confirmed to be the direct target of miR-129-5p through dual-luciferase reporter assay. Meanwhile, we discovered that TGIF2 overexpression stimulated glioma cell viability and metastasis. Our study demonstrated the suppressing effect of miR-129-5p on glioma cell progression might contribute to the improvement of glioma therapy.

A growing body of evidence revealed the aberrant expression of various mRNAs in glioma tissues and cells, which functioned as either tumour suppressors or promotors by targeting diverse genes. For example, mRNAs like miR-105 and miR-592 were reported to inhibit glioma cell malignancy,\textsuperscript{20,21} while some other mRNAs like miR-21 and miR-183 promoted the metastasis of glioma cells.\textsuperscript{22,23} Previous studies had revealed the suppressive role of miR-129-5p in several malignancies such as chondrosarcoma, gastric cancer and laryngeal cancer.\textsuperscript{24-26} In our study, we attempted to elucidate the biological function of miR-129-5p in human glioma. We revealed the down-regulation of miR-129-5p in human glioma tissues and cells and the remarkable suppressive effects of miR-129-5p.
overexpression on glioma cell progression and tumour growth. The results we obtained were consistent with what Xu et al demonstrated in their study,9 signifying the repressive effects of miR-129-5p in human glioma.

To explicate the specific regulatory mechanism of miR-129-5p in glioma cell progression, Xu et al identified its targeting gene FNDC3B, while we centred on the interaction between miR-129-5p and another targeting gene TGIF2. Previous studies had also revealed that TGIF2 could be targeted and regulated by various mRNAs in different cancers. For example, Wang et al observed that the up-regulated TGIF2 would be restrained by miR-34c restoration in hepatocellular carcinoma27 and miR-148a was reported to moderate ovarian cancer cell multiplication and invasion by interacting with TGIF2.28 Our study first demonstrated the role of TGIF2 in human glioma. In this study, we ascertained the targeting relationship between miR-129-5p and established TGIF2 overexpression and knockdown cells to investigate its impact on glioma cell processes. The experiment results showed that TGIF2 overexpression promoted the propagation of glioma cell. Collectively, all the results suggested the negative correlation between miR-129-5p and TGIF2 and their opposite effects on glioma initiation and development.

Undoubtedly, insufficiencies still existed in our study. For example, in vivo experiments directly detecting the impact of TGIF2 modulation on glioma tumorigenesis was lack. Besides, TGIF2 is not the only target gene of miR-129-5p, and other molecular mechanisms should be further investigated to adequately understand the functions of miR-129-5p in human glioma.

5 | CONCLUSION

Collective data supported the suppressive role of miR-129-5p on glioma both in vitro and in vivo. By targeting TGIF2, miR-129-5p proved to suppress the cell proliferation, migration, invasion, induced cell apoptosis and G0/G1 cell cycle arrest. The novel findings shed new light on potential therapeutic strategies for glioma treatment.

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CONFLICT OF INTEREST

The authors confirm that there are no conflict of interests.

AUTHOR CONTRIBUTION

Baozhe Jin involved in substantial contributions to research design, or the acquisition, analysis or interpretation of data. Yuling Diao and Liyong Huang drafted the manuscript or revised it critically. Wenke Zhou performed approval of the submitted and final versions.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the First Affiliated Hospital of Xinxiang Medical University.

Informed consent: Approval was obtained from the ethic committee of the First Affiliated Hospital of Xinxiang Medical University.

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