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Regular paper

MicroRNA-151 regulates the growth, drug sensitivity and epithelial mesenchymal transition of human glioma cells by targeting profilin 2

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The microRNA-151 (miR-151) has been reported to be involved in the growth, development, and tumorigenesis of different types of human cancers. This study was designed to unravel the role and therapeutic potential of miR-151 in glioma. The results showed glioma was found to be associated with significant ($P<0.05$) downregulation of miR-151. Low expression of miR-151 was also associated with poor survival of the glioma patients. Overexpression of miR-151 resulted in a significant ($P<0.05$) decline of glioma cell proliferation and colony formation. The sensitivity of the glioma cells to adriamycin also increased significantly ($P<0.05$) upon miR-151 overexpression. Additionally, overexpression of miR-151 also suppressed the migration and invasion of the human glioma cells. This was also associated with alteration in the expression of epithelial mesenchymal transition proteins. The expression of E-cadherin was increased while as that of N-cadherin, vimentin, and Snail was considerably decreased upon miR-151 overexpression. Bioinformatic analysis and ducal luciferase assay showed miR-151 targets profilin 2 (PFN2) in human glioma cells. The expression of PFN2 was found to be significantly ($P<0.05$) upregulated in human glioma tissues cells and cell lines. Nonetheless, the PFN2 expression was considerably suppressed upon miR-151 overexpression. Knockdown of PFN2 resulted in decrease of glioma cells proliferation. In contrary, overexpression of PFN2 could avoid the tumor-suppressive effects of miR-151. Taken together, present study points towards the tumor-suppressive effects of miR-151 and prospective therapeutic implications in human glioma.

Key words: Glioma, micro-RNA, adriamycin, epithelial-to-mesenchymal transition, migration, invasion.

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Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; EMT, epithelial-to-mesenchymal transition; PBS, phosphate buffered saline, PFN, Profilin 2

INTRODUCTION

The glioma, representing the malignant form of brain tumor originating from neuro-epithelial tissues, is considered as the most dominant tumor of human brain (Schwartzbaum *et al.,* 2006). Nearly 30% cases of the total malignant tumors of central nervous system and more than 80% of intra-cranial tumor malignancies system are believed to correspond to glioma (Goodenberger & Jenkins, 2012). Moreover, sufficient level of annual fatality is seen to be caused by human glioma (Shi *et al.,* 2018). Although, the advanced surgical approaches along with chemo/radio-therapeutic modalities have reduced the proportion of annual glioma deaths, the mortality data still demand exploration of better treatment strategies. There are scientific reports that highlight the role of micro-RNAs (miRs) as effective prognostic and therapeutic agents against cancer (To *et al.,* 2018). The miRs which represent a heterogenous class of endogenously synthesized RNA oligonucleotides act at the translational level to regulate eukaryotic gene expression (Sharma *et at.,* 2019). According to the estimates, miRs regulate the expression of more than 30% protein coding genes in eukaryotes (O'Brien *et al.,* 2018). The crucial cellular processes like cell division, differentiation, apoptosis etc. have been shown to be controlled by miRs (Shirjang *et al.,* 2019). Moreover, miRs are linked with the process of human tumorigenesis (Rupaimoole & Slack, 2017). Human cancers are usually associated with altered expression patterns of one or more miRs (Wong *et al.,* 2018). The miRs are thus valued for their prognostic role (Qadir & Faheem, 2017). The researchers have even proposed the concept of miR- correctional theory as a vital anti-cancer strategy (Chen *et al.,* 2018). The miR-151 has been elucidated to exhibit transcriptional downregulation or upregulation in several human cancers (Liu *et al.,* 2019; Lv *et al.,* 2016). The miR-151 was shown to target various protein coding genes in human cancers to modulate the growth and proliferation of cancer cells (Liu *et al.,* 2019; Tommasi *et al.,* 2016). Besides, its involvement in regulating cancer metastasis has been established from multiple studies (Krell *et al.,* 2012). The miR-151 has also been shown to affect the epithelial-to-mesenchymal transition (EMT) of cancer cells (Yeh *et al.,* 2016). Previously, the exosomal transfer of miR-151 was found to enhance the chemo-sensitivity of drug-resistant glioblastoma cells to temozolomide (Zeng *et al.,* 2018). In the present study, miR-151 was significantly downregulated in human glioma. Low expression of miR-151 was shown to be linked with poor patient survival. The re-expression of miR-151 in glioma cells declined the growth, migration, and invasion of cancer cells. EMT was also shown to be inhibited under miR-151 overexpression. Additionally, the glioma cancer cells over-expressing miR-151 exhibited higher susceptibility to adriamycin (ADR) treatment *in vitro*. Profilin 2 (PFN2) was shown to act as the functional target of

miR-151 in glioma and the regulatory effects of miR-151 in glioma were shown to be exerted through miR-151/PFN2 axis. Collectively, the study represents the tumor-suppressive role of miR-151 in glioma and highlights its potential to enhance the chemo-sensitivity of glioma cells.

MATERIALS AND METHODS

Clinical specimens

Tissue samples, 40 glioma tissues and non-cancerous brain tissues were obtained from May 2013 to June 2016 at Tianjin Medical University General Hospital after informed consent from the patients. Further, the serum specimens were collected from peripheral blood smear from the corresponding patients, which were recognized either as high miR-151 expression group or low miR-151 group based on mean miR-151 expression. The characteristics of the patients are listed in Table 1. The study was approved by the ethics committee of Tianjin Medical University General Hospital.

Cells lines and culture conditions

The human glioma cancer cell lines (U87, U118 and M059K) and the normal astrocytes were obtained from China Center for Type Collection (CCTCC, Wuhan, China). The cell lines were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, PAN Biotech). The culture media were supplemented with 10% fetal bovine serum together with 1% penicillin/streptomycin (PAN Biotech). For cell transfection, Lipofectamine 2000 (Thermo Fisher Scientific) was used.

Expression study

The human tissue and serum samples and cell lines were treated with TRIzol reagents to isolate the total RNA. RNA was reverse transcribed using ABI Reverse Transcription Kit (Thermo Fisher Scientific) and cDNA was synthesized. The quantitative real-time polymerase chain reaction (qRT-PCR) was performed on QuantStudio 5.0 Real-Time PCR System (Thermo Fisher Scientific) with the help of SYBR Green PCR mix (Thermo Fisher Scientific). U6 and GAPDH were used as internal controls for miR-151 and PFN2, respectively. The relative gene expression was measured through 2^{-ddCt} method. The sequences of the primers used in the study are listed in Table 2.

Table 1. Clinical characteristics of glioma patients that participated in the present study

Variables	Glioma patients (n= 40)
Age	
<40	17
>40	23
Sex	
Male	21
Female	19
WHO grades	
Grade I	4
Grade II	9
Grade III	12
Grade IV	15
Histological sub-type	
Astrocytoma	9
Anaplastic astrocytoma	10
Oliodendroglioma	7
Glioblastoma	5
Gliocytoma	8
Meduloblastoma	1

Table 2. List of primers used in the study.

Primer	Direction	Sequence
miR-151	Forward	5′-GGATGCTAGACTGAAGCTCCT-3′
	Reverse	5′-CAGTGCGTGTCGTGGAGT-3′
PFN2	Forward	5′-ATGATTGTAGGAAAAGACCGGGA-3′
	Reverse	5′-GCAGTCACCATCGACGTATAGAC-3′
U6	Forward	5′-GTCCGGTTTCAGCATGTTT-3′
	Reverse	5′-CTCGCTTCGGCAGCACA-3′
GADPH	Forward	5′-CAATGACCCCTTCATTGACC -3′
	Reverse	5′-TGGAAGATGGTGATGGGATT -3′

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The transfected cancer cells were placed in 96-well plates and cultured for 0, 12, 24, 48, or 96 h at 37°C. Then, MTT solution was added, and plates were again incubated for 4 h. After the addition of dimethyl sulfoxide (DMSO) the cells were harvested, washed with PBS, and the absorbance was measured at 570 nm using a micro-plate spectrometer (BioRad Laboratories).

Colony formation assay

For analyzing the colony formation, the transfected glioma cells were treated with Trypsin-EDTA solution (Sigma). The cell culturing was performed in 12-well non-adherent plates for 12 days at 37°C. Finally, the colonies were stained with 0.1% crystal violet after being ethanol fixed. The colonies were photographed and manually counted under light microscope.

5-ethynyl-2′-deoxyuridine (EdU) staining assay

The proliferative capability of transfected glioma cells was assessed with EdU assay. The labeling of transfected glioma cells was performed using EdU labeling solution from EdU labeling/detection kit (RiboBio, China), as per the manufacturer's instructions. 70% ethanol was used for fixing the cells. The fixed cells were incubated with glycine, washed with PBS, and then administered with anti-EdU solution. The cell permeation was performed using 0.5% Triton X-100 in phosphate buffered saline (PBS). The nuclei of glioma cells were stained using 0.5% DAPI (4',6-diamidino-2-phenylindole). The fluorescent microscope was used for visualizing the cells.

Wound healing assay

The migration of glioma cells was determined with the help of wound-healing method. Briefly, the cells were cultured till confluence in 6-well plates. Subsequently, the cell surface was wounded by drawing a perpendicular scratch using pipette tip. Following 24 h incubation

at 37°C, the scratch width was observed and compared with the scratch width at 0 h. The scratch was photographed under light microscope at 0 h and after 24 h.

Cell invasion assays

The matrigel (BD biosciences) coated transwell chambers (Millipore) were prepared as per the manufacturer's instructions. Post starvation, approximately 10^5 cells (in 200 µL of serum-free culture medium) were placed into the top chamber. The lower chamber received only 700 µL culture medium with 10% FBS. After 24 h incubation at 37°C, 4% paraformaldehyde was used for fixing the cells invading the lower chamber. The staining of cells was performed with 0.1% crystal violet solution (Beyotime). Photographs were taken and cells were counted under light microscope from five randomly selected fields.

Target identification and dual luciferase reporter assay

TargetScan (http://www.targetscan.org) was used to identify the potential target of miR-151. A 3'-UTR fragment of PFN2 was synthesized carrying either wild-type (WT) or mutant (MUT) binding site and cloned into the psiCHECK-2 reporter vector (Applied Biosystems, USA). The reporter plasmid (WT or MUT) was co-transfected with miR-151 mimics or miR-NC into U118 glioma cells. Cells were collected after 48 h of transfection. The Dual Luciferase Reporter Assay System (Promega, Madison, USA) was used to determine the luciferase activity.

Western blotting

The proteins from glioma cells were isolated using RIPA lysis and extraction buffer (Thermo Fisher Scientific). Then proteins were resolved on SDS-PAGE gel and transferred to PVDF membranes. After blotting, membranes were treated with primary antibodies against specific proteins at 4°C overnight after blocking with 5% skimmed milk. Afterwards, the membranes were exposed to horseradish peroxidase-conjugated goat anti-rabbit secondary antibody for 2 h at room temperature. Finally, the proteins were visualized using BeyoECL Plus Kit (Beyotime).

Statistical analysis

The statistical data were analyzed with SPSS 20.0 software and results were given as mean ± standard deviation (S.D.). Student's *t*-test or one-way ANOVA were used for statistical analyses. The $P<0.05$ were taken as the measure of statistically significant difference.

RESULTS

Downregulation of miR-151 corresponds with low patient survival

The qRT-PCR expression analysis revealed that miR-151 was significantly ($P<0.05$) repressed in glioma tissues (Fig. 1A). The expression analysis from paired glioma and normal tissues showed that glioma tissues had significantly ($P<0.05$) lower miR-151 expression as compared to the normal adjacent tissue specimens (Fig. 1B). The Kaplan-Meier survival analysis showed that the patients with higher miR-151 expression exhibit higher disease survival as compared to the patients with lower miR-151 expression (Fig. 1C). The glioma cell lines were also seen to possess significantly lower ($P<0.05$) miR-151

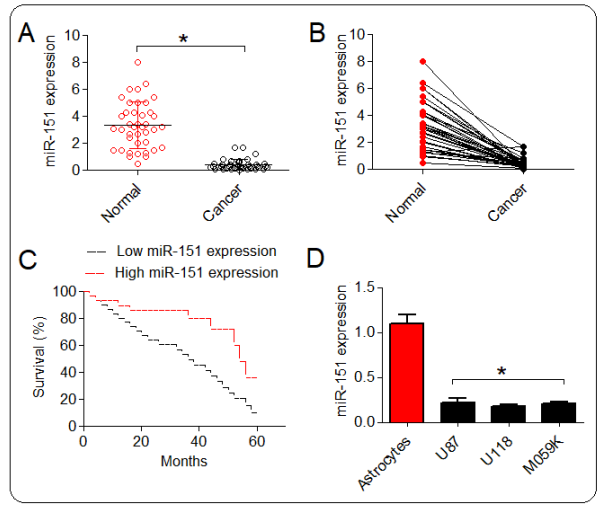

Figure 1. miR-151 is significantly repressed in glioma and predicts the patient survival.
(**A**) qRT-PCR analysis of miR-151 in glioma and normal adjacent tissues (**B**) qRT-PCR analysis of miR-151 in paired glioma and normal adjacent tissues (**C**) Kaplan-Meier survival analysis of glioma with reference to miR-151 expression (**D**) qRT-PCR expression analysis of miR-151 in glioma cell lines (U87, U118 and M059K) and normal astrocytes. The experiments represent the mean of three independent replicates ± S.D. (*$P<0.05$)

transcript levels (Fig. 1D) as compared to the normal astrocytes. Together, the results are suggestive of miR-151 repression in glioma, which associates with the lower patient survival.

miR-151 overexpression declined the growth and viability of glioma cells

In order to analyze the molecular role of miR-151 in glioma, miR-151 was, at first, over-expressed in U118 glioma cells (Fig. 2A). As revealed by MTT assay, glio-

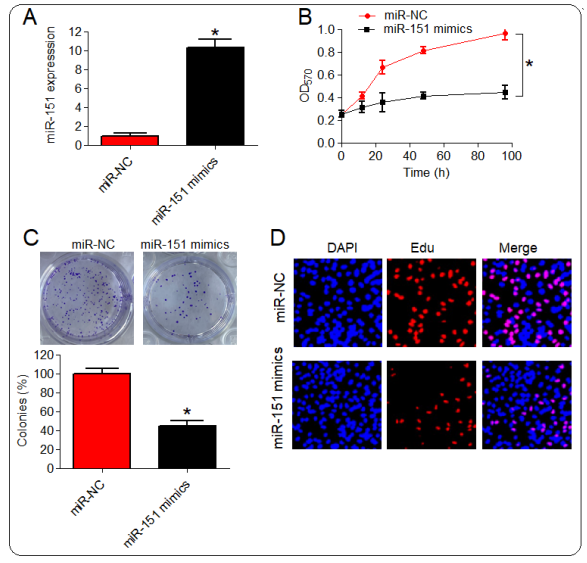

Figure 2. Glioma cell growth and viability significantly declines under miR-151 up-regulation.
(**A**) qRT-PCR analysis of miR-151 from U118 cells transfected with miR-151 mimics or miR-NC (**B**) MTT assay for the analysis of growth of U118 cells transfected with miR-151 mimics or miR-NC (**C**) analysis of colony formation of U118 cells transfected with miR-151 mimics or miR-NC (**D**) EdU assay for the analysis of proliferative viability of U118 cells transfected with miR-151 mimics or miR-NC. The experiments represent the mean of three independent replicates ± S.D. (*$P<0.05$).

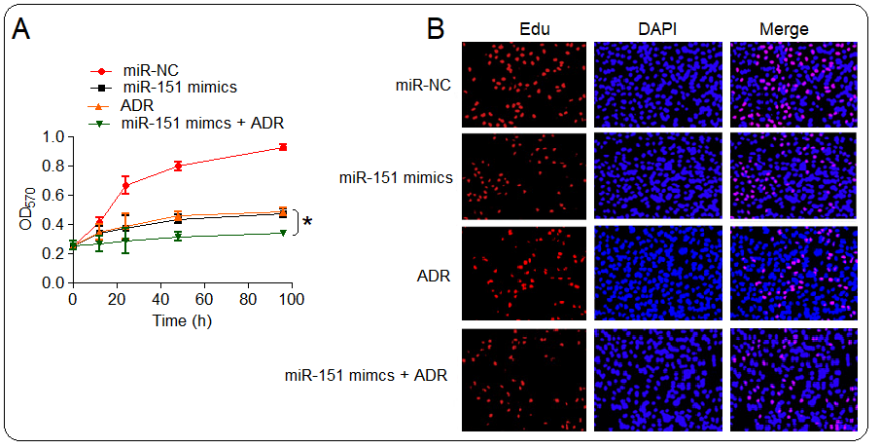

Figure 3. miR-151 overexpression enhances chemo-sensitivity of glioma cells.
(**A**) MTT assay for the analysis of growth of U118 cells administered with ADR, miR-151 over-expression, ADR plus miR-151 over-expression, or miR-NC control transfection (**B**) EdU assay showing proliferation of U118 cells administered with ADR, miR-151 over-expression, ADR plus miR-151 over-expression, or miR-NC control transfection. The experiments represent the mean of three independent replicates ± S.D. (*P<0.05)

ma cancer cells over-expressing miR-151 exhibited significantly lower growth potential in comparison to the negative control cells (Fig. 2B). The colony formation was also markedly lower under miR-151 overexpression (Fig. 2C). The EdU assay showed that glioma cells had lower proliferative ability under miR-151 overexpression (Fig. 2D). The results therefore indicate the tumor-suppressive role of miR-151 in human glioma.

Glioma cells exhibited higher ADR-susceptibility under miR-151 re-expression

To analyze if miR-151 regulates drug sensitivity of glioma cells, the glioma cells over-expressing miR-151 were treated with low dose of ADR (10 μM). The glioma cells were assessed for proliferation analysis through MTT assay with or without ADR. It was found that the overexpression of miR-151 led to higher growth inhibition of glioma cells growth by ADR than ADR only (Fig. 3A). The results were also confirmed by the EdU staining. The proportion of EdU stained cells was significantly higher when cells were treated with ADR with endogenous miR-151 expression. However, the glioma cells treated with 10 μM ADR and under overexpression of miR-151 exhibited significantly lower proportion of EdU positive cells (Fig. 3B). The results therefore confirm that miR-151 overexpression sensitizes the glioma cells to external drug application.

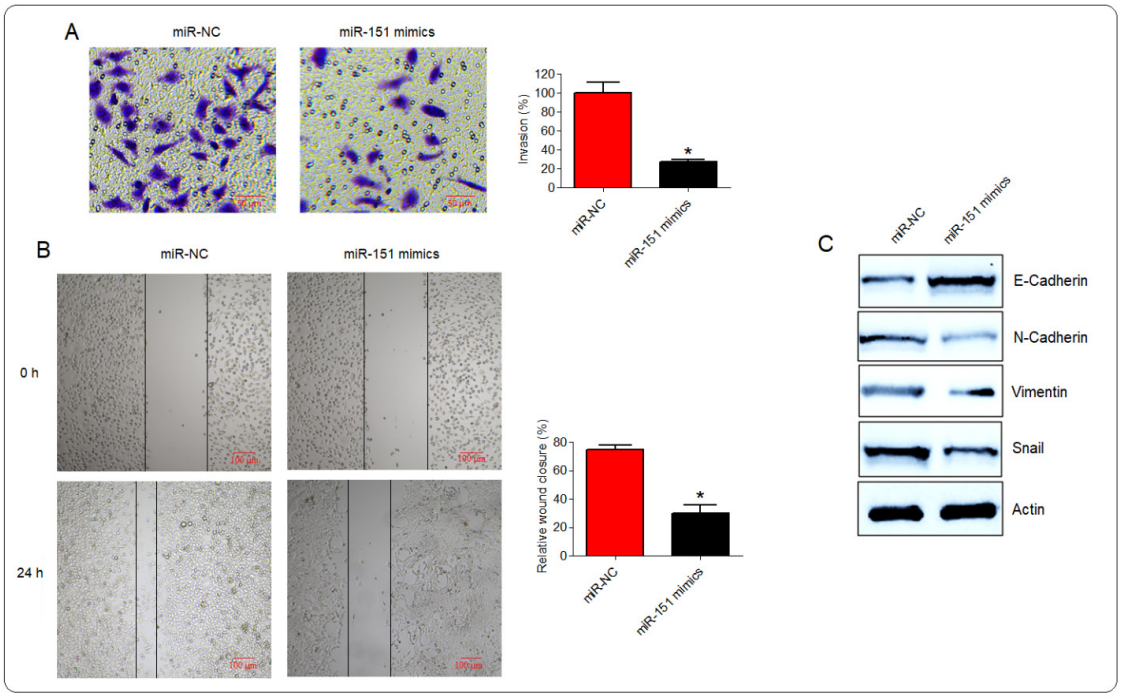

Figure 4. Invasion, migration and EMT of glioma cells is inhibited by miR-151 over-expression.
(**A**) Transwell assay for the analysis of invasion of glioma cells transfected with miR-151 mimics or miR-NC (**B**) wound-healing assay for the analysis of migration of glioma cells transfected with miR-151 mimics or miR-NC (**C**) western blotting of markers of EMT from glioma cells transfected with miR-151 mimics or miR-NC. The experiments represent the mean of three independent replicates ± S.D. (*P<0.05)

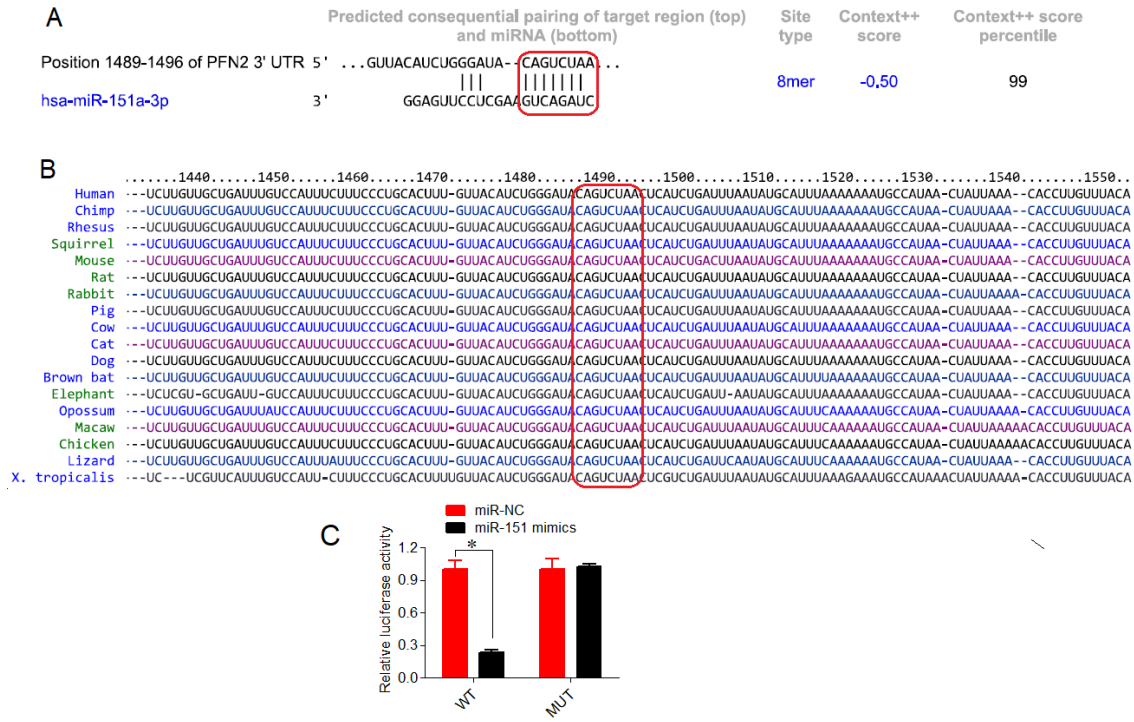

Figure 5. PFN2 was predicted as miR-151 target in glioma.
(**A**) Prediction of molecular target along with binding site for miR-151 (**B**) sequence analysis of miR-151 binding site form different animals (**C**) Dual luciferase assay showing the interaction between miR-151 and PFN2. The experiments represent the mean of three independent replicates ± S.D. (*P<0.05)

Overexpression of miR-151 inhibited migration, invasion, and the epithelial to mesenchymal transition of human glioma cells

From the transwell invasion assay, it was deduced that miR-151 overexpression in U118 glioma cells restricted their invasion significantly (P<0.05) in comparison to the control transfection (Fig. 4A). Again, the glioma cells over-expressing miR-151 showed limited migration in comparison to the negative control cells (Fig. 4B). The analysis of expression of markers of EMT showed that miR-151 led to an increase in E-cadherin protein, while the proteins N-cadherin, Snail and Vimentin were found to decrease considerably under miR-151 overexpression (Fig. 4C). In sum, the results indicate that miR-151 negatively regulates EMT of glioma cells and thus their metastasis.

PFN2 is the functional target of miR-151 in glioma

The online bio-informatics analysis showed that miR-151 interacts with 3'-UTR of profilin 2 (PFN2) at sequence site complementary with its 8-mer nucleotide region (Fig. 5A). The sequence analysis revealed that the binding site of PFN2 3'-UTR is highly conserved in the animal kingdom (Fig. 5B). The interaction between miR-151 and PFN2 was confirmed by the dual luciferase assay (Fig. 5C). The relative expression of PFN2 showed that glioma tissues exhibit significantly higher PFN2 transcript level corresponding to lower miR-151 expression levels (Fig. 6A and 6B). Further, all the glioma cell lines were shown to possess significantly higher PFN2 expression in comparison to normal astrocytes (Fig. 6C). Also, the PFN2 protein expression was significantly lower in glioma cells over-expressing miR-151 in comparison to the negative control cells (Fig. 6D). To confirm whether miR-151 regulates glioma cell growth via PFN2, PFN2 was silenced in U118 glioma cells (Fig. 6E). The silencing of PFN2 in glioma cells remarkably reduced the cell growth like miR-151 overexpression (Fig. 6F). The overexpression of miR-151 together with PFN2 silencing led to the cell growth inhibition mimicking PFN2 silencing itself (Fig. 6G). Lastly, the upregulation of PFN2 restored the normal proliferation potential of glioma cells even under miR-151 overexpression (Fig. 6H). The results indicate PFN2 as a function target of miR-151, which exerts the regulatory effects of the latter in glioma.

DISCUSSION

The miRs, with their tremendous potential to serve as the prognostic biomolecules in human cancer, have been proposed to act as the leading therapeutic targets against several cancer types (To *et al.,* 2018; Qadir & Faheem, 2017; Chen *et al.,* 2018). There are also reports that miRs might enhance the clinical outcomes of presently employed chemo-therapy approaches (Garofalo *et al.,* 2014). Various miRs have been shown to improve the chemo-sensitivity of cancer cells (Zeng *et al.,* 2018; Zhang *et al.,* 2016). In the present study, the regulatory control of miR-151 was assessed in glioma. It has been previously reported that miR-151 is repressed in glioma (Xiao *et al.,* 2014). The results of the present study also revealed the similar inference regarding the expression pattern of miR-151 in glioma. MiR-151 was shown to act as a tumor-suppressor in human tumors such as breast cancer (Lv *et al.,* 2016; Liu *et al.,* 2019). The overexpression of miR-151 was reported to decline the growth and viability of cancer cells. The growth and viability of glioma cells was also affected in the similar fashion in the present study. MiR-151 was previously

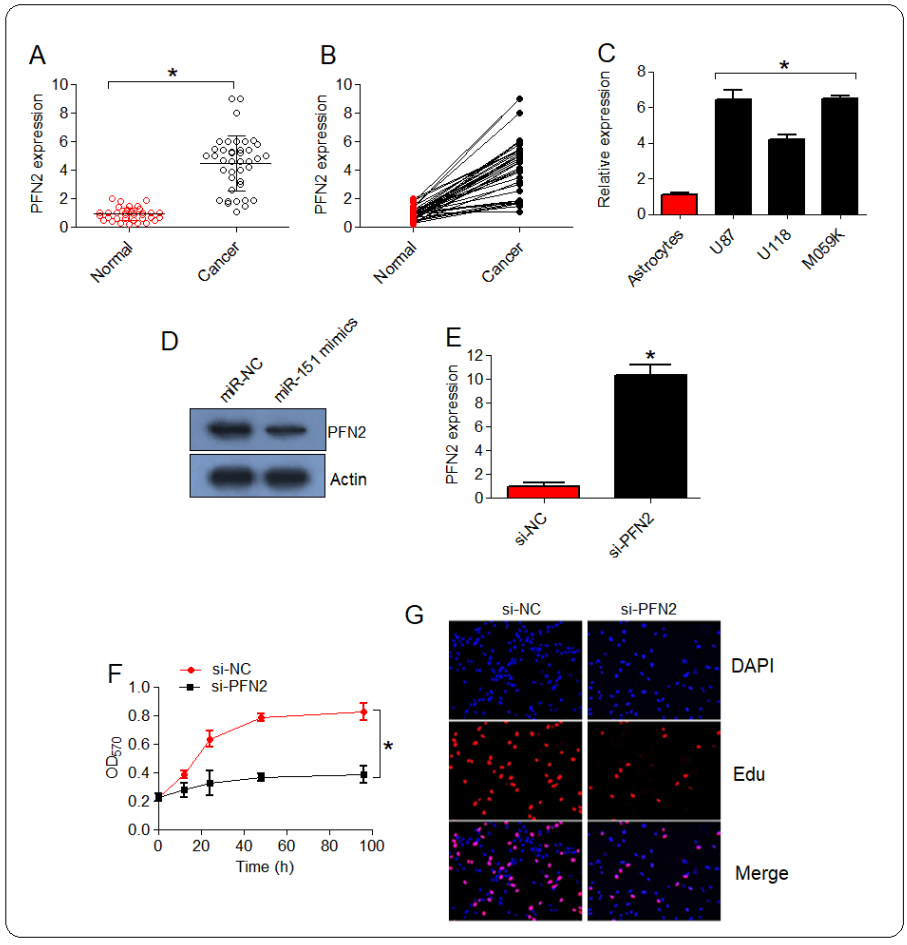

Figure 6. miR-151 targets PFN2 in glioma to exert its role.
(**A**) qRT-PCR analysis of PFN2 in glioma and normal adjacent tissues (**B**) qRT-PCR analysis of PFN2 in paired glioma and normal adjacent tissues (**C**) qRT-PCR expression analysis of PFN2 in glioma cell lines (U87, U118 and M059K) and normal astrocytes (**D**) western blotting of PFN2 from U118 glioma cells transfected with miR-151 mimics or miR-NC (**E**) qRT-PCR expression analysis of PFN2 from glioma cells transfected with si-PFN2 or si-NC (**F**) MTT assay for the analysis of growth of glioma cells transfected with si-PFN2 or si-NC (**G**) MTT assay for the analysis of growth of glioma cells transfected with si-PFN2, si-PFN2 plus miR-151 mimics or si-NC (H) MTT assay for the analysis of growth of glioma cells transfected with miR-151 mimics, miR-151 mimics plus pcDNA-PFN2 or miR-NC. The experiments represent the mean of three independent replicates ± S.D. (*P<0.05)

shown to increase the susceptibility of glioblastoma cells to temozolomide (TMZ) (Zeng *et al.*, 2018). Coinciding with the same, the overexpression of miR-151 enhanced the chemo-sensitivity of glioma cells to ADR in the present study. The epithelial-to-mesenchymal transition (EMT), a complex process of cellular development, enhances the motility of cancer cells and thus enables them to invade the secondary tissues referring to metastasis (Smith & Bhowmick, 2016). The EMT involves reduction in expression of E-cadherin protein, while N-cadherin protein expression is seen to increase (Zhu *et al.,* 2018). The transcriptional repressor snail is reported to repress the expression of E-cadherin (Peinado *et al.*, 2004). Vimentin, a type-III intermediate filamentary protein, is highly expressed in mesenchymal state and thus used as an EMT marker (Kokkinos *et al.,* 2007). The miR-151 was shown to positively regulate EMT of non-small lung cancer cells (Daugaard *et al.,* 2017). However, in the present study, EMT was found to be negatively regulated by miR-151, which indicates that miRs like miR-151 exhibit specific molecular roles for tissues. Evidently, the migration and invasion of glioma cells was significantly restricted by miR-151 overexpression. Similar, anti-metastatic role of miR-151 has been confirmed in the previous studies (Yeh *et al.,* 2016). Importantly, miR-151 was shown to repress the expression of profilin 2 (PFN2) in glioma, and PFN2 was proved to act as the mediator of regulatory role of miR-151 in glioma. PFN2, a regulator of cytoskeleton, behaves like an oncogene in human tumors such as breast cancer, regulates EMT of cancer cells, and is involved in tumorigenesis of human cancers (Jaing *et al.*, 2019; Zhou *et al.*, 2019; Yan *et al.*, 2017). Concluding, the miR-151 targets PFN2 in glioma and regulates the growth, metastasis, and drug sensitivity of the glioma cells. The study thus advocates the combinatorial application of miR-151 overexpression and chemo-therapy administration against glioma for better clinical outcomes.

CONCLUSIONS

The present study showed that miR-151 repression is associated with the development and progression of human glioma, suggesting that miR-151 might be utilized for the diagnosis of glioblastoma. The results further indicated the tumor-suppressive role of miR-151 against glioma together with potential to enhance the drug sensitivity of glioma cells, suggesting its therapeutic utility.

Acknowledgements

Not applicable

Conflict of interest

All the authors declare that there is no conflict of interest.

Ethical approval

Informed consent was sought from the patients before inclusion in the present study. The research ethics committee of Tianjin Medical University General Hospital, Tianjin, China. approved the study with approval number TMU-55-2019.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author contributions

Conceptualization: WZ and HN; methodology: WZ, HN, and YW; formal analysis and investigation: WZ, HN, RW and CL; writing – original draft preparation: WZ and HN; writing – review and editing critically for important intellectual content: WZ; supervision: YW.

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