ORIGINAL RESEARCH

MiR-601 Promotes Cell Proliferation of Human Glioblastoma Cells by Suppressing TINP1 Expression

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ABSTRACT

Objective • It has been well documented that microRNAs (miRNAs) play essential roles in cancer initiation and development. In this study, we aimed to provide a better understanding of the mechanism of cell proliferation in glioblastoma (GBM).

Methods • Levels of miR-601 expression were detected in GBM tissues and cells. The effects of miR-601 dysregulation on GBM cell proliferation by mean transit time (brain tissue blood flow) (MTT) assays, colony formation, anchorage-independent growth assay, bromodeoxyuridine (BrdU) labeling and immunofluorescence assay. Bioinformatics analysis, luciferase reporter system and Western blot assays were used to predict and confirm the target gene miR-601.

Results • MiR-601 levels were identified as significantly up-regulated in GBM primary tumors and cell lines. Ectopic expression of miR-601 suppressed cell proliferation of GBM. Moreover, miR-601 showed its function by suppressing potential target TINP1 and TINP1 suppression reversed the effects of miR-601-in in U87MG GBM cells.

Conclusion • Taken together, our results indicate that miR-601 promotes proliferation of GBM via inhibition of TINP1. (*Altern Ther Health Med.* 2022;28(2):102-108).

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INTRODUCTION

Glioblastoma (GBM) is reportedly the most common and aggressive central nervous system malignancy in adults worldwide.[1] However, the causes of GBM carcinogenesis and progression are largely unknown. Therefore, understanding the molecular mechanisms of GBM is important for developing better diagnostic strategies and new approaches for molecular therapeutics in GBM.

MicroRNAs (miRNAs) are a class of small RNAs that play essential roles in various physiological and pathological processes.[2-5] A large body of evidence has shown that dysregulation of miRNA expression has been found in diverse cancers.[6-8] Compelling evidence has indicated that miRNAs are novel modulators of cancer progression and new targets for cancer therapy, including GBM.[9-13] MiR-137 was reported to suppress cell growth of GBM through epidermal growth factor repression (EGFR) suppression[14]; MiR-520c was found to inhibit GBM cell migration and invasion by suppressing TGFBRII.[15] Findings from a 2017 study by Wang, et al indicated that miR-217 promotes the proliferation and invasion of GBM by repressing YWHAG.[16]

However, the biological function of miR-601 in GBM has still not been fully investigated. Our study investigated higher levels of miR-601 expression in GBM tissues and cells compared with normal brain tissue and novel hormonal agents (NHAs). MiR-601 overexpression increased *in vitro* GBM cell proliferation. Mechanistically, the results of bioinformatics analysis and luciferase-reporter assay showed that miR-601 promoted cell proliferation by targeting TINP1.

MATERIALS AND METHODS

Clinical Specimens and Cell Culture

A total of 8 paired surgically removed human GBM tissue samples and 2 normal brain tissue samples were obtained from individuals who had died in traffic accidents. All patients gave written informed consent and the study was approved by the local Ethics Committee.

Human GBM cell lines A-172, LN-229, LN-18, LN-443, U87MG and LN-340 were purchased from the National

Rodent Laboratory Animal Resource (Shanghai, People's Republic of China) and maintained in DMEM media (Gbico Service, Amarillo, Texas, USA) supplemented with 10% fetal bovine serum, and NHAs were purchased from Lonza (Basel, Switzerland) and maintained in the provided astrocyte growth media supplemented with rhEGF, insulin, ascorbic acid, GA-1000, L-glutamine and 5% FBS. All cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37° C.

Plasmids and Transfection

The miR-601 mimic, miR-601-in and the corresponding negative controls were purchased from Gene Copoeia™ (Guangzhou, China). Small interference RNA of TNIP1 (TNIP1-siRNAs) and their corresponding negative controls (NCs) were purchased from RiboBio Co. Ltd. (Guangzhou, China). Cell transfection was accomplished using Lipofectamine™ 2000 (Invitrogen, ThermoFisher, California, USA) according to the manufacturer's instructions.

RNA Extraction and Real-Time Quantitative PCR

To detect miRNA expression, total miRNA was isolated using miRNeasy mini-Kit (Qiagen). MiR-601 expression was quantified with miRNA-specific TaqMan miRNA assay kit (Applied Biosystems/Thermo Fisher Scientific, USA), with U6 snRNA as an internal control.

To examine mRNA expression, total RNA was isolated from fresh tissues and cells using TRIzol Reagent (Invitrogen), according to the manufacturer's protocol. Gene levels were examined by SYBR Kit (Qiagen, China) using a Light Cycler system. The following PCR primers were synthesized by Gene Copoeia™: MYC (HQP011597) and P21 (HQP000331). GAPDH were used as controls for normalization; relative expression levels were quantified using the $2^{-\Delta\Delta CT}$ method.

Cell Proliferation Assays

Cell proliferation was assayed by mean transit time (brain tissue blood flow) (MTT) assay according to the manufacturer's instructions. Briefly, transfected cells were seeded in a 96-well plate at approximately 5×10^3 cells/well. After incubation for 1, 2, 3, 4 and 5 days, 20 µl of 5 mg/ml MTT solution (Sigma-Aldrich) was added to each well and incubated for 4 h at 37° C, and then the culture medium was removed and 150 µl DMSO (Sigma-Aldrich) was added and absorbance at 490 nm was examined.

For the colony formation assay, transfected cells (500 cells/well) were seeded in a fresh 6-well plate and incubated for 14 days in medium containing 10% FBS. Cells were fixed with 4% paraformaldehyde for 10 minutes and stained with 0.1% crystal violet for 1 minute. Visible colonies were counted under a microscope.

A total of 1000 transfected cells were suspended in 2 ml complete medium plus 0.3% agar (Sigma) and then plated on top of a bottom layer consisting of 1% agar in complete medium. Dishes were incubated in a 37° C incubator for 2 weeks and viable colonies larger than 0.1 mm in diameter were photographed and counted under a microscope. This process was performed 3 times.

Bromodeoxyuridine Labeling and Immunofluorescence

Transfected cells grown on coverslips (Fisher, Pittsburgh, Pennsylvania, USA) were incubated with bromodeoxyuridine (BrdU) for 1 h and then BrdU antibodies (Upstate, Temecula, California, USA) were added according to the manufacturer's protocol. Gray level images were acquired using an Axioskop 2 plus laser scanning microscope (Carl Zeiss Co., Ltd., Jena, Germany).

Luciferase Assays

Wild-type and mutant 3'UTR of TNIP1 were then cloned downstream of the luciferase open reading frame in the pGL3 vector (Invitrogen). Cells (5×10^4) in each group were seeded in 24-well plates and transfected with co-transfected luciferase reporter wild-type 3'-UTR region of TNIP1 (TNIP1-3'UTR) and miR-601 or miR-601-in or the corresponding relative controls. Cells were harvested 48 h after post-transfection, and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay system (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol.

Western Blotting

Total cellular proteins were extracted with RIPA lysis buffer on ice. Then, proteins (40 µg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, California, USA) by electroblotting. The membranes were incubated with primary antibody TNIP1 (SAB1100720, Sigma), P21 (SAB4500065, Sigma) and C-MYC (SAB4501941, Sigma); α-Tubulin (SAB4500087, Sigma) served as the loading control. The bands were visualized by using an enhanced chemiluminescence Kit (Pierce™ Thermo Fisher).

Statistical Analysis

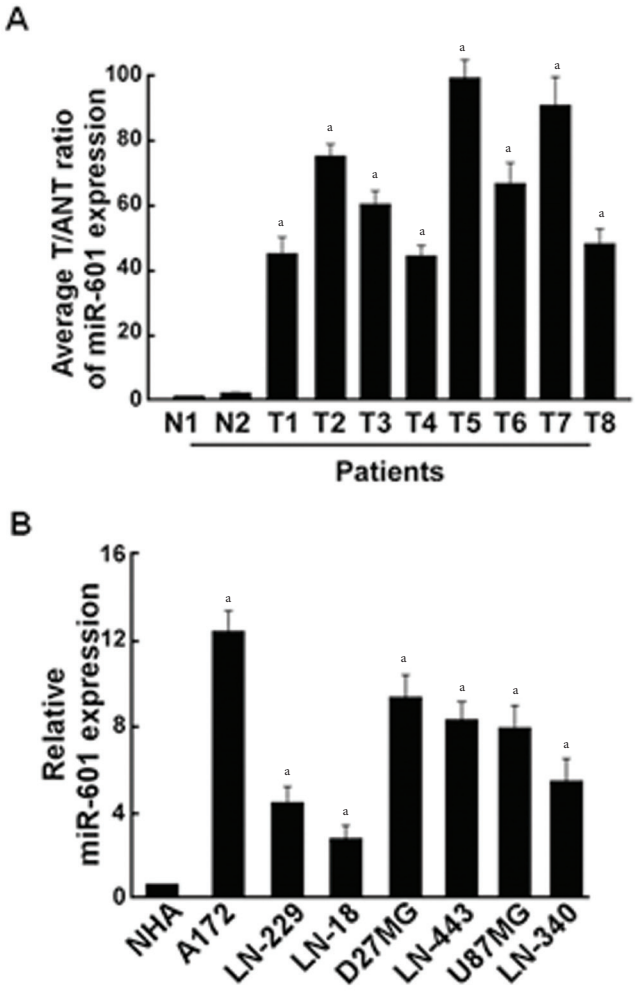
Data were expressed as mean ± standard deviation (SD) and performed using IBM® SPSS 18.0 software. The student's t-test or one-way ANOVA analysis was used to evaluate the statistical significance of differences. $P < .05$ was perceived as statistically significant.

RESULTS

MiR-601 Was Upregulated in Human GBM Tissues and GBM Cell Lines

To evaluate the expression level of miR-601in GBM, we further examined the expression level of miR-601 in GBM tissues and cell lines by qRT-PCR. The result showed that miR-601 was significantly up-regulated in GBM tissues compared with normal brain tissues (Figure 1A). In agreement with these observations, upregulation of miR-601was also confirmed in 7 human GBM cell lines (A-172, LN-229, LN-18, LN-443, U87MG and LN-340) compared with NHAs (Figure 1B). Taken together, these data

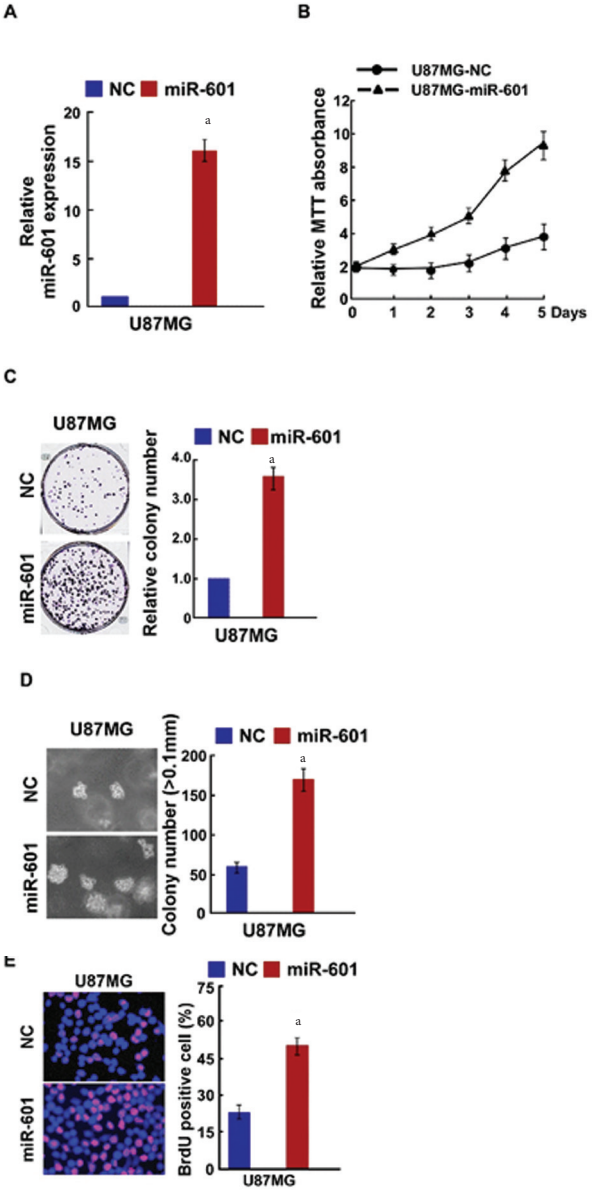
Figure 1. Expression of miR-601 in human GBM tissues and cell lines. (A) Relative miR-601 mRNA expression levels in 8 primary GBM tissues (T) and normal brain tissues (N) were detected by PCR analysis. (B) Real-time PCR analysis of miR-601 expression in normal human astrocyte (NHA) and GBM cell lines, including LN229, LN443, LN18, U87MG, A172 and LN340. Each bar represents the mean of 3 independent experiments.



[a]$P < .05$

Abbreviations: GMB, glioblastoma; NHR, normal human astrocyte; PCR, polymer chain reaction.

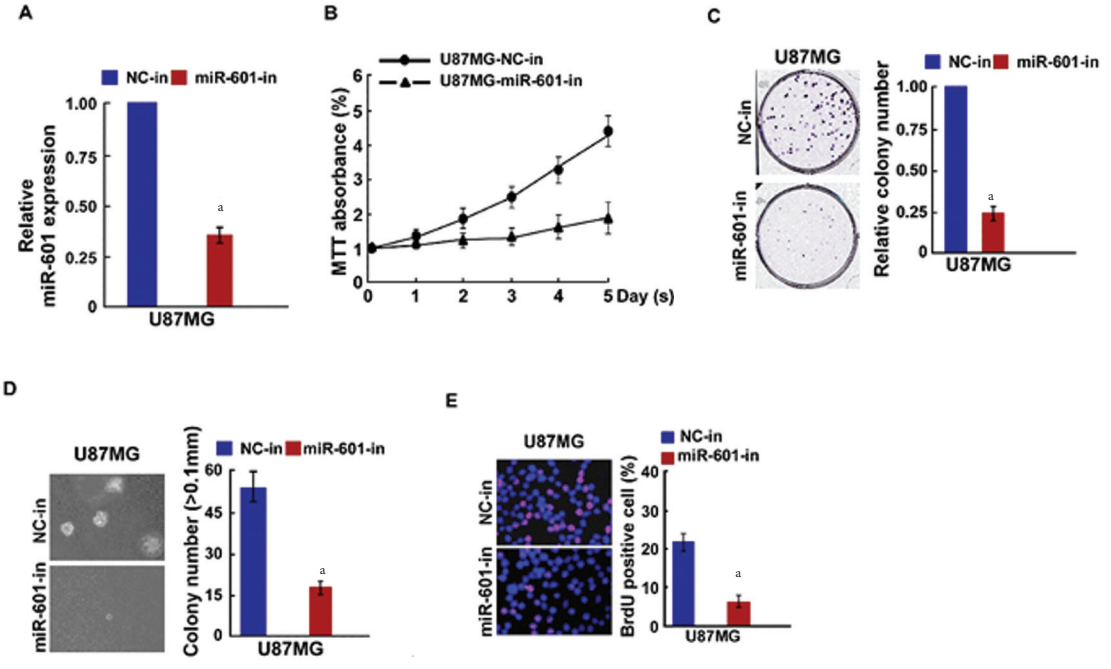
Figure 2. MiR-601 overexpression promoted GBM cell proliferation. (A) PCR analysis was used to validate miR-601 expression levels after transfection. (B) MTT assays revealed that upregulation of miR-601 promoted growth of U87MG cells. (C) Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. (D) Upregulation of miR-601 promoted the anchorage-independent growth of U87MG cells. Representative micrographs (left) and quantification of colonies that were >0.1 mm (right). (E) Representative micrographs (left) and quantification (right) of the BrdU incorporation assay in U87MG cells. Each bar represents the mean of 3 independent experiments.



[a]$P < .05$

Abbreviations: BrdU, bromodeoxyuridine; GMB, glioblastoma; MTT, mean transit time (brain tissue blood flow); PCR, polymer chain reaction.

Figure 3. Inhibition of miR-601 inhibited GBM cell proliferation. (A) Validation of miR-601 expression levels after transfection by PCR analysis. (B) MTT assays revealed that inhibition of miR-601 inhibited growth of U87MG cells. (C) Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. (D) Inhibition of miR-601 suppressed the anchorage-independent growth of U87MG cells. Representative micrographs (left) and quantification of colonies that were >0.1 mm (right). (E) Representative micrographs (left) and quantification (right) of the BrdU incorporation assay in U87MG cells. Each bar represents the mean of 3 independent experiments.



[a]$P < .05$

Abbreviations: BrdU, bromodeoxyuridine; GMB, glioblastoma; MTT, mean transit time (brain tissue blood flow); PCR, polymer chain reaction.

demonstrated that miR-601 expression was elevated in GBM tissues and cell lines.

MiR-601 Promoted GBM Cell Proliferation

To examine the biological function of miR-601 in GBM progression, U87MG cells stably overexpressing miR-601or down-regulated miR-601 were established (Figure 2A and 3A). Analysis by MTT assay indicated that ectopic expression of miR-601 increased, while downregulation of miR-601 significantly decreased the U87MG cell growth rate (Figure 2B and 3B). Colony formation assay and an anchorage-independent growth assays revealed that U87MG cells after transection with miR-601 promoted the colony formation capacity of U87MG cells and formed more numerous and larger colonies than the control cells, while miR-601-in showed the opposite effect (Figure 2C, 2D and 3C, 3D). Furthermore, the BrdU results demonstrated that a decreased percentage of BrdU incorporation after miR-601 overexpression, while miR-601-in decreased the percentage of BrdU incorporation of U87MG cells (Figure 2E and 3E). Collectively, these data clearly indicated that miR-601 is a promoter of proliferation in U87MG GBM cells.

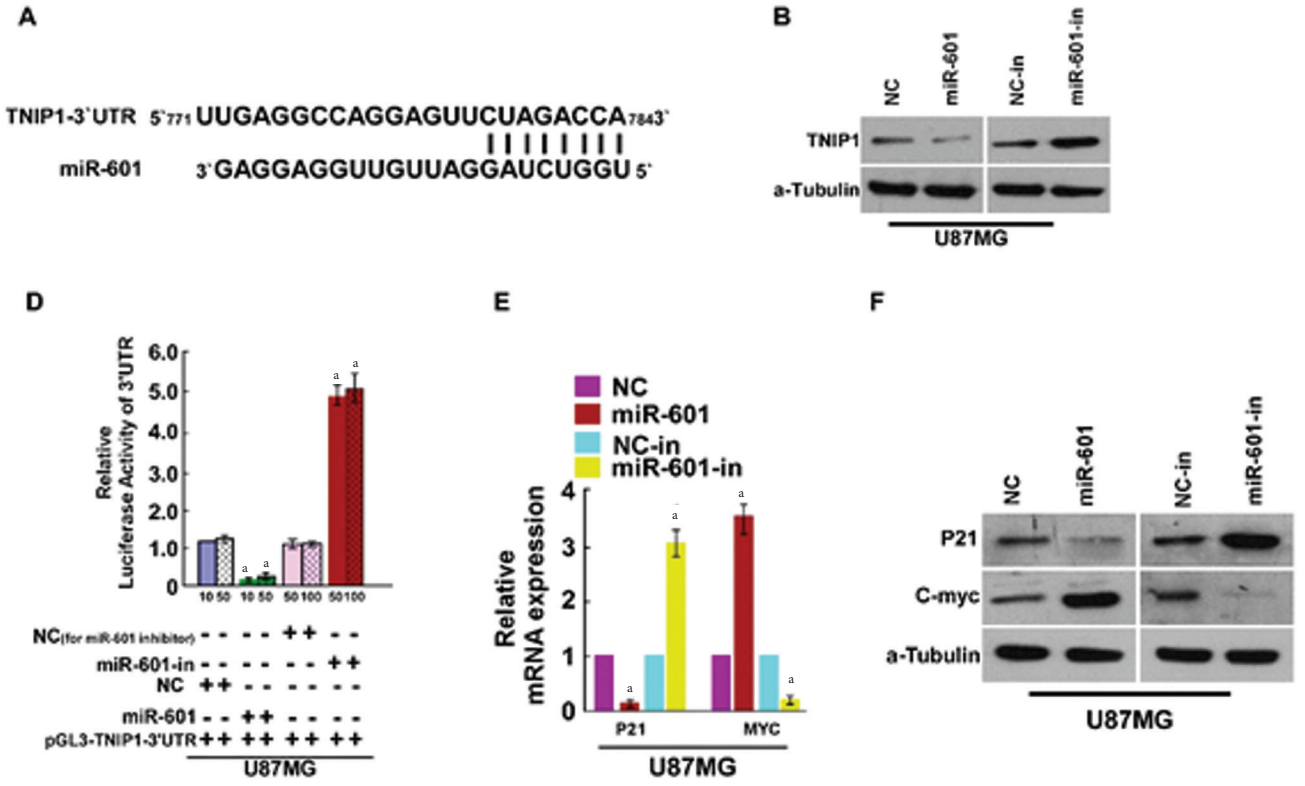
MiR-601 Directly Targets TINP1 by Binding to its 3'-UTR and TINP1 Was a Direct Target of miR-601 in GBM Cells

As predicted by TargetScan, TINP1 was a potential target gene of miR-601 (Figure 4A). Western blot assay results indicated that TINP1 expression was down-regulated in the miR-601-overexpressing U87MG cells but up-regulated in the cells transfected with miR-601-in (Figure 4B).

To further confirm the regulation of TINP1 by miR-601, luciferase report assay was used to evaluate whether miR-601 directly binds to TINP1 3'UTR. As shown in Figure 4C, co-transfection of miR-601 with pGL3-TINP1-3'UTR luciferase reporter plasmid caused a remarkable decrease in luciferase activity, whereas miR-601-in led to increased luciferase activity. Collectively, these results suggest that miR-601 directly targets TINP1 in GBM cells.

As miR-601 promoted cell proliferation, we next examined the effect of miR-601 on the expression of the genes that regulate cell proliferation, including P21 and MYC. The results of RT-PCR and Western blot assays revealed that compared with NC transfected cells, mRNA and protein expression of P21 were down-regulated and

Figure 4. MiR-601 suppresses TINP1 expression by directly targeting the TINP1 3′-UTR and altered levels of proteins related to cell proliferation and cell cycle in GBM U87MG cells. (A) Predicted miR-601 target sequence in the 3′-UTR of TINP1 (TINP1-3′-UTR). (B) TINP1 protein expression in U87MG cells transfected with miR-601 or the miR-601 inhibitor were detected by Western blot analysis. α-Tubulin served as the loading control. (C) Luciferase reporter assay of U87MG cells transfected with the pGL3-TINP1-3′-UTR reporter and miR-601, miR-601-in, the relative controls. (D) Real-time PCR analysis of expression of P21 and MYC in U87MG cells. (E) Western blot analysis of protein expression of P21 and C-MYC in U87MG cells. α-Tubulin was used to serve as the loading control.



[a]$P < .05$

Abbreviations: GMB, glioblastoma; MTT, mean transit time (brain tissue blood flow); PCR, polymer chain reaction.

MYC was up-regulated in miR-601-transfected cells, while P21 was up-regulated and MYC was down-regulated in miR-601-in transfected cells (Figure 4E and 4F).

Silencing of TINP1 reversed miR-601-in suppressed cell proliferation in GBM cells.

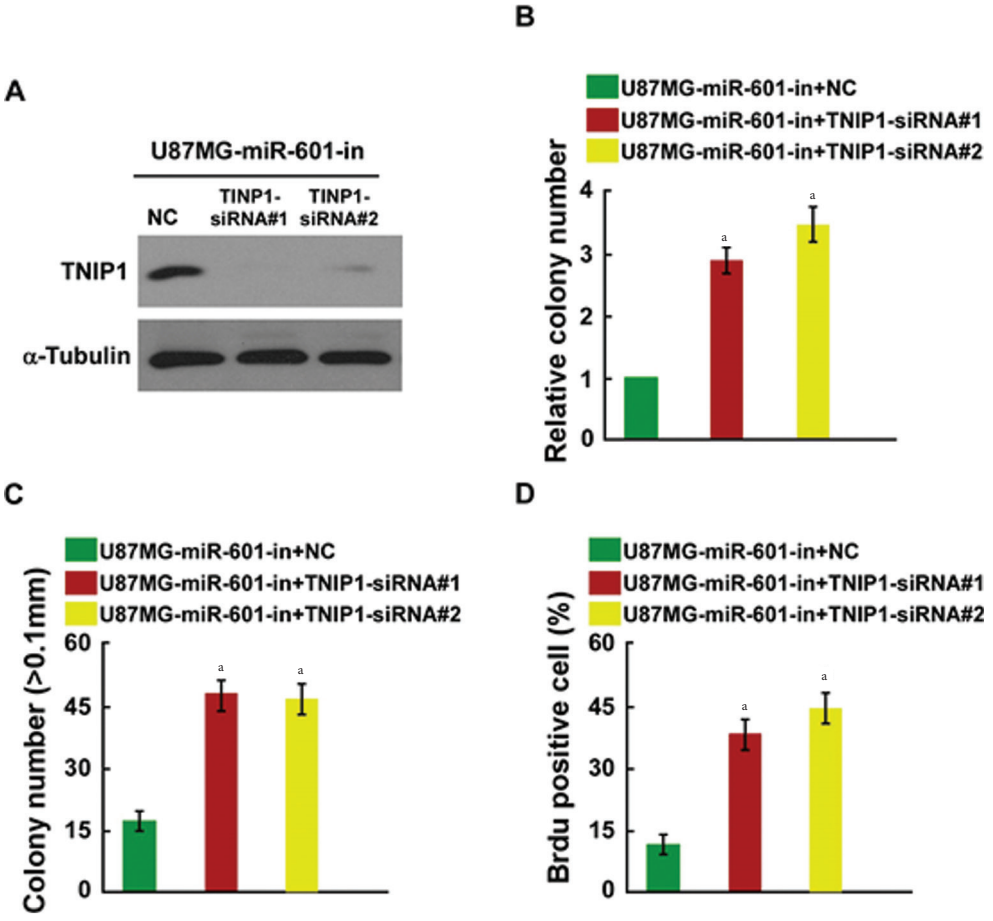
To confirm the functional relevance of miR-601 suppressing TINP1 in GBM, we performed loss-of-function studies by transfecting siRNA-TINP1 into miR-601-in transfected U87MG GBM cells. Western blot assay indicated that knockdown of TINP1 suppressed miR-601-in promoted TINP1 expression (Figure 5A). The colony formation and anchorage-independent growth assays showed that miR-601-in transfected U87MG cells transfected with TINP1 siRNAs formed considerably more numerous and larger colonies than those transfected with NC (Figure 5B and 5C). Moreover, BrdU assay showed a significant increase in the positive cells in U87MG-miR-601 inhibitor cells after transfection with TINP1 siRNAs (Figure 5D). These data confirmed that miR-601 promoted GBM cell proliferation by repressing TINP1expression.

DISCUSSION

Growing evidence indicates that miRNAs bind to the 3'-untranslated regions (3'-UTR) of their target mRNAs and then play an essential role in cellular processes related to the biological behavior of tumors, including cell proliferation, cell migration, cell invasion and cell apoptosis.[17-21]

In this study, we investigated the role of miR-601 in GBM cell proliferation and clarified the possible molecular mechanisms. Previous studies reported that miR-601 could serve as a putative tumor suppressor gene in pediatric embryonal central nervous system neoplasms.[22] Cao, et al. indicated that miR-601 expression was significantly down-regulated in pancreatic cancer and suppressed PC cell proliferation and migration by inhibiting Sirtuin 1[23], while Yao, et al. revealed that miR-601 was significantly up-regulated

Figure 5. Silencing of TINP1 reversed miR-601-in suppressed cell proliferation of GBM cells. (A) Western blot analysis verified that silencing TINP1 effectively decreased the expression of TINP1 in miR-601-in transfected U87MG cells. (B) miR-601-in transfected U87MG cells reversed the cell colonies formation after transfection with TINP1-siRNAs. (C) Representative quantification of colonies >0.1 mm in diameter. Each bar represents the mean of 3 independent experiments. (D) Representative micrographs (left) and quantification (right) of the BrdU incorporation assay in U87MG cells.



[a]$P < .05$

Abbreviations: BrdU, bromodeoxyuridine.

in gastric cancer and involved in cancer development and progression.[24] miR-601 was reported to suppress cell proliferation and metastasis of esophageal squamous cell carcinoma via targeting HDAC6.[25] However, the underlying mechanisms of miR-601 that modulate GBM carcinogenesis remain obscure. Our data indicated that the expression of miR-601 increased significantly in GBM tissues and cells, suggesting miR-601might be a potential oncogene in GBM. Furthermore, overexpression of miR-601 was found to promote cell proliferation and colony formation capacity of GBM.

To clarify the molecular mechanisms of miR-601, TINP1 was identified as a potential target of miR-601 by the bioinformatics approach. This is the first time a study confirmed the ability of miR-601 to target 3'UTR of TINP1 by luciferase reporter assay. And our data indicated that upregulation of miR-601 can effectively suppress TNIP1 expression, suggesting that TNIP1 was negatively affected by miR-601. Furthermore, Western blot assay indicated that downstream genes of TINP1, P21 were down-regulated, while MYC was up-regulated in miR-601 transfected U87MG GBM cells. Moreover, silencing TINP1 expression reversed miR-601-in suppressed cell proliferation of GBM cells, indicating that TINP1 functioned as a mediator of miR-601 in regulating cell proliferation.

CONCLUSION

In this study, we found that miR-601 may be involved in GBM cell proliferation by targeting TINP1. Taken together, our findings provide new insights into the roles of miR-601 in GBM and implied that miR-601 may be a potential therapeutic agent for GBM.

CONFLICT OF INTEREST

None.

REFERENCES

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin.* 2014;64(1):9-29. doi:10.3322/caac.21208
2. Reddy KB. MicroRNA (miRNA) in cancer. *Cancer Cell Int*. 2015;15:38. doi:10.1186/s12935-015-0185-1
3. Areeb Z, Stylli SS, Koldej R, et al. MicroRNA as potential biomarkers in glioblastoma. *J Neuro-Oncol*. 2015;125(2):237-48. doi:10.1007/s11060-015-1912-0
4. Ohtsuka M, Ling H, Doki Y, Mori M, Calin GA. MicroRNA processing and human cancer. *J Clin Med*. 2015;4(8):1651-1667. doi:10.3390/jcm4081651
5. Di Bari M, Bevilacqua V, De Jaco A, et al. Mir-34a-5p mediates cross-talk between M2 muscarinic receptors and notch-1/EGFR pathways in U87MG glioblastoma cells: Implication in cell proliferation. *Inter J Mol Sci*. 2018;19(6):1631. doi:10.3390/ijms19061631
6. Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. *Ann Rev Pathol*.2014;9:287-314. doi:10.1146/annurev-pathol-012513-104715
7. Lei ST, Shen F, Chen JW, et al. MiR-639 promoted cell proliferation and cell cycle in human thyroid cancer by suppressing CDKN1A expression. *Biomed Pharmacother.* 2016;84:1834-1840. doi:10.1016/j.biopha.2016.10.087
8. Zhao X, Lu C, Chu W, et al. MicroRNA-124 suppresses proliferation and glycolysis in non-small cell lung cancer cells by targeting AKT-GLUT1/HKII. *Tumour Biol.* 2017;39(5):1010428317706215. doi:10.1177/1010428317706215
9. Wang H, Xu T, Jiang Y, Yan Y, Qin R, Chen J. MicroRNAs in human glioblastoma: from bench to beside. *Front Bioscience (Landmark edition)*. 2015;20:105-118.
10. Lei BX, Liu ZH, Li ZJ, Li C, Deng YF. miR-21 induces cell proliferation and suppresses the chemosensitivity in glioblastoma cells via downregulation of FOXO1. *Int J Clin Exper Med.* 2014;7(8):2060-2066.
11. Chen H, Lu Q, Fei X, Shen L, Jiang D, Dai D. miR-22 inhibits the proliferation, motility, and invasion of human glioblastoma cells by directly targeting SIRT1. *Tumour Biol*. 2016;37(5):6761-6768. doi:10.1007/s13277-015-4575-8
12. Conti A, Romeo SG, Cama A, et al. MiRNA expression profiling in human gliomas: upregulated miR-363 increases cell survival and proliferation. *Tumour Biol.* 2016;37(10):14035-14048. doi:10.1007/s13277-016-5273-x
13. Liu S, Li X, Zhuang S. miR-30c Impedes glioblastoma cell proliferation and migration by targeting SOX9. *Oncol Res*. 2019;27(2):165-171. doi:10.3727/096504018X15193506006164
14. Zhang Z, Song X, Tian H, et al. MicroRNA-137 inhibits growth of glioblastoma through EGFR suppression. *Amer J Translat Res.* 2017;9(3):1492-1499.
15. Hu S, Chen H, Zhang Y, et al. MicroRNA-520c inhibits glioma cell migration and invasion by the suppression of transforming growth factor-beta receptor type 2. *Oncology Rep*. 2017;37(3):1691-1697. doi:10.3892/or.2017.5421
16. Wang H, Zhi H, Ma D, Li T. MiR-217 promoted the proliferation and invasion of glioblastoma by repressing YWHAG. *Cytokine*. 2017;92:93-102. doi:10.1016/j.cyto.2016.12.013
17. Farazi TA, Hoell JI, Morozov P, Tuschl T. MicroRNAs in human cancer. *Adv Exper Med Biol*. 2013;774:1-20. doi:10.1007/978-94-007-5590-1_1
18. Gu Y, Cheng Y, Song Y, et al. MicroRNA-493 suppresses tumor growth, invasion and metastasis of lung cancer by regulating E2F1. *PloS One*. 2014;9(8):e102602. doi:10.1371/journal.pone.0102602
19. Jin S, Dai Y, Li C, Fang X, Han H, Wang D. MicroRNA-544 inhibits glioma proliferation, invasion and migration but induces cell apoptosis by targeting PARK7. *Amer J Translat Res*. 2016;8(4):1826-1837.
20. Zhang J, Fei B, Wang Q, et al. MicroRNA-638 inhibits cell proliferation, invasion and regulates cell cycle by targeting tetraspanin 1 in human colorectal carcinoma. *Oncotarget*. 2014;5(23):12083-12096. doi:10.18632/oncotarget.2499
21. Li L, Shao M-Y, Zou S-C, Xiao Z-F, Chen Z-C. MiR-101-3p inhibits EMT to attenuate proliferation and metastasis in glioblastoma by targeting TRIM44. *J Neurooncol*.2019;141(1):19-30. doi:10.1007/s11060-018-2973-7
22. Braoudaki M, Lambrou GI, Giannikou K, et al. Microrna expression signatures predict patient progression and disease outcome in pediatric embryonal central nervous system neoplasms. *J Hematol Oncol*. 2014;7:96. doi:10.1186/s13045-014-0096-y
23. Cao W, Jin H, Zhang L, Chen X, Qian H. Identification of miR-601 as a novel regulator in the development of pancreatic cancer. *Biochem Biophys Res Communicat*. 2017;483(1):638-644. doi:10.1016/j.bbrc.2016.12.090
24. Yao Y, Suo AL, Li ZF, et al. MicroRNA profiling of human gastric cancer. *Molec Med Rep*. 2009;2(6):963-970. doi:10.3892/mmr_00000199
25. Liu C, Tian X, Sun HB, Wang ZF, Jiang LF, Li ZX. MiR-601 inhibits the proliferation and metastasis of esophageal squamous cell carcinoma (ESCC) by targeting HDAC6. *Eur Rev Med Pharmacol Sci*.2019;23(3):1069-1076. doi:10.26355/eurrev_201902_16995