MiR-218 inhibits malignant phenotypes of glioma by targeting TNC/AKT/AP-1/TGFβ1 feedback signaling loop

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Abstract

**Background:** Gliomas are the most malignant and common tumors in human brains, and the prognosis of glioma patient is very poor. MicroRNAs (miRNAs) play critical roles in different types of cancer by performing posttranscriptional regulation of gene expression. Although miR-218 has been demonstrated decreased in gliomas, its role in gliomas still remains largely unknown.

**Methods:** MiR-218 expression were analyzed in gliomas and normal brain tissues (control subjects) using TCGA dataset. A series of *in vitro* and *in vivo* studies was performed to determine the biological roles of miR-218 in glioma cells. Potential targets of miR-218 were identified using dual-luciferase reporter system. Western blot and dual-luciferase reporter system were performed to evaluate the regulatory effect of miR-218 on TNC/AKT/AP-1/TGF-β1 pathway.

**Results:** We demonstrated miR-218 was significantly downregulated in gliomas compared to control subjects, and played potent tumor suppressor roles in glioma cells by inhibited cell proliferation, colony formation, migration, invasion and tumorigenic potential in nude mice, as well as inducing cell cycle arrest and apoptosis. Mechanistically, miR-218 inhibited malignant phenotypes of glioma cells by binding to the 3’ UTR of its target TNC and subsequently repressing its expression. As a result, it could reduce AKT phosphorylation and subsequently inhibit transcriptional activity of AP-1 by reducing JNK phosphorylation, downregulating the expression of TGF-β1, while TGF-β1 is able to, in turn, activate the TNC/AKT/AP-1 signaling axis.

**Conclusions:** Our data uncover a previously unknown tumor suppressor role of miR-218 by blocking the TNC/AKT/AP-1/TGF-β1 positive feedback loop in glioma.

**Background**

Gliomas, which represent about 70% of all brain tumors, are the most malignant and common tumors in human brains [1]. Currently, a combination of chemotherapy and radiation following maximal safe surgical resection is the treatment standard for newly diagnosed gliomas patients [2, 3]. However, despite these treatment, overall survival rate of glioma patients continues to be among the lowest of all the main types of cancer [4]. Thus, in order to develop effective therapeutic strategies for glioma,
there is an urgent need to illustrate the mechanism of its pathogenesis. MicroRNAs (miRNAs) can bind to the 3′-untranslated region (3′-UTR) of the target mRNAs and induce translational repression, mRNA destabilization or cleavage to perform posttranscriptional regulation of gene expression [5]. Growing evidence has indicated that miRNAs, as small noncoding single-stranded RNA molecules, are critical in the tumorigenesis and development of multiple cancers including gliomas [6-8]. Among them, decreased expression of miR-218 has been reported in gliomas, but not in normal brain tissues [9-11], and it is strongly correlated with poor overall survival and disease-free survival in glioma patients [12]. Notably, miR-218 contains miR-218-1 and miR-218-2, located on chromosome 4p15.31 and 5q35.1 respectively, which are difference in 3p sequence, but same in 5p as miR-218-5p [13]. The role of miR-218 still needs to be further explored in glioma. In this study, we identify miR-218’s novel target, tenascin C (TNC), which is a major constituent of the extracellular matrix in developing brain, and can be re-expressed in wound healing, inflammation and tumors [14-17]. TNC is found to be upregulated in gliomas, and is significantly correlated with poor patient survival and malignant progression [18]. There is evidence showing that TNC may be a promising therapeutic target for glioma[19]. MiR-218 was confirmed as a potential tumor suppressor in glioma by blocking TNC/AKT/AP-1/TGFβ1 positive feedback loop through a series of in vitro and in vivo studies.

Methods
quantitative RT-PCR (qRT-PCR)
The mRNA expression of the indicated genes was tested by qRT-PCR, which were performed using primers (Supplementary Table 1) as described previously [20], and normalized to 18S rRNA cDNA. The gene-specific RT primers (Supplementary Table 2) were synthesized to reverse transcribed the indicated miRNAs to cDNA. Supplementary Table 3 shown the primer sequences for miRNAs. And the miRNA reference used U6. We ran each sample in triplicate.

Cell culture
Human glioma cell lines U251 and SHG44 (American Type Culture Collection, ATCC) were cultured in DMEM medium (Invitrogen Technologies Inc., CA) with 10% fetal bovine serum (Biological Industries
Inc., BI) at 37°C routinely. Recombinant human TGFβ1 proteins (Sino Biological Inc.) (10 ng/mL) were used to treat cells for 24 h in some experiments. The control was defined as the same volume of the vehicle.

**Mimcs and lentivirus transfection**

miR-218 mimics and NC (RiboBio, Guangzhou, China) (25 nM) were transfected into cells using Lipofectamine 3000 (Invitrogen, Grand Island, NY) in three replicates.

Lentivirus encoding miR-218 (Ubi-MVC-SV40-EGFP-IRES-Puro-miR-218) and control lentivirus (Ubi-MVC-SV40-EGFP-IRES-Puro) (Shanghai Genechem Co., Ltd, China) were transfected into cells with 20-100 final lentivirus multiplicity of infection (MOI) at 50% confluence.

**In vitro functional studies**

We performed MTT assay to assess the proliferation ability of glioma cells. Monolayer culture was used to perform colony formation assay. Flow cytometer was used to evaluate cell cycle and apoptosis. We used transwell chambers to evaluate migration and invasion ability of glioma cells. We performed each experiments as described previously in triplicate [20].

**Western blot analysis**

The experiments were executed using antibody (Supplementary Table 4) as described previously[20].

**Dual-luciferase reporter assay**

The wild type TNC 3’UTR was amplified from cDNA of U251 cells to construct luciferase reporter plasmids. TNC 3’UTR region with mutant binding site of miR-218 was synthesized from Sangon Biotech (Shanghai, China). These two fragments were inserted into pre-digested pmirGLO luciferase vector (a gift from Dr. Yanke Chen, Xi’an Jiaotong University Health Science Center) to produce the luciferase reporter plasmids pmirGLO-TNC 3’UTR-WT and pmirGLO-TNC 3’UTR-MUT. Supplementary Table 5 presented the primers for plasmid constructs. The 3xAP in pGL3-Basic luciferase reporter plasmid was obtained from Addgene (plasmid #40342), which contains three canonical AP-1 binding sites (TGACTCA) upstream of the luciferase reporter plasmid pGL3-Basic promoter fragment.

To test the 3’UTR activity of TNC mRNA modulated by miR-218, U251 and SHG44 cells were transfected with NC or miR-218-mimics in 6-well plates and were co-transfected with pmirGLO-TNC
3'UTR-WT or pmirGLO-TNC 3'UTR-MUT using Lipofectamine 3000 (Invitrogen). To test transcriptional activity of AP-1 regulated by miR-218 or TGFβ1, U251 and SHG44 cells treated with TGFβ1 or transfected with NC/ miR-218-mimics, and were then co-transfected with pRL-TK plasmids and the 3xAP in pGL3-Basic luciferase reporter plasmid. We analyzed luciferase activity using dual-luciferase reporter assay system (Promega) on EnSpire Multimode Plate Reader (PerkinElmer) after a 36-h transfection. pRL-TK plasmid containing Renilla luciferase was chose to normalize transfection efficiency. We executed each assay in triplicate.

**Animal studies**

Athymic mice (three- to four-week-old male), purchased from SLAC laboratory Animal Co., Ltd, were subcutaneously inoculated 6×10^6 U251 cells with miR-218 stably overexpressing or control cells into the root region of right hind leg to establish tumor xenografts. From day 3 post-injection, the formula (width^2 x length x 0.5) was used to calculate tumor volumes every 2 days. After 13 days, we sacrificed the mice and harvest tumors in accordance with Institution Guidelines. The Laboratory Animal Center of Xi’an Jiaotong University approved our experimental procedures involving animals.

**Immunohistochemistry (IHC)**

We performed IHC assay as described previously [21] to assess the Ki67 levels of the xenograft tumors.

**Statistical analysis**

We used the unpaired t test to compare gene expression in control subjects and tumor tissues. The association of TNC expression with miR-218 expression was analyzed by linear regression. We constructed and compared survival curves using the Kaplan-Meier method and the log-rank test respectively. And we performed all statistical analyses using SPSS statistical package (11.5, Chicago, IL) and considered P values <0.05 as significantly.

**Results**

**MiR-218 is frequently downregulated in gliomas**

To investigate miR-218 function in glioma tumorigenesis, miR-218-1 and miR-218-2 expression was analyzed in gliomas and normal brain tissues (control subjects) using The Cancer Genome Atlas
(TCGA) dataset. As shown in Fig. 1a, compared to control subjects, both miR-218-1 and miR-218-2 was significantly downregulated. Moreover, miR-218-2 expression levels was found significantly higher than miR-218-1 expression levels in gliomas (4.99 ± 1.95 vs. 0.25 ± 0.43, $P < 0.001$), indicating that mature miR-218 in gliomas was mostly constituted by miR-218-2, which was consistent with a previous study in thyroid cancers [22]. We further analyzed miR-218-1 and miR-218-2 expression in gliomas with different histologic grades. As shown in Fig. 1b (left panel), miR-218-1 expression were not significantly different between gliomas with histologic grade 2 (G2) and grade 3 (G3) ($P =0.71$). However, the gliomas with histologic G3 had a significant lower miR-218-2 expression than those with histologic G2 ($P =0.002$) (Fig. 1b, right panel).

Next, a large cohort of gliomas in TCGA dataset was analyzed by Kaplan-Meier method. As shown in Fig. 1c, the expression of miR-218-1 or miR-218-2 almost did not affect the survival of glioma patients when their survival time were less than 2000 days. However, downregulation of miR-218-2 but not miR-218-1 was significantly correlated with poor patient survival when their survival time more than 2000 days (Fig. 1d). The above findings suggest that miR-218-2 may be a potential biomarker to predict glioma patients’ long-term survival.

**MiR-218 inhibits glioma cell growth**

To explore biological function of miR-218 in glioma, we performed a series of *in vitro* experiments with miR-218 gain-of-function in glioma cells (Fig. 2a). Our data showed that miR-218 mimics significantly suppressed the proliferation of U251 and SHG44 cells compared to the control (Fig. 2b). Next, we assessed the effect of miR-218 mimics on cell growth using soft agar colony formation assay. The results showed that, compared with control cells, the colonies formed by miR-218 overexpressing cells were fewer (Fig. 2c). *In vivo* tumor-suppressing effect of miR-218 was also evaluated in nude mice. The results showed that, the U251 cells with miR-218 stably expressing induced tumors having significantly smaller mean tumor volume and longer latency relative to control (Fig. 2d). We isolated and weighed the xenograft tumors at the end of the experiments. As shown in Fig. 2e, compared with control tumors, weight of the tumors with miR-218 stably expressing was significantly less ($P =0.0009$). As expected, miR-218 stably expressing significantly decreased the
percentage of Ki-67 positive cells in the tumors (Fig. 2f).

Then, the effect of miR-218 mimics on cell cycle contributions and apoptosis in U251 and SHG44 cells was tested. We found that, compared to control cells, the cell cycle of miR-218 overexpressing cells was arrested at the G₀/G₁ phase (Fig. 3a). The percentage of G₀/G₁ phase was respectively rose from 51.7 ± 2.4% to 62.3± 2.0% in U251 cells (P =0.004) and from 52.3± 2.7% to 66.6 ± 3.7 % in SHG44 cells (P =0.005). In addition, compared with the control, miR-218 mimics transfection showed elevation in both early and late apoptosis (20.5 ± 1.1% vs. 28.9 ± 1.8 % in U251 cells, P <0.002; 7.2 ± 1.3 % vs. 16.0 ± 2.1 % in SHG44 cells, P =0.003) (Fig. 3b). Collectively, our results further support miR-218 as a tumor suppressor in glioma cells.

**MiR-218 inhibits glioma cell migration and invasion**

The effect of miR-218 on migration and invasion potential was assessed in U251 and SHG44 cells. We found that the miR-218 overexpressing cells which migrated were significantly less than migrated control cells (Fig. 4). Moreover, miR-218 mimics were found to clearly downregulate the ability of cells passing through the matrigel-coated membrane (Fig. 4). These data indicate that miR-218 expression was closely associated with metastatic phenotypes of glioma cells.

**TNC is identified as a new target of miR-218**

A panel of candidate genes, which potentially targeted by miR-218, were identified by target predicting tools such as miRanda, TargetScan and miRDB. Among them, genes involved in vital signal pathways were selected to accept further detection, including *IKBKB*, *TNC* and *WNT2B*. As shown in Fig. 5a, b; Supplementary Fig. 1, among these three genes, only TNC was dramatically downregulated by miR-218 mimics in these two cell lines at both mRNA and protein levels. We further determined that miR-218 modulated TNC by direct interacting with it. Two *TNC* 3’ UTR (attached to luciferase coding region) luciferase reporter plasmid, which containing putative miR-218 binding sites: wild type (WT) 5’-AAGCACA-3’ or mutated (MUT) 5’-ACGAATA-3’, were constructed (Fig. 5c). We found that, in U251 and SHG44 cells transfected with WT luciferase reporter plasmid, luciferase activity was significantly suppressed by miR-218 mimics (Fig. 5d). While, in these cells transfected with mutated MUT luciferase reporter plasmid, the luciferase activity almost wasn’t affected (Fig. 5d). Our results
demonstrated TNC as a direct target of miR-218.

Next, by analyzing TNC expression in gliomas and normal brain tissues using TCGA dataset, we confirmed TNC to be significantly elevated in gliomas (Fig. 5e), which was consistent with a previous study [18]. In addition, the relationship between miR-218-1/miR-218-2 and TNC expression in gliomas was also investigated. As shown in Fig. 5f, TNC expression was not significantly correlated with miR-218-1 expression ($P = 0.08, r = 0.08$; Pearson’s correlation coefficient) (left panel), while was strongly correlated with miR-218-2 expression ($P < 0.0001, r = 0.18$; Pearson’s correlation coefficient) (right panel).

**MiR-218 functions as a tumor suppressor in gliomas cells by inhibiting TNC/AKT/AP-1/TGFβ1 positive feedback loop**

Then, the mechanism of malignant phenotypes of glioma cells inhibited by miR-218 was explored. There is evidence revealing that TNC can increase phosphorylation of AKT at Ser 473 by interacting with intergrins, thereby activating the PI3K/AKT pathway [23-25]. Thus, we speculated that miR-218 inhibited the PI3K/AKT signaling via targeting TNC. It was demonstrated that miR-218 mimics expectedly downregulated TNC expression, and clearly inhibited phosphorylation of AKT at Ser 473, while almost did not affect phosphorylation of AKT at Thr 308 in U251 and SHG44 cells (Fig. 6a). Evidently, targeted by PI3K/AKT signaling pathway, transcription factor AP-1 is constitutively activated in glioma and important in cell proliferation [26-29]. AP-1, which can bind to a common DNA binding sequence, is heterodimers composed primarily by the FOS and JUN families. AP-1 activation contains complex process, such as increased expression or phosphorylation of FOS and JUN [30]. As shown in Fig. 6a, we found that miR-218 mimics strongly inhibited JNK phosphorylation, while almost did not affect FOS and JUN expression in U251 and SHG44 cells. Considering that TGFβ1 is a well-known target of AP-1 [31-33], thus we speculated that miR-218 could downregulate TGFβ1 expression by suppressing AP-1 activity. As shown in Fig. 6a, compared to the control, miR-218 mimics expectedly decreased TGFβ1 expression in U251 and SHG44 cells. These results suggest that transcriptional activity of AP-1 can be inhibited by miR-218, as supported by the AP-1 luciferase reporter assay (Fig. 6b).
Next, to confirm the above observations in vivo, we performed western blot analysis to detect the indicated gene expression in the xenograft tumors. Our data demonstrated that, compared with control tumors, TNC expression was significantly decreased in miR-218-overexpression tumors (Fig. 6c). As expected, phosphorylation of Akt at Ser 473 and JNK and TGFβ1 expression in miR-218-overexpression tumors were dramatically lower than that of control tumors, but phosphorylation of Akt at Thr 308 and the expression of FOS and JUN were not different between two kinds of tumors, further supporting the in vitro results.

It should be noted that TGFβ1 has been reported to, in turn, induce TNC expression involving Smad3/4, Sp1, Ets1 and CBP300 [34]. Thus, we suppose that TGFβ1 is able to activate AKT/AP-1 signaling axis by increasing TNC expression, thereby forming a positive feedback loop. To prove this, we treated U251 and SHG44 cells with recombinant human TGFβ1 proteins. The results showed that TGFβ1 treatment markedly induced TNC expression and subsequently increased phosphorylation of Akt at Ser 473 and JNK expression, while this effect could be reversed by miR-218 mimics (Fig. 6d). This was also supported by the AP-1 luciferase reporter assay (Fig. 6e). Altogether, those results indicate that miR-218 exerts tumor suppressor roles in glioma cells by blocking the TNC/AKT/AP-1/TGFβ1 positive feedback loop.

Give the above, a model is proposed to explore the mechanism of miR-218 inhibiting malignant progression of glioma (Fig. 6f). Briefly, miR-218 represses TNC expression by binding to its 3’ UTR. This will reduce AKT phosphorylation and subsequently suppress transcriptional activity of AP-1 by decreasing JNK phosphorylation, thereby downregulating the expression of TGFβ1, which is able to, in turn, activate the TNC/AKT/AP-1 signaling axis. Thus, miR-218 acts as a potent tumor suppressor in glioma by blocking the TNC/AKT/AP-1/TGFβ1 positive feedback loop.

Discussion
MiR-218 has been widely reported to play putative tumor suppressor roles and be silenced in multiple human cancers, including gastric, nasopharyngeal, lung, cervical, oral and brain tumors [9-11, 35-39]. Besides, low miR-218 expression was strongly correlated with poor overall survival and disease-free survival in glioma patients [12]. Nonetheless, the role and exact mechanism of miR-218 in glioma
needs to be further explored.

In our study, strong evidences were provided to support that miR-218 play potent tumor suppressor roles in glioma. First, systemically analyze of TCGA dataset demonstrated that both miR-218-1 and miR-218-2 in gliomas was highly downregulated compared to control subjects. Moreover, we also confirmed that miR-218-2 constituted most of mature miR-218 in gliomas. Besides, miR-218-2 expression is negatively correlated with gliomas’ histologic grading. Importantly, miR-218-2 downregulating is closely related to poor long-term survival of glioma patients. These observations imply miR-218-2 as a potential prognostic biomarker for glioma patients. Second, miR-218 mimics ectopic expressing significantly suppressed malignant phenotypes of glioma cells. These data further validate its tumor suppressor role in glioma cells.

To further understand miR-218’s tumor suppressor activity in gliomas, we first identified TNC as miR-218’s novel target using target prediction tools, western blot and dual-luciferase reporter system. Similar analyze of TCGA dataset showed that TNC expression in gliomas was significantly increased compared to control subjects, and was negatively associated with miR-218 expression, particularly miR-218-2. TNC, which characterized by a modular construction and a six-armed quaternary structure, is a large secreted oligomeric extracellular matrix glycoprotein that binds to integrin cell adhesion receptors, periositin, syndecan membrane proteoglycans and fibronectin [40-42]. We validated that miR-218 mimics expectedly downregulated both TNC mRNA and protein expression by in vitro and in vivo studies.

TNC, which is miR-218’s new target, has been demonstrated to activate the PI3K/AKT signaling pathway by interacting with intergrins [23-25]. Thus, we tested the impact of miR-218 on PI3K/AKT pathway activity. Expectedly, we observed that miR-218 mimics strongly inhibited phosphorylation of AKT at Ser 473 but not Thr 308 in glioma cells. Meanwhile, we also demonstrated that transcriptional activity of AP-1, a downstream target of the PI3K/AKT pathway, was markedly inhibited by miR-218 via reducing JNK phosphorylation. Considering transcriptionally inducing function of AP-1 to TGF-β1 by binding to its promoter region [31-33], we thus suppose that miR-218 downregulates TGF-β1 expression by suppressing AP-1 activity via the blockade of PI3K/AKT signaling. As expected, our
results confirmed that miR-218 mimics distinctly decreased TGF-β1 expression in both glioma cell lines and xenograft tumors, accompanied by the reduced TNC expression and inhibition of AKT/JNK phosphorylation.

It is clear that TGFβ1 has been demonstrated to, in turn, induce TNC expression[34]. Thus, we speculate that TGFβ1 is able to form a positive feedback loop with TNC/AKT/AP-1 signaling axis. Indeed, our data showed that exogenous TGFβ1 clearly increased TNC expression and subsequently enhanced phosphorylation of AKT at Ser 473 and AP-1 activity, while this effect could be effectively reversed by miR-218 mimics. These findings further support the above hypothesis.

Conclusions

In summary, we validate miR-218 highly decreased and exerting tumor suppressor roles in glioma by a series of systematic in vitro and in vivo experiments. what’s more, we identify TNC as miR-218’s novel target, and demonstrate that miR-218 inhibits malignant phenotypes of glioma cells by blocking the TNC/AKT/AP-1/TGFβ1 positive feedback loop. This study uncovers miR-218’s previously unknown tumor suppressor function in glioma, improving our knowledge of molecular pathogenesis of glioma.

Abbreviations

ATCC, American Type Culture Collection; CNS, central nervous system; ECM, extracellular matrix; FBS, fetal bovine serum; IHC, immunohistochemistry; miRNAs, microRNAs; qRT-PCR, quantitative RT-PCR; TCGA, The Cancer Genome Atlas; TNC, tenascin C; UTR, untranslated region.

Declarations

Acknowledgements

Not applicable.

Authors’ contributions

M. J. and G. L. conceived and designed the study. S. D., R. Z. and S. T. performed experimental work. S. D. and P. H. performed data analyses. S. D. and M. J. produced the text and the figures. M. J., G. L. and P. H. provided patient materials and data. M. J. provided leadership for the project. The final manuscript was approved and contributed by all authors.

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**Availability of data and materials**

The data of miR-218-1 expression, TNC expression and survival of glioma patients analyzed during the current study are available in the TCGA database. Other data generated or analyzed during this study are included in this article.

**Ethics approval and consent to participate**

All experimental procedures involving animals were conducted in accordance with Institution Guidelines and were approved by the Laboratory Animal Center of Xi’an Jiaotong University.

**Consent of publication**

Not applicable.

**Competing Interests**

The authors have declared that no competing interest exists.

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Figures

a

b


c

Survival probability (%)

Survival time after surgery (days)

miR-218-1

Low

High

P = 0.39

P = 0.84

Low

High

P = 0.11

P = 0.03

miR-218-2

d
Figure 1

Downregulation of miR-218 in gliomas. a Expression of miR-218-1 and miR-218-2 in a panel of gliomas (T) and normal brain tissues (N). b The expression of miR-218-1 and miR-218-2 in gliomas with different histologic grades. c The association of miR-218-1/2 expression with short-term survival (< 2000 days) of glioma patients. d The association of miR-218-1/2 expression with long-term survival (> 2000 days) of glioma patients. The data were obtained from TCGA database and expressed as mean ± SD.
Inhibitory effect of miR-218 on glioma cell growth. a U251 and SHG44 cells were transfected with miR-218 mimics and the control (NC), and qRT-PCR was performed to monitor miR-218 expression. U6 was used as a reference gene. b The MTT assay was used to evaluate inhibitory effect of miR-218 mimics on the proliferation of the indicated glioma cells. c The effect of miR-218 mimics on colony formation ability of the indicated glioma cells. Left panels show the representative images of colony formation, and right panel represents quantitative analysis of colony numbers. d Tumor growth curves were compared between U251 cells stably expressing miR-218 and control cells in nude mice (n =5/group). Tumor cells were injected at day 0th. e Photographs (left panel) and scatter diagram of tumor weight (right panel) of dissected tumors from miR-218-overexpression and control groups. f Representative Ki67 staining of dissected tumors from the indicated groups was shown in left panel. Histogram represents the percentage of Ki67-positive cells from 5 microscopic fields in each group (×400; right panel). Scale bars, 200 μm. The data were expressed as mean ± SD. *, P <0.05; **, P <0.01; ***, P <0.001.
Figure 3

Inhibitory effect of miR-218 on glioma cell growth. a U251 and SHG44 cells were transfected with miR-218 mimics and the control (NC), and qRT-PCR was performed to monitor miR-218 expression. U6 was used as a reference gene. b The MTT assay was used to evaluate inhibitory effect of miR-218 mimics on the proliferation of the indicated glioma cells. c The effect of miR-218 mimics on colony formation ability of the indicated glioma cells. Left panels show the representative images of colony formation, and right panel represents
quantitative analysis of colony numbers. d Tumor growth curves were compared between U251 cells stably expressing miR-218 and control cells in nude mice (n = 5/group). Tumor cells were injected at day 0th. e Photographs (left panel) and scatter diagram of tumor weight (right panel) of dissected tumors from miR-218-overexpression and control groups. f Representative Ki67 staining of dissected tumors from the indicated groups was shown in left panel. Histogram represents the percentage of Ki67-positive cells from 5 microscopic fields in each group (×400; right panel). Scale bars, 200 μm. The data were expressed as mean ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 4

Inhibitory effect of miR-218 on glioma cell migration and invasion. The representative pictures of migrated/invaded U251/SHG44 cells were shown in the left panels, and statistical data of cell numbers were shown in the right panels. The data were expressed as mean ± SD. Scale bars, 50 μm. ***, P < 0.001.
Figure 5

Identification of TNC as a target of miR-218. The effect of miR-218 on TNC expression in
U251 and SHG44 cells was assessed by qRT-PCR (a) and western blot (b) assays. c

Sequence of the TNC 3’UTR showing miR-218 binding sites. Matching regions are highlighted by lines. Shown are wild-type (WT) and mutant (MUT, red bases indicating mutation sites) TNC 3’UTR fragments. d The indicated cells were co-transfected with miR-218 mimics/NC and WT/MUT luciferase reporter plasmids, and luciferase activity was then analyzed in these cells with empty vector as the control. Transfection efficiency was normalized by measuring renilla luciferase. e TNC expression in a panel of gliomas (T) and normal brain tissues (N) (data from TCGA database). f The association of TNC expression with the expression of miR-218-1 (left panel) and miR-218-2 (right panel) in gliomas was assessed by linear regression analysis. The data were expressed as mean ± SD. *, P <0.05; **, P <0.01
Figure 6

Blockade of the TNC/AKT/AP-1/TGFβ1 positive feedback loop by miR-218. a The effect of
miR-218 on the expression or phosphorylation of the indicated proteins in U251 and SHG44 cells was assessed by western blot analysis. β-actin was used as a loading control. b Dual-luciferase reporter system was used to assess the effect of miR-218 on AP-1 activity in U251 and SHG44 cells. Empty vector was used as the control. The ratio of the Luc/Renilla activity was shown as mean ± SD of three independent assays. c The effect of miR-218 on the expression or phosphorylation of the indicated proteins in the xenograft tumors was evaluated by western blot analysis. U251 and SHG44 cells transfected with miR-218 mimics or NC were treated with exogenous TGFβ1 or not. d Western blot analysis was performed to detect the expression or phosphorylation of the indicated proteins. β-actin was used as a loading control. e Dual-luciferase reporter system was performed to assess AP-1 activity. Empty vector was used as the control. The ratio of the Luc/Renilla activity was expressed as mean ± SD. f A schematic model illustrating the mechanism of miR-218 inhibiting malignant phenotypes of glioma cells. *, P <0.05; **, P <0.01.

Supplementary Files
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Supplementary Data.docx