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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 common and malignant tumors in the brain of humans, and the prognosis of glioma patient is very poor. MicroRNAs (miRNAs) play critical roles in different types of cancer by regulating gene expression at the posttranscriptional levels. Although miR-218 has been reported to be downregulated in gliomas, its role in gliomas still remains largely unknown.

Methods: MiR-218 expression in gliomas and normal brain tissues (control subjects) were analyzed using TCGA dataset. The biological roles of miR-218 in glioma cells were determined by a series of *in vitro* and *in vivo* studies. The dual-luciferase reporter system was performed to identify potential targets of miR-218. The regulatory effect of miR-218 on TNC/AKT/AP-1/TGFβ1 pathway was evaluated by dual-luciferase reporter system and western blot.

Results: We demonstrated miR-218 was significantly downregulated in gliomas compared to control subjects, and exerted a potent tumor suppressor in glioma cells by inhibiting 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 TGFβ1, while TGFβ1 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 in glioma by blocking the TNC/AKT/AP-1/TGFβ1 positive feedback loop.

Background

Gliomas are the most common and malignant tumors in the brain of humans, which represent about 70% of all brain tumors [1]. The current standard of care for newly diagnosed gliomas patients includes maximal safe surgical resection, followed by a combination of radiation and chemotherapy [2, 3]. However, despite these treatment, overall survival rate of the patients suffering from glioma is among the lowest of all the main types of cancer and has not improved during recent decades [4]. Thus, there is an urgent need to illustrate the mechanism of glioma pathogenesis and develop effective therapeutic strategies for this disease.

MicroRNAs (miRNAs) are small noncoding single-stranded RNA molecules that regulate gene expression at the posttranscriptional levels and induce translational repression, mRNA cleavage or destabilization by binding to the 3’-untranslated region (3’-UTR) of the target mRNAs [5]. Growing evidence has indicated a critical role of miRNAs in the tumorigenesis and development of different types of cancer including gliomas [6–8]. Among them, miR-218 has been reported to be highly downregulated in gliomas compared

to normal brain tissues [9–11], and decreased expression of miR-218 is strongly associated with poor disease-free survival and overall survival in glioma patients [12]. Notably, miR-218 can be transcribed from two loci located on chromosome 4p15.31 (miR-218-1) and 5q35.1 (miR-218-2), and they share the same 5p sequence as miR-218-5p, just different in 3p [13]. Although miR-218 has been demonstrated to be a tumor suppressor in glioma, its role in glioma still remains largely unclear.

In this study, we identify a novel target of miR-218, tenascin C (TNC), which is a major constituent of the extracellular matrix (ECM) of the developing brain, persisting in the neurogenic regions of the adult central nervous system (CNS), and can be re-expressed in wound healing, inflammation and tumors [14–17]. TNC is found to be highly expressed in gliomas, but not in normal brain tissues, and is significantly correlated with malignant progression and poor patient survival [18]. There is evidence showing that TNC may be a promising therapeutic target for glioma[19]. Through a series of in vitro and in vivo studies, we demonstrate that miR-218 is a potential tumor suppressor in glioma by blocking TNC/AKT/AP-1/TGFβ1 positive feedback loop.

Methods

RNA extraction and quantitative RT-PCR (qRT-PCR)

RNA isolation, cDNA preparation and qRT-PCR were performed as described previously [20]. The mRNA expression of the indicated genes was normalized to 18S rRNA cDNA. The primer sequences were presented in Supplementary Table 1. The indicated miRNAs were reverse transcribed to cDNA by gene-specific RT primers (Supplementary Table 2). U6 was used as the miRNA reference. The primer sequences for miRNAs were presented in Supplementary Table 3. Each sample was run in triplicate.

Cell culture

Human glioma cell lines U251 and SHG44 were obtained from American Type Culture Collection (ATCC). Cells were all routinely cultured at 37 °C in DMEM medium (Invitrogen Technologies, Inc., CA) with 10% fetal bovine serum (FBS). In some experiments, cells were treated with 10 ng/mL recombinant human TGFβ1 proteins (Sino Biological Inc.) for 24 h. The same volume of the vehicle was used as the control.

Mimcs and lentivirus transfection

miR-218 mimics and NC were obtained from RiboBio (Guangzhou, China). Cells were transfected at 50% confluence using Lipofectamine 3000 (Invitrogen, Grand Island, NY) according to the instructions of the manufacturer, with a final mimics concentration of 25 nM. All experiments were performed in three replicates.

Lentivirus encoding miR-218 (Ubi-MVC-SV40-EGFP-IRES-Puro-miR-218) and control lentivirus (Ubi-MVC-SV40-EGFP-IRES-Puro) were obtained from Shanghai Genechem Co., Ltd (China). Cells were transfected

at 50% confluence with a final lentivirus multiplicity of infection (MOI) of 20–100 according to the instructions of the manufacturer.

In vitro functional studies

MTT assay was performed to evaluate cell proliferation. Colony formation assay was performed using monolayer culture. Cell cycle and apoptosis were evaluated by flow cytometer. Cell migration and invasion assays were assessed by transwell chambers. The detailed protocols were performed as described previously [20], and each experiment was carried out in triplicate.

Western blot analysis

The detailed protocol was described previously [20]. The antibody information was presented in Supplementary Table 4.

Dual-luciferase reporter assay

To construct luciferase reporter plasmids, the wild type 3'UTR region of TNC was amplified from cDNA of U251 cells. The 3'UTR region of TNC with mutant miR-218 binding site 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. The primers for plasmid constructs were presented in Supplementary Table 5. The 3xAP in pGL3-Basic luciferase reporter plasmid was obtained from Addgene (plasmid #40342), which contains three canonial AP-1 bingding sites (TGACTCA) upsream of a minimal promoter fragment containing a TATA box in the luciferase reporter plasmid pGL3-Basic.

To test the 3'UTR activity of TNC mRNA modulated by miR-218, U251 and SHG44 cells were transfected with miR-218-mimics or NC 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 transfected with miR-218-mimics/NC or treated with TGFβ1, and were then co-transfected with the 3xAP in pGL3-Basic luciferase reporter plasmid and pRL-TK plasmids. The pRL-TK plasmid containing Renilla luciferase was used to normalize transfection efficiency. After a 36-h transfection, luciferase activity was analyzed on EnSpire Multimode Plate Reader (PerkinElmer) using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Data were expressed as relative luciferase activity (Firefly luciferase activity/Renilla luciferase activity). Each assay was run in triplicate.

Animal studies

Three- to four-week-old male athymic mice were purchased from SLAC laboratory Animal Co., Ltd. The mice were randomly divided into two groups (six mice per group). Tumor xenografts were established by subcutaneous inoculation of 6×10^6 U251 cells stably expressing miR-218 or control cells into the root region of nude mice right hind leg. From day 3 post-injection, tumor size was measured every 2 days.

Tumor volumes were calculated by the formula (length × width^2 × 0.5). The mice were sacrificed after 13 days, and tumors were harvested and weighted. Next, tumors obtained from representative animals were embedded in paraffin and sectioned at 5 μm until use. 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.

Immunohistochemistry (IHC)

IHC assay was carried out to evaluate the levels of Ki67 in the xenograft tumors. The detailed protocol was described previously [21].

Statistical analysis

Gene expression in tumor tissues and control subjects were compared by the unpaired t test. The association of TNC expression with miR-218 expression was tested by linear regression. Survival curves were constructed using the Kaplan-Meier method, and compared using the log-rank test. All statistical analyses were performed using the SPSS statistical package (11.5, Chicago, IL). P values < 0.05 were considered significantly.

Results

MiR-218 is frequently downregulated in gliomas

To investigate the functional role of miR-218 in glioma tumorigenesis, we analyzed the expression of miR-218-1 and miR-218-2 in gliomas and normal brain tissues (control subjects) using The Cancer Genome Atlas (TCGA) dataset. As shown in Fig. 1a, compared to control subjects, the expression of both miR-218-1 and miR-218-2 was significantly downregulated in gliomas. Moreover, we found that the expression levels of miR-218-2 were significantly higher than those of miR-218-1 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 the expression of miR-218-1 and miR-218-2 in gliomas with different histologic grades. The results showed that expression levels of miR-218-1 were not significantly different between gliomas with histologic grade 2 (G2) and grade 3 (G3) (P = 0.71) (Fig. 1b, left panel). 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, Kaplan-Meier analysis of survival was performed according to their expression levels in a large cohort of gliomas using TCGA dataset. The results showed that 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 (Fig. 1c). However, decreased expression of miR-218-2 but not miR-218-1 was significantly associated 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 for predicting long-term survival of glioma patients.

MiR-218 inhibits glioma cell growth

To determine biological role of miR-218 in glioma, a series of in vitro experiments were performed with gain-of-function of miR-218 in glioma cells. First, we validated ectopic expression of miR-218 mimics in U251 and SHG44 cells by qRT-PCR assay (Fig. 2a). Next, we evaluated the effect of miR-218 mimics on malignant phenotypes of glioma cells. The results showed that miR-218 mimics significantly inhibited the proliferation of U251 and SHG44 cells compared to the control (Fig. 2b). The inhibitory effect of miR-218 mimics on cell growth was further confirmed by soft agar colony formation assay. As shown in Fig. 2c, the colonies formed in miR-218 mimics-transfected cells were fewer than those formed in control cells. We also evaluated in vivo tumor-inhibitory effect of miR-218 in nude mice. As shown in Fig. 2d, the tumors induced by U251 cells stably expressing miR-218 showed significantly longer latency and smaller mean tumor volume than those induced by control cells. At the end of the experiments, the xenograft tumors were isolated and weighed. The mean weight of the tumors stably expressing miR-218 was significantly less relative to control tumors ($P = 0.0009$) (Fig. 2e). As expected, our data showed that the percentage of Ki-67 positive cells was significantly decreased in the tumors stably expressing miR-218 relative to control tumors (Fig. 2f).

Next, we tested the effect of miR-218 mimics on cell cycle contributions and apoptosis in U251 and SHG44 cells. As shown in Fig. 3a, compared to control cells, cell cycle was arrested at the G_0/G_1 phase in miR-218 mimics-transfected cells. The percentage of G_0/G_1 phase was increased 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 ($P = 0.005$), respectively. In addition, we found that miR-218 mimics transfection showed an increase in both early and late apoptosis in comparison with the control (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 tumor suppressor role of miR-218 in glioma cells.

MiR-218 inhibits glioma cell migration and invasion

We attempted to assess the effect of miR-218 on migration and invasion potential in U251 and SHG44 cells. As shown in Fig. 4, there were a significantly lower number of migrated cells in the miR-218 mimics-transfected cells than that in control cells. In addition, by in vitro invasion assay, we found that miR-218 mimics clearly decreased the ability of cells to pass through the matrigel-coated membrane compared to the control (Fig. 4). These data indicate that there is a close correlation between miR-218 expression and metastatic phenotypes of glioma cells.

TNC is identified as a new target of miR-218

Using target prediction tools such as miRDB, miRanda and TargetScan, we identified a panel of candidate genes that are potentially targeted by miR-218. Among them, genes involved in vital signal pathways were selected to accept further detection, including IKBKB, TNC and WNT2B. Next, we assessed the effect of miR-218 on their expression in U251and SHG44 cells. The results showed that, among these three genes, only TNC was dramatically downregulated by miR-218 mimics in these two cell lines at both mRNA and protein levels (Fig. 5a, b; Supplementary Fig. 1). To determine whether TNC is a target of miR-

218, we constructed a luciferase reporter plasmid, where the 3' UTR (containing putative miR-218 binding sites: 5'-AAGCACA-3') of TNC was attached to the coding region of luciferase (Fig. 5c). Meanwhile, to further determine that the modulation of TNC by miR-218 is caused by direct interaction, we also constructed a miR-218 binding sites of TNC mutated (5'-ACGAATA-3') luciferase reporter plasmid (Fig. 5c). As shown in Fig. 5d, relative to the control, miR-218 mimics significantly suppressed luciferase activity in U251 and SHG44 cells transfected with wild-type (WT) luciferase reporter plasmid, while almost did not affect luciferase activity in these cells transfected with mutated (MUT) luciferase reporter plasmid. These data support TNC as a direct target of miR-218.

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

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

We next attempted to explore the mechanism of miR-218 inhibiting malignant phenotypes of glioma cells. There is evidence revealing that TNC can increase phosphorylation of AKT at Ser 473 by interacting with intergrins, thereby activating the PI3K/AKT signaling pathway [23–25]. Thus, we speculated that miR-218 plays its tumor suppressor role in glioma cells by inhibition of the PI3K/AKT signaling via targeting TNC. As shown in Fig. 6a, miR-218 mimics expectedly decreased 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.

Evidently, transcription factor AP-1 as a target of the PI3K/AKT signaling pathway plays an important role in cell proliferation and is constitutively activated in glioma [26–29]. AP-1 is composed primarily of heterodimers of various proteins of the FOS and JUN families, that bind to a common DNA binding sequence. 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 the expression of FOS and JUN 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, thereby inhibiting malignant phenotypes of glioma cells. The results showed that miR-218 mimics expectedly decreased TGFβ1 expression in U251 and SHG44 cells compared to the control (Fig. 6a). 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 in the xenograft tumors using the indicated antibodies. As shown in Fig. 6c, TNC expression was significantly

downregulated in miR-218-overexpression tumors relative to control tumors. As expected, phosphorylation of Akt at Ser 473 and JNK and TGFβ1 expression were dramatically downregulated in miR-218-overexpression tumors relative to control tumors, but not phosphorylation of Akt at Thr 308 and the expression of FOS and JUN, 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 compared to the control, 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, our data indicate that miR-218 exerts its tumor suppressor function in glioma cells by blocking the TNC/AKT/AP-1/TGFβ1 positive feedback loop.

Give the above, we propose a model to explore the mechanism of miR-218 inhibiting malignant progression of glioma (Fig. 6f). Briefly, miR-218 binds to the 3’ UTR of its target TNC, and represses its expression. This will reduce AKT phosphorylation and subsequently suppress transcriptional activity of AP-1 by decreasing JNK phosphorylation, thereby downregulating the expression of TGFβ1, which be 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 be silenced in multiple human cancers, and function as a putative tumor suppressor, including gastric, nasopharyngeal, lung, cervical, oral and brain tumors [9–11, 35–39]. Besides, glioma patients with low miR-218 expression had poorer disease-free survival and overall survival [12]. However, its role and exact mechanism in human cancers including glioma still has not been clearly elucidated until now.

In this study, we provided strong evidences supporting that miR-218 is a potent tumor suppressor in glioma. First, by systemically analyzing TCGA dataset, we found the expression of both miR-218-1 and miR-218-2 in gliomas was highly downregulated compared to control subjects. Moreover, we also demonstrate that mature miR-218 in gliomas is mostly constituted by miR-218-2. Besides, we found that miR-218-2 expression is negatively associated with histologic grading of gliomas. Importantly, low miR-218-2 expression is closely related to poor long-term survival of glioma patients. These observations suggest that miR-218-2 may be a potential prognostic biomarker for glioma patients. Second, ectopic expression of miR-218 mimics in glioma cells significantly inhibited cell proliferation, colony formation, migration, invasion, and tumorigenic potential in nude mice, and strongly induced cell cycle arrest and apoptosis. These data further validate its tumor suppressor role in glioma cells.

To better understand tumor suppressor activity of miR-218 in gliomas cells, we first identified TNC as a target of miR-218 by using target prediction tools, western blot and dual-luciferase reporter system. By similarly analyzing TCGA dataset, we found that TNC expression was significantly upregulated in gliomas compared to control subjects, and was negatively correlated with miR-218 expression, particularly miR-218-2. TNC is a large secreted oligomeric ECM glycoprotein that characterized by a six-armed quaternary structure and a modular construction, and binds to fibronectin, periostin, integrin cell adhesion receptors and syndecan membrane proteoglycans [40–42]. In vitro and in vivo studies showed that miR-218 mimics expectedly downregulated TNC expression at both mRNA and protein levels.

As a new target of miR-218, TNC has been demonstrated to activate the PI3K/AKT signaling pathway by interacting with intergrins [23–25]. Thus, we tested the effect of miR-218 on the activity of PI3K/AKT signaling pathway. 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 TGFβ1 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 that miR-218 is highly downregulated and exerts a tumor suppressor role in glioma by a series of systematic *in vitro* and *in vivo* studies. In addition, we identify TNC as a new target of miR-218, 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 a previously unknown tumor suppressor role of miR-218 in glioma, improving our knowledge of molecular pathogenesis of glioma.

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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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.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69-90.
2. Clarke J, Butowski N, Chang S. Recent advances in therapy for glioblastoma. Arch Neurol. 2010;67(3):279-283.
3. Park DM, Sathornsumetee S, Rich JN. Medical oncology: treatment and management of malignant gliomas. Nat Rev Clin Oncol 2010;7(2):75-77.
4. Castro MG, Candolfi M, Kroeger K, et al. Gene therapy and targeted toxins for glioma. Curr Gene Ther. 2011;11(3):155-180.
5. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-297.
6. Park S, James CD. ECop (EGFR-coamplified and overexpressed protein), a novel protein, regulates NF-kappaB transcriptional activity and associated apoptotic response in an IkappaBalpha-dependent manner. Oncogene. 2005;24(15):2495-2502.
7. Frampton AE, Castellano L, Colombo T, et al. Integrated molecular analysis to investigate the role of microRNAs in pancreatic tumour growth and progression. Lancet. 2015;385 Suppl 1:S37.
8. Dvinge H, Git A, Graf S, et al. The shaping and functional consequences of the microRNA landscape in breast cancer. Nature. 2013;497(7449):378-382.
9. Song L, Huang Q, Chen K, et al. miR-218 inhibits the invasive ability of glioma cells by direct downregulation of IKK-beta. Biochem Biophys Res Commun. 2010;402(1):135-140.
10. Setty M, Helmy K, Khan AA, et al. Inferring transcriptional and microRNA-mediated regulatory programs in glioblastoma. Mol Syst Biol. 2012;8:605.
11. Xia H, Yan Y, Hu M, et al. MiR-218 sensitizes glioma cells to apoptosis and inhibits tumorigenicity by regulating ECOP-mediated suppression of NF-kappaB activity. Neuro Oncol. 2013;15(4):413-422.
12. Cheng MW, Wang LL, Hu GY. Expression of microRNA-218 and its clinicopathological and prognostic significance in human glioma cases. Asian Pac J Cancer Prev. 2015;16(5):1839-1843.
13. Tatarano S, Chiyomaru T, Kawakami K, et al. miR-218 on the genomic loss region of chromosome 4p15.31 functions as a tumor suppressor in bladder cancer. Int J Oncol. 2011;39(1):13-21.
14. Erickson HP. Tenascin-C, tenascin-R and tenascin-X: a family of talented proteins in search of functions. Curr Opin Cell Biol. 1993;5(5):869-876.
15. Jones PL, Jones FS. Tenascin-C in development and disease: gene regulation and cell function. Matrix Biol. 2000;19(7):581-596.
16. Fassler R, Sasaki T, Timpl R, Chu ML, Werner S. Differential regulation of fibulin, tenascin-C, and nidogen expression during wound healing of normal and glucocorticoid-treated mice. Exp Cell Res. 1996;222(1):111-116.
17. Zagzag D, Friedlander DR, Miller DC, et al. Tenascin expression in astrocytomas correlates with angiogenesis. Cancer Res. 1995;55(4):907-914.
18. Zamecnik J. The extracellular space and matrix of gliomas. Acta Neuropathol. 2005;110(5):435-442.

19. Rolle K, Nowak S, Wyszko E, et al. Promising human brain tumors therapy with interference RNA intervention (iRNAi). Cancer Biol Ther. 2010;9(5):396-406.
20. Shi J, Liu W, Sui F, et al. Frequent amplification of AIB1, a critical oncogene modulating major signaling pathways, is associated with poor survival in gastric cancer. Oncotarget. 2015;6(16):14344-14359.
21. Shi J, Qu Y, Li X, et al. Increased expression of EHF via gene amplification contributes to the activation of HER family signaling and associates with poor survival in gastric cancer. Cell Death Dis. 2016;7(10):e2442.
22. Guan H, Wei G, Wu J, et al. Down-regulation of miR-218-2 and its host gene SLIT3 cooperate to promote invasion and progression of thyroid cancer. J Clin Endocrinol Metab. 2013;98(8):E1334-1344.
23. Jang JH, Chung CP. Tenascin-C promotes cell survival by activation of Akt in human chondrosarcoma cell. Cancer Lett. 2005;229(1):101-105.
24. Ding H, Jin M, Liu D, et al. TenascinC promotes the migration of bone marrow stem cells via tolllike receptor 4mediated signaling pathways: MAPK, AKT and Wnt. Mol Med Rep. 2018;17(6):7603-7610.
25. Paron I, Berchtold S, Voros J, et al. Tenascin-C enhances pancreatic cancer cell growth and motility and affects cell adhesion through activation of the integrin pathway. PLoS One. 2011;6(6):e21684.
26. Xu Z, Liu D, Fan C, Luan L, Zhang X, Wang E. DIXDC1 increases the invasion and migration ability of non-small-cell lung cancer cells via the PI3K-AKT/AP-1 pathway. Mol Carcinog. 2014;53(11):917-925.
27. Wu D, Peng F, Zhang B, et al. PKC-beta1 mediates glucose-induced Akt activation and TGF-beta1 upregulation in mesangial cells. J Am Soc Nephrol. 2009;20(3):554-566.
28. Peterziel H, Muller J, Danner A, et al. Expression of podoplanin in human astrocytic brain tumors is controlled by the PI3K-AKT-AP-1 signaling pathway and promoter methylation. Neuro Oncol. 2012;14(4):426-439.
29. Ho E, Ames BN. Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkappa B, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. Proc Natl Acad Sci U S A. 2002;99(26):16770-16775.
30. Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol. 1997;9(2):240-246.
31. Kim SJ, Angel P, Lafyatis R, et al. Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex. Mol Cell Biol. 1990;10(4):1492-1497.
32. Kim SJ, Jeang KT, Glick AB, Sporn MB, Roberts AB. Promoter sequences of the human transforming growth factor-beta 1 gene responsive to transforming growth factor-beta 1 autoinduction. J Biol Chem. 1989;264(12):7041-7045.
33. Yue J, Mulder KM. Requirement of Ras/MAPK pathway activation by transforming growth factor beta for transforming growth factor beta 1 production in a Smad-dependent pathway. J Biol Chem. 2000;275(40):30765-30773.

34. Jinnin M, Ihn H, Asano Y, Yamane K, Trojanowska M, Tamaki K. Tenascin-C upregulation by transforming growth factor-beta in human dermal fibroblasts involves Smad3, Sp1, and Ets1. Oncogene. 2004;23(9):1656-1667.
35. Tie J, Pan Y, Zhao L, et al. MiR-218 inhibits invasion and metastasis of gastric cancer by targeting the Robo1 receptor. PLoS Genet. 2010;6(3):e1000879.
36. Alajez NM, Lenarduzzi M, Ito E, et al. MiR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2-ROBO1 pathway. Cancer Res. 2011;71(6):2381-2391.
37. Shi ZM, Wang L, Shen H, et al. Downregulation of miR-218 contributes to epithelial-mesenchymal transition and tumor metastasis in lung cancer by targeting Slug/ZEB2 signaling. Oncogene. 2017;36(18):2577-2588.
38. Martinez I, Gardiner AS, Board KF, Monzon FA, Edwards RP, Khan SA. Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells. Oncogene. 2008;27(18):2575-2582.
39. Uesugi A, Kozaki K, Tsuruta T, et al. The tumor suppressive microRNA miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer. Cancer Res. 2011;71(17):5765-5778.
40. Jones FS, Jones PL. The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling. Dev Dyn. 2000;218(2):235-259.
41. Kii I, Nishiyama T, Li M, et al. Incorporation of tenascin-C into the extracellular matrix by periostin underlies an extracellular meshwork architecture. J Biol Chem. 2010;285(3):2028-2039.
42. Orend G, Chiquet-Ehrismann R. Tenascin-C induced signaling in cancer. Cancer Lett. 2006;244(2):143-163.

Figures

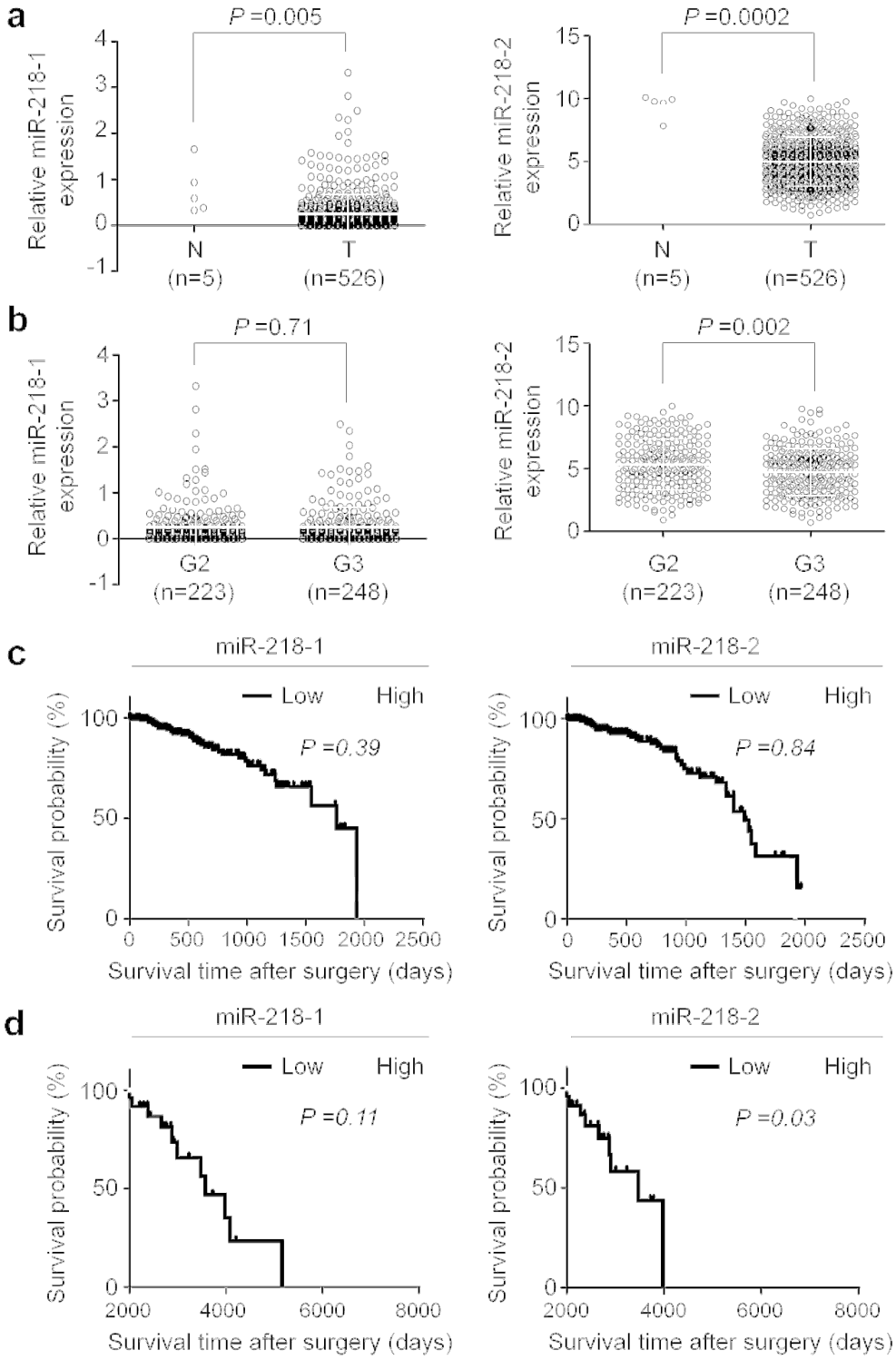

Figure 1

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.

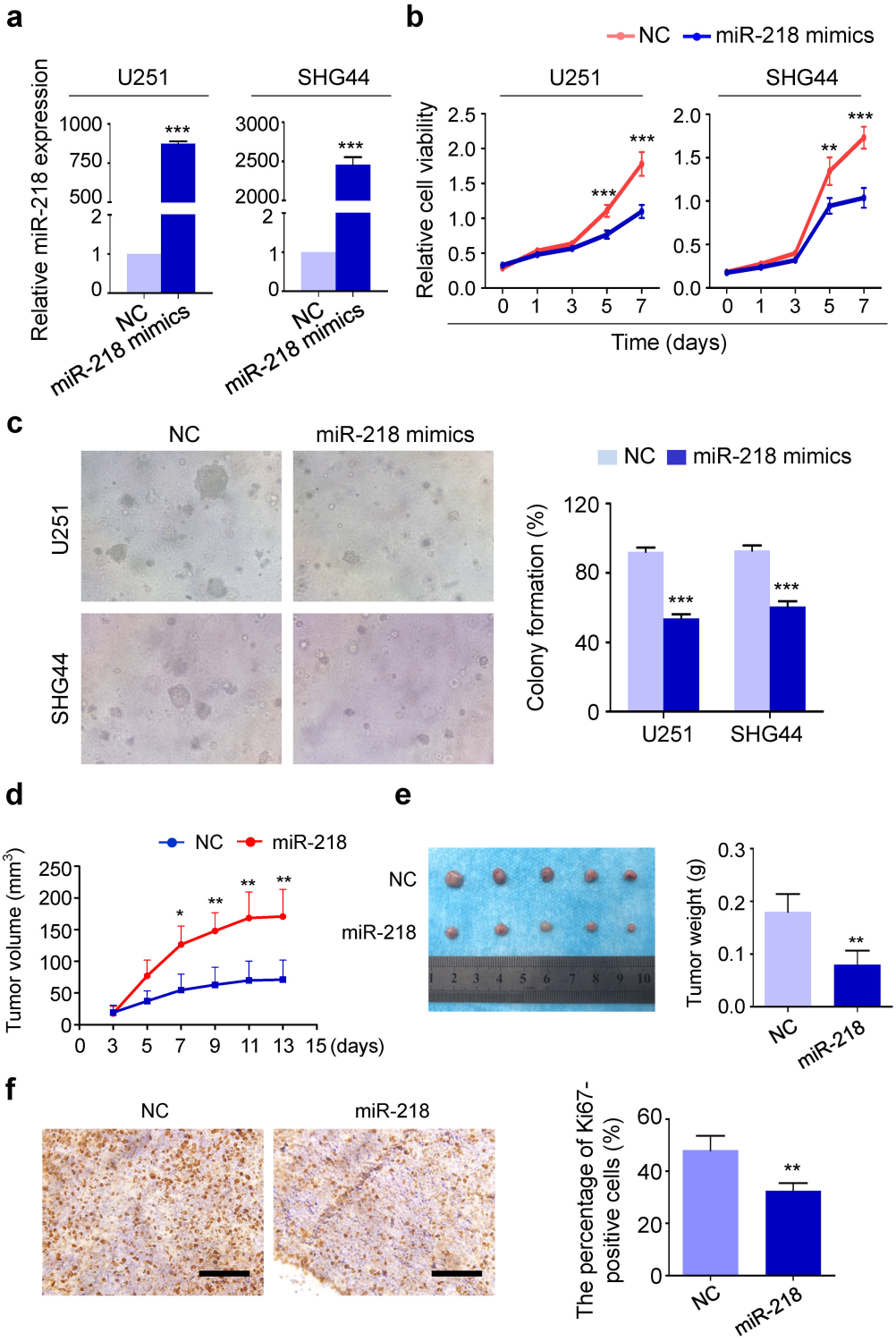

Figure 2

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.

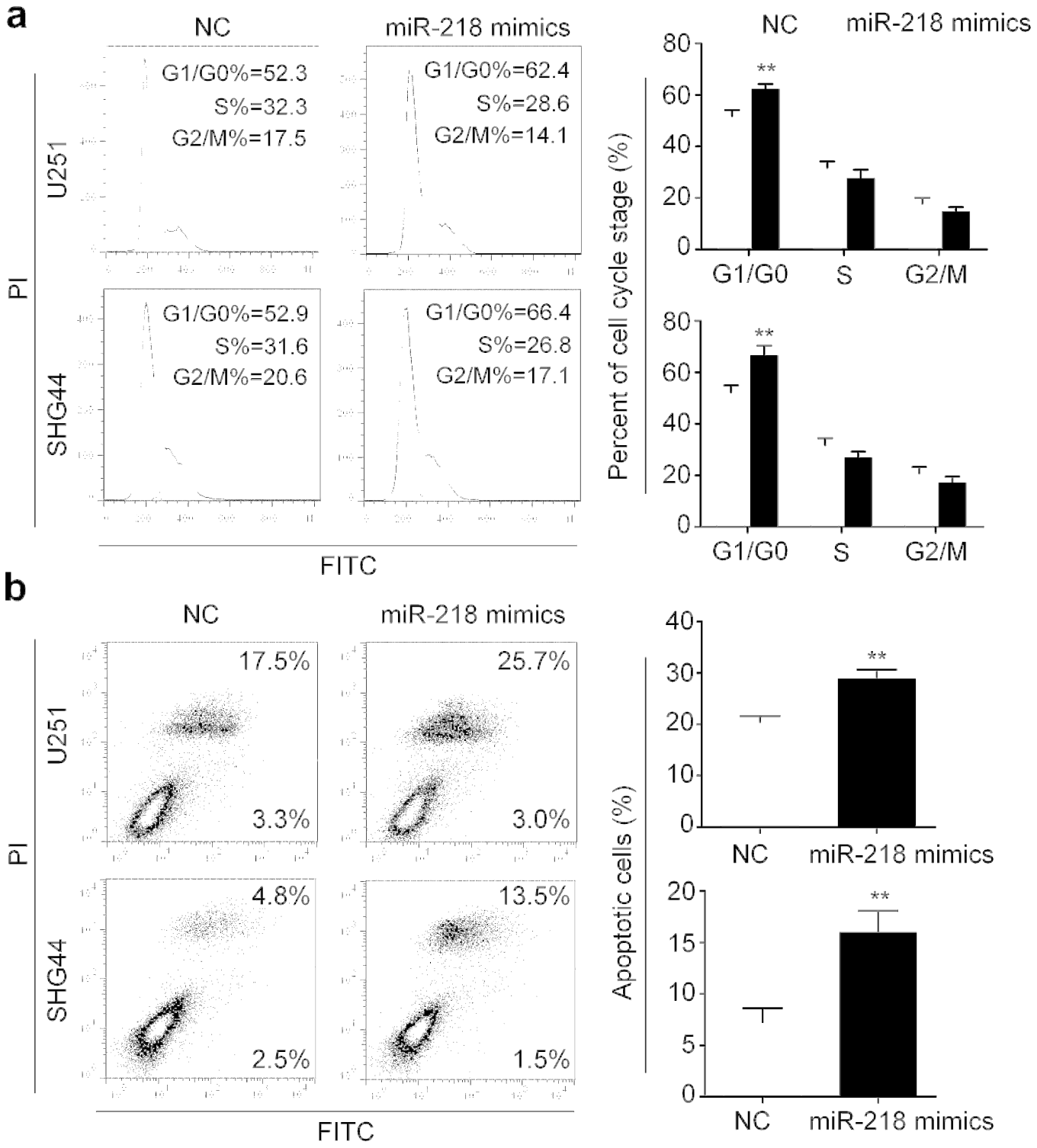

Figure 3

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.

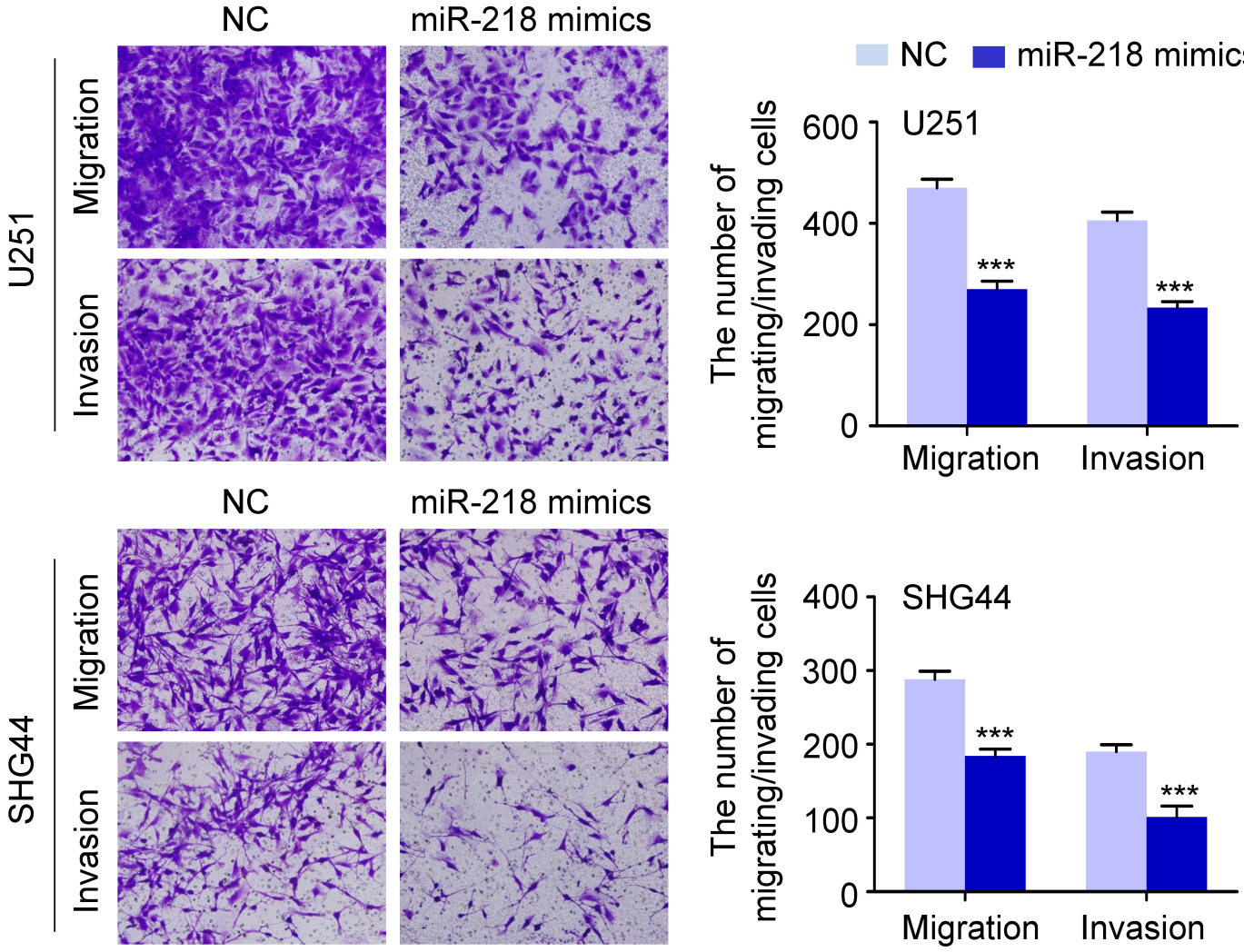

Figure 4

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.

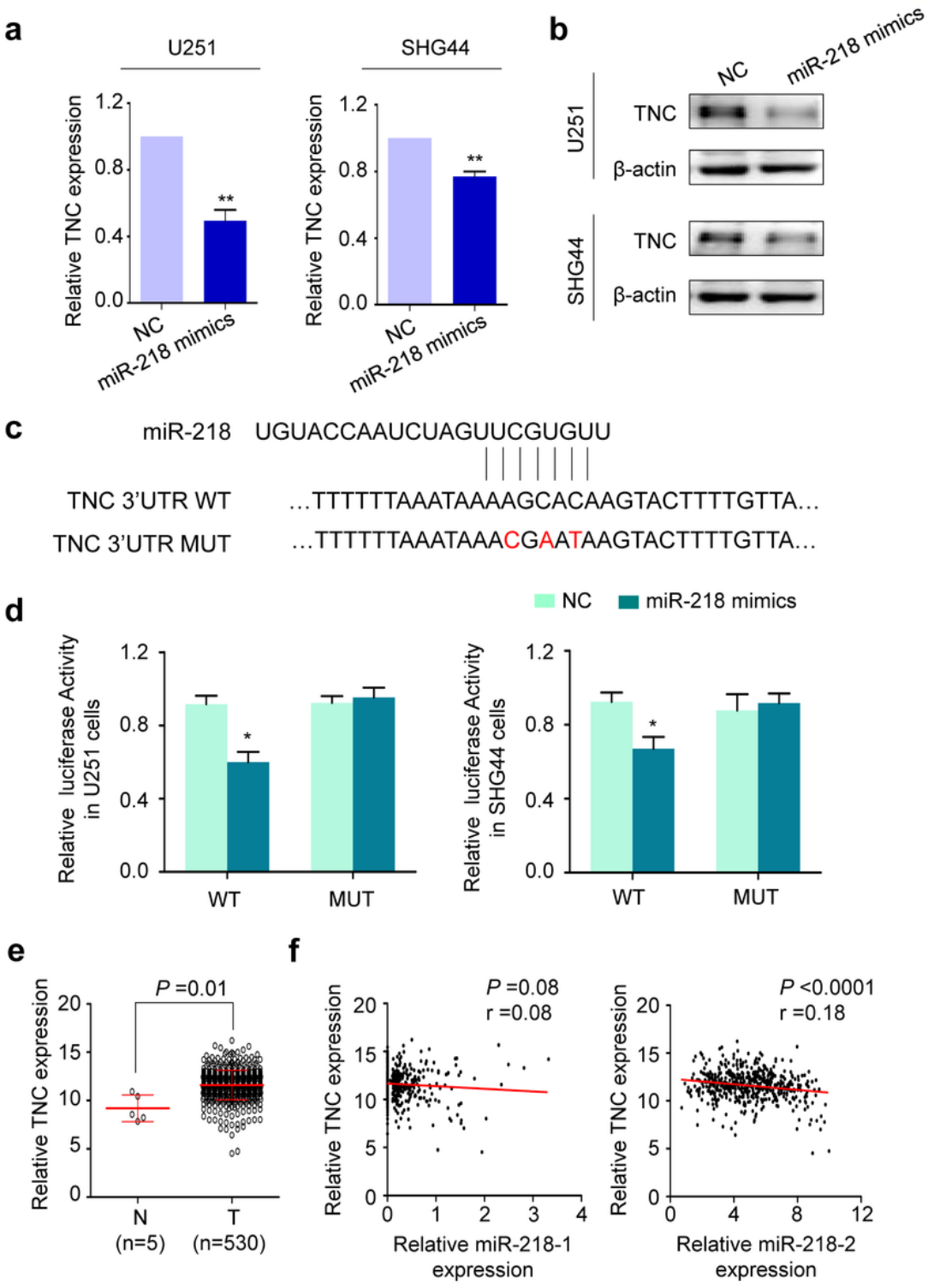

Figure 5

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. *, $P < 0.05$; **, $P < 0.01$

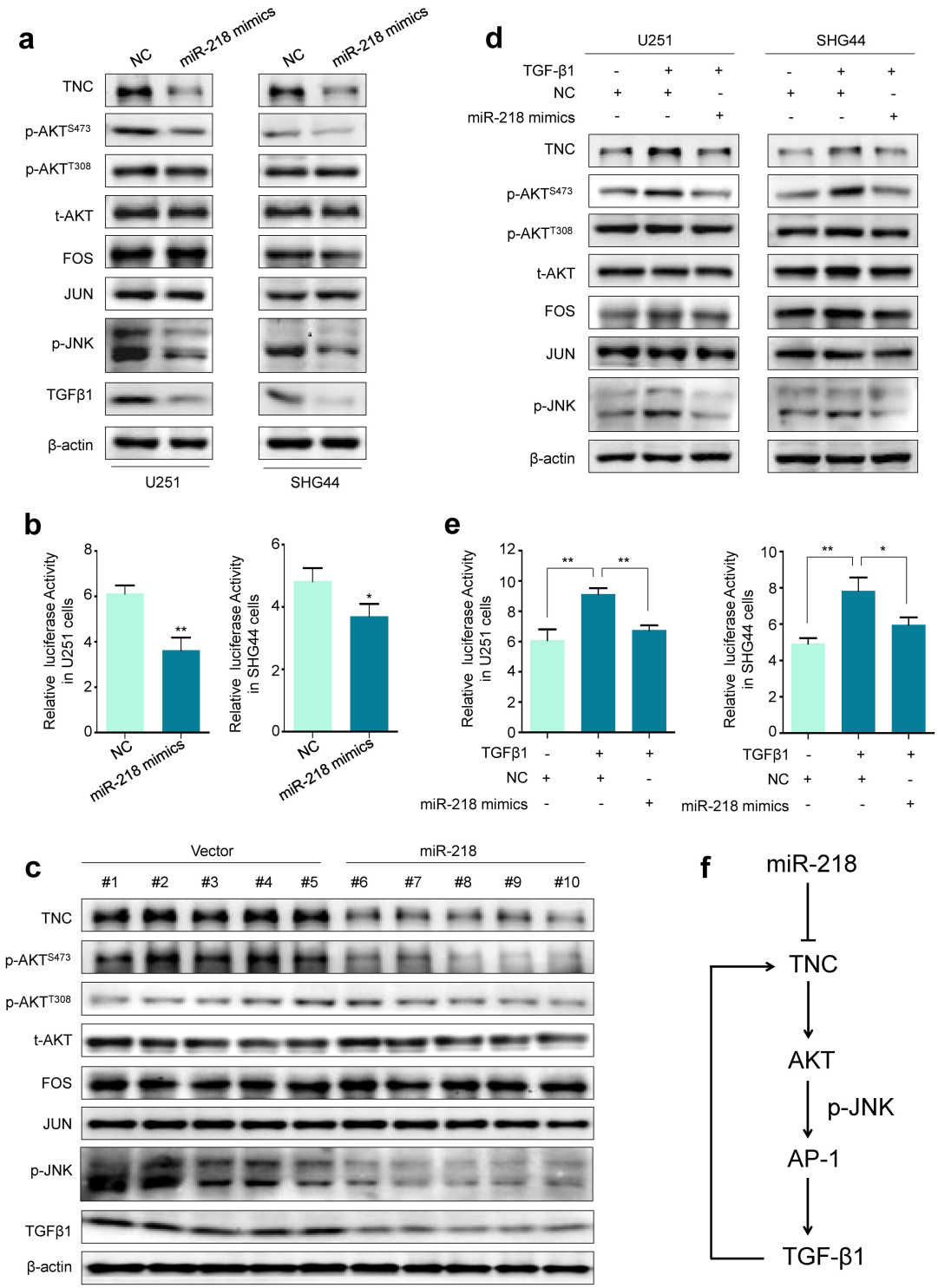

Figure 6

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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- SupplementaryData.docx