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miR-218 inhibits the proliferation of glioma U87 cells through the inactivation of the CDK6/cyclin D1/p21$^{Cip1/Waf1}$ pathway

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Abstract. Malignant gliomas are the most common and deadly primary brain tumors in adults and the high proliferative ability of these cells is one of the most important causes of the poor prognosis of this cancer. Suppressing the proliferation of malignant gliomas cells by altering effector molecules can significantly improve the prognosis of a patient. microRNAs (miRNAs) are small non-coding RNA molecules ~22 nucleotides in length that are able to function as oncogenes or tumor suppressors in human cancer. In the present study, it was demonstrated that the expression level of miRNA-218 (miR-218) is markedly downregulated in glioma cell lines and human primary glioma tissues. Upregulation of miR-218 in glioma U87 cells dramatically inhibited the proliferation by inducing G_1-S checkpoint arrest. Furthermore, it was demonstrated that ectopically expressing miR-218 in glioma U87 cells results in the downregulation of the expression of cyclin dependent kinase (CDK)6 and cyclin D1 and upregulation of the expression of p21$^{Cip1/Waf1}$. In addition, it was identified that miR-218 inactivated the CDK6/cyclin D1/p21$^{Cip1/Waf1}$ pathway by downregulating CDK6 expression through the direct targeting of the 3'-untranslated region of CDK6. The present results suggest that miR-218 plays an important role in the prevention of the proliferation of glioma cells, and the present study also revealed a novel mechanism for miRNA-mediated direct suppression of the CDK6/cyclin D1/p21$^{Cip1/Waf1}$ pathway in glioma cells.

Introduction

Human glioma is the most common primary tumor in the central nervous system and is characterized by a high proliferative and invasive ability (1). Standard therapies for glioma, including surgery, radiation and chemotherapy, are only effective in treating patients with a high-grade condition. Numerous glioma patients have already developed metastasis at the onset of clinical symptoms (2). The mechanism of glioma tumorigenesis remains unclear, and the molecular determinants of the aggressiveness of glioma have been the subject of numerous studies, but these investigations have not yet reached full fruition (3-6). Therefore, there is an acknowledged requirement for novel approaches based on increased understanding of the biological and molecular nature of these tumors.

microRNAs (miRNAs) are short non-coding, single-stranded RNA molecules that are 22-25 nucleotides in length and negatively regulate gene expression by post-transcriptional silencing of target messenger RNAs (mRNAs) through complementary binding (7,8). An increasing number of studies have indicated that miRNA plays an important role in the development of various cancers, including glioma, and miRNA has been associated with tumor suppressor and oncogenic activities (9,10). Out of these miRNA molecules, miRNA 218 (miR-218) has been revealed to be downregulated in human glioblastoma multiforme (GBM) specimens compared with the adjacent brain tissue that is devoid of tumor cells (11-14). Accumulated data have demonstrated that the upregulation of miR-218 is able to inhibit tumor cell invasion and proliferation in glioma cells by altering the expression of multiple target genes (14-17).

In the present study, the expression of miR-218 was upregulated by transient transfection of the human glioma U251, U87, SNB19 and LN229 cells with miR-218 mimics. This was performed with the aim of affecting the cyclin dependent kinase (CDK)6/cyclin D1/p21$^{Cip1/Waf1}$ pathway and demonstrating that miR-218 inactivates the CDK6/cyclin D1/p21$^{Cip1/Waf1}$ pathway by directly targeting the 3'-untranslated region (UTR) of CDK6. Furthermore, it was not only found that the stable expression of miR-218 inhibited proliferation *in vitro* and suppressed tumorigenicity of glioma cells *in vivo*, but it was also found that the expression of CDK6 and cyclin D1 in xenograft tumor tissues was significantly decreased, in contrast to the expression of p21$^{Cip1/Waf1}$, which was significantly increased. In summary, the present results suggest that miR-218 inhibits the proliferation of glioma cells through the inactivation of the CDK6/cyclin D1/p21$^{Cip1/Waf1}$ signaling pathway.

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Materials and methods

Clinical samples. Tumor specimens were obtained from patients that underwent positive debulking surgery in the Neurosurgery Department of The First Affiliated Hospital of Soochow University (Suzhou, China) between 2011 and 2013. The diagnosed gliomas were reviewed on histological slides by an experiential neuropathologist, according to the 2007 World Health Organization classification (18), resulting in 20 glioma samples being classified as grades I and II, 20 as grade III and 20 as grade IV. In total, 10 normal brain tissue samples were obtained from the internal decompression of patients with cerebral injury. For the use of these clinical materials for research purposes, patient consent and approval from the Ethics Committee of Taizhou People's Hospital (Taizhou, China) and The First Affiliated Hospital of Soochow University was obtained prior to the present study.

Cell lines and transfection. Primary normal human astrocytes (NHAs) were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). The glioma U251, U87, SNB19 and LN229 cell lines were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Beijing, China). The cells were maintained in RPMI-l640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, 50 units/ml penicillin G and 250 μg/ml streptomycin (Invitrogen) in a humidified atmosphere containing 5% CO_2 at 37˚C. Transfections with miR-218 were performed in serum-free medium 24 h subsequent to plating, using Lipofectamine 2000 (Invitrogen). After 6 h, the cells were placed in complete medium and maintained at 37˚C in a 5% CO_2 atmosphere.

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA, including miRNA, was extracted from cultured cells or fresh glioma cancer tissues using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The expression of miR-218 was quantified using the miRNA-specific TaqMan miRNA Assay kit (Applied Biosystems Life Technologies, Foster City, CA, USA). U6 small nuclear RNA was used as an internal control. The mRNA expression of CDK6, cyclin D1 and p21$^{Cip1/Waf1}$ was analyzed by qPCR using the SYBR-Green method (7500 ABI; Applied Biosystems Life Technologies). The protocols were performed according to the manufacturer's instructions and the results were normalized to the expression of GAPDH. The primers used were as follows: CDK6 forward, 5'-CTGAATGCTCTTGCTCCTTT-3' and reverse, 5'-AAAGTTTTGGTGGTCCTTGA-3'; cyclin D1 forward, 5'-TCCTCTCCAAAATGCCAGAG-3' and reverse, 5'-GGCGGATTGGAAATGAACTT-3'; p21 forward, 5'-CGATGCCAACCTCCTCAACGA-3' and reverse, 5'-TCGCAGACCTCCAGCATCCA-3'; and GAPDH forward, 5'-TCGGAGTCAACGGATTTGG-3' and reverse, 5'-CATGGGTGGAATCATATTGGA-3'.

Western blotting. Cells were lysed using 1% nonidet P-40 lysis buffer 48 h subsequent to exposure of the cells to LY294002 or vehicle treatment. Homogenates were clarified by centrifugation at 20,000 x g for 15 min at 4˚C, and the protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). SDS-PAGE was performed on 40 μg of protein from each sample. The gels were then transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) and incubated with polyclonal rabbit anti-human CDK6 (dilution, 1:200; cat. no. sc-177; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), polyclonal mouse anti-human cyclin D1 (dilution, 1:200; cat. no. sc-29287; Santa Cruz Biotechnology, Inc.) and polyclonal mouse anti-human p21$^{Cip1/Waf1}$ (dilution, 1:100; cat. no. sc-44271; Santa Cruz Biotechnology, Inc.) primary antibodies, which was followed by incubation with horseradish peroxidase-conjugated monoclonal goat anti-rabbit or anti-mouse IgG secondary antibodies (dilution, 1:1,000; cat. no's. W10804 and W10815; Zymed Life Technologies, Carlsbad, CA, USA). The membranes were stripped and reprobed with a primary polyclonal mouse anti-human GAPDH antibody. The protein bands were quantitated by densitometry using the gel analysis ImageJ software (National Institutes of Health, Bethesda, MA, USA). The values were normalized to the expression of GAPDH (dilution, 1:1,000; cat. no. sc-48167; Santa Cruz Biotechnology, Inc.).

Luciferase assay. The CDK6 3'-UTR sequence that was predicted to interact with miR-218 and a mutated sequence containing the predicted target sites were synthesized and inserted into the *Xba*I and *Fse*I sites of a pGL3 control vector (Promega, Madison, WI, USA). These constructs were termed pGL3-CDK6-3'UTR and pGL3-CDK6-3'UTR-mut. For the reporter assay, the U87 cells were plated onto 24-well plates and transfected with pGL3-CDK6-3'UTR or pGL3-CDK6-3'UTR-mut, and P-miR-218 or P-miR-control vectors using FuGENE HD (Promega, Madison, WI, USA). A *Renilla* luciferase vector, pRL-SV50 (Promega), was cotransfected to normalize the differences in transfection efficiency. Subsequent to transfection for 48 h, the cells were harvested and assayed using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. Transfection was repeated three times in triplicate.

Proliferation assay. The proliferative ability of cells was measured by using the cell counting kit-8 (CCK-8) at 24, 48 and 72 h post-transfection, according to the manufacturer's protocol. Briefly, 10 μl of CCK-8 solution was added to each well. Following incubation at 37˚C for 4 h in 5% CO_2, the absorbance of each well at a wavelength of 490 nm was detected using a microplate reader (Multiskan Spectrum; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Clonogenicity assay. The stably-transfected glioma cells were seeded into six-well plates and cultured in cell culture medium for two weeks to allow colony formation. The culture medium was changed every third day. The colonies were then fixed in 100% methanol and stained with crystal violet solution. Subsequently, the number of macroscopically observable colonies was recorded.

Cell cycle assay. The cells were harvested by trypsinization 48 h subsequent to transfection, washed three times with ice-cold phosphate-buffered saline (PBS), and fixed with 70% ethanol overnight at 4˚C. The fixed cells were rehydrated in PBS and subjected to propidium iodide/RNase staining

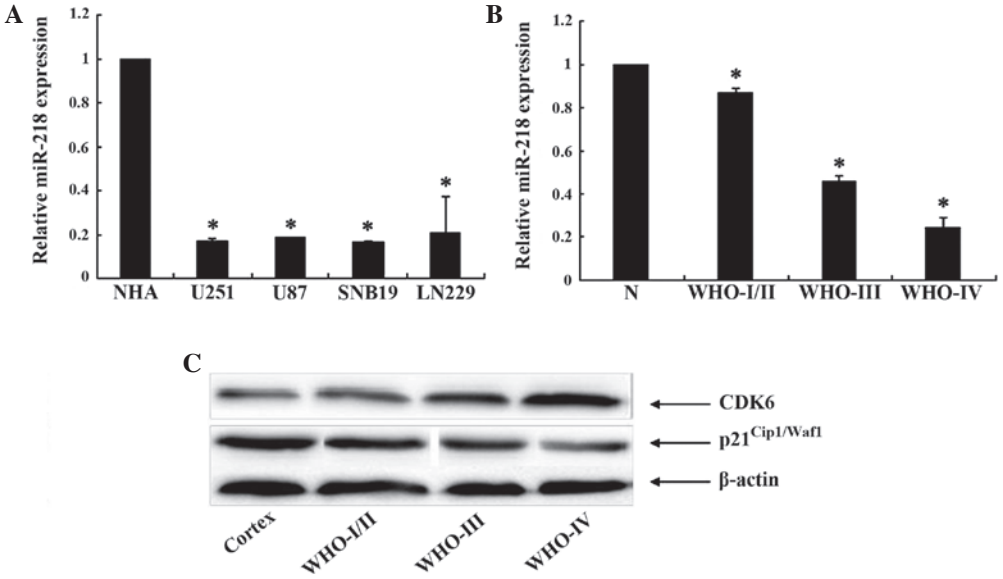

Figure 1. Level of miR-218 expression in glioma cell lines and tissues, and the expression of miR-218 and CDK6 in glioma tissues. (A) Expression of miR-218 was detected in the glioma U251, U87, SNB19 and LN229 cell lines and in the primary NHAs. (B) miR-218 was detected in 10 normal brain tissue and 60 glioma tissues by quantitative polymerase chain reaction. (C) Expression of CDK6 and $p21^{Cip1/Waf1}$ was detected by western blot analysis. The data are expressed as the mean + standard deviation of three independent experiments. *P<0.05 vs. NHAs. NHAs, normal human astrocytes; WHO, World Health Organization; CDK6, cyclin dependent kinase; miR-218, microRNA-218.

followed by fluorescence-activated cell sorter scan (FACS) analysis (Becton Dickinson, Mountain View, CA, USA). The percentage of cells in each phase of the cell cycle was estimated using PV ELITE software (Integraph Corporation, Madison, AL, USA).

Animal studies. Stably transfected U87 cells were resuspended in PBS and implanted into the left flanks of the BALB/c athymic mice, with $1.5x10^6$ cells being administered per flank by subcutaneous injection. The tumor volumes were determined by measuring the length (*a*) in mm, and the width (*b*) in mm^3, of the mass. The tumor volume (V) was calculated according to the formula $V=ab^2/2$. The statistical significance of the difference between the P-miR-218 and P-miR-control transfected groups was evaluated using Student's *t*-test. All procedures that involved animals were performed according to the guidelines of The First Affiliated Hospital of Soochow University, Soochow University and Chinese Academy of Medical Sciences.

Statistical analysis. Experimental data were presented as the mean ± standard deviation. All statistical analyses were performed using a two-tailed Student's *t*-test performed by SPSS software, version 12.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-218 is downregulated in glioma tissues. To analyze the expression levels of miR-218, qPCR was performed to assess miR-218 expression in the glioma U251, U87, SNB19 and LN229 cell lines. The results revealed that miR-218 was downregulated in all glioma cell lines compared with the NHAs (P<0.05). In addition, when miR-218 expression was measured in 10 normal brain and 60 glioma tissue samples, the expression level of miR-218 was observed to be significantly decreased in glioma tissues, particularly in grade III/IV tissues (Fig. 1A and B). This result demonstrated that miR-218 expression decreases markedly from normal brain tissue to low-grade to GBM tissue. Overall, the present results indicate that miR-218 was downregulated in glioma cell lines and glioma tissues.

CDK6 is upregulated and $p21^{Cip1/Waf1}$ is downregulated in glioma tissues. The expression levels of CDK6 and $p21^{Cip1/Waf1}$ were identified in glioma tissues using western blot analysis, and it was observed that CDK6 was expressed at extremely low levels in normal brain tissue, but at extremely high levels in human glioma tissues. In addition, with the progression of malignant glioma, the expression of CDK6 is gradually increased. By contrast, $p21^{Cip1/Waf1}$ was expressed at very high levels in normal brain tissue, but at very low levels in human glioma tissues (Fig. 1C).

Upregulation of miR-218 regulates expression of CDK6/cyclin D1/$p21^{Cip1/Waf1}$ in glioma U87 cells. In the present study, the possibility that cell cycle regulators may be modulated by miR-218 was investigated. Quantitative PCR analysis revealed that treatment with the miR-218 mimic for 24 h significantly upregulated the mRNA levels of $p21^{Cip1/Waf1}$, followed by a decrease in the expression of CDK6 and cyclin D1 (Fig. 2A-C). Notably, western blot analysis revealed similar results to the quantitative PCR analysis by miR-218 overexpression (Fig. 2D). Overall, these results indicated that miR-218 is able to transcriptionally regulate the expression of CDK6, cyclin D1 and p21.

CDK6 is a functional downstream target of miR-218. Using the publicly available algorithms in TargetScan (Whitehead

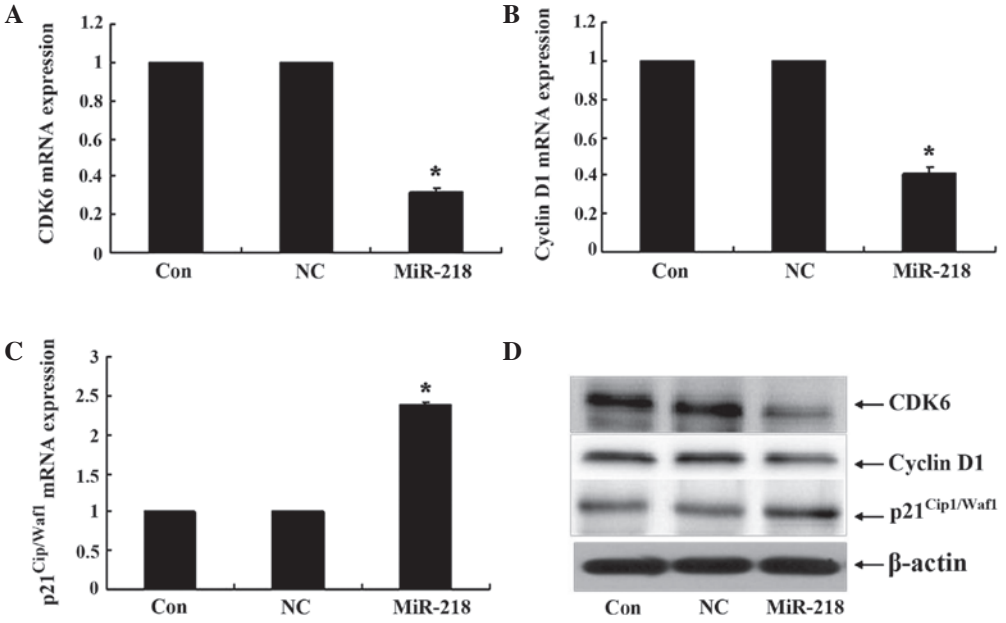

Figure 2. Upregulation of miR-218 reduces CDK6 expression through the inactivation of the CDK6/cyclin D1/p21$^{Cip1/Waf1}$ pathway. (A-C) The U87 cells were treated with control, NC and miR-218 mimics, and the levels of CDK6, cyclin D1 and p21$^{Cip1/Waf1}$ mRNA were measured by quantitative polymerase chain reaction. (D) The levels of the CDK6, cyclin D1 and p21$^{Cip1/Waf1}$ proteins in the U87 cells were measured by western blot analysis. The data are expressed as the mean + standard deviation of three independent experiments. *P<0.05 vs. control. CDK6, cyclin dependent kniase 6; miR-218, microRNA-218; Con, control, NC, negative control.

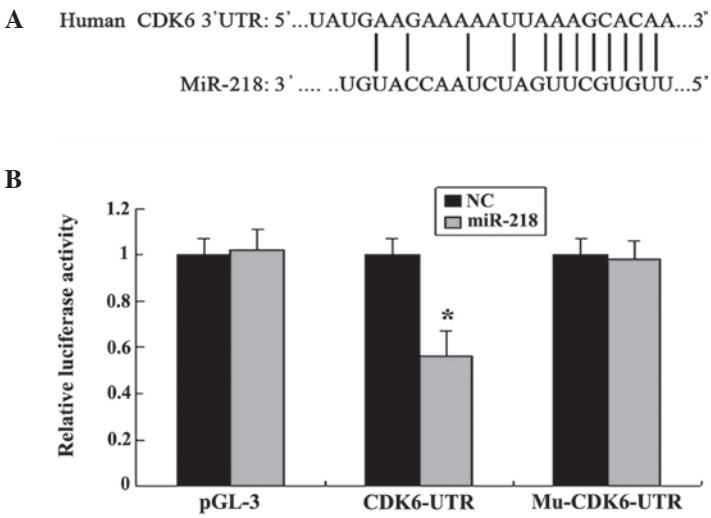

Figure 3. CDK6 is a direct target of miR-218. (A) Identification of target sites in the 3'-UTR of CDK6 was performed in TargetScan. (B) A luciferase assay was performed to determine the targeting association in the U87 cells. The data are expressed as the mean + standard of three independent experiments.*P<0.05 vs. control. CDK6, cyclin dependent kinase 6; miR-218, microRNA-218; NC, negative control; UTR, untranslated region.

Institute for Biomedical Research, Cambridge, MA, USA), CDK6 was theoretically identified as the target gene of miR-218 (Fig. 3A). A luciferase reporter assay further confirmed the direct association between miR-218 and the 3'-UTR of CDK6 mRNA. The luciferase activity for the wild-type 3'-UTR of CDK6 was significantly inhibited by cotransfection with miR-218 mimics compared with constructs containing mutated 3'-UTRs. This demonstrated that CDK6 is a direct target of miR-218 (Fig. 3B).

Upregulation of miR-218 inhibited cell proliferation in U87 cells. To determine the effects of miR-218 on glioma cell proliferation *in vitro*, cell proliferation and plate clonogenic assays were used. As shown in Fig. 4A, the cell growth inhibition rate was evidently increased in the miR-218 mimic group compared with the control groups at 48 and 72 h post-transfection (P<0.05). In Fig. 4B and C, stable overexpression of miR-218 markedly reduced the number of surviving colonies from the U87 cell line compared with the control and NC groups (P<0.05). This finding indicates that miR-218 is able to significantly inhibit the proliferation of glioma cells. Consequently, a cell cycle analysis was conducted in the U87 cells transiently transfected with the miR-218 mimics. The results revealed that the cells

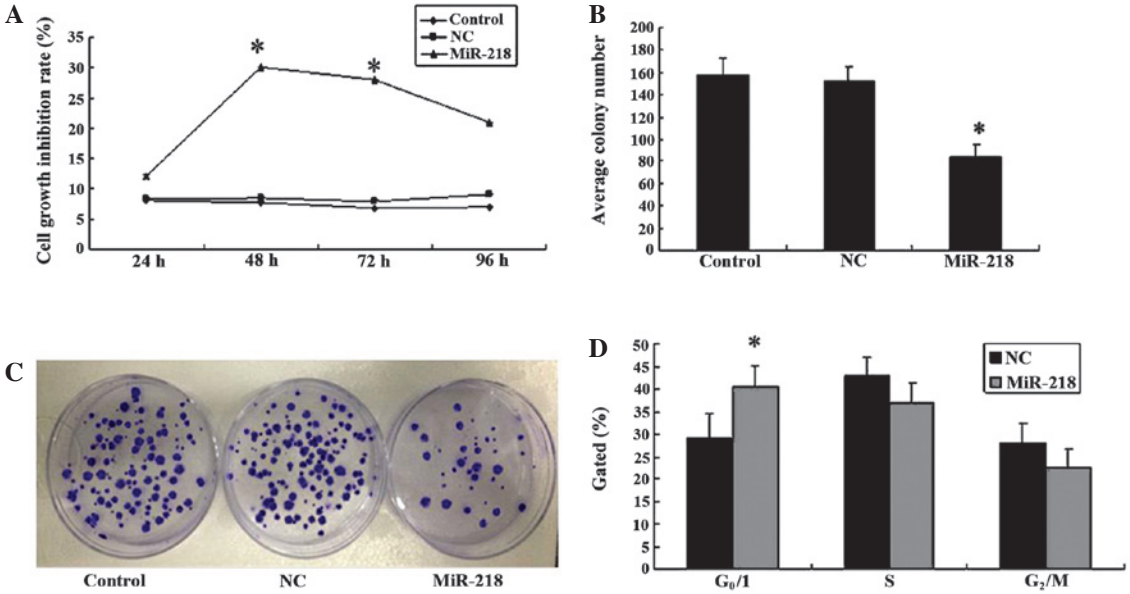

Figure 4. miR-218 inhibits cell proliferation in U87 cells. (A) miR-218 significantly inhibited the proliferation of U87 glioma cells at various time points. (B and C) Upregulation of miR-218 dramatically reduced the number of surviving colonies from the U87 cell lines compared with the control and NC groups. (D) Upregulation of miR-218 resulted in an increased accumulation at the G_0-G_1 phase with a decreased accumulation at the S phase. The data are the mean + standard deviation of three independent experiments. *P<0.05 vs. control. miR-218, microRNA-218; NC, negative control.

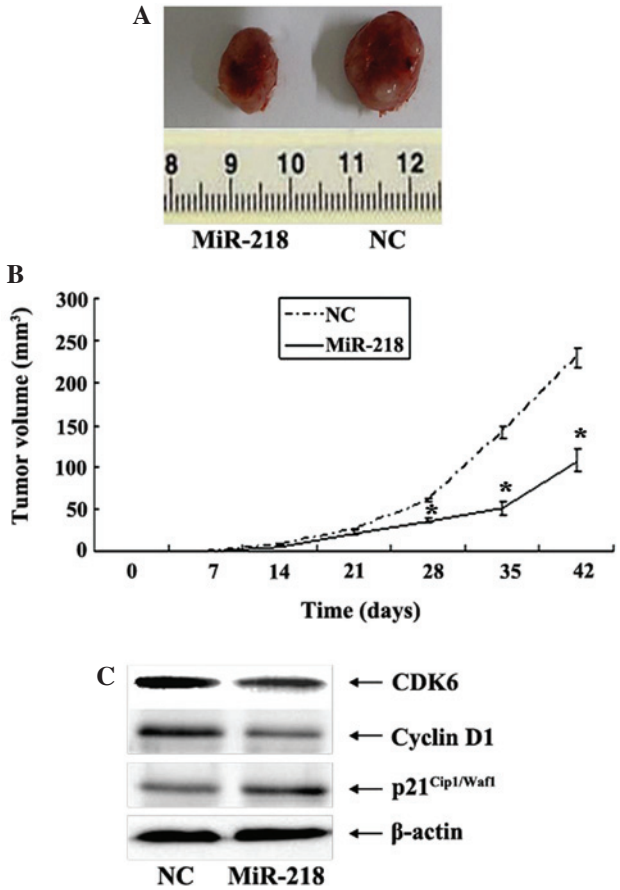

Figure 5. Upregulation of miR-218 suppresses the tumorigenicity of U87 cells *in vivo*. (A) Representative image of tumor growth. Nude mice were subcutaneously injected with $1.5x10^6$ U87 cells that were stably transfected with miR-218 mimic or mimic NC. (B) Determination of the tumor growth. Tumor volume was calculated every week subsequent to injection. (C) Western blot-analysis of the expression of the CDK6, cyclin D1 and $p21^{Cip1/Waf1}$ proteins in xenograft tumor tissues. The data are presented as the mean + standard deviation from five mice in each group. *P<0.05 vs. NC. miR-218, microRNA-218; NC, negative control; CDK6, cyclin dependent kinase 6.

transfected with the miR-218 mimics exhibited an increased accumulation in the G_0-G_1 phase with a decreased number of cells in the S phase (Fig. 4D).

Upregulation of miR-218 suppresses tumorigenicity of U87 cells in vivo. To substantiate the role of miR-218 in glioma carcinogenesis, the effects of miR-218 on tumorigenicity of glioma cells were assessed *in vivo*. Cells that were stably transfected with U87-miR-218 mimic or U87-mimic-NC were implanted into the left flanks of BALB/c athymic mice by subcutaneous injection. At 28 days post-injection, the mean volumes of tumors generated from the miR-218 group were significantly smaller compared with those originating from the NC group (Fig. 5A and B). Western blot analysis of the glioma xenograft tissues revealed that the expression of CDK6 and cyclin D1 was decreased in the xenograft tumor tissues derived from the miR-218 group compared with the NC group. By contrast, the expression of $p21^{Cip1/Waf1}$ was significantly increased (Fig. 5C).

Discussion

Rapid and unrestrained cell proliferation is a fundamental component of the malignant phenotype of cancer, not only for the development and growth of primary tumors, but also for the colonization of metastatic tumor cells in their target organs (19). Cell cycle progression involves sequential activation of CDKs, which possess an association with corresponding regulatory cyclins that is necessary for their activation (20). As a critical modulator of the G_1 to S phase transition, increased expression of cyclin D1 in cancer cells results in an uncontrolled growth advantage. The cyclin D-CDK4/CDK6 and cyclin E-CDK2 complexes regulate cell cycle transition from the G_1 to S phase by phosphorylating and inactivating the retinoblastoma (Rb) protein. However, aberrant activation of the

cyclin/CDK complexes can be partly ascribed to the loss or inactivation of endogenous CDK inhibitors, including $p15^{Ink4b}$, $p16^{Ink4a}$, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ (21). In addition, miR-153 overexpression in prostate cancer cells has been found to increase the expression of the G_1/S transitional promoter cyclin 1 and to decrease the expression of the cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1}$ (22). However, the underlying mechanism that modulates the abundance of the cyclins and CDKs in glioma has yet to be elucidated.

An increasing number of studies have indicated that microRNAs are associated with proliferation and apoptosis by negative regulation of the expression of oncogenes or tumor suppressor genes (23-25). Numerous studies have demonstrated that the expression level of miR-218 is frequently downregulated in several human cancers, including gastric cancer, lung squamous cell carcinoma, malignant astrocytomas and medulloblastomas, which suggests that miR-218 may function as a tumor suppressor (26-28). Previous studies have demonstrated that the ectopic expression of miR-218 contributes to the inhibition of the proliferation, invasion and migration of glioma cells, and induces apoptosis by downregulating the directly targeted gene of miR-218. miR-218 inhibits the expression of target genes that include IKK-β, and inhibits the expression of NF-κB in a dose-dependent manner. However, miR-218 also reduces the expression of matrix metalloproteinase-9 (MMP-9) and inhibits the invasive and migratory ability of glioma cells (14). EGFR-coamplified and overexpressed protein (ECOP) has also been identified as a functional downstream target of the genes targeted by miR-218. ECOP is able to regulate the transcriptional activity of NF-κB and is associated with the apoptotic response. Overexpression of miR-218 restrains the activity of NF-κB through the target gene ECOP, thus inducing glioma cell apoptosis and inhibiting the activity, proliferation and tumorigenicity of glioma cells (15). The expression of lymphoid enhancer-binding factor 1 (LEF1) and MMP-9 in the high-grade glioma group was extremely high, while the expression in the low-grade glioma group was extremely low, and the expression of LEF1 and MMP-9 was negatively correlated with the expression of miR-218. Overexpression of miR-218 has been found to inhibit the Wnt/LEF1 signaling pathways that lead to a reduction in MMP-9 synthesis and inhibit tumor invasion (16). The abnormal expression of miR-218 in glioma cells decreased, but abnormal increase CDK6 expression, the expression level of both negatively correlated. Overexpression of miR-218 in glioma cell line can inhibit CDK6 expression and glioma cell proliferation and promote its apoptosis (16).

In the present study, the expression levels of miR-218, CDK6 and $p21^{Cip1/Waf1}$ were detected in 70 tissue samples obtained from normal brain tissue and low- and high-grade glioma tissues by RT-qPCR and western blot analysis. It was found that the expression of miR-218 and $p21^{Cip1/Waf1}$ was always inversely associated with the expression of CDK6. Notably, the protein and mRNA levels of CDK6 and cyclin D1 were significantly decreased and the levels of $p21^{Cip1/Waf1}$ were significantly increased subsequent to the transfection of U87 cells with miR-218 mimics. CDK6 was identified as a direct functional target of miR-218 using bioinformatics analysis, and this finding was experimentally confirmed using a luciferase reporter assay. Therefore, it was found that miR-218 was involved in the modulation of the CDK6/cyclin D1/$p21^{Cip1/Waf1}$ pathway and downregulation of CDK6 expression by directly targeting the 3'-UTR of CDK6. In the gain of function investigation, the overexpression of miR-218 was found to inhibit the proliferation of glioma cells proliferation and result in a G_0/G_1 phase arrest of the cell cycle *in vitro*. miR-218 may also suppress the tumorigenicity of glioma cells *in vivo*. The results of western blot analysis substantiate that the expression of CDK6 and cyclin D1 in xenograft tumor tissues was significantly decreased. By contrast, the expression of $p21^{Cip1/Waf1}$ was significantly increased. Overall, the present results indicate that miR-218 inhibits the proliferation of glioma cells through targeting CDK6 to inhibit cyclin D1 activity and activate endogenous CDK inhibitors of $p21^{Cip1/Waf1}$ activity.

In conclusion, the present study reveals an association between miR-218-mediated downregulation of glioma cell proliferation and the inactivation of CDK6/cyclin D1/$p21^{Cip1/Waf1}$ signaling. The present results suggest that miR-218 is critical for the inhibition of proliferation of glioma cells, and understanding the role of miR-218 may provide important insights into the treatment of gliomas.

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