Co-suppression of miR-221/222 cluster suppresses human glioma cell growth by targeting p27^{kip1} *in vitro* and *in vivo*

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Abstract. MicroRNAs are short regulatory RNAs that negatively modulate protein expression at a post-transcriptional level. Emerging evidence suggests that altered regulation of miRNA may be involved in the pathogenesis of several types of cancers. In the current study, an inverse relationship between the expression of miR-221/miR-222 and the cell cycle inhibitor p27^{Kip1} was identified in U251 glioma cells. Co-suppression of miR-221/222 directly resulted in the up-regulation of p27^{Kip1} in the tested cells, consequently, affects their growth potential by reducing a G1 to S shift in the cell cycle. Consistently, miR-221/222 knocked-down through antisense 2'-OME-oligonucleotides increased p27^{Kip1} in U251 glioma subcutaneous mice and strongly reduced tumor growth *in vivo* through up regulation of p27^{Kip1}. Our results suggest that miR-221/222 is a regulator of the tumor suppressor gene p27^{Kip1}, and co-suppression of miR-221/222 expression in advanced gliomas may inhibit glioma cell proliferation by a mechanism involving the up-regulation of p27^{Kip1} *in vitro* and *in vivo*.

Introduction

The microRNAs (miRNAs or miRs) are a class of small, non-coding RNA molecules that negatively regulates protein expression at the post-transcriptional level in a sequence specific manner, through the binding to the target mRNA 3'UTRs (3' untranslated regions) and subsequent inhibition of protein translation (1-5). Accumulating evidence indicates that miRNAs play important roles in malignant progression. Specific miRNAs have been demonstrated to be deregulated in diverse cancer subtypes including lymphoma, colorectal cancer, lung cancer, breast cancer, papillary thyroid carcinoma, hepatocellular carcinoma and glioblastoma. As well as these deregulated miRNAs, it has been proved that miR-221/222 cluster was overexpressed in patients with malignancy (6-14).

Gliomas are the most common primary brain tumors (15). According to widely used classification by the World Health Organization, human gliomas are divided into four grades with high grade gliomas being represented by grade III (anaplastic astrocytoma) and grade IV (glioblastoma multiforme, GBM) tumors. GBM is the highest grade, as well as the most aggressive and frequent human glioma (16). After extensive tumor resection, combined with radiotherapy and chemotherapy, GBM confers a dismal prognosis with a median survival of 9-12 months (16-18). While the exact molecular mechanisms underlying the formation of glioma remain unclear, recent studies have reported several miRNA abnormalities, including miR-221 and miR-222 (14).

The p27^{Kip1} gene is a member of the cip/kip family of cyclin-dependent kinase (CDK) inhibitors that negatively control the cell cycle progression. The p27^{Kip1} binds to CDK2 and cyclin E complexes to prevent cell cycle progression from G1 to S phase (19,20). The p27^{Kip1} does not follow Knudson's classic 'two-hit hypothesis' of tumor suppression. The inactivation of p27^{Kip1} is not caused by direct mutations in the gene encoding p27^{Kip1}. The homozygous loss or silencing of the locus in human tumors is extremely rare (21). A recent study demonstrated that the miR-221/222 cluster is an endogenous regulator of p27^{Kip1}, confirming the miRNA modulation may play a key role in the inactivation of p27^{Kip1} (22-25). However, that the miR-221/222 cluster affects tumor cell proliferation by regulating p27^{kip1} *in vivo* has not been well documented.

In the current study, we aimed to confirm whether a similar pathway might also function in human glioma cells, and to demonstrate the tumor-suppressive effect of the miR-221/222 cluster *in vivo*. We identified an inverse relationship

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between the expression of miR-221/222 and CDK inhibitor $p27^{Kip1}$. Co-suppression of miR-221/222 directly resulted in the up-regulation of $p27^{Kip1}$ and affected their growth potential by reducing the G1 to S shift in the cell cycle. Consistently, miR-221 and miR-222 knocked-down through antisense 2'-OME-oligonucleotides up regulated $p27^{Kip1}$ in U251 subcutaneous mice and strongly reduced tumor growth *in vivo*. These observations suggest that $p27^{Kip1}$ is an important functional target for the miR-221/222 cluster in glioma cells, and co-suppression of the miR-221/222 cluster might be used as a potential therapeutic strategy in brain cancer.

Materials and methods

Cell culture and culture conditions. Human U251 glioblastoma cells were obtained from China Academia Sinica cell repository, Shanghai, China. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine (Sigma), 100 Units of penicillin/ml (Sigma), and 100 μg of streptomycin/ml (Sigma), and incubated at 37˚C with 5% CO_2.

Oligonucleotides and cell transfection. 2'-O-methyl (OMe)-oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography by GenePharma Co., Ltd. (Shanghai, China). All the bases were 2'-OMe modified entirely and had the following sequences: 2'-OMe-anti-miR-221 (As-miR-221), 5'-GUCAACAUCAGUCUGAUAAGCUA-3'; 2'-OMe-anti-miR-222 (As-miR-222), 5'-GUCAACAUCAGUCUGAUAAGCUA-3'. The 2'-OMe-oligonucleotides (200 pmol) were transfected using Lipofectamine 2000 (Invitrogen). Scrambled oligonucleotides (Scr) were also transfected as a control and had the following sequence: 5'-UCUACUCUUUCUAGGAGGUUGUGA-3'. Cell growth was monitored on day 1, 2, 3, 4, 5 and 6 after transfection. RNAs and proteins were extracted 3 days after transfection for further analysis.

RNA extraction and Northern blot analysis. Total RNA was extracted from U251, U251-Scr, As-miR-221, As-miR-222 and As-miR-221/222 cells with TRIzol reagent (Invitrogen). The protocol of Northern blot analysis of miRNA was adopted from Ramkissoon *et al* (26). Total RNAs (20 μg) were separated on a 12% denaturing polyacrylamide gel, and then transferred to Hybond N^+ nylon membrane (Ambion, USA). The membrane was dried, UV cross-linked, hybridized with Digoxigenin labeled probes overnight at 37˚C in a buffer containing 5X SSC, 20 mmol/l Na_2HPO_4 (pH 7.2), 7% SDS, 1X Denhardt's, and 0.2 mg/ml salmon sperm DNA. The specific probes, end-labeled with DIG, were miRNA-221, 5'-GUCAACAUCAGUCUGAUAAGCUA-3'; miRNA-222, 5'-GUCAACAUCAGUCUGAUAAGCUA-3'; U6, 5'-GUCAACAUCAGUCUGAUAAGCUA-3'. The probes were purchased from Proligo Primer and Probes (Sigma, USA). The membrane was washed with 1X SSC/1% SDS buffer at 50˚C. After equilibration in detection buffer, blots were detected with Dig Luminescent detection kit (Roche, USA) and analyzed by GeneGenius.

Bioinformatics method. Combined with reference (23), the miRNA targets predicted by computer-aided algorithms were obtained from PicTar (http://pictar.bio.nyu.edu/cgi-bin/PicTar_vertebrate.cgi), TargetScan Release 4.0 (http://www.targetscan.org).

Luciferase activity assay. The pGL3-p27-3'UTR, pGL3-p27-3'UTR-DM (mutant) and pGL3 control plasmids were kindly provided by Reuven Agami (Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands). For reporter assays, cells were cultured in 96-well plates and transfected with 0.2 μg each of three plasmids and 5 pmol of the As-miR of interest (As-miR-221 and/or As-miR-222) by Lipofectamine 2000. Luciferase activity was measured 72 h after transfection using a Dual-luciferase reporter assay system (Promega).

Western blot analysis. Western immunoblot analysis was used to examine the protein expression after gene knock-down by co-transfection with AS-miR-221 and AS-miR-222 in U251 cells. Parental and transfected cells were washed with pre-chilled phosphate-buffered saline (PBS) three times. The cells were then solubilized in 1% Nonidet P-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and a protease inhibitor mixture). Homogenates were clarified by centrifugation at 20,000 x g for 15 min at 4˚C, and protein concentrations were measured by Nanodrop spectrophotometer (Gene, USA). Equal amounts (40 μg) of lysates were subjected to SDS-PAGE on 8% SDS-acrylamide gels. Separate proteins were transferred to PVDF membranes (Millipore, USA) and incubated with primary antibody against $p27^{Kip1}$ (Zhongshan, China) followed by incubation with an HRP-conjugated secondary antibody (Zymed, USA). The specific protein was detected by using a SuperSignal protein detection kit (Pierce, USA). The membrane was stripped and reprobed with an antibody against ß-actin (Santa Cruz, USA). In As-miR-221/222 transfected group, cells were collected at 0, 1, 2, 3 or 4 days and Western blotting was performed. Moreover, 40, 80, 120, 160 or 200 pmol of AS-miR221 and AS-miR-222 were co-transfected. Cells were collected 2 days after transfection and Western blotting was performed.

Cell viability assay. Cells were seeded into 96-well plates at 4000 cells/well. After transfection as described previously, 20 μl of MTT (5 g/l) was added into each well, each day for 6 consecutive days after treatment and incubated for 4 h, and the supernatant was then discarded. Finally, 200 μl of DMSO was added to each well to dissolve the precipitate. Optical density (OD) was measured at the wavelength of 570 nm. The data are presented as the mean ± SD, derived from triplicate samples of at least three independent experiments.

Cell cycle assay. For cell cycle analysis by flow cytometry, transfected and control cells in the log phase of growth were harvested, washed with PBS, fixed with absolute ethanol overnight at 4˚C, and then incubated with RNase at 37˚C for 30 min. Nuclei of cells were stained with propidium iodide

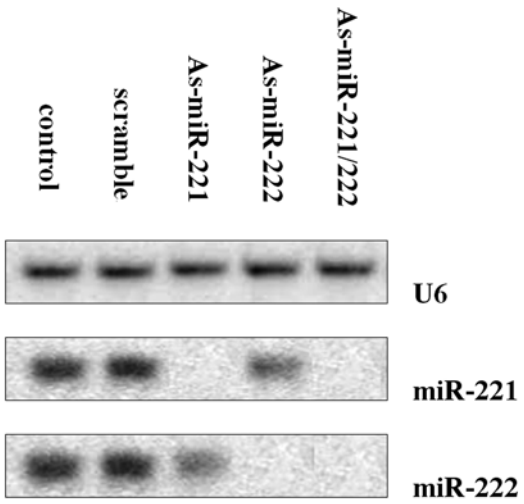

Figure 1. Expression profile of miR-221, miR-222 in four transfected groups of cells and U251 cells *in vitro*. Northern blot analysis of total RNA from four transfected groups of cells and U251 cells were probed for miR-221 and miR-222 *in vitro*. The snRNA U6 is included as a loading control.

for additional 30 min. A total of 10,000 nuclei were examined in a FACSCalibur flow cytometer (Becton-Dickinson, USA) and DNA histograms were analyzed by Modifit software.

Subcutaneous tumor model and As-miR-221/222 therapy. Five-week-old female immune-deficient nude mice (BALB/C-nu) were purchased from animal center of the Cancer Institute of Chinese Academy of Medical Science, bred at Tianjin Medical University and housed in microisolator individually ventilated cages with water and food. All experimental procedures were carried out according to the regulations and internal biosafety and bioethics guidelines of Tianjin Medical University and the Tianjin Municipal Science and Technology Commission. U251 glioma subcutaneous model was established as previously described (27). When the subcutaneous tumor reached 50 mm^3 in size the mice were randomly divided into four groups (10 mice/group). A mixture of 5 μl oligonucleotides containing Scr, AS-miR-221, AS-miR-222 or equal amount of AS-miR-221/222 cluster and 10 μl Lipofectamine was injected into the xenograft tumor model in a multi-site injection manner. Mice in U251 control group received 10 μl of PBS only. Treatment was conducted every three days, five times. The tumor volume was measured with a caliper every two days, using the formula volume = length x $width^2/2$.

Fluorescence in situ hybridization and immunohistochemistry. Twenty-eight days after subcutaneous implantation of tumor cells, mice were sacrificed and paraffin-embedded tissue sections were used for examination of miR-221, miR-222 and Ki67, $p27^{Kip1}$ expression in the tumors.

Using an antisense locked nucleic acid (LNA/DNA) modified oligonucleotide probe, *in situ* hybridization was performed with fluorescence *in situ* hybridization kit (CYBRDI, China). LNA/DNA oligos contained locked nucleic acids at five located bases (indicated by underline) and had the following sequences: LNA-miR-221 5'-GAAACCCAGCAGACAATGTAGCT-3'; LNA-miR-222 5'-GAGACCCAGTAGCCAGATGTAGCT-3'. Moreover, LNA-miR-221 uses DIG modification and LNA-miR-222 uses BIO modification in 3' distal end. Sections were deparaffinized and deproteinated, and then prehybridized for 2 h in hybridization liquid in a humidified chamber (50% formamide, 5 x SSC). The probes (miR-221, miR-222 and 10 ng) were added to the sections and incubated overnight at 40˚C in a water bath. After washing 3 times, the same volume of anti-digoxigenin-rhodamine and streptavidin-FITC solution was added and incubated for 2 h at room temperature in the dark. Nuclei were counterstained with a DAPI karyotyping kit (Genmed, USA). After washing 3 times, sections were sealed and detected under a fluorescence microscope with OptiGrid system and analyzed by IPP6.1 (Olympus, Tokyo, Japan).

For the immunohistochemistry study, sections were incubated with appropriate primary antibody (1:200 dilution) overnight at 4˚C. Biotinylated secondary antibody (1:200 dilution) was then added at room temperature for 1 h, followed by incubation with ABC-peroxidase for an additional 1 h. After washing with Tris-buffer, the sections were incubated with DAB (3,3' diaminobenzidine, 30 mg dissolved in 100 ml Tris-buffer containing 0.03% H_2O_2) for 5 min, rinsed in water and counterstained with hematoxylin.

Statistical analysis. Data are expressed as means ± SE, and $P<0.01$ is considered as statistically significant by ANOVA, χ^2 test and SNK t-test.

Results

As-miR-221 and As-miR-222 inhibit miR-221 and miR-222 in U251 cell lines. To knockdown endogenous miR-221 and miR-222, chemically engineered oligonucleotides were synthesized and transfected into U251 cells. Northern blot analysis showed that As-miR-221 and As-miR-222 efficiently and specifically silenced endogenous miR-221 and miR-222, respectively. In five experimental groups, the control and scramble groups expressed miR-221 and miR-222, As-miR-221 group only expressed miR-222, As-miR-222 group only expressed miR-221 and As-miR-221/222 group did not have evident expression of miR-221 and miR-222. U6 was present in the five groups (Fig. 1).

Detection of miR-221 and miR-222 target mRNA by luciferase assay. By bioinformatics analysis, we found miR-221 and miR-222 specific binding sequence of the $p27^{kip1}$ 3'UTR. To further confirm if the 3'UTR of $p27^{kip1}$ has miR-221 and miR-222 specific binding sequences, we transfected the cells with pGL3-p27-3'UTR, pGL3-p27-3'UTR-DM (mutant) and pGL3 control plasmids. As-miR-221/222 significantly enhanced the luciferase activity (Fig. 2A and B).

Western blot assay demonstation of the As-miR-221/222 enhancement of $p27^{kip1}$ expression. Western blot analysis demonstrated that the $p27^{kip1}$ expression in As-miR-221/222 group was significantly up-regulated than the other four groups. The $p27^{kip1}$ expression levels in As-miR-221 and As-miR-222 groups were more up-regulated than the scramble and control groups (Fig. 2C).

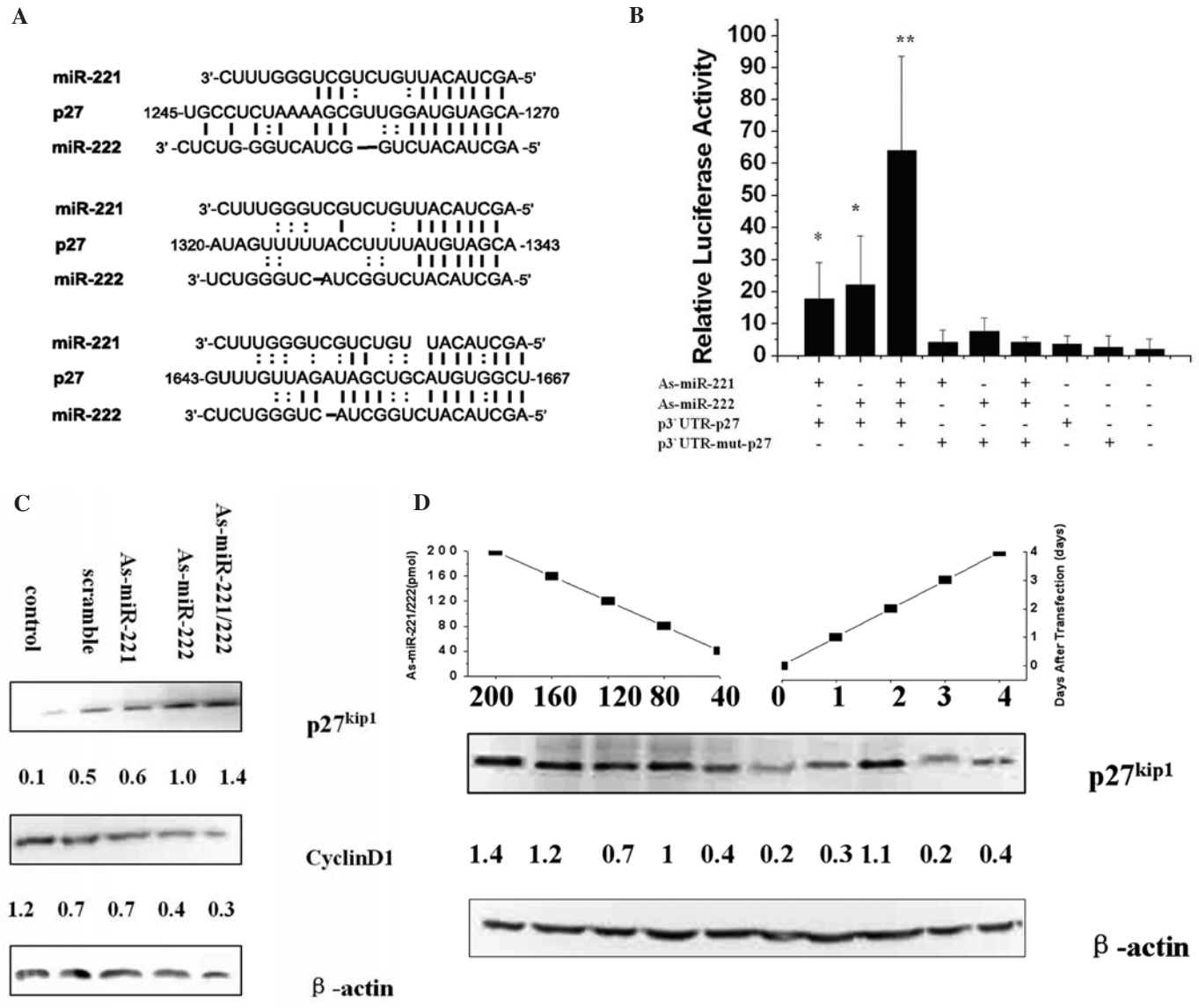

Figure 2. The relationship between the expression of miR-221/222 and CDK inhibitor $p27^{Kip1}$. (A) By bioinformatics analysis, we found that $p27^{Kip1}$ is a potent target of miR-221/222. We also found miR-221 and miR-222-specific binding sequence of the $p27^{Kip1}$'s 3'UTR. (B) p3'UTR-p27 or p3'UTRmut-p27 luciferase constructs containing a wild-type or a mutated p27 3'UTR were transfected into U251 cells stably transduced with As-miR-221 and/or As-miR-222. Luciferase activity was determined 48 h after transfection. The ratio of normalized sensor to control luciferase activity is shown. Error bars represent standard deviation and were obtained from three independent experiments. (C) Western blot analysis demonstrated that the $p27^{Kip1}$ expression level in As-miR-221/222 group was up regulated significantly compared to the other four groups. (D) MiR-221/222 regulates $p27^{Kip1}$ expression in a dose- and time-dependent manner.

Of the five groups transfected with different amounts of As-miR-221/222 (40, 80, 120, 160 and 200 pmol), the $p27^{kip1}$ expression level in 200 pmol group was up-regulated the most. In the other four groups, the $p27^{kip1}$ expression was gradually decreased or disappeared. In As-miR-221/222 group, the $p27^{kip1}$ expression was up-regulated most significantly 2 days after transfection (Fig. 2D).

As-miR-221/222 cluster decreases cell proliferation. The cell proliferation rate in different transfected cells was measured by MTT assay. It was shown that the cells transfected with As-miR-221/222 proliferated at a significantly lower rate than the other four groups (Fig. 3).

Cell cycle shows G1 arrest in the As-miR-221/222 group. The cell cycle distribution of transfected cells were analyzed by flow cytometry. The G0/G1 phase percentage of control, scrambled treated cells, As-miR-221 treated cells, As-miR-222 treated cells was 28.6, 26.0, 30.6 and 29.8%, respectively; while the fraction of As-miR-221/222 treated cells was 46.2%. The S phase fraction in control, scrambled treated cells, As-miR-221 treated cells, As-miR-222 treated cells was 56.8, 57.2, 54.3 and 50.6%, respectively; while in the As-miR-221/222 treated cells it was 26.8%. No statistical significance of G2/M phase percentage was detected among the five groups (Fig. 4).

As-miR-221/222 inhibits xenograft tumor growth in vivo. Fluorescence *in situ* hybridization proved that As-miR-221 and As-miR-222 efficiently and specifically silenced endogenous miR-221 and miR-222, respectively, in xenograft tumor *in vivo* (Fig. 5A). Moveover, we have found that

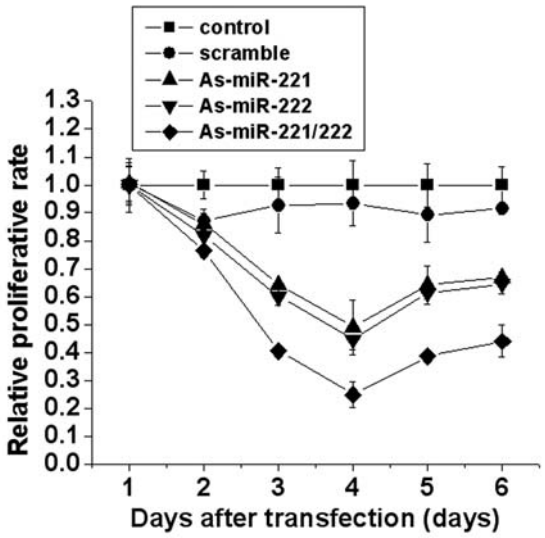

Figure 3. As-miR-221/222 decrease cell proliferation. MTT assay shows that the cells transfected with As-miR-221/222 proliferated at a significantly lower rate than the other four groups. The latitudinal axis represents days after cell implantation in the 96-well plate. The cell proliferation rate in parental U251 glioma cells is presented as 100%.

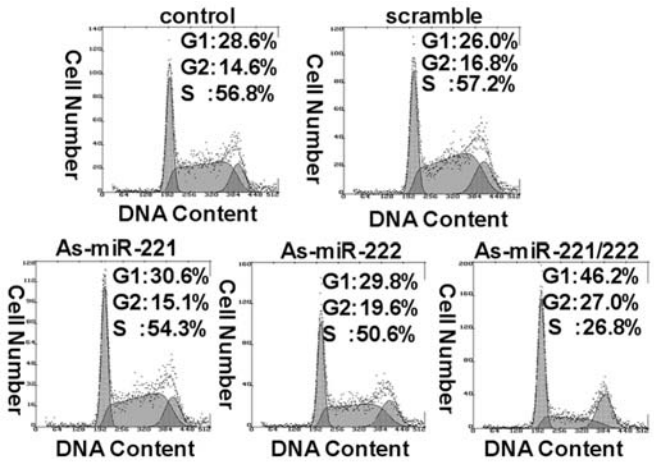

Figure 4. Co-suppression of miR-221/222 expression by As-miR-221/222 induces G1 arrest in U251 cells. Transfected and control cells were harvested by trypsinization, washed and fixed overnight. Nuclei of cells were stained with propidium iodide, a total of 10,000 nuclei were examined. By flow cytometry analysis, the rate of cells in G1 in the As-miR-221/222 group was significantly higher than the other four groups.

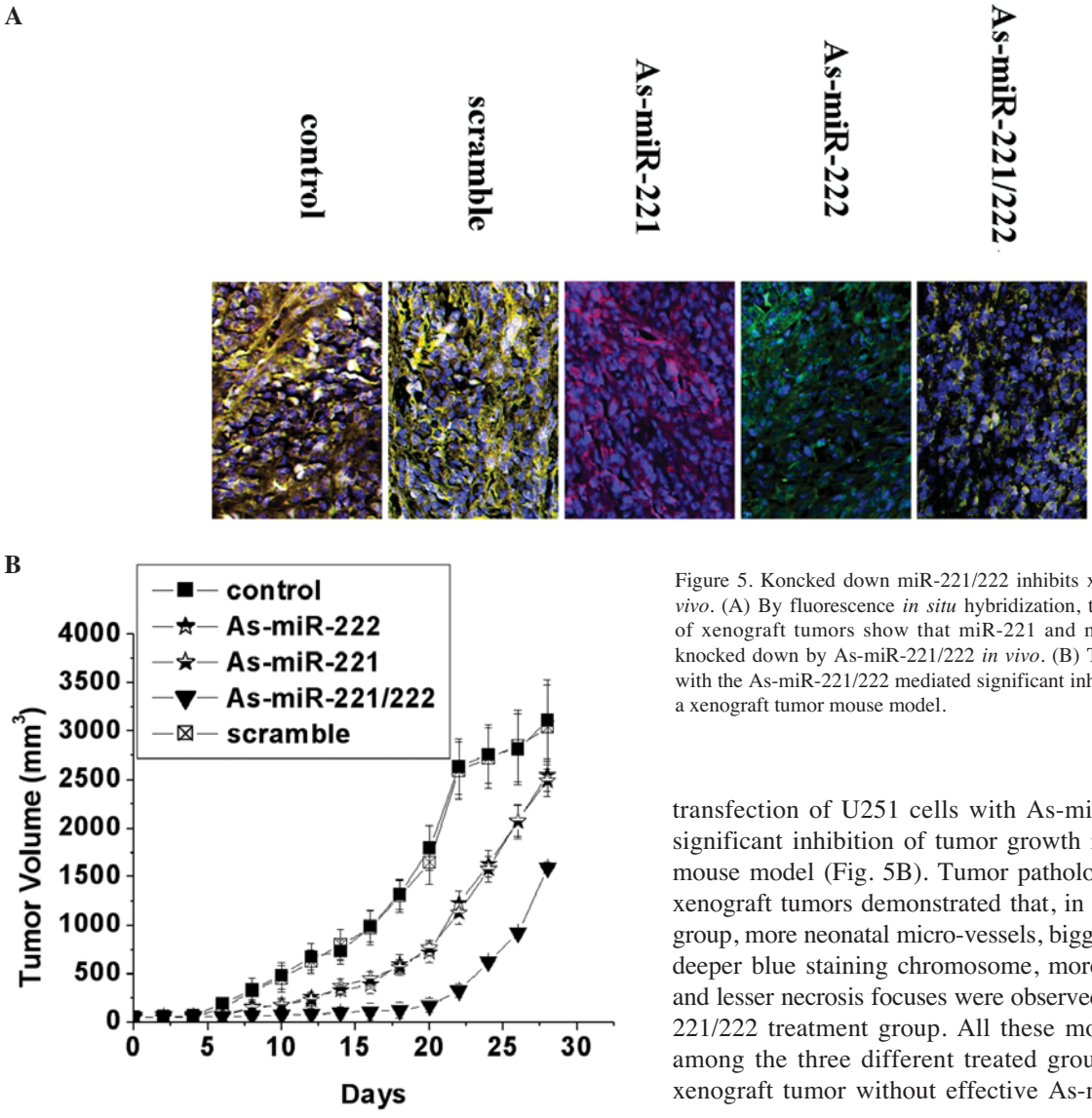

Figure 5. Koncked down miR-221/222 inhibits xenograft tumor growth *in vivo*. (A) By fluorescence *in situ* hybridization, tumor pathological section of xenograft tumors show that miR-221 and miR-222 expression were knocked down by As-miR-221/222 *in vivo*. (B) Transfection of U251 cells with the As-miR-221/222 mediated significant inhibition of tumor growth in a xenograft tumor mouse model.

transfection of U251 cells with As-miR-221/222 mediated significant inhibition of tumor growth in a xenograft tumor mouse model (Fig. 5B). Tumor pathological examination of xenograft tumors demonstrated that, in control and scramble group, more neonatal micro-vessels, bigger tumor cell nucleus, deeper blue staining chromosome, more mitotic tumor cells and lesser necrosis focuses were observed than in the As-miR-221/222 treatment group. All these morphological changes among the three different treated groups indicate that, the xenograft tumor without effective As-miR-221/222 therapy

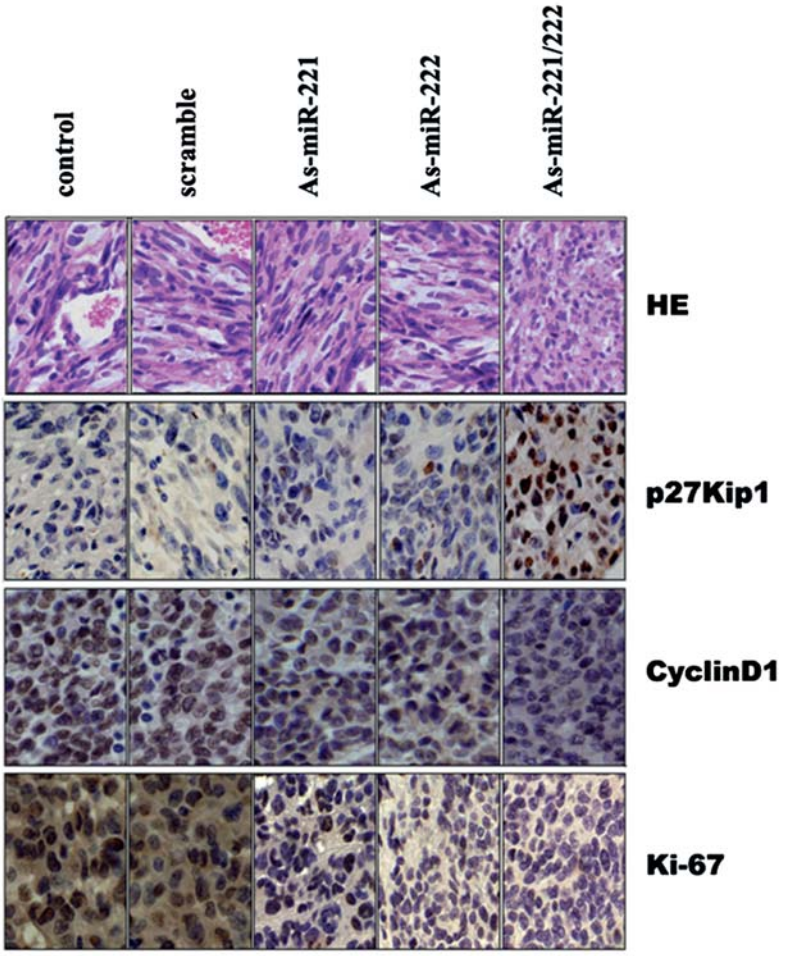

Figure 6. Transfection of the As-miR-221/222 induces up-regulation of the $p27^{kip1}$ expression in xenograft tumors ($p<0.001$).

can perform relatively more malignant pathological manifestations. The As-miR-221/222 treated tumors had a lower level of Ki-67 expression compared to the other four groups. Tumor immunochemistry examination of xenograft tumors demonstrated that the $p27^{kip1}$ expression level in As-miR-221/222 group was up regulated significantly compared to the other four groups and the Cyclin D1 expression levels in As-miR-221/222 group was down-regulated significantly more than the other four groups (Fig. 6).

The results of above-mentioned cell proliferation assays not only revealed that miR-221/miR-222 is related to the tumor cell growth, but also indicate that AS-miR-221/222 can be a potential therapeutic approach for glioblastoma by up-regulating the $p27^{kip1}$.

Discussion

Growing number of studies have demonstrated a link between several forms of human cancer and the expression of miRNAs (9). Accumulating evidence indicates that miR-221/222 are deregulated in certain types of human tumors, such as thyroid papillary carcinomas, prostate carcinomas, lung cancer and glioblastoma (11,14,23,24). Ciafre *et al* used microarray method to compare the global expression levels of 245 miRNAs in glioblastoma tissue with normal brain tissue and found that the expression of miR-221 was strongly up regulated in glioblastoma cells. They identified nine overexpressing miRNAs including the miR-221/222 cluster (14). Furthermore, elevated expression of miR-221/222 in normal thyroid tissue adjacent to tumors was proposed to be an early event in carcinogenesis. In addition, blockade of miR-221/ 222 function and overexpression of miR-221/222 in human PTC-derived cell lines suggest a critical role of miR-221/222 in thyroid carcinogenesis (11). These observations strongly support the notion that miR-221/222 is an important factor in glial carcinogenesis, and thus should be considered as OncomiRs.

The $p27^{kip1}$ was successfully identified as a direct target of miR-221/222 cluster in carcinogenesis *in vitro*. Knocked-down miR-221/222 expression can inhibit tumor cell proliferation (22,23), but single miR-221 antagomiR or single miR-222 antagomiR does not affect cellular growth in U87 glioblastoma cells *in vitro* (22). This indicates a functional overlap between miR-221/222 in controlling cell proliferation. In the current study, we show that co-suppression of miR-221/222 directly results in the up regulation of $p27^{kip1}$ and affects the growth potential in U251 glioma cells. Consistently, U251 cells co-transfected with As-miR-221/222 proliferates at the lowest rate. Flow cytometry analysis shows that cells are blocked in G1 phase in the As-miR-221/222 transfected group. MiR-221/222 knocked-down through antisense 2'-OME-oligonucleotides increases $p27^{kip1}$ in U251

glioma subcutaneous mice and strongly reduces tumor growth *in vivo* through the up-regulation of $p27^{kip1}$. These data prove that As-miR-221/222 inhibit cancer cell growth.

Previous studies indicated that the CDK inhibitor $p27^{kip1}$ is a target of miR-221 and miR-222 (22-24). The $p27^{kip1}$ has a critical role in the control of mammalian cell proliferation. The $p27^{kip1}$ negatively regulates the action of CDKs that are necessary for DNA replication. The levels of $p27^{kip1}$ are higher in quiescent cells, but following growth stimulation by mitogenic stimuli, $p27^{kip1}$ is degraded which allows CDKs to drive cells into S phase. The negative regulatory role of $p27^{kip1}$ in cell cycle progression, as a tumor suppressor in human cancer, is validated by the impairment of $p27^{kip1}$ function in many types of human cancer, which is correlated with tumor aggressiveness. In turn, the increase in cancer cell growth is tightly linked to the G1 to S phase shift, which is in agreement with the modulation of $p27^{kip1}$ (20). By co-transfecting As-miR-221/222, miR-221 and miR-222 are knocked down in U251 glioma cells, resulting in the up regulation of $p27^{kip1}$. Consequently, $p27^{kip1}$ trigers G1 arrest to slow cancer cell growth. In addition, our results indicate that miR-221/222 regulates $p27^{kip1}$ expression in a dose- and time-dependent manner. This is in agreement with the typical regulatory mode of miRNAs acting by modulating the expression levels of their targets, rather than knocking them out completely (6).

Our data show that xenograft tumor growth was inhibited according to As-miR-221/222 treatment and the expression of tumor cell proliferation-related factors, Ki-67 and Cyclin D1, was also suppressed. Cyclin D1 plays an important role in the progression of G1 phase in the cell cycle to mediate glioma cell proliferation. In brief, after knocking down the expression of miR-221 and miR-222, glioma cell malignancies are partially reversed. We assessed that miR-221 and miR-222 control the glioma cell prolifer-ation through regulating $p27^{kip1}$.

In summary, inhibition of specific endogenous miRNAs was achieved by the administration of synthetic anti-sense oligonucleotides that are complementary to the mature endogenous miRNAs. These anti-miRNA oligonucleotides have been shown to specifically inactivate endogenous target miRNAs, although quite inefficiently (18,19). The *in vitro* and *in vivo* evidence demonstrate that antisense approach targeting miR-221/222 can suppress cell proliferation in U251 *in vitro* and *in vivo*. The $p27^{kip1}$ is an important functional target for the miR-221/222 cluster in glioma cells, and co-suppression of miR-221/222 cluster might be used as a potential therapeutic strategy in glioma through up-regulating the $p27^{kip1}$ expression.

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