miR-221/222 promote malignant progression of glioma through activation of the Akt pathway

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Abstract. MicroRNAs (miRNAs) are short regulatory RNAs that negatively modulate protein expression at a post-transcriptional level. Emerging evidence suggests that miRNAs play important roles in the pathogenesis of several types of cancers. However, the further mechanisms of miRNA remain unknown. In this study, we aimed to explore the coordinated function of miR-221/222 in glioma by bioinformatics and experiment methods. Bioinformatics analysis revealed that miR-221/222 had the potential to regulate about 70 common target genes and may exert a cooperative effect on regulation and function via Akt signaling pathway. Overexpression of miR-221/222 increased glioma cell proliferation and invasion *in vitro* and induced glioma growth in a subcutaneous mouse model. Furthermore, miR-221/222 overexpression resulted in an obvious activation of p-Akt and significant changes of Akt-related gene expression in glioma cells. Our results suggest that miR-221/222 co-enhance the glioma malignant phenotype via activation of the Akt pathway mediated by regulation of common gene expression.

Introduction

MicroRNAs (miRNAs) are short non-coding RNA located in non-coding regions or the introns of the genome, and regulate gene expression by binding to the 3'-untranslated regions (3'-UTRs) of specific mRNAs. They are found to regulate apoptosis, proliferation, differentiation, development, and metabolism in worm, fly, fish, mouse, and human cells (1). Each type of cell is likely to have a specific miRNA milieu to control gene expression (2). Each miRNA has the potential to regulate about 200 target genes according to recent computational predictions. Extensive studies have indicated that miRNAs could function as oncogenic miRNAs (oncomiRs) or tumor suppressor miRNAs, playing crucial roles in transformation and carcinogenesis (3). miRNA expression signatures have been used to classify cancers and define miRNA markers that might predict a favorable prognosis (4,5).

Among oncomiRs, miR-221/222 have been proved to be overexpressed in several different types of human cancers, such as glioblastoma (6,7), prostate carcinoma (8,9), papillary thyroid carcinoma (10), hepatocellular cancer (11) and pancreatic cancer (12) suggesting that miR-221/222 are associated with the development and progression of cancer. miR-221/222, located in a cluster on chromosome Xp11.3, are considered part of the same family. They share the same 'seed' sequence, short regions at their 5' ends through which they bind their target sites in mRNA 3'-UTRs. The seed sequence is very well evolutionarily conserved, indicating an important role in coordinated regulation and function. Several genes have been evidenced to be common targets of these two miRNAs, such as $p27^{kip1}$ (6), p57 (13) and kit (10).

Taken together, we aimed to clarify whether overexpression of miR-221/222 might co-enhance the malignant phenotype in glioma cells through a similar pathway. By bioinformatics analysis, 70 common target genes of miR-221/222 were generated and 16 of these genes represented direct or indirect interaction with Akt. Overexpression of miR-221/222 increased glioma cell proliferation and invasion *in vitro* and induced glioma growth in a subcutaneous mouse model. Furthermore, miR-221/222 activated the Akt pathway by regulation of common gene expression in gliomagenesis.

Materials and methods

Cell culture and culture conditions. Human U251 glioblastoma cells and rat C6 glioma cells were obtained from China

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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, and incubated at 37˚C with 5% CO_2.

Bioinformatics method. The common targets of miR-221/222 predicted by computer-aided algorithms were obtained from multiple target prediction programs (PicTar http://pictar.mdc-berlin.de/, TargetScan http://www.targetscan.org/ and miRanda http://www.microrna.org/). For enrichment analysis, the common target gene list was set as a test dataset and the human gene proteome was set as a reference. All gene ontology (GO) terms that were significant with adjusted $P<0.05$ (after correcting for multiple term testing by using the FDR procedure of Bonferroni-Hochberg) were selected as overrepresented or underrepresented. Pathway Studio software (Ariadne Genomics, MD, USA), a specialized graph visualization engine, was used to determine the relevant pathways of the common target proteins. Each identified pathway was confirmed via the PubMed/Medline hyperlink embedded in each node.

Generation of retroviral recombinants and transduction. pMSCV-miR-221 vector and pMSCV-miR-222 vector were kindly provided by Reuven Agami (Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands). The procedures used for production of the retroviral recombinants have been described previously (14). The retroviral recombinants pMSCV-miR-221 and pMSCV-miR-222 used for transduction were produced by transfection of packaging cells PT67 by Lipofectamine 2000. The titers of the homogeneous viruses were calculated after infection of NIH3T3 cells.

Once cells grew to 70-80% confluence, U251 and C6 cells were infected with pMSCV-miR-221 and/or pMSCV-miR-222 at a multiplicity of infection (MOI) of 50 at 37˚C. At 4 h after the infection, the medium was replaced with fresh DMEM with 10% fetal bovine serum, and the cells were incubated for an additional 48 h. Then the infected cells were harvested for further study, and divided into 4 groups: control group, miR-221group, miR-222 group and miR-221/222 group.

Cell viability assay. Cells were seeded into 96-well plates at 2000 cells/well. After transfection, 20 μl of MTT (5 g/l) was added into each well and incubated for 4 h, and then the supernatant was discarded, for 5 consecutive days after treatment. Finally, 200 μl of DMSO was added to each well to dissolve the precipitate. Optical density (OD) was measured at the wavelength of 490 nm. The data are presented as the mean ± SD, derived from triplicate samples of three independent experiments.

In vitro invasion assays. Transwell membranes coated with Matrigel (BD Biosciences, San Jose, CA) were used to assay invasion of glioma cells *in vitro*. Transfected cells were plated at $5x10^4$ per well in the upper chamber in serum-free medium. FBS (10%) was added to the medium in the lower chamber. After incubating 24 h, non-invading cells were removed from the top well with a cotton swab while the bottom cells were fixed with 3% paraformaldehyde, stained with hematoxylin, and photographed in three independent 400x fields for each well. Three independent experiments were done and the penetration proportion of cells in Matrigel was calculated.

For wound-healing migration assay, the cells were seeded on 6-well plates at a density of $5x10^5$ cells/well in culture medium. Cells were placed in serum-free medium 24 h prior to the assay. The confluent monolayer was then disrupted with a cell scraper. After incubating for 48 h, five random 200x fields of each well were recorded. Experiments were done in triplicate. The rate of wound closure was calculated by the average cell number of migration between the two wound edges.

Western blot analysis. Western immunoblot analysis was used to examine the protein expression after transfection. Parental and transfected cells were washed with pre-chilled phosphate-buffered saline (PBS) 3 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). Protein concentrations were measured by Nanodrop spectrophotometer (Gene, USA). Equal amounts of protein per lane were separated by 8% SDS-polyacrylamide gel and transferred to PVDF membrane. The membrane was blocked in 5% skim milk for 1 h and then incubated with a specific antibody for 2 h. The antibodies used for Western blot analysis in this study were: antibodies to p-Akt (Ser473), PTEN (Santa Cruz, USA), $p27^{kip1}$, MMP-2, MMP-9, TIMP-3 (Zhongshan, China). 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).

Subcutaneous tumor assay. BALB/c-A nude mice at 6 weeks of age were purchased from animal center of the Cancer Institute of Chinese Academy of Medical Science. 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 (15). When the subcutaneous tumor reached 50 mm^3 in size, the mice were randomly divided into 2 groups (5 subcutaneous tumors/group). A mixture of 20 μl PBS, equal amount of pMSCV-miR-221 and pMSCV-miR-222 was injected into the xenograft tumor model in a multi-site injection manner. Treatment was conducted every 4 days. The tumor volume was measured with a caliper every 4 days, using the formula volume = length x $width^2/2$ (16).

Immunohistochemistry assay. Twenty-eight days after subcutaneous implantation of tumor cells, mice were sacrificed and paraffin-embedded tissue sections were used for examination of p-Akt, MMP-2, MMP-9, PCNA, $p27^{kip1}$, PTEN and TIMP-3 expression in the tumors. 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 30 min, followed by incubation with ABC-peroxidase

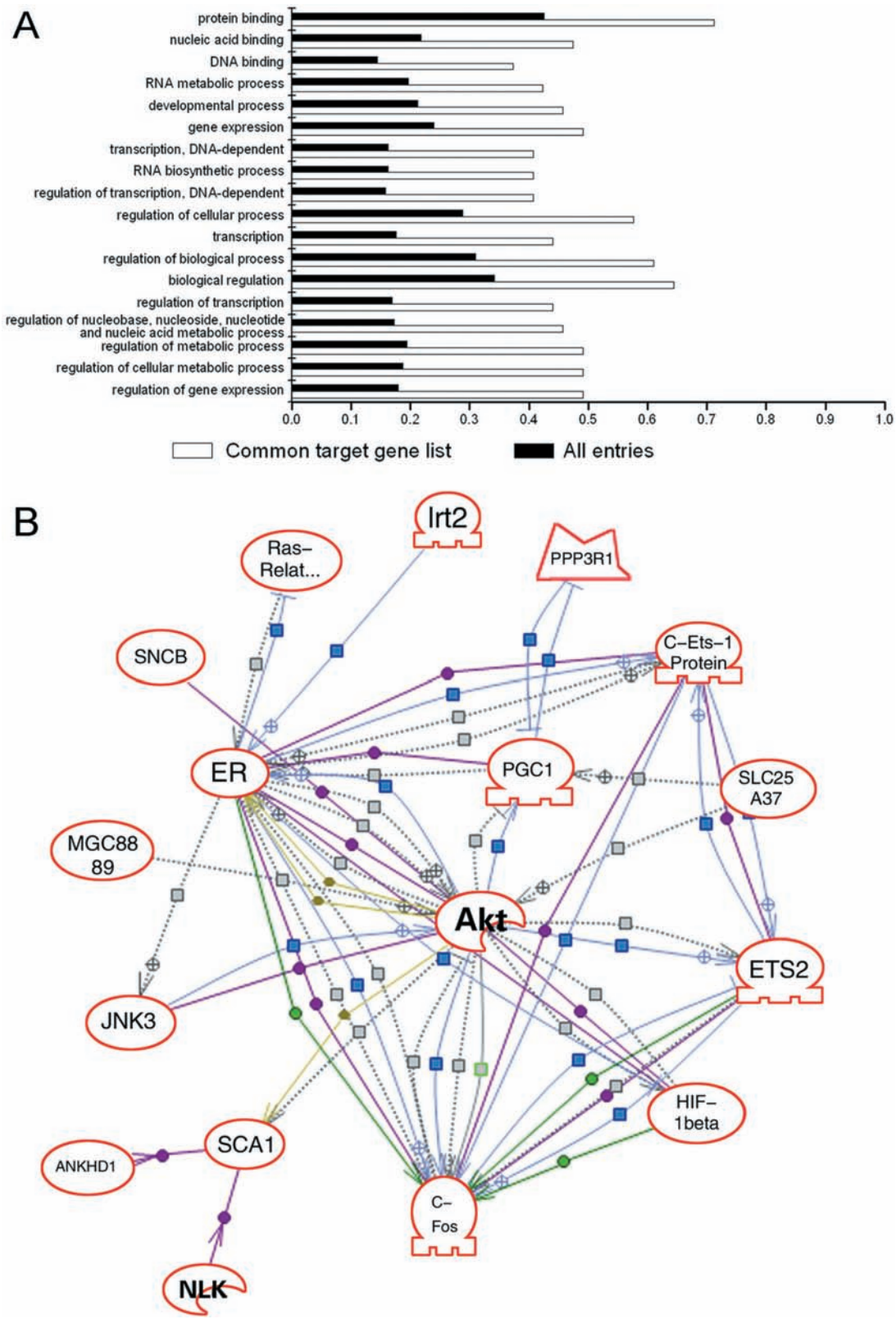

Figure 1. Bioinformatics analysis of miR-221/222. (A) Overrepresented annotations of the common targets of miR-221/222. The set of the common targets was compared with the proteomic database, and significantly GO molecular function and biological process terms ($P<0.05$) are illustrated. (B) Pathway analysis of the common targets of miR-221/222. By Pathway Studio, 16 common targets of miR-221/222 represented direct or indirect interaction with Akt. Proteins are indicated as red ovals, and regulation events are displayed with arrows and documented by literature citations.

for an additional 30 min. 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 were analyzed with SPSS 10.0. ANOVA and t-test were used to analyze the significance between groups. Statistical significance was assigned to P-values <0.05.

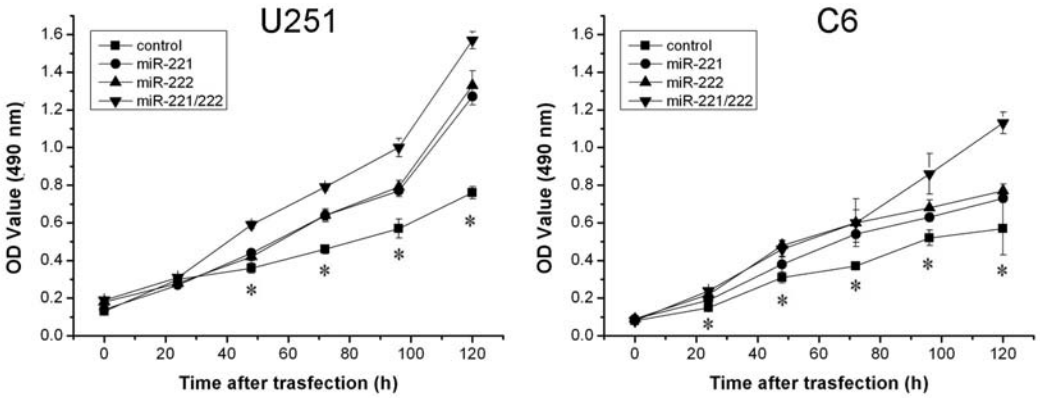

Figure 2. miR-221/222 increase cell proliferation. MTT assay showed that U251 and C6 cells transfected with miR-221/222 proliferated at a significantly higher rate than the other three groups. There was no difference between miR-221 group and miR-222 group in the experiment. *P<0.05 compared with control group.

Results

Bioinformatics analysis of miR-221/222. Because each target prediction program uses a different computer-aided algorithm for prediction, encompassing all these methods will probably produce a more reliable model of target prediction. Thus, a common target gene list of miR-221/222 was generated from 3 target prediction programs (PicTar, TargetScan and miRanda). This procedure obtained 70 common targets. To examine what biologically important entries are enriched in the common targets of miR-221/222, we compared our dataset with the proteomic database, 3 GO terms (protein binding, nucleic acid binding, DNA binding) for molecular function appeared to be significantly overrepresented, and none significantly underrepresented. In the biological process category, 15 GO terms appeared to be significantly overrepresented, and none significantly underrepresented (Fig. 1A). To further explore the common pathway in the common targets of miR-221/222, Pathway Studio was performed. Pathway Studio includes an automated text-mining tool which enables the software to generate pathways from the PubMed database and other public sources. Fig. 1B revealed that 16 (22.8%) gene products represented direct or indirect interaction with Akt, suggesting that miR-221/222 were both related with Akt signaling pathway. Therefore, the data of bioinformatics analysis indicated that miR-221/222 might exert a coordinated effect on regulation and function through a similar pathway.

miR-221/222 increase cell proliferation. To study the biological function of miR-221/222 on cell proliferation of glioma cells, cell viability of U251 and C6 glioma cells was measured by MTT assay. As shown in Fig. 2, statistically significant cell proliferation of U251 cells was found in miR-221, miR-222 and miR-221/222 groups compared with control group at 48 h after transfection, while cell proliferation of C6 cells started to reach statistical significance at 24 h after transfection. There was no difference between miR-221 group and miR-222 group in the whole experiment. However, miR-221 or miR-222 exerted lower effects on U251 and C6 cells than miR-221/222, indicating that combination treatment with miR-221/222 had the most robust effect on cell proliferation.

miR-221/222 promote cell invasion. To measure effects of miR-221/222 on the invasive ability of glioma cells, the *in vitro* Matrigel invasion assay and wound-healing migration assay were employed. As observed in the Matrigel invasion assay, the penetration proportions of U251 cells in Matrigel were 0.33±0.02, 0.40±0.03, 0.51±0.01, 0.80±0.03, respectively, for control, miR-221, miR-222 and miR-221/222 group (P<0.01). Significant penetration proportion of C6 cells in Matrigel was also observed. Next we found that the Matrigel invasion capacity of miR-221/222 group was significantly increased compared with miR-221 or miR-222 group, respectively (Fig. 3A). In wound-healing migration assay, similar trend of cell migration was detected in U251 and C6 cells (Fig. 3B). Taken together, the results showed a cooperative effect of miR-221/222 on glioma cell invasion.

Gene regulation of miR-221/222 at the protein level in vitro. In order to further explore the molecular mechanism of miR-221/222 involved in glioma cell proliferation and invasion, Western blot analysis was used. As is well-known, Akt signaling pathway is involved in cell survival and invasion. We determined Akt-related pathway after miR-221/222 overexpression. After transfection with miR-221/222, U251 and C6 cells were solubilized and subjected to Western blot analysis. As shown in Fig. 4, an obvious activation of phosphorylated-Akt was observed both in U251 and C6 cells. Consistently, overexpression of miR-221/222 led to a marked induction of MMP-2 and MMP-9 protein expression, which were involved in downstream pathway of Akt. These results suggested that introduction of miR-221/222 promoted glioma cell proliferation and invasion via the Akt pathway.

Our previous study and other studies have demonstrated that $p27^{kip1}$ is a common target gene of miR-221/222 (6,17). There was an inverse correlation between $p27^{kip1}$ and miR-221/222 expression level. Therefore, we investigated whether $p27^{kip1}$ expression level was down-regulated after miR-221/222 overexpression. Coordinately, overexpression of miR-221/222 resulted in a notable decrease of $p27^{kip1}$ protein expression in U251 and C6 cells (Fig. 4). Additionally, some miRNAs may function as oncomiRs or tumor suppressor miRNAs involved in the pathogenesis of tumors by regulating oncogenes or tumor-suppressor genes (2). In this regard, we further explored whether other genes correlated with oncogenes or tumor-suppressor genes participated in miR-221/222 regulation. Western blot analysis showed that a lower protein expression level of PTEN was seen in miR-221/222 group, although this

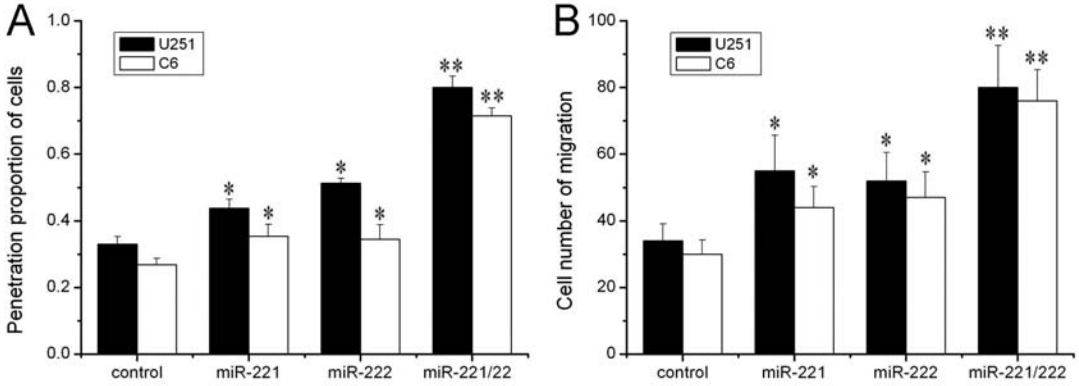

Figure 3. miR-221/222 promote cell invasion. (A) In the Matrigel invasion assays, penetration proportion of U251 and C6 cells was significantly increased in miR-221/222 group. (B) In wound-healing migration assay, similar trend of cell migration was also detected. *P<0.05 compared with control group, **P<0.01 compared with control group.

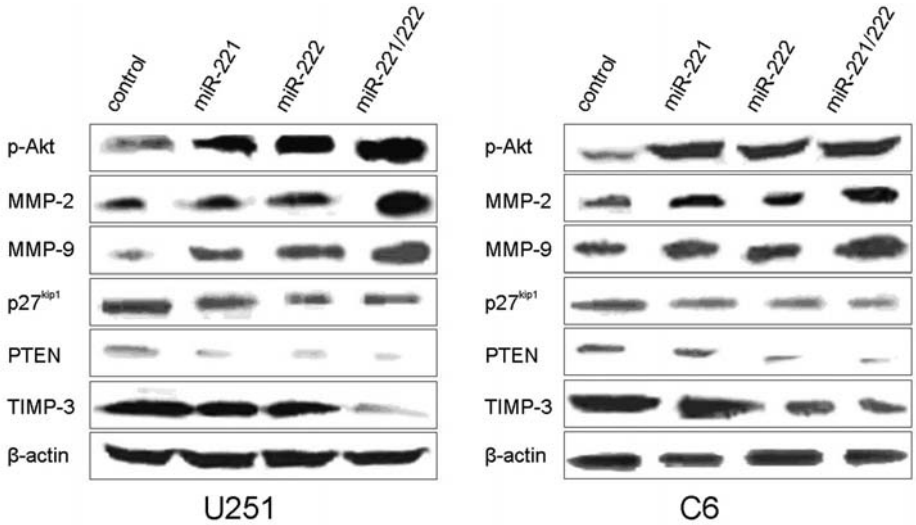

Figure 4. Gene regulation of miR-221/222 at the protein level *in vitro*. U251 and C6 cells were transfected with miR-221/222, and p-Akt, MMP-2, MMP-9, $p27^{kip1}$, PTEN and TIMP-3 were analyzed by Western blotting. Expression of ß-actin was used as a loading control.

difference did not reach statistical significance (P=0.054). This may be because U251 cells have mutant PTEN and very low PTEN expression. However, overexpression of miR-221/222 led to a remarkable decrease of PTEN in C6 cells. TIMP-3 expression was observed to decrease significantly in miR-221, miR-222 and miR-221/222 group compared with control group (Fig. 4).

miR-221/222 enhance xenograft tumor growth in vivo. Given that cell proliferation and invasion were induced in glioma cells *in vitro* following infection with pMSCV-miR-221 and pMSCV-miR-222, next we evaluated the effect of miR-221/222 on tumor growth in a xenograft glioma mouse model. On day 12, tumors of miR-221/222 group started to reach statistical significance compared with control group (355.5±35.3 versus 231.4±48.6 mm^3, P<0.05). At the termination of the study, the difference in tumor mass between miR-221/222 group and control group was marked (P<0.01). As shown in Fig. 5, the average tumor volumes on day 28 were 914.5 and 1478.7 mm^3 in control group and miR-221/222 group, respectively.

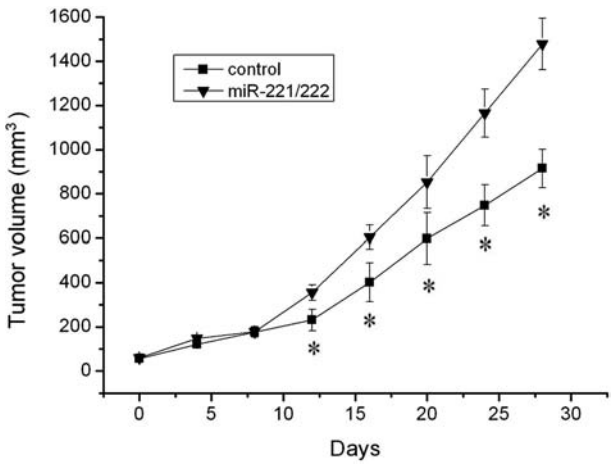

Figure 5. miR-221/222 enhance xenograft tumor growth *in vivo*. When subcutaneous tumors were established, pMSCV-miR-221 and pMSCV-miR-222 were injected in a multi-site injection manner every 4 days. Tumor volumes were measured every 4 days during treatment. miR-221/222 efficiently enhanced tumor growth *in vivo*. *P<0.05 compared with control group.

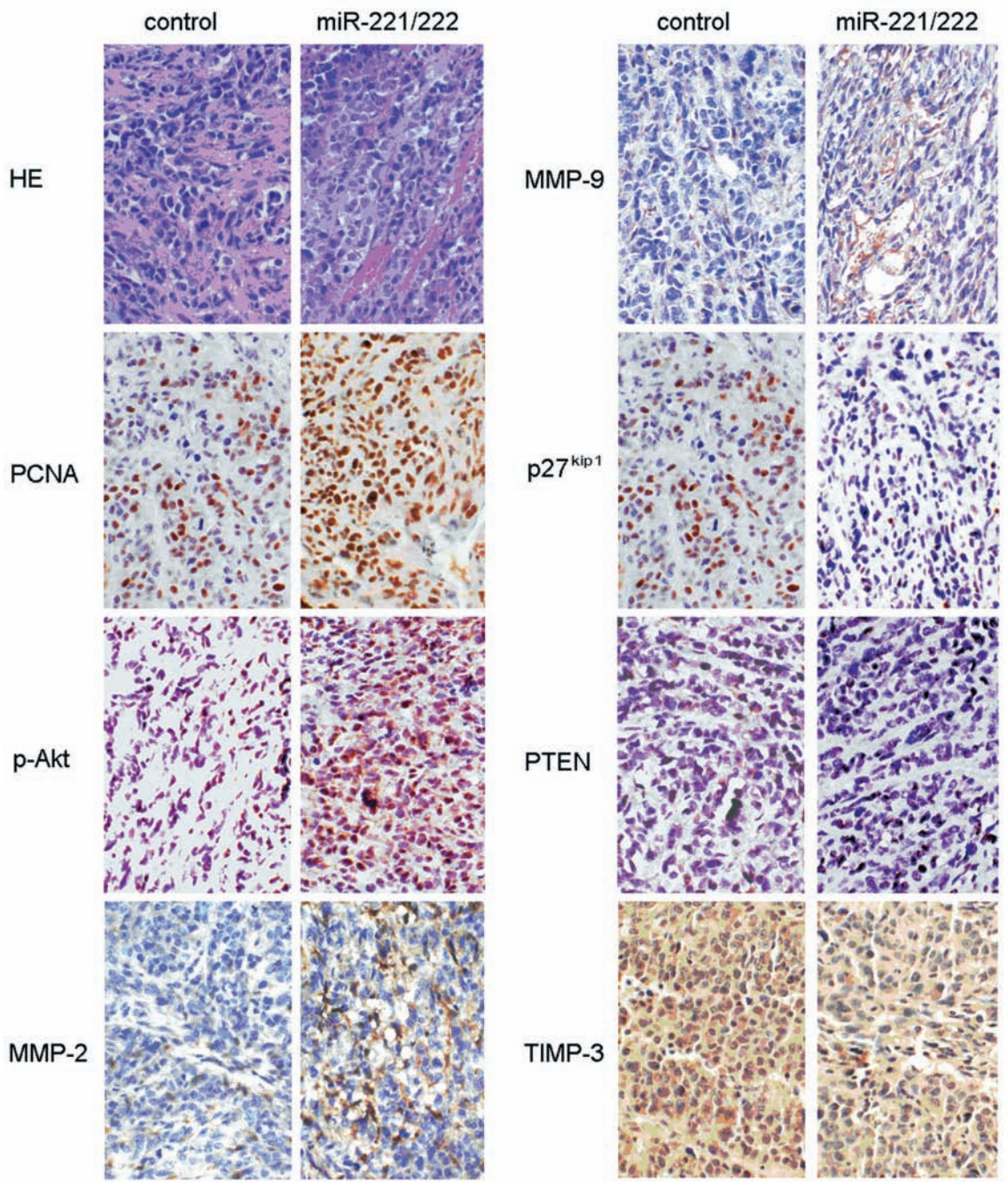

Figure 6. Immunohistochemistry analysis of xenograft tumors after miR-221/222 treatment. The expression levels of PCNA, p-Akt, MMP-2 and MMP-9 were increased in U251 xenograft tumors of miR-221/222 group. Additionally, the expression levels of $p27^{kip1}$, PTEN and TIMP-3 were suppressed.

Gene regulation of miR-221/222 at the protein level in vivo. Tumor pathological examination of xenograft tumors demonstrated that in miR-221/222 group, more neonatal microvessels, bigger tumor cell nucleus and deeper blue staining chromosome were observed than those in control group (Fig. 6). All these morphological changes indicated that the xenograft tumor with effective miR-221/222 combination treatment could perform more malignant pathological phenotypes. miR-221/222 treated tumors had a higher level of PCNA expression compared to control group. In order to verify the data of the gene expression level induced by miR-221/222 overexpression *in vitro*, we determined the protein expression of these genes by immunohistochemistry assay. Tumor immunohistochemistry examination of xenograft tumors displayed that the phosphorylation of Akt in miR-221/222 group was up-regulated significantly compared with control group (Fig. 6). Consistently, the expression levels of MMP-2 and MMP-9 in miR-221/222 group increased significantly. Additionally, similar effects on $p27^{kip1}$, PTEN and TIMP-3 were achieved in miR-221/222 group, in line with the *in vitro* experiments.

Discussion

Accumulating evidence indicates that miR-221/222 participate in development and progression of human tumors, such as

thyroid papillary carcinomas, prostate carcinomas and glioblastoma. In our results, overexpression of miR-221/222 cooperated to enhance malignant phenotype of U251 and C6 glioma cells *in vitro*. miR-221/222 exerted higher effects on glioma cell proliferation and invasion than single miR-221 or single miR-222, consistent with previous data. Knocked-down miR-221/222 expression inhibited tumor cell proliferation, but single miR-221 antagomiR or single miR-222 antagomiR did not affect cellular growth in U87 glioma cells *in vitro* (17). Our previous study showed that single suppression of miR-221 or miR-222 induced lower glioma growth inhibition *in vivo* than co-suppression of miR-221/222 (6). In our *in vivo* study, miR-221/222 overexpression promoted glioma growth in a xenograft tumor mouse model, confirming the experiment *in vitro*.

miR-221/222 are highly conserved and share the same seed regions at their 5' ends. It has been shown that $p27^{kip1}$, p57 and kit are the common targets of miR-221/222 in carcinogenesis. However, the further mechanisms are not clear and still under investigation. Therefore, we used bioinformatics method to clarify the common targets of miR-221/222 in human. Data suggested that miR-221/222 had the potential to regulate about 70 common target genes according to computer prediction. By examining the GO database, these common targets impacted strongly on 18 GO terms for molecular function and biological process. Pathway Studio revealed that 16 of 70 common targets represented direct or indirect interaction with Akt. Taken together, the common pathways, especially Akt signaling pathway, appeared to be involved in miR-221/222 regulation. Akt, the serine-threonine protein kinase, mediates many of the downstream effects of PI3K and consequently plays an important role in human malignancy. Akt phosphorylates a variety of substrates involved in the regulation of oncogenesis. Akt promotes glioma cell survival by activating mTOR, TSC2, and S6. Akt also contributes to glioma cell invasion by increasing expression of MMPs (18,19). Akt can be activated by a number of growth factors and their receptors, especially EGFR, and is negatively regulated by PTEN (20-22). Overexpression or mutation of EGFR may also potentially activate AkT in glioblastoma. Loss of PTEN in glioma is highly correlated with activation of AkT. Therefore, Akt activation frequently occurs in the majority of malignant gliomas. Our Western blot analysis revealed that overexpression of miR-221/222 led to an obvious activation of phosphorylated-Akt in U251 cells. To confirm these data, we determined Akt activation after overexpression miR-221/222 in C6 cells and similar results were obtained. Thus, our study indicated that Akt pathway contributed to miR-221/222 regulation in gliomagenesis.

$p27^{kip1}$, successfully identified as a direct target of miR-221/222, has a major contribution to the control of cancer cell proliferation and invasion. In the human androgen-independent prostate cancer PC3 cell line, exogenous expression of $p27^{kip1}$ inhibits the proliferation of PC3 cells through induction of G1 arrest and apoptosis, and this process correlates with inhibition of EGFR/PI3K/Akt signaling pathway (23). In the human breast cancer cell line MDA-MB-231, up-regulation of $p27^{kip1}$ remarkably inhibits the invasion of the breast cancer cells, in part due to the reduced expression of MMP-9 (24). In contrast, Akt regulates cell proliferation in breast cancer cells by preventing $p27^{kip1}$-mediated growth arrest (25). EGFR can enhance cell proliferation in part by down-regulation of $p27^{kip1}$ through activation of PI3K/Akt pathway (22). Thus, we are unable to convincingly show which effector, $p27^{kip1}$ or Akt was involved and how miR-221/222 regulated the Akt signalling pathway. This could be the result of a direct or indirect interaction between miR-221/222 and more genes that contribute to the Akt signaling pathway. For example: i) miR-221/222 could suppress targets that inhibit or buffer the oncogenic signals generated from the Akt pathway. ii) miR-221/222 could augment the effects of Akt signaling, by repressing a transcriptional inhibitor of a subset of Akt target genes. To verify this hypothesis, we evaluated the uptream regulators of the Akt signaling pathway *in vitro* and *in vivo*. The expression levels of PTEN and TIMP-3 were observed to decrease after miR-221/222 overexpression. PTEN, as a tumor suppressor, led to inhibition of cell growth by negatively regulating the PI3K/Akt cascade (26). TIMP-3, a target of miR-221/222 by computer prediction, had an antitumor effect via inhibition of PI3K/Akt activity (27). Therefore, these results may provide a model to explain the complex pathways of miRNA regulation.

In conclusion, we showed that 70 genes appeared to be common targets of miR-221/222 by computer prediction. GO and Pathway Studio analysis of these common targets indicate that miR-221/222 may exert a coordinated effect on regulation and function via Akt signaling pathway. Furthermore, overexpression of miR-221/222 promotes U251 cell growth and invasion *in vitro* and xenograft tumor growth *in vivo*, cooperatively. Finally, miR-221/222 may co-regulate multiple common genes and then activate the Akt pathway in gliomagenesis. Therefore, the results of the present study suggest that the modulation of the mechanism responsible for miR-221/222 in glioma could be used as a therapeutic strategy to treat glioma and warrants further investigation.

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