Co-inhibition of microRNA-10b and microRNA-21 exerts synergistic inhibition on the proliferation and invasion of human glioma cells

CHANG G. DONG[1*], WILLIAM K.K. WU[2], SU Y. FENG[3*], XIAO J. WANG[2], JUN F. SHAO[4] and JIAN QIAO[1]

[1]Department of Pathophysiology, College of Veterinary Medicine, China Agricultural University, Beijing 100094; [2]Institute of Digestive Disease, Department of Medicine and Therapeutics and LKS Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong; [3]Department of Neurosurgery, Wuxi No. 4 People's Hospital affiliated to Soochow University, Wuxi 214062; [4]Department of Neurosurgery, Wuxi People's Hospital of Nanjing Medical University, Wuxi 214023, P.R. China

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Abstract. MicroRNAs (miRNAs) are small non-coding RNAs that function as negative gene regulators. Alterations in the expression of miRNAs have been implicated in the pathogenesis and development of most human malignancies. Recent data indicate that microRNA-21 and microRNA-10b are significantly elevated in glioblastoma multiforme (GBM) suggesting their role in the regulation of multiple genes associated with cancer. In this study, U87MG human glioblastoma cells were treated with miRNA inhibitors targeting miR-10b and miR-21, alone or in combination. The results showed that the miR-21 inhibitor additively interacted with miR-10b inhibitor on U87MG cells. The 50% inhibitory concentration values were dramatically decreased in cells treated with the combination of miR-10b and miR-21 inhibitors. Furthermore, inhibitors synergistically combined, enhanced apoptosis significantly and reduced invasion ability assessed by flow cytometry and Transwell migration assay. Thus, the miR-21 inhibitor may interrupt the activity of EGFR pathways, increasing PDCD4 and TPM1 expression and reducing MMP activities, independently of PTEN status. Meanwhile, miR-10b inhibitor reduced by Twist proceeds to inhibit translation of the mRNA encoding HOXD10 leading to the increase of the expression of the well-characterized pro-metastatic gene RHOC. Taken together, these data strongly suggest that a combination of miR-21 inhibitor and miR-10b inhibitor could be an effective therapeutic strategy for controlling the growth of GBM by inhibiting oncogene expression and overexpressing tumor suppressor genes. Moreover, a regulatory strategy based on the combination of miRNA inhibitors may provide insights into the mechanisms of the modulation of signaling genes involved in tumor cell apoptosis and invasiveness.

Correspondence to: Dr Jun F. Shao, Department of Neurosurgery, Wuxi People's Hospital of Nanjing Medical University, Wuxi 214023, P.R. China
E-mail: shaojunfei@sina.com

Dr Jian Qiao, Department of Pathophysiology, College of Veterinary Medicine, China Agricultural University, Beijing 100094, P.R. China
E-mail: qiaojiancau@126.com

*Contributed equally

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Introduction

Malignant gliomas are the most common primary brain tumors with high mortality and morbidity. Despite recent advancements in chemotherapy, radiotherapy and neurosurgery, the prognosis of patients with malignant gliomas remains dismal (1). So there is a urgent need to develop novel and effective therapeutic strategies for this disease.

MicroRNAs (miRNAs) are a large family of endogenous non-protein-coding small RNAs with 20-25 nucleotides in length (2). miRNAs control gene expression through binding to the 3'-untranslated regions (UTRs) of target mRNAs, resulting in translational repression and/or mRNA degradation (3). Emerging evidence supports that miRNAs are novel players in carcinogenesis (4,5). In this regard, miRNAs are dysregulated in most, if not all, types of human cancer examined so far. Dysregulated miRNAs take part in tumorigenesis by regulating cellular processes important to cancer development, including cell proliferation, cell survival, cell motility and invasiveness, angiogenesis, and drug resistance (6-8). Targeting upregulated miRNAs have been shown to suppress malignant phenotypes of cancer cells and may therefore represent a novel treatment approach (6).

Accumulating evidence suggests that miR-21 and miR-10b may behave as novel oncogenes in human cancers. miR-21 has been identified as one of the most upregulated miRNAs in human cancers. Increased level of plasma miR-21 has also been demonstrated in some cancer types (9). In human gliomas, miR-21 is upregulated during the progression of the tumor.

Importantly, when miR-21 is targeted in glioma tissues, inhibition of cell proliferation and caspase-dependent apoptosis occur (10,11). Several tumor-suppressors, including programmed cell death 4 (PDCD4) and tropomyosin (TPM1), have been identified as direct targets of miR-21 (12,13). Regarding miR-10b, this putatively oncogenic miRNA was initially found to be highly expressed in metastatic breast cancer cells. miR-10b also positively regulates the migration and invasion of breast cancer cells (14,15). Overexpression of miR-10b in otherwise non-metastatic breast tumors initiates robust invasion and metastasis. Recently, miR-10b was reported to be upregulated in glioma tissues compared with non-tumoral brain tissues. Upregulation of miR-10b was also associated with higher grade gliomas (16). In relation to the intracellular signaling network, miR-10b has been shown to target the tumor suppressor HOXD10, which in turn regulates the expression of metastasis-related genes, such as RhoC and urokinase-type plasminogen activator receptor (14,17).

Although miR-21 and miR-10b are upregulated in glioma tissues, their interactions in the regulation of oncogenic phenotypes and pathways are unknown. Moreover, targeting these two oncogenic miRNAs simultaneously may exert a more potent anti-cancer effect as compared with inhibition of either miRNA alone. In the present study, using synthetic miRNA inhibitors, we demonstrate that inhibition of miR-21 and miR-10b synergistically induced cell cycle arrest and apoptosis and inhibited cell invasiveness in a human glioma cell line. Effects of co-inhibition of miR-21 and miR-10b on the expression of several putative cellular targets were also examined.

Materials and methods

Cell line and reagents. The human glioblastoma cell line U87MG was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The 2'-O-methyl (2'-OMe-) miR-10b and miR-21 inhibitors were chemically synthesized by Invitrogen (Carlsbad, CA, USA) with the following sequences: miR-10b inhibitor: 5'-CAC AAA UUC GGU UCU ACA GGG UA-3'; miR-21 inhibitor: 5'-UCA ACA UCA GUC UGA UAA GCU A-3'; scrambled control: 5'-UCU UCA UGA GUC AGA UUA CCU A-3'. The sequence of scrambled control has been analyzed by BLAST search to exclude potential hits in the human transcriptome. The oligonucleotides were purified by high-pressure liquid chromatography, dissolved in diethylpyrocarbonate water, and frozen at -20˚C. The final working concentrations of scrambled control, miR-10b inhibitor, miR-21 inhibitor and combined miRNA inhibitors were 100 nmol/ml.

Cell culture and transfection. U87MG cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine (Sigma, St. Louis, USA), 100 units of penicillin/ml (Sigma), and 100 μg of streptomycin/ml (Sigma). Cells were seeded in 25-cm^2 flasks and incubated at 37˚C with 5% CO_2. Twenty-four hours before transfection, cells at 70-90% confluence were detached, transferred to 96-well or 6-well plates, and cultured in fresh medium without antibiotics. Oligonucleotides (5-100 pmol) were transfected into U87MG cells at 70% confluence using Lipofectamine-2000 (0.25-5 μl) according to the manufacturer's instructions (Invitrogen). Transfection efficiency was examined by fluorescence microscopy detection of FAM green fluorescence signal (Abs 495nm, Em 520nm) that was labeled to the scrambled control.

RNA extraction and real-time reverse transcription-PCR for miRNA detection. miRNA was isolated 48 h after transfection with Ambion mir-Vana™ miRNA isolation kit (Ambion, USA). A nanodrop spectrophotometer (Gene, USA) was used to measure the concentration of total RNA. Relative levels of miRNA were examined using SYBR green real-time quantitative reverse transcription-PCR (qRT-PCR) (Applied Biosystems) and normalized with U6 snRNA. RT and PCR primers were purchased from Ambion. The amplification reaction was performed using MJ real-time PCR (Bio-Rad, Hercules, CA, USA) for 40 cycles. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (18). All qRT-PCRs were performed in triplicate, and analyzed initially using Opticon Monitor Analysis software V2.02 software (MJ Research, USA).

Protein extraction and western blotting. After transfection, total proteins were extracted after solubilization in lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1 mM sodium vanadate, and 1 mM sodium fluoride. Protein extracts were resolved on a Tris-HCl 11% gradient gel. Western blotting was performed using standard methodologies as previously described (19). Blots were developed using the enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia, Buckinghamshire, UK) and visualized using the Gene-Genius Imaging System (Syngene, Frederick, MD, USA).

Cell viability assay. Cell viability was determined by the MTT assay. Briefly, 5,000 transfected cells per well were seeded in 96-well plates and allowed to attach overnight. On each day of five consecutive days, 20 μl of MTT (0.5 mg/ml) was added to the wells and the cells were incubated at 37˚C for 4 h. The reaction was then stopped by lysing the cells with 200 μl of dimethyl sulfoxide (DMSO) for 15 min. Measurements of optical density were obtained at the wavelength of 570 nm using spectrophotometric analysis. The half maximal inhibitory concentration (IC_{50}) values were calculated from the linear regression line of the plot of percentage inhibition versus log inhibitor concentration.

Cell cycle analysis. Cell cycle was analyzed flow cytometry. In brief, transfected and control cells in the log phase of growth were harvested, washed with phosphate-buffered saline, fixed with 90% ethanol overnight at 4˚C, and then incubated with RNase at 37˚C for 30 min. The nuclei of cells were stained with propidium iodide (PI) for another 30 min. A total of 10^4 cells were examined in a FACSCalibur flow cytometer (Becton-Dickinson, USA). Samples were analyzed by flow cytometry for the FL-2 area and DNA histograms were analyzed by Modifit software. Experiments were performed in triplicate. Results are presented as percentage of cells in a particular phase.

Quantitation of apoptosis. To quantify miRNA inhibitor-induced apoptosis, annexin V/PI staining was performed, and apoptosis was evaluated by flow cytometry analysis. Briefly,

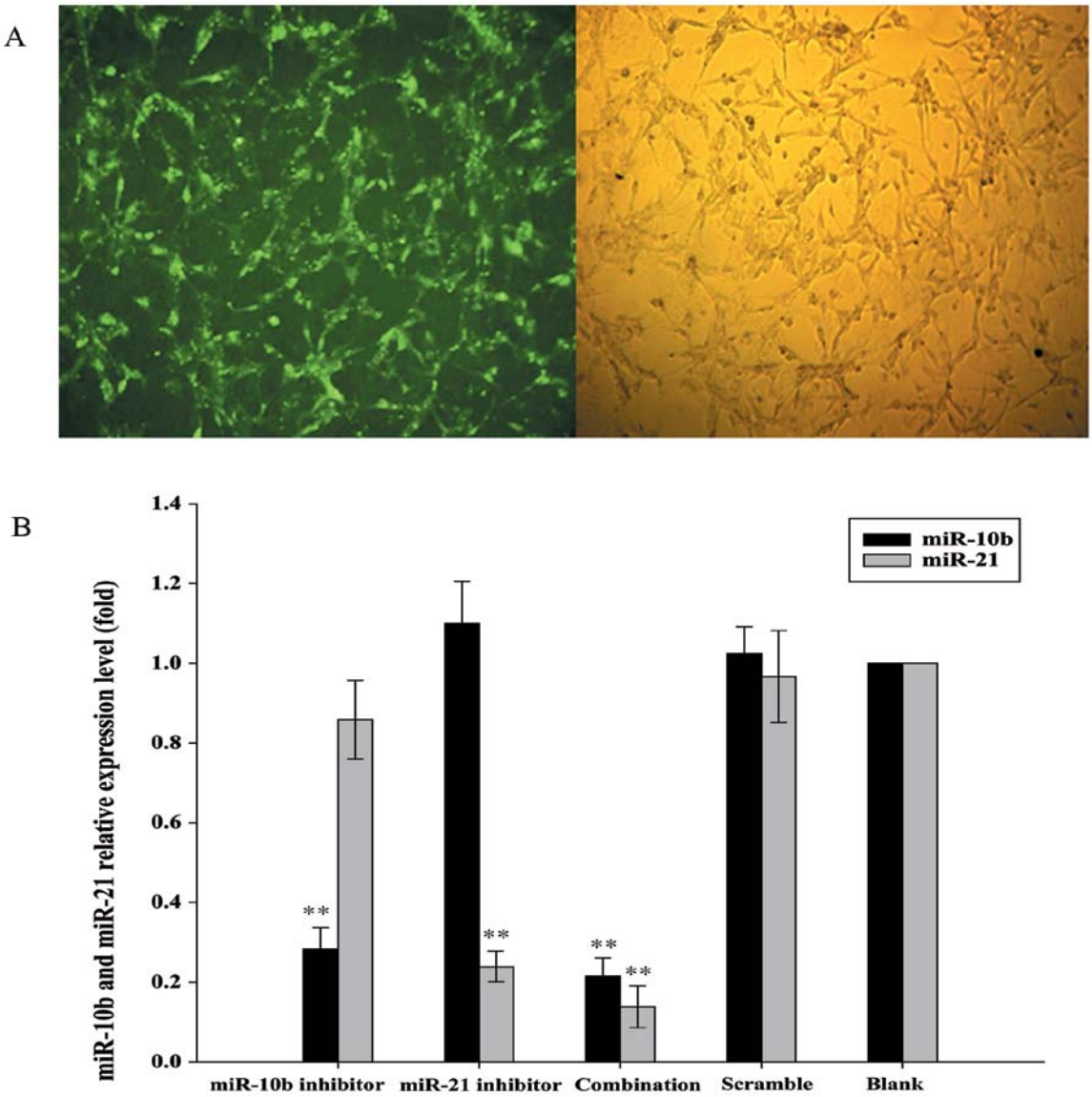

Figure 1. miR-10b and miR-21 expression in glioma progression. (A) Transfect ration (200x), using the scramble sequences was maked Fam (Ab 450nm, Em 490nm) to evaluate transfect ration. (B) U87MG cells was treated with miR-10b inhibitor, the miR-21 inhibitors alone and in combination, and qRT-PCRs for miR-21 and miR-10b have been performed with primers specific for mature miRNAs. The reactions were performed in duplicates, and the data are represented as means ± SD. (**P<0.01).

after transfection, both floating and attached cells were collected and subject to annexin V/PI staining using an annexin V-FITC Apoptosis Detection kit (BioVision, Palo Alto, CA), according to the manufacturer's protocol. The resulting fluorescence was measured by flow cytometry using a FACS flow cytometer (BD Biosciences, San Jose, CA).

Measurement of cell invasiveness. Cell invasiveness was examined using six-well transwell chambers and a reconstituted extracellular matrix membrane (Matrigel, USA). The cell invasion chambers were prepared by placing 100 μl of a 1:5 dilution of Matrigel onto the filter, and incubating the filter at 37˚C for 30 min to allow Matrigel polymerization. Transfected cells were resuspended at 5x10^5 cells/ml in serum-free medium. Afterwards, 200 μl of cell suspension from each treatment group was added to the upper chambers. The chambers were incubated for 48 h in 37˚C with 5% CO_2. The filters were then fixed in 95% ethanol and stained with hematoxylin. The upper surfaces of the filters were scraped twice with cotton swabs to remove non-migrated cells. The experiments were repeated in triplicate wells, and the migrated cells were counted microscopically (200x) in five different fields per filter.

Statistical analysis. Results were analyzed using SPSS software 11.0 and compared using one-way analysis of variance (ANOVA) with Fisher's post hoc test. Data were presented as mean ± standard deviation (SD) of separate experiments (n≥3). P-values <0.05 were considered to be significant.

Results

miR-10b and miR-21 inhibitors reduced the expression of their miRNA targets in U87 glioma cells. FAM-labeled scrambled control was used to determine the transfection efficiency of

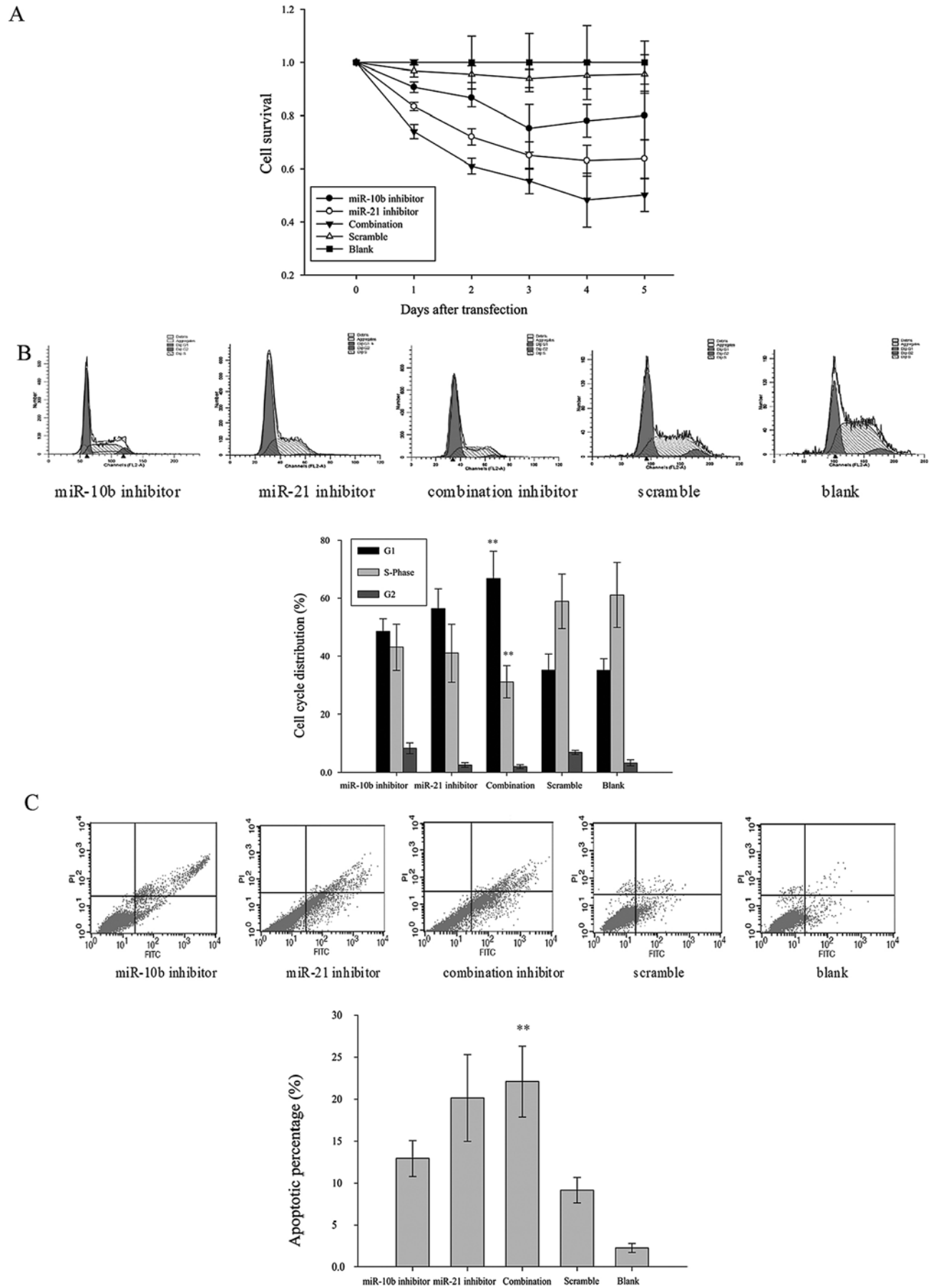

Figure 2. Effect of miR-10b and miR-21 inhibitor on U87 glioma cells. (A) The proliferation of U87 cells was inhibited by the miR-10b and miR-21 inhibitor, and the cell number was measured using the MTT assay. Values represent the mean ± SD. (B) Flow cytometry analyses the cell-cycle of U87 cell distribution after PI staining. (C) Cell apoptosis profiles after annexin V/PI staining. Combination of the miR-10b and miR-21 inhibitors enhanced cell apoptosis. Percentages of apoptotic cells are shown in the histogram. Flow cytometry analyses of propidium iodide-stained cells were performed in triplicate.

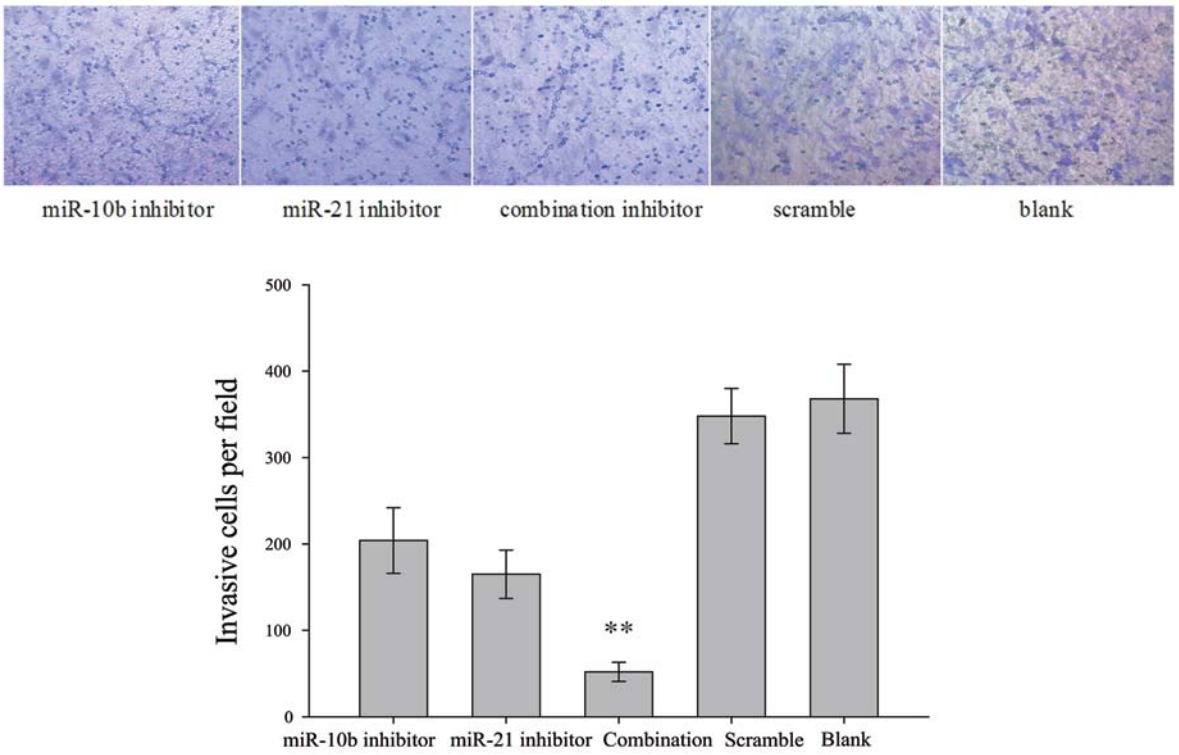

Figure 3. miR-10b and miR-21 inhibitor, alone or in combination inhibits U87 cell invasion. U87 cells were infected with miR-10b inhibitor, miR-21 inhibitor alone or combination, then tested for invasion ability in Matrigel chambers. *In vitro* cell invasion assays with representative fields of invaded cells, were performed in triplicate and the data are presented as means ± SD. (**P<0.01).

Lipofectamine-mediated entry of RNA oligos into human glioma U87 cells. As shown in Fig. 1A, green fluorescent signals were detected in more than 95% FAM-labeled scrambled control-transfected U87 cells, indicating that RNA oligonucleotides could readily gain access to the cells. RNA sequences that showed complementarity with the sequences of miR-10b and miR-21 were then transfected into U87 cells to inhibit the functions of these two miRNAs. Since miRNA inhibitors have been shown to induce the degradation of their endogenous miRNA targets (20), we determined if U87 cells transfected with miR-10b and miR-21 inhibitors, alone or in combination, showed downregulation of miR-10b and miR-21 by qRT-PCR. Inhibitors of miR-10b and miR-21 specifically reduced the levels of their respective target miRNA in U87 cells when compared with scrambled control. Moreover, co-transfection of miR-10b and miR-21 inhibitors effectively reduced the levels of both miRNAs (Fig. 1B).

miR-10b and miR-21 inhibitors lowered cell proliferation in U87. Cell viability was measured in miRNA inhibitor-transfected U87 cells up to 5 days after transfection by MTT assay. Results revealed that miR-10b and miR-21 inhibitors, alone or in combination, exerted inhibitory effect on U87 cell viability. The growth-inhibitory effect peaked on day 3-4 post-transfection (Fig. 2A). The inhibitory effect of miR-21 was more prominent than that of miR-10b (35.00±5.12% inhibition vs 24.80±9.00% inhibition on day 3). Notably, when U87 cells were co-transfected with both miR-10b and miR-21 inhibitors, cell viability was further reduced (48.20±10.18% inhibition on day 3). These data suggest that inhibiting the functions of miR-10b and miR-21 reduced the cell proliferation in glioma cells.

miR-10b and miR-21 inhibitors induced G_0/G_1-phase cell cycle arrest in U87. To determine if decreased cell viability was a result of cell cycle arrest, we analyzed the cell cycle distribution of miRNA inhibitor- or scrambled control-transfected U87 cells by flow cytometry. At 48 h post-transfection, miR-21 inhibitor and miR-10b inhibitor, alone or in combination, increased the proportion of U87 cells in G_0/G_1-phase when compared with the scrambled control-transfected group. A reciprocal reduction of cells in S-phase was also observed in these treatment groups. The effect on cell cycle was more prominent in U87 cells co-transfected with miR-10b and miR-21 inhibitors as compared with those transfected with either miRNA inhibitor. These data suggest that miR-10b and miR-21 inhibitors, especially in combination, induced G_0/G_1-phase cell cycle arrest in glioma cells (Fig. 2B).

miR-10b and miR-21 inhibitors induce apoptosis in U87. Loss of phosphatidylserine asymmetry is a molecular hallmark of apoptotic cell death. To determine if miR-10b and miR-21 inhibitors induced apoptosis in addition to cell cycle arrest, phosphatidylserine externalization was assayed by flow cytometry of Annexin V/PI double-stained U87 cells. As shown in Fig. 2C, the percentage of the Annexin V-positive apoptotic cells were significantly higher in cells transfected with inhibitors of miR-10b and miR-21, alone or in combination, when compared with the scrambled control transfected group. The pro-apoptotic

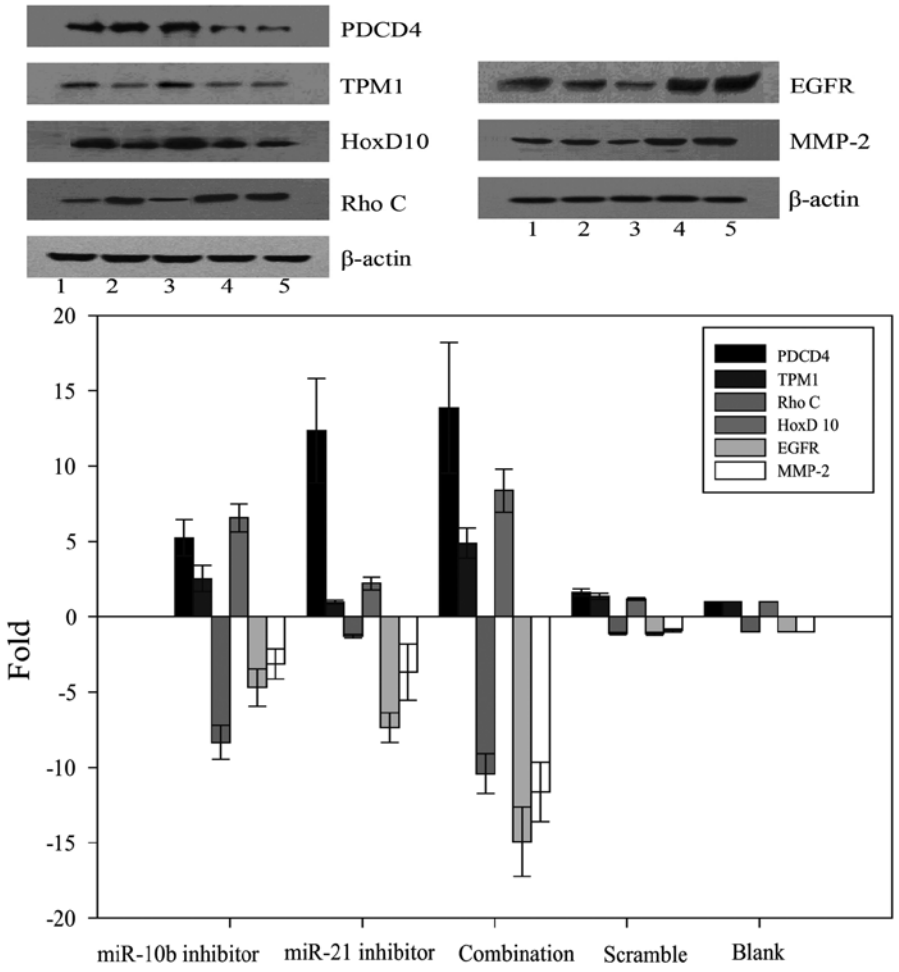

Figure 4. Valuation of the expression of PDCD4, TPM1, RhoC, HoxD10, EGFR and MMP-2 in human glioblastoma U87 cells. Western blot analysis of protein extracts from cells treated with the miR-10b inhibitor, miR-21 inhibitor alone or combination. The expression of β-actin was examined to ensure uniform protein loading in the lanes. (line: 1, miR-10b inhibitor; 2, miR-21 inhibitor; 3, combination inhibitor; 4, scramble; 5, blank).

effect of combined transfection of miR-10b and miR-21 inhibitors was significantly stronger than that of miR-10b inhibitor alone ($p<0.05$).

miR-10b and miR-21 inhibitors impair U87 cell invasiveness. To measure the effects of miR-10b and miR-21 inhibitors, alone or in combination, on glioma cell invasiveness, a transwell invasion assay was employed. The system consists of two fluid-filled stacked compartments, separated by a porous membrane filter coated with Matrigel. Cells were grown in the upper chamber and assessed for invasion through the Matrigel toward a chemo-attractant (10% serum) in the lower chamber. The number of invasive U87 cells co-transfected with both miR-10b and miR-21 inhibitors was substantially reduced when compared with the scrambled control-transfected cells (Fig. 3). miR-10b inhibitor or miR-21 inhibitor also exerted minimal-to-moderate inhibitory effect on U87 cell invasiveness.

miR-10b and miR-21 inhibitors increase the protein expression of respective miRNA targets and synergistically repress the expression of EGFR and MMP-2. PDCD4 and TPM1 have been reported to be the direct targets of miR-21 in other cell types (12,13). We therefore determined if these two proteins could be upregulated by miR-21 inhibitor in U87 cells. Results revealed that miR-21 inhibitor alone upregulated the protein expression of PDCD4, but not TPM1. Unexpectedly, miR-10b inhibitor exerted minimal-to-moderate stimulatory effect on PDCD4 and TPM1 protein expression. In this regard, miR-21 inhibitor potentiated the stimulatory effect of miR-10b inhibitor on TPM1 protein expression.

miR-10b has been shown to target HoxD10 to induce RhoC protein expression in breast cancer cells to enhance tumor cell invasiveness and metastasis (14). We therefore determined whether miR-10b inhibitor could reverse this metastatic cascade. Results showed that miR-10b inhibitor but not miR-21 inhibitor increased HoxD10 and reduced RhoC protein expression. Similar effect could be observed in U87 cells co-transfected with both miR-10b and miR-21 inhibitors. In addition to the abovementioned mediators, we measured the protein expression levels of matrix metalloproteinase (MMP)-2 and epidermal growth factor receptor (EGFR), both of which have been reported to be associated with malignant phenotypes of glioma cells (21,22). As shown in Fig. 4 and 5, both miR-10b inhibitor and miR-21 inhibitor reduced the protein expression of both MMP-2 and EGFR. When these miRNA inhibitors were co-transfected into U87 cells, a synergistic inhibitory effect on MMP-2 and EGFR protein expression was observed.

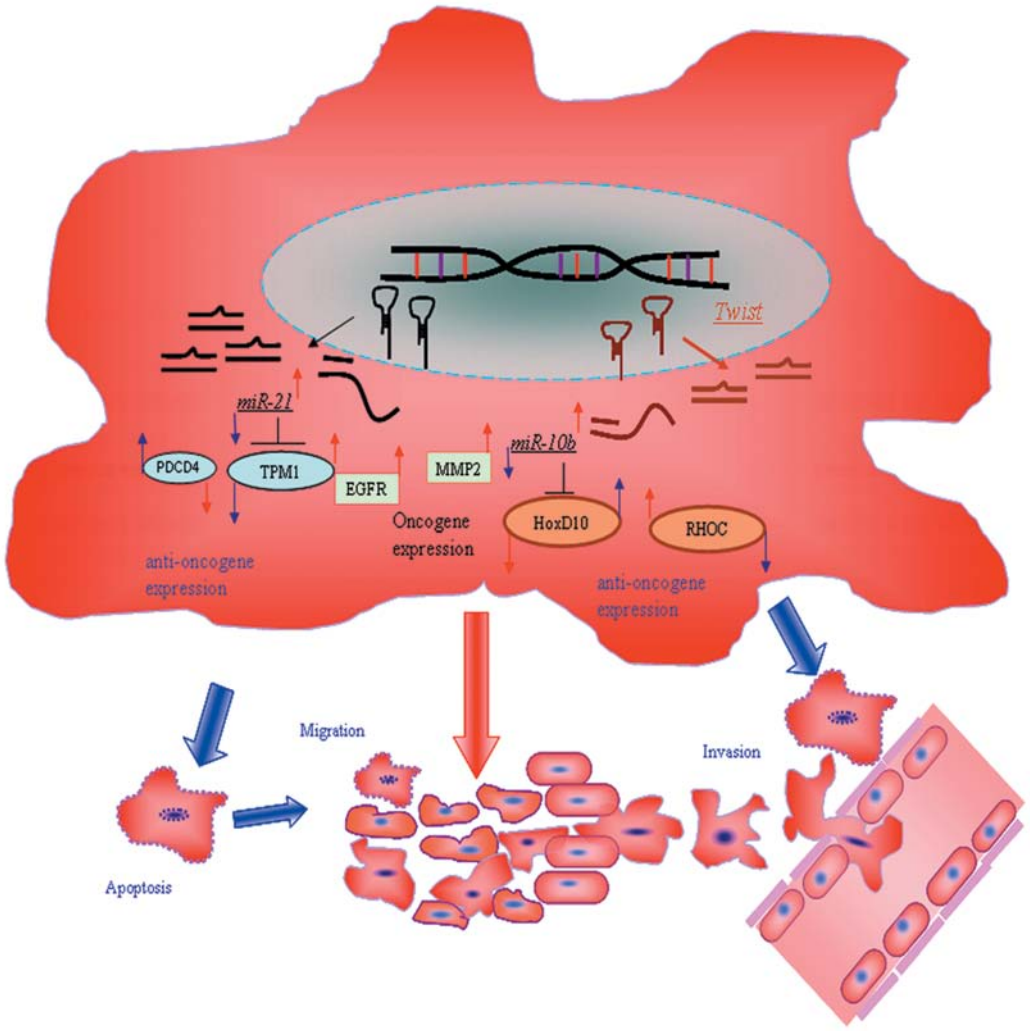

Figure 5. A model of miR-21 and miR-10b regulation of the expression of tumor suppressor and oncogenes. Overexpression of miRNAs leads to decreased expression of targets and the under expression of miRNAs by their inhibitors results in increased expression of targets. miR-21 and miR-10b overexpressed in glioma cells, interact with TPM1 and HoxD10. Using the miR-21 and miR-10b inhibitors may downregule the oncogene expression (red arrow) and upregulate the suppressor gene overexpression (blue arrow), leading to promotion of cancer cell apoptosis and inhibition of cancer cell invasion and metastasis.

Discussion

miRNAs have been considered as emerging key players in carcinogenesis due to their widespread dysregulation in cancer and important roles in the control of gene expression. The mechanism by which miRNAs are dysregulated in cancer is complex and may involve genetic and epigenetic abnormalities as well as altered activity of certain transcription factors. Dysregulated miRNAs contribute to tumorigenesis by regulating the levels of their target genes post-transcriptionally. Importantly, a single miRNA very often represses multiple targets to mediate its biological function. The orchestrated alterations of gene expression then modulates cancer-related phenotypes thereby promoting or suppressing tumor formation.

Using high-throughput profiling of miRNA expression, miR-10b and miR-21 were identified as two of the most strongly upregulated miRNAs in a large proportion of human glioma specimens (17,23). By miRNA microarray, miR-10b was shown to be upregulated 1.97-fold to 13.6-fold in 5 out of 9 glioblastomas samples. The finding was also confirmed by northern blotting (16). miR-21 was also identified as one of the most overexpressed miRNAs in a large-scale profiling experiments designed for the characterization of miRNA expression in human cancers. Aberrant upregulation of miR-21 in human glioblastoma has been reported (10). In this regard, compared with normal brain tissue, miR-21 expression was elevated 7- to 11-fold in low-grade astrocytomas, anaplastic astrocytomas, and glioblastoma multiforme (24). In the present study, miR-10b and miR-21 antagonized by their respective inhibitors in U87 glioma cells, in which their downregulation was confirmed by qRT-PCR. Functionally, inhibition of miR-10b and miR-21 exert potent anti-glioma effects, as evidenced by inhibition of cell cycle progression, enhanced apoptosis and reduced cell invasion. The anticancer actions of anti-miR-10b and anti-miR-21 are accompanied by the upregulation of their respective targets, including PDCD4, TPM1 and HOXD10.

PDCD4, a novel tumor suppressor, is downregulated in various types of cancer. It has been shown that post-transcriptional downregulation of PDCD4 by miR-21 in T98G glioma cells stimulates cell proliferation and inhibits apoptosis (12). Besides PDCD4, TPM1 has been identified as a potential miR-21 target in tumors (13). TPM1 exhibits an anti-oncogenic function

through binding microfilament and regulating cytoskeleton (25). Downregulation of TPM1 is consistently observed in transformed breast epithelial cells. Inhibition of TPM1 function caused by miR-21 overexpression leads to enhanced cell migration and invasion (13). MiR-10b has been reported to inhibit the translation of HOXD10, which in turn modulates expression of downstream targets involving in cell invasion, migration, extracellular matrix remodeling and tumor progression, including uPAR, RhoC, integrins, and MMP-14 (26,27). MiR-10b is known to be induced by TWIST, a master regulator of morphogenesis and tumor metastasis. TWIST has been detected in a large proportion of human gliomas and increased TWIST mRNA level is associated with increased glioma grading.

About 30-50% of gliomas show dysregulated EGFR signaling, including aberrant amplification of the EGFR gene and/or auto-secretion of EGFR ligands (28). Enhanced EGFR signaling results in increased cell proliferation, angiogenesis and cell invasion. The latter involves the infiltration of tumor cells through the extracellular matrix by local proteolysis mediated by MMPs. In this study, inhibition of miR-10b and miR-21 reduces the expression of EGFR and MMP-2. These two mediators may be important for the anti-proliferative and anti-metastatic effects of anti-miR-10b and anti-miR-21 in glioma cells.

In conclusion, inhibition of miR-10b and miR-21, alone and in combination, can effectively retard the growth of glioma through inhibition of cell cycle progression and induction of apoptosis. Inhibition of these two oncogenic microRNAs also impairs the invasiveness of glioma cells. Our findings provide a proof-of-concept that microRNA inhibitors could serve as therapeutic agents to exert their anticancer effects on glioma cells. Importantly, besides derepressing the expression of their verified targets, we here demonstrate that inhibition of miR-10b and miR-21 synergistically suppress the expression of MMP-2 and EGFR. These findings suggest that inhibition of miR-10b and miR-21 could affect common downstream signaling components, which may be important for the synergism between these two microRNA inhibitors. Taken together, our study provides functional and mechanistic insights into the anti-glioma effects of anti-miR-10b and anti-miR-21. Whether our findings can be translated into clinical benefits of glioma patients, however, awaits further investigation.

Acknowledgements

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