miR-221/222 is the regulator of Cx43 expression in human glioblastoma cells

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Received October 13, 2011; Accepted December 8, 2011

DOI: 10.3892/or.2012.1652

Abstract. The miR-221/222 cluster is significantly upregulated in malignant glioma cells and regulates the expression of multiple genes associated with glioma cell proliferation, invasion and apoptosis, which was shown in our previous studies. Cx43 has been identified as a tumor suppressor and major component for the establishment of gap junction intercellular communication (GJIC) in glial cells, which is frequently reduced or deleted in high-grade gliomas. According to bioinformatic analysis, connexin 43 (Cx43) may be one of the target genes of miR-221/222. The aim of the present study was to validate Cx43 as a target gene of miR-221/222 and to determine whether overexpression of miR-221/222 is one of the molecular mechanisms for the reduced expression of Cx43 in malignant gliomas. We transfected miR-221/222 antisense oligonucleotides (AS-miR-221/222) into U251 human glioblastoma cells using a lipofectamine method. Northern blot analysis was conducted to detect the expression of the miR-221/222 cluster. Luciferase reporter assays were exploited to confirm Cx43 as a target gene of miR-221/222. Cx43 expression was assessed by western blotting and immunofluorescence staining. Scrape loading and dye transfer (SLDT) assays were used for examination of GJIC. Proliferation and invasion of U251 cells were evaluated by MTT and transwell assays, respectively. Cell cycle kinetics and apoptosis were determined with flow cytometry. We found that expression of the miR-221/222 cluster was significantly reduced while Cx43 expression was upregulated in U251 cells transfected with AS-miR-221/222, and the GJIC deficiency in parental U251 cells was re-established. Moreover, the luciferase activity determined by the luciferase reporter assay was enhanced in AS-miR-221/222-treated cells, and cell proliferation and invasion were suppressed while apoptosis was induced. We conclude that miR-221/222 function as oncogenic microRNAs in human gliomas, at least in part, by targeting Cx43.

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Key words: glioma, microRNA-221/222, connexin 43, gap junction intercellular communication, cell proliferation, apoptosis, antisense oligonucleotides

Introduction

Glioblastoma (GBM) is the most frequent and the most aggressive primary brain tumor in adults (1), and despite the currently available therapeutic interventions, the prognosis for GBM patients remains dismal. The median survival of patients with GBM is less than one year (1,2). Therefore, it is imperative to further study the molecular pathogenesis of GBM for the development of novel, effective therapeutic approaches. In recent years, microRNAs (miRNAs, miRs) were newly recognized as gene expression regulators. microRNAs work at post-transcriptional and/or translational levels and have been found to be associated with numerous biological processes, including development, cell differentiation, proliferation and apoptosis (3-7). It is speculated that each miR is capable of targeting hundreds of transcripts by directly binding to target mRNA 3'UTRs, and multiple miRs can converge on a single protein coding gene target (8-10). Therefore, the potential effect of miRs on regulation of gene expression is enormous. Accumulated evidence indicates that dysregulation of miRs contributes to a range of human tumors, including glioma (11). The discovery of miRs has offered great potential for correcting the regulation of aberrantly expressed and cancer-related genes, as well as in the development of novel therapeutic agents for GBMs. Studies on miR expression profiles in human GBMs have been carried out by microarray analysis (12-15), and the results revealed a series of miRs overexpressed or weakly expressed. Among them, miR-21 and miR-221/222 were the most common and significantly overexpressed in GBMs (12,16). Our previous study on miRs expression profiles of six GBM cell lines also showed that overexpression of the miR-221/222 cluster was the

most common characteristic of these cell lines (17). miRanda algorithm was used for predicting targets of miR-221/222, and Cx43 was identified as a putative target gene. Reduced Cx43 expression and loss of GJIC is consistently found in malignant gliomas (18,19), therefore, the aim of this study was to confirm Cx43 as a target gene of miR-221/222, and to confirm if upregulation of Cx43 expression in GBM cell lines by knocking down overexpressed miR-221/222 will re-establish GJIC, or further inhibit cell growth.

Materials and methods

Cell culture. Human U251 glioblastoma cells were provided by Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine (Sigma), 100 units/ml of penicillin/ml (Sigma), and 100 μg/ml of streptomycin (Sigma) at 37˚C in 5% CO_2, and subcultured every other day.

Oligonucleotides and cell transfection. Oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography (GenePharma Co., Ltd., Shanghai, China). The antisense oligonucleotide sequences against miR-221 (AS-miR-221) and miR-222 (AS-miR-222) were 5'-AGCUACAUUGUCUGCUGGGUUUC-3' and 5'-AGCUACAUCUGGCUACUGGGU-3', respectively. A scramble oligonucleotide, 5'-UCUACUCUUUCUAGGAGGUUGUGA-3', was used as control. Cells were cultured in 6-well plates in DMEM supplemented with 10% fetal bovine serum. At 24 h after plating, the complete medium was replaced with serum-free medium. Cells were then transfected with 200 pmol oligonucleotides using Lipofectamine 2000 (Invitrogen, USA). After 6 h, serum-free medium was replaced again with complete medium and cultured at 37˚C in 5% CO_2.

Northern blot analysis. Total RNA was extracted from U251 cells transfected with As-miR-221/222 and scramble oligonucleotides using TRIzol reagent (Invitrogen, USA). The protocol for Northern blot analysis of miRNA was used as Ramkissoon *et al* (20) reported. Briefly, total RNA (20 μg) was 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 (DIG) 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 probe end-labeled with DIG is 5'-GAAACCCAGCAGACAATGTAGCT-3' for detecting miRNA-221; 5'-GAGACCCAGTAGCCAGATGTAGCT-3' for detection of miR-222; and 5'-ATTTGCGTGTCATCCTTGCG-3' for U6 as internal control. 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.

Luciferase reporter assay. The miRanda algorithm (http://www.microrna.org/microrna) was used to predict the human microRNA binding sites in 3' UTR alignments of CX43 mRNA (NCBI gene ID 2697). The human 3'-UTR of the Cx43 gene was then amplified by PCR using the following primers: CX43-3'UTR-forward: 5'-TCAGGAATTCTTGTTTGACATTCCATGTTAAACACGGTCATGTTCAGCTTCATTGCAT-3' and Cx43-3'UTR-reverse: 5'-AACACTCTCCAGAACACATGATCTGATGGACTAGTCTACATTACATGCAATGAAGCTG-3'. The cDNA was cloned into the pGL3-control vector (Promega, USA), downstream of the luciferase gene, to generate the vector pGL3-Cx43-3'UTR. For the luciferase reporter assay, U251 cells were cultured in 96-well plates and co-transfected with 0.2 μg of the pGL3-Cx43-3'UTR and 5 pmol of AS-miR-221/222 or scramble oligonucleotide mediated by Lipofectamine 2000. Luciferase activity was measured at 72 h after transfection with the Luciferase Reporter Assay System (Promega).

Scrape loading and dye transfer (SLDT) assay. U251 cells were seeded in 6-well plates, then transfected with AS-miR-221/222. After 48 h, cells grew to confluency. Scrape loading was performed using a micropipette tip to draw several clear straight lines on the monolayer of cells. After rinsing with PBS, cells were immersed in 0.05% Lucifer Yellow (Sigma) in PBS for 3 min. The Lucifer Yellow solution was removed, cells were washed with PBS, and transfer of Lucifer Yellow was observed under an inverted fluorescence microscope. Cells competent in GJIC demonstrated the transfer of Lucifer Yellow to neighboring cells from the scraped line border, whereas cells incompetent in GJIC did not show dye transfer.

Western blot analysis. At 48-72 h after transfection with As-miR-221/222 and scramble oligonucleotide, total proteins from control and transfected U251 glioma cells were extracted and the protein concentration was determined by Nanodrop spectrophotometer (Gene Co., USA). Protein (40 μg) from each sample was subjected to SDS-PAGE on 12% SDS-polyacrylamide gel. Separated proteins were transferred to a PVDF membrane (Millipore, USA). The membrane was incubated with primary antibodies against Cx43 (Catalog: sc-6560, Santa Cruz Biotechnology, USA; 1:500 dilution), followed by HRP-conjugated secondary protein (cat. no. 81-1620, Zymed, USA; 1:1000 dilution). The Cx43 protein was detected using a SuperSignal protein detection kit (Pierce, USA). The membrane was stripped and reprobed with an antibody against β-actin (Catalog: sc-130301, Santa Cruz Biotechnology; 1:500 dilution), followed by HRP-conjugated secondary protein (cat. no. 81-6720, Zymed, USA; 1:1000 dilution). The band density of Cx43 protein was quantified after normalization with the density of β-actin.

Immunofluorescence staining. Forty-eight hours after transfection with oligonucleotides, transfected and parental cells growing on coverslips were washed with PBS, fixed with -20˚C pre-cooled methanol for 5-10 min, blocked with 1% BSA in PBS for 30 min at room temperature and incubated overnight at 4˚C with primary antibodies against Cx43 (Catalog: sc-6560, Santa Cruz Biotechnology; 1:500 dilution). The cells were then incubated in a FITC (fluorescein isothiocyanate) conjugated secondary antibody (cat. no. 61-1611, Invitrogen, USA; 1:100

dilution) at 37˚C for 30 min, then washed with PBS. The slides were sealed with 0.5 M Na_2CO_3, 50% glycerol, and the Cx43 expression was observed using a laser confocal microscope.

MTT assay. The proliferation activity of control and AS-miR-221/222 transfected cells was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) assay. Briefly, $5x10^3$ cells were plated in each well of a 96-well plate. Each day for six consecutive days, 20 μl MTT (5 mg/l) was added into each well and cells were incubated for an additional 4 h. After the medium containing MTT was removed, the formazan crystals were dissolved in 200 μl of dimethyl sulfoxide (DMSO) for 5 min. The quantification measurement (optical density) was recorded using a Teacan 96-well spectrophotometer at a wavelength of 570 nm. The data were derived from triplicate samples of at least three independent experiments and expressed as the percentage of control.

Flow cytometric analysis of the cell cycle. Parental and transfected cells in the log phase of growth were washed with PBS, fixed with ethanol and incubated with RNase at 37˚C for 30 min. Nuclei of cells were stained with propidium iodide for an additional 30 min. A total of 10,000 nuclei were examined using FACSCalibur (Becton-Dickinson, USA) and DNA histograms were analyzed by Modifit software.

Transwell invasion assay. The upper surface of polycarbonic membranes (pore size 8 μm) within transwell filters were coated with matrigel (3.9 μg/μl, 60 μl). Control and transfected cells ($1x10^5$) suspended in 200 μl of serum free DMEM were added to the upper chamber and conditional medium of these cells was placed in the lower chamber as chemo-attractant. After 24 h of incubation at 37˚C in 5% CO_2, the medium was removed from the upper chamber. The non-invaded cells on the upper surface of the inserted filters were gently scraped with a moist cotton swab, and the cells which had invaded into the lower surface of the filters were stained with hematoxylin and counted using a light microscope. The average number of invaded cells from five randomized fields for each well was calculated.

Detection of cell apoptosis by Annexin-V staining. For the evaluation of apoptosis in control and transfected cells, Annexin-V-FITC labeled Apoptosis Detection Kit (Abcam, USA) was used according to the manufacturer's protocol.

Statistical analysis. All data were analyzed using SPSS 11.5 software. Measurement data are expressed as mean ± SE, and p-values <0.05 was considered significant using ANOVA and LSD-t test.

Results

As-miR-221/222 knocks down the expression of miR-221/222 in U251 cells. U251 cells exhibit upregulation of miR-221 and miR-222, and this overexpression was specifically and effectively knocked down by transfection with the chemically synthesized antisense oligonucleotide, As-miR-221/222 (Fig. 1).

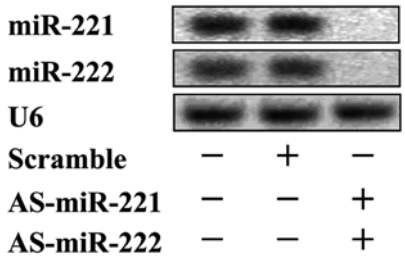

Figure 1. As-miR-221/222 downregulates the expression of miR-221/222 in U251 cells. miR-221/222 expression level in U251 cells transfected with As-miR-221/222 was significantly knocked down as assesed by Northern blot analysis. U6 RNA was used as internal control.

CX43 is the target gene of miR-221/222. The luciferase reporter assays demonstrated that downregulation of miR-221/222 with AS-miR-221/222 markedly enhanced luciferase activity of pGL3-Cx43-3'UTR (Fig. 2B). This result indicated that the Cx43-3'UTR contained direct binding sites of miR-221/222 and the binding between them was inhibited by AS-miR221/222. Moreover, after trasfection with AS-miR-221/222, Cx43 expression was upregulated significantly in U251 cells as shown by western blotting (Fig. 2C) and immunofluorescence staining (Fig. 2D), additionally, SLDT assay showed the re-establishment of GJIC (Fig. 2E).

AS-miR-221/222 inhibits U251 cell growth. The MTT assay revealed that the viability of U251 cells transfected with AS-miR-221/222 decreased starting 48 h after transfection and continued declining to nearly 30-40% of control cells during the 6-day observation period; whereas the viability of cells transfected with scramble oligonucleotide was not suppressed significantly when compared with parental cells (Fig. 3A). The cell cycle kinetics of parental and transfected cells was analyzed by flow cytometry. As shown in Fig. 3B, the S phase fraction of parental cells and cells transfected with scramble oligonucleotide was 36.2 and 37.7%, respectively, whereas AS-miR-221/222 treated cells had a decreased S phase fraction of 25%. The G0/G1 phase of AS-miR-221/222 transfected cells was elevated at 65%, while it was 55.4 and 51.8% in control and scramble oligonucletide transfected cells. These results implicate that cells were arrested in G0/G1 phase, cell cycle progression was delayed, and proliferation activity in U251 cells transfected with AS-miR-221/222 was decreased.

AS-miR-221/222 inhibits cell invasion. The invasive ability of U251 cells transfected with AS-miR-221/222 was significantly decreased when compared to that of control and scramble oligonucletide-transfected cells. The average number of invaded cells for each well containing AS-miR-221/222 transfected cells was 15±2.39, while it was 40±1.58 and 39±2.07 in control and scramble oligonucleotide-transfected cells, respectively (Fig. 4).

AS-miR-221/222 induces cell apoptosis. Annexin-V staining showed that apoptotic cells in U251 cells transfected with AS-miR-221/222 were significantly increased when compared with those in parental cells and cells transfected with scramble oligonucleotides (Fig. 5). These findings suggest that lack of

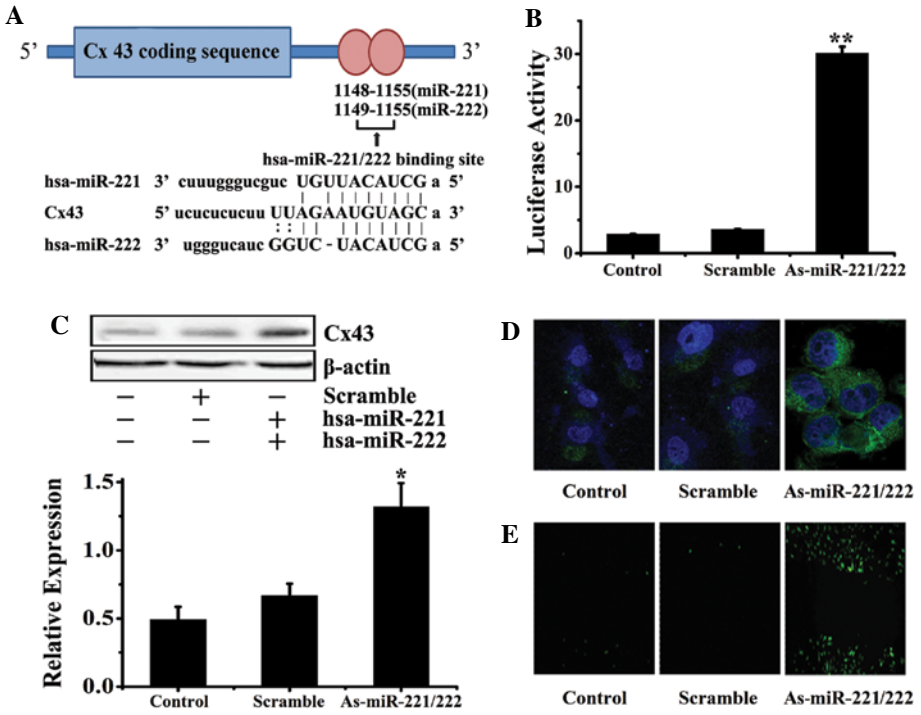

Figure 2. Connexin 43 is a direct target gene of miR-221/222. (A) Schematic representation of the putative binding sites in Cx43 mRNA 3'UTR for miR-221/222 (identical seed sequence AGCUACAU) and miR-221 and miR-222-specific binding sequence of the Cx43 3'UTR. (B) The luciferase activity increased after co-transfecting pGL3-Cx43-3'UTR and AS-miR-221/222 in U251 glioblastoma cells ($^{**}p<0.01$). (C) Western blot analysis demonstrated that the Cx43 expression level in As-miR-221/222 group was upregulated significantly compared to the other two groups ($^{*}p<0.05$). (D) Immunofluoresence staining revealed the Cx43 expression elevated in U251 cells transfected with AS-miR-221/222. (E) SLDT assay showed that the functional GJIC in AS-miR-221/222 treated U251 cells were re-established, lucifer yellow was transferred to multiple rows of cells near the scrape border.

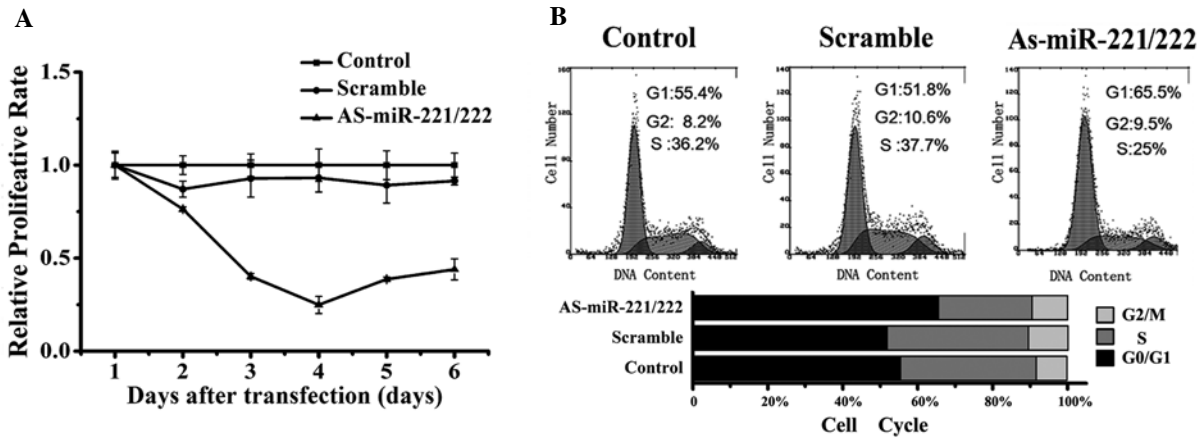

Figure 3. AS-miR-221/222 suppresses U251 cell growth. (A) MTT assay showed that the viability of U251 cells treated with AS-miR-221/222 was significantly decreased during the sixth day of the observation period as compared to the control cells. (B) Flow cytometric analysis demonstrated that the S-phase fraction of U251 cells transfected with AS-miR-221/222 was decreased and the cell cycle was arrested in G0/G1 phase.

miR-221/222 expression is able to induce apoptosis in glioma cells.

Discussion

MiRs are speculated to regulate up to 30% of the human genome via induction of mRNA degradation or inhibition of translation by targeting the 3' untranslated regions of genes through specific base pairing (21-25). More than half of the known human miR genes are located in cancer-associated genomic regions, including fragile sites of chromosomes, common breakpoint regions, minimal regions of heterozygosity (LOH) loss, and minimal regions of amplification (26,27). Alteration of miR expression has been found in essentially every cancer type studied (6). miRs can be aberrantly expressed in cancer through a variety of mechanisms, such as chromosomal aberrations, epigenetic defects, mutations, and alterations in the process of miR biogenesis (28).

Taken together, this evidence indicates that miRs are involved in tumorigenesis and may function as oncogenes or

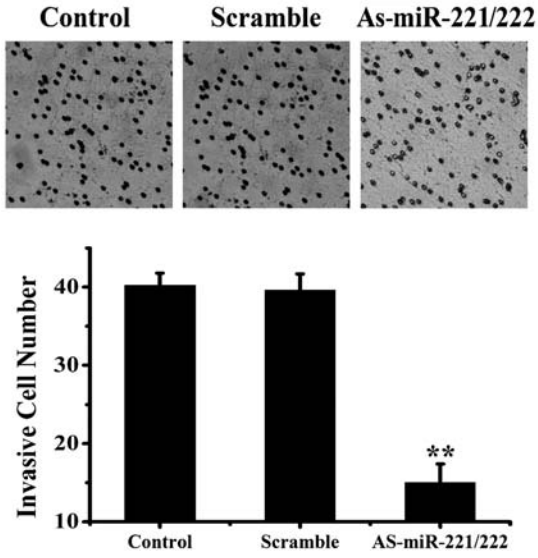

Figure 4. AS-miR-221/222 inhibits cell invasion. Transwell assay revealed the average number of invading cells significantly decreased in AS-miR-221/222 treated group (**p<0.01; original magnification, x100).

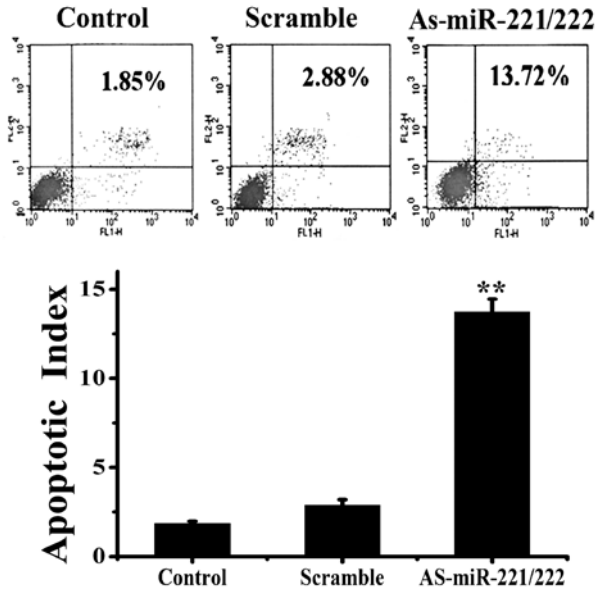

Figure 5. AS-miR-221/222 induces cell apoptosis. Apoptotic cell index was significantly increased in U251 cells tranfected with AS-miR-221/222 in comparison with that of the other control cell groups (**p<0.01).

tumor suppressor genes (29). Recently reported, miR-221 and miR-222 were strongly upregulated in primary glioblastomas and cell lines, especially miR-221 (12,30-32). Our previous studies on the miR expression profiles of six GBM cell lines and fresh samples of high grade gliomas have also shown concordant upregulation of miR-221/222 (10, and our unpublished data). Therefore, miR-221/222 could be a molecular signature of GBMs. miR-221 and miR-222 are clustered on chromosome Xp11.3. They have identical core seed sequences AGCUACAU (GeneDoc, www.psc.edu/biomed/genedoc), as well as same target specificity (33). Additionally, our previous studies confirmed that miR-221 and miR-222 must be simultaneously downregulated for the effective regulation of targeted genes and suppression of glioma cell growth (34,35).

Cx genes have been demonstrated as tumor suppressor genes. To date, twenty members of Cx genes have been identified in mammalian tissues and are expressed in a tissue specific manner. Cx43 is the predominant form of Cxs expressed in astrocytes and is also the constituent of connexons which compose GJIC in the brain (36,38). Our previous study and other studies showed that Cx43 expression is inversely correlated with the degree of malignancy of astrocytomas (19,39). In most malignant gliomas, Cx43 expression was reduced or deleted and GJIC was deficient (39). Using a bioinformatics algorithm, Cx43 is predicted to be one of the target genes of miR-221/222 (Fig. 2A). In the present study, the luciferase reporter assay showed that luciferase activity was enhanced following transfection with AS-miR-221/222 in U251 cells (Fig. 2B). Meanwhile, Cx43 expression was significantly upregulated and GJIC was re-established as detected by western blotting (Fig. 2C) and immunofluorescence staining (Fig. 2D) as well as SLDT assay (Fig. 2E). Therefore, Cx43 has been validated as the target gene of miR-221/222 and is negatively regulated by overexpression of miR-221/222 in GBM cells. It has been reported that Cx43 mRNA contains two binding sites for miR-206 and miR-1 in its 3'-UTR. miR-206 and miR-1 are capable of inhibiting the expression of Cx43 protein during myoblast differentiation without altering Cx43 mRNA levels. However, they are muscle specific miRs, and thus no aberrant expression of miR-206 or miR-1 has currently been found in GBMs (40,41).

Transfection of a specific Cx gene into transformed cells deficient in the same Cx gene has been suggested to reverse the transformed phenotypes (42-45). We transfected Cx43 cDNA into rat C6 glioma cells deficient in Cx43 and GJIC, and found that Cx43 overexpression exihibited a potent suppressive effect on cell proliferation, invasion and tumorigenicity *in vitro* and *in vivo* (46-48). Meanwhile, deficient GJIC was restored. Whether or not the ability of Cx43 to suppress tumor cell growth depends on the formation of functional GJIC is controversial and should be explored further (49,50).

As shown in this study, the cell proliferation (Fig. 3A) and invasion (Fig. 4) abilities of U251 cells transfected with AS-miR-221/222 were repressed while cell apoptosis (Fig. 5) was induced. The miR-221/222 cluster has been identified to target multiple important tumor suppressor and proapoptotic genes, such as p27, p57, and PUMA (34-37), and/or more unidentified genes associated with cell proliferation and apoptosis. Therefore, it is speculated that upregulation of Cx43 after transfection with AS-miR-221/222 may be one of the contributors to the suppression of GBM cell growth. Collectively, these data strongly suggest that the miR-221/222 cluster is involved in gliomagenesis. The impact of miR-221/222 antisense oligonucleotides or inhibitors on the adjustments of protein output might be a potential therapeutic strategy in malignant glioma.

Acknowledgements

This study was supported by The China National Natural Scientific Fund (Grant no. 30901772) and 211 Project Innovation Foundation of Tianjin Medical University for PhD Graduations (Grant no. 2009GSI17).

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