

miR-422a Inhibits Glioma Proliferation and Invasion by Targeting IGF1 and IGF1R

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Glioma is a common type of malignant brain tumor characterized by aggressive metastasis capability. Recent evidence has suggested that noncoding RNAs, including microRNAs, have important functions in the pathophysiology of glioma development. In this study, we investigated the biological function of miR-422a in human glioma. We found that miR-422a was downregulated in glioma tissues. We also demonstrated that expression of miR-422a in glioma cells markedly suppressed cell proliferation, migration, and invasion. In addition, we identified insulin-like growth factor 1 (IGF1) and IGF1 receptor (IGF1R) as inhibitory targets of miR-422a in glioma cells. We established that the expression levels of miR-422a were negatively correlated with the expression levels of IGF1/IGF1R and the clinical parameters in glioma patients. An IGF1R inhibitor, AG1024, completely blocked the activity of miR-422a on glioma cell proliferation and invasion, which further confirmed that miR-422a functions through IGF1 and IGF1R.

**Key words: miR-422a; Insulin-like growth factor 1 (IGF1);
Insulin-like growth factor 1 receptor (IGF1R); Glioma**

INTRODUCTION

Glioma is a common type of malignant brain tumor characterized by aggressive metastasis capability (1). Although the detailed molecular and cellular mechanisms responsible for glioma initiation and progression are still under debate, it is generally accepted that abnormal proliferation and migration of glial cells are key factors contributing to glioma malignancy (2,3). Therefore, with increased understanding of these mechanisms, we will be able to design improved diagnostic approaches as well as targeted therapies for glioma patients.

Recent evidence has suggested that noncoding RNAs, including microRNAs, have important functions in the pathophysiology of cancer development by regulating the expression of oncogenes and tumor suppressors (4,5). MicroRNAs (miRNAs) are small noncoding RNAs that are 19–24 nucleotides in length. They function primarily by binding to the 3′-UTR of target mRNAs, resulting in translational inhibition and/or mRNA destabilization (6,7). The role of miR-422a in cancers has been suggested by a series of recent studies. miR-422a levels in the serum were found to be one of the most differentially

expressed miRNAs in colorectal adenocarcinoma patients and could potentially serve as a biomarker for early diagnosis (8). The expression of miR-422a was significantly downregulated in hepatic cell carcinoma, and miR-422a levels were negatively correlated with pathological grading, recurrence, and metastasis (9). In osteosarcoma bone tissue, the expression levels of miR-422a were strongly decreased compared with noncancerous bone tissues (10). The biological function of miR-422a in glioma, however, has not been investigated.

Insulin-like growth factors (IGFs) and their receptors (IGFRs) play well-characterized roles in glucose, protein, and lipid metabolism (11) and are thereby involved in the regulation of energy balance and cell growth (12,13). Recently, an expanding body of research has suggested that IGF/IGFR signaling could also have a critical function in cancer biology (14,15). On the basis of the function of IGF/IGFR signaling in cancer development, molecular targeting therapies including anti-IGF1R antibodies, anti-IGF1/IGF2 antibodies, and small molecule inhibitors that suppress IGF1R kinase activity have been used in solid tumor treatment (16–19).

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In this study, we investigated the biological function of miR-422a in human glioma and the underlying mechanisms.

MATERIALS AND METHODS

Human Samples

Glioma tissues were collected from 54 patients by surgical resection (13 WHO grade II, 11 grade III, and 30 grade IV cases) at the Department of Neurosurgery, The First Affiliated Hospital of Harbin Medical University. None of the patients had received chemotherapy or radiotherapy before surgical resection. All specimens were immediately frozen in liquid nitrogen after resection and stored at -80°C until analysis. The study was approved by the Ethics Committee of the Harbin Medical University. Written consent was obtained from all participants.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions, and was reverse transcribed to cDNA with the PrimeScriptTM RT Reagent Kit (Takara, Dalian, P.R. China). qRT-PCR was performed for miR-422a expression with the SYBR Premix Ex Taq kit (Takara) and for gene expression with the SYBR Premix Ex TaqTM II kit. PCR primers used were as follows: *IGF1*, 5'-GCTCTTCAGTTCGTGTGTGGA-3' (forward) and 5'-CGACTGCTGGAGCCATACC-3' (reverse); *IGF1R*, 5'-AGGGGAATTTCATCCCAAAT-3' (forward) and 5'-AGGAAAAGTTCCTCCGCAGTG-3' (reverse); *β-actin*, 5'-CAGAGCCTCGCCTTTGCC-3' (forward) and 5'-GTCGCCACATAGGAATC-3' (reverse).

Cell Lines

The glioma cells (U87 and U251) were maintained at 37°C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Shanghai, P.R. China), supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 mg/ml) (Gibco).

3-(4,5-Dimethylthiazol-2-yl)-2,5-di-Phenyltetrazolium Bromide (MTT) Assay

Cell proliferation was determined by MTT assay. U87 and U251 cells (3×10^3 /well) were transfected with miR-422a or miR-Ctrl for 48 h and then plated in 96-well plates overnight. Cell proliferation was assessed at 0, 1, 2, 3, 4, and 5 days. MTT solution (20 μl , 5 mg/ml) was added in each well and incubated for 4 h at 37°C . The medium was removed, and 100 μl of dimethyl sulfoxide (DMSO) was added. The OD at 490 nm was detected by a microplate spectrophotometer after 10 min of gentle shaking. The absorbance value was calculated as raw data subtracting reading from DMEM+10% FBS+DMSO

blank control. MTT assay was used to analyze cell viability under AG1402 (Sigma-Aldrich, St. Louis, MO, USA) treatment. The results were presented as the percentage compared to the control group.

Colony Formation Assay

Colony formation assay was used to analyze cell proliferation. Glioma cells were transfected with miR-422a or miR-Ctrl for 48 h, and then cells were trypsinized and seeded for colony formation assay in six-well plates at 400/well. The medium was changed every 3 days. On the 10th day after seeding, the cells were fixed by methanol and then stained with 0.1% crystal violet, and the number of colonies was counted.

Transwell Assays of Migration and Invasion

Cell migration and invasion were detected using an 8- μm pore Transwell chamber system (Corning, Cambridge, MA, USA) with (invasion assay) or without (migration assay) Matrigel (BD Biosciences, San Diego, CA, USA). Briefly, 500 μl of DMEM containing 10% FBS was added to the lower chamber, while glioma cells (1×10^4) transfected with miR-422a or miR-Ctrl for 48 h were added to the upper chamber in 100 μl of DMEM with 1% FBS. After 24 h, the cells on the top surface of the membrane were removed using a cotton swab, and the cells on the bottom surface of the membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, washed three times with running water, and then photographed under a light microscope (Nikon, Tokyo, Japan).

Plasmid Construction and Cell Transfection

pCDNA3.1 (+) plasmid backbone (Life Technologies) was used to construct the pCDNA3.1-IGF1 and pCDNA3.1-IGF1R expression vectors under CMV promoter together with *NotI* and *XbaI* restriction enzymes. For transfection, oligonucleotides were allowed to form transfection complexes with Lipofectamine 2000 (Life Technologies), subsequently added to glioma cells at a final concentration of 100 nmol/L, and left to incubate for 8 h before medium change. For cotransfections, cells seeded in six-well plates were transfected with 1.6 μg of oligonucleotides and 1.6 μg of control or overexpression vectors with the addition of 8 μl of Lipofectamine 2000. Functional experiments were performed 2 days after the transfection.

Luciferase Assay

Cells were cultured in a 96-well plate and cotransfected with luciferase reporter plasmids encoding wild-type 3'-UTR of IGF1 or IGF1R (RiboBio, Guangzhou, P.R. China) with miR-422a or miR-Ctrl using Lipofectamine 2000 (Life Technologies) following the manufacturer's information. After 48 h, luciferase activities were analyzed

using a dual-luciferase reporter analysis system (Promega, Madison, WI, USA). The relative luciferase activity was normalized to *Renilla* luciferase activity.

Western Blot

The total protein was extracted from the cell lysates using RIPA buffer. Equal amounts of cell lysates were loaded on a 10% sodium dodecyl sulfate–polyacrylamide gel for electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 1 h at room temperature using 5% skimmed milk, and then the following primary antibodies were applied overnight at 4°C: anti-IGF1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IGF1R (Santa Cruz Biotechnology), and anti- β -actin (Boster, Wuhan, Hubei, P.R. China). After washing three times with TBST, the membranes were incubated with secondary antibody at room temperature for 1 h and washed again with TBST. Images of target proteins were detected by a chemiluminescence ECL kit (Santa Cruz Biotechnology).

Statistical Analysis

The data shown represent the mean \pm standard deviation (SD) values of at least three independent experiments. Comparisons between groups were analyzed by the *t*-test and one-way ANOVA. The relationship between miR-422a and IGF1/IGF1R was explored by Spearman's correlation. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

miR-422a Is Downregulated in Glioma Tissues

In order to determine the possible function of miR-422a in glioma development, we first examined the expression levels of miR-422a in human glioma tissues. We

performed qRT-PCR analysis on 13 grade II, 11 grade III, and 30 grade IV glioma and 10 normal brain tissues. We found that miR-422a was significantly downregulated in glioma tissues of all grades and that the expression levels of miR-422a decreased progressively with the advancement of disease stages (Fig. 1A). We next detected miR-422a expression in the serum of patients with grade IV glioma. As shown in Figure 1B, miR-422a levels were significantly downregulated in these patients. In addition, we performed RT-PCR to analyze miR-422a expression in three glioma cell lines (U87, U251, and LN229). Compared with normal brain tissues, miR-422a decreased in these glioma cell lines (Fig. 1C). These data provided initial indication that miR-422a could play a suppressive role in glioma development.

miR-422a Inhibits Glioma Cell Proliferation, Migration, and Invasion

In order to further investigate the function of miR-422a in human glioma, we transfected miR-422a into two independent glioma cell lines: U87 and U251 (Fig. 2A). Using U87 and U251 cells with ectopic miR-422a expression, we performed an MTT cell proliferation assay. In both glioma cells, overexpression of miR-422a led to markedly decreased cell proliferation compared to cells transfected with a control miRNA (Fig. 2B). Furthermore, we performed colony formation assay on these cells. As expected, miR-422a overexpression resulted in less cell colony formation, indicating the fact that cell proliferation was suppressed (Fig. 2C). To examine the function of miR-422a in affecting glioma cell metastasis, we performed Transwell cell migration and invasion assays on U87 and U251 cells transfected with miR-422a versus a control miRNA. We found that in both cell migration (Fig. 3A) and cell invasion (Fig. 3B) assays, cells transfected with miR-422a demonstrated a decreased ability to move from the upper

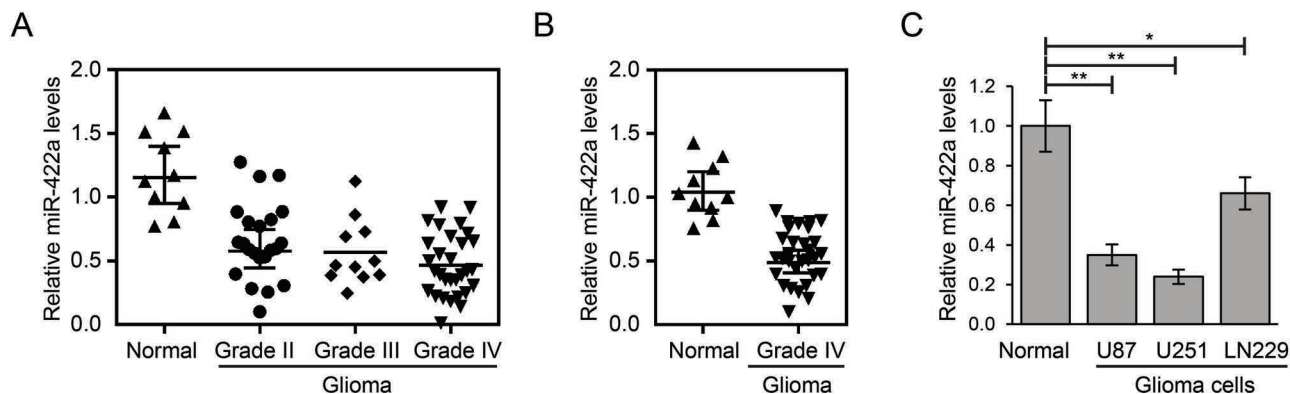


Figure 1. miR-422a is downregulated in glioma. (A) Relative miR-422a expression levels as determined by qRT-PCR in glioma tissues from patients designated WHO grade II ($n = 13$), grade III ($n = 11$), and grade IV ($n = 30$), and in normal brain samples ($n = 10$). $p < 0.001$. (B) Relative miR-422a expression levels in the serum of 30 glioma grade IV patients and 10 normal participants. $p < 0.001$. (C) Expression levels of miR-422a in U87, U251, and LN229 cells compared with normal brain tissues. * $p < 0.05$ and ** $p < 0.01$.

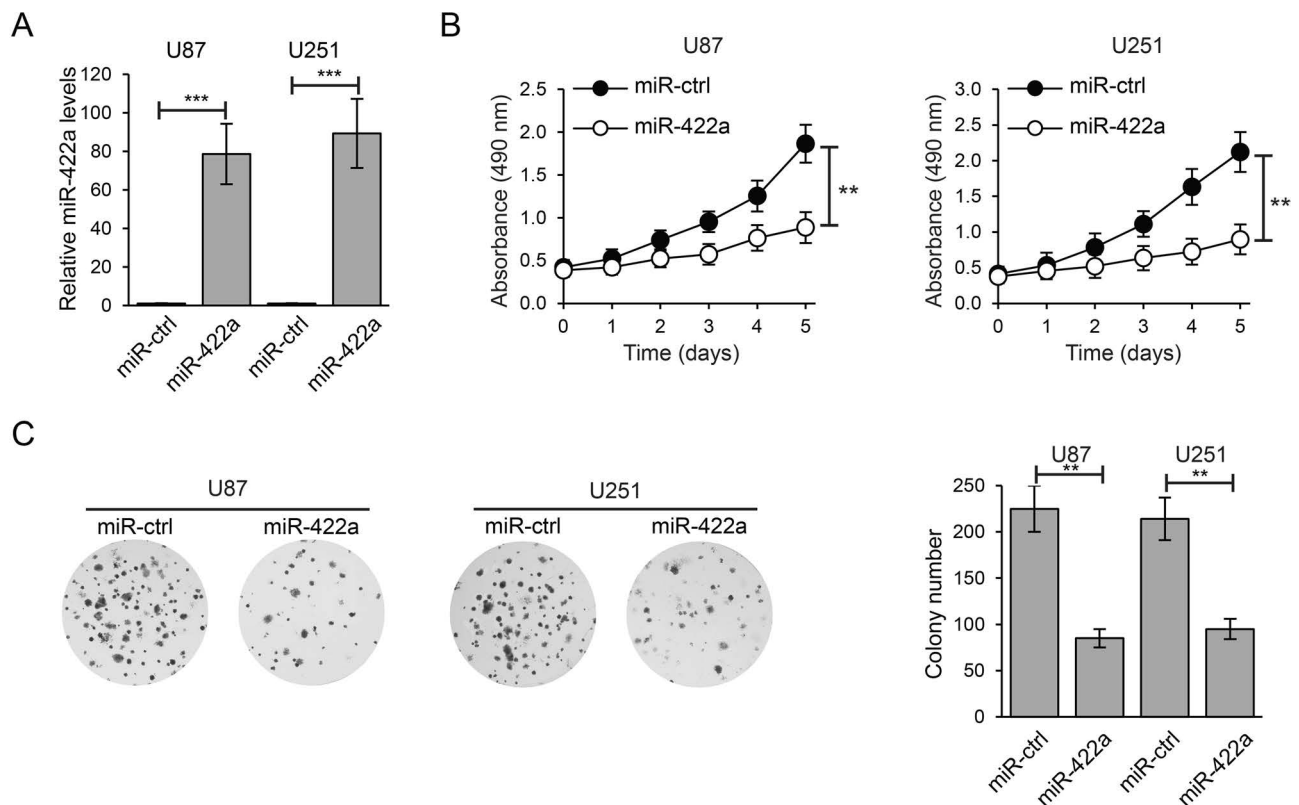


Figure 2. miR-422a inhibits glioma cell proliferation, migration, and invasion. (A) Expression levels of miR-422a in U87 and U251 cells transfected with miR-422a or a control microRNA (miR-Ctrl), as determined by qRT-PCR. *** $p < 0.001$. (B) MTT cell proliferation and (C) colony formation assays performed in U87 and U251 cells transfected with miR-422a or a control microRNA. ** $p < 0.01$.

chamber to the lower chamber of the Transwell. Taken together, these results indicated that miR-422a expression negatively regulated the proliferation, migration, and invasion of glioma cells.

IGF1 and IGF1R Are Direct Targets of miR-422a

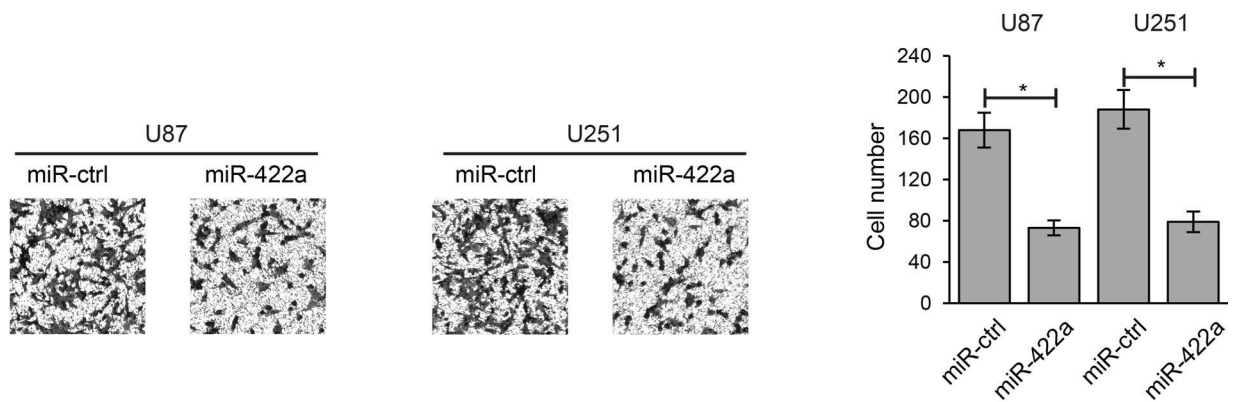
In order to study the regulatory mechanism of miR-422a in glioma cells, we used TargetScan bioinformatics tool (<http://www.targetscan.org>) to analyze potential direct targets of miR-422a, and we found that both IGF1 and IGF1R were predicted to have multiple miR-422a binding sites in their mRNA (Fig. 4A). To directly test the target relationship, we performed luciferase activity assays and found that overexpression of miR-422a led to a reduction in luciferase activity of the reporter plasmid containing the 3'-UTR of IGF1 or IGF1R in both U87 and U251 cells (Fig. 4B). By qRT-PCR and Western blot analysis, we found that both the mRNA (Fig. 4C) and the protein (Fig. 4D) levels of IGF1 and IGF1R were significantly reduced in cells transfected with miR-422a, compared to those transfected with a control miRNA. We next sought to investigate the correlation between miR-422a and IGF/IGF1R expression levels in glioma patient

samples. We examined the mRNA expression levels of IGF1 and IGF1R in glioma patient samples by qRT-PCR and then performed Spearman's rank correlation analysis to compare the expression levels of IGF1 and IGF1R to those of miR-422a in these samples. As shown in Figure 5, the statistical analysis revealed reverse correlation between miR-422a and IGF1 ($r = -0.55$, $p < 0.001$) and between miR-422a and IGF1R ($r = -0.59$, $p < 0.001$).

IGF1 and IGF1R Are Required for the Biological Effects of miR-422a in Glioma Process

To further verify the functional connection between miR-422a and its targets (IGF1 and IGF1R), U87 cells were transfected with miR-Ctrl, miR-422a, and miR-422a+IGF1 (Fig. 6A). When IGF1 was ectopically overexpressed in miR-422a-transfected U87 cells, the inhibition of cell proliferation, migration, and invasion induced by miR-422a was partially reversed (Fig. 6B–D). In addition, overexpressing IGF1R (Fig. 6E) could also partially block the repression of cell malignance induced by ectopic miR-422a (Fig. 6F–H). Furthermore, we blocked IGF1/IGF1R cascade using the IGF1R inhibitor AG1024 to confirm that miR-422a functions through the

A



B

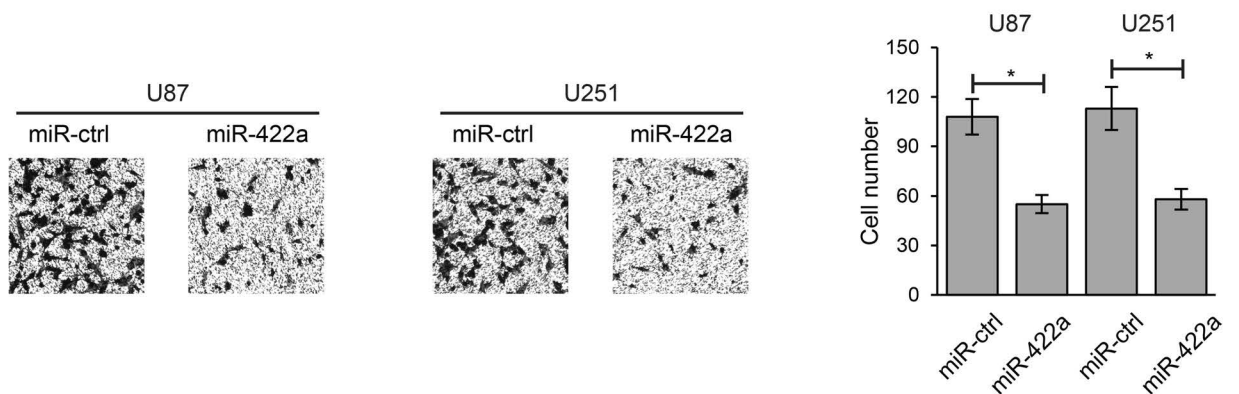


Figure 3. miR-422a represses glioma cell proliferation, migration, and invasion. (A) Cell migration and (B) cell invasion assays performed in U87 and U251 cells transfected with miR-422a or a control microRNA. * $p < 0.05$.

IGF1/IGF1R pathway. Functional experiments including MTT, migration, and invasion assays were performed after U87 cells were transfected with miR-422a or miR-ctrl for 48 h and then treated with 25 μ M AG1024. As shown in Figure 6I–K, AG1024 significantly reduced U87 cell proliferation, migration, and invasion, while miR-422a could not further decrease U87 cell malignance in the presence of AG1024. These data further confirmed that miR-422a functions via IGF1/IGF1R.

DISCUSSION

In this study, we first demonstrated that the expression levels of miR-422a were significantly downregulated in glioma tissues and that the expression levels of miRNA-422a were inversely correlated with disease progression in glioma patients. These results were consistent with recent findings that the expression levels of tumor-suppressing miRNAs were often downregulated in glioma (20). Noticeably, the expression levels of miR-422a itself were also demonstrated by recent studies to be correlated with cancer risk and could serve as biomarkers for the diagnosis of multiple types of cancers. In hepatic cell carcinoma,

miR-422a expression was significantly downregulated, and miR-422a levels were negatively correlated with pathological grading, recurrence, and metastasis (9). The expression levels of miR-422a were also strongly decreased in osteosarcoma bone tissue, and the expression levels of miR-422a were inversely correlated with tumor size and metastatic potential (10). In colorectal adenocarcinoma patients, the levels of miR-422a in serum were specifically altered and could potentially serve as a biomarker for the early diagnosis of this disease (8). However, our study is among the first to reveal the association of miR-422a with glioma development.

Here we examined the function of miR-422a on cell proliferation, cell migration, and cell invasion in cultured glioma cells. We used two independent cell lines for these assays, so that we could confirm that the effects of miR-422a were not restricted to a specific glioma cell line. We found that overexpression of miR-422a in glioma cells significantly inhibited cell proliferation, migration, and invasion. These aspects of cell behavior represent the key steps in the progression of tumor malignancy, and therefore these results were consistent

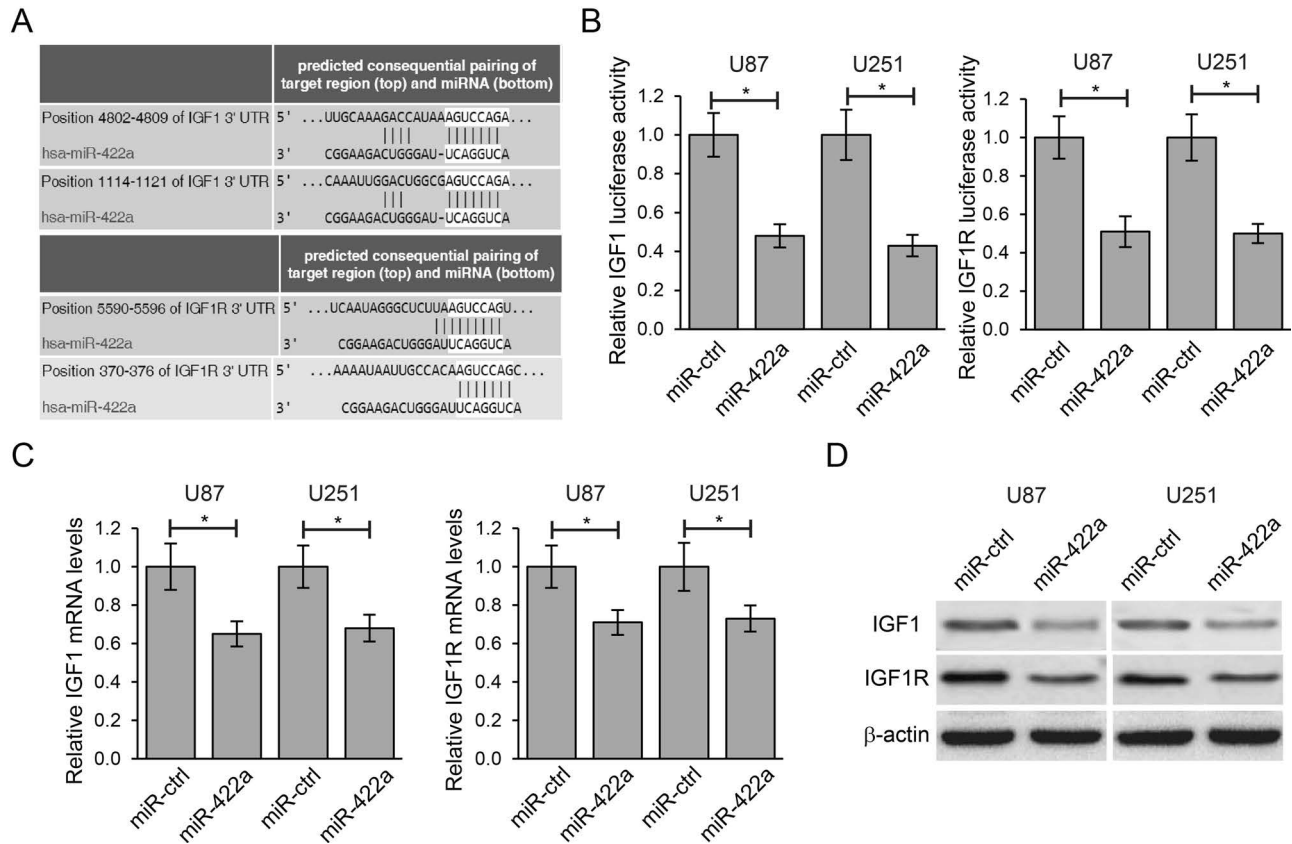


Figure 4. IGF1 and IGF1R are direct targets of miR-422a. (A) Prediction results by bioinformatics analysis demonstrating the nucleotide complementarity between miR-422a and IGF1 and between miR-422a and IGF1R. (B) Luciferase activity in U87 and U251 cells transfected with miR-422a and reporter plasmids containing the 3'-UTR of IGF1 or IGF1R. (C) mRNA levels as determined by qRT-PCR and (D) protein levels as determined by Western blot analysis of IGF1 and IGF1R in U87 and U251 cells transfected with miR-422a or miR-Ctrl. * $p < 0.05$.

with the discovery that miR-422a expression was down-regulated in glioma. However, the inhibition of glioma cell proliferation by miR-422a might negatively affect the ability of cells to migrate and invade, so further evaluation of the exact effect of miR-422a on migration and invasion will be needed.

The results of our study were consistent with the hypothesis that miR-422a inhibits glioma progression via directly targeting and inhibiting the expression of IGF1 and IGF1R. The IGF1/IGF1R signaling pathway plays an important role in regulating glucose, protein, and lipid metabolism (11) and has recently emerged as

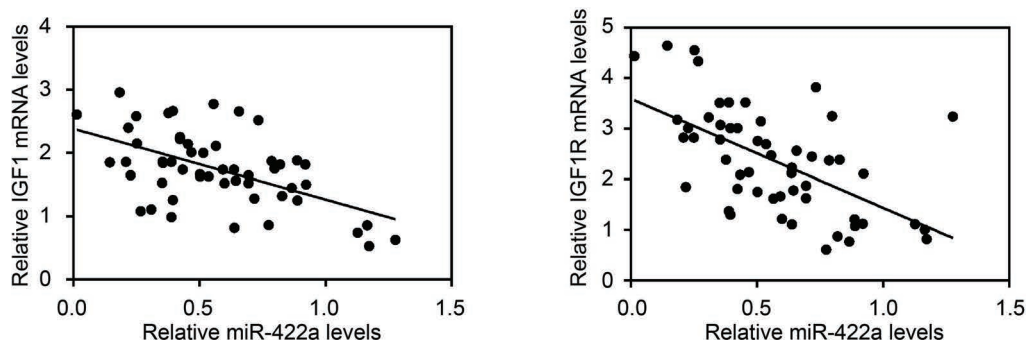


Figure 5. Correlation between miR-422a and IGF1/IGF1R in glioma. Spearman's rank correlation between miR-422a and IGF1 ($r = -0.55$, $p < 0.001$) and between miR-422a and IGF1R ($r = -0.59$, $p < 0.001$) in samples from glioma patients.

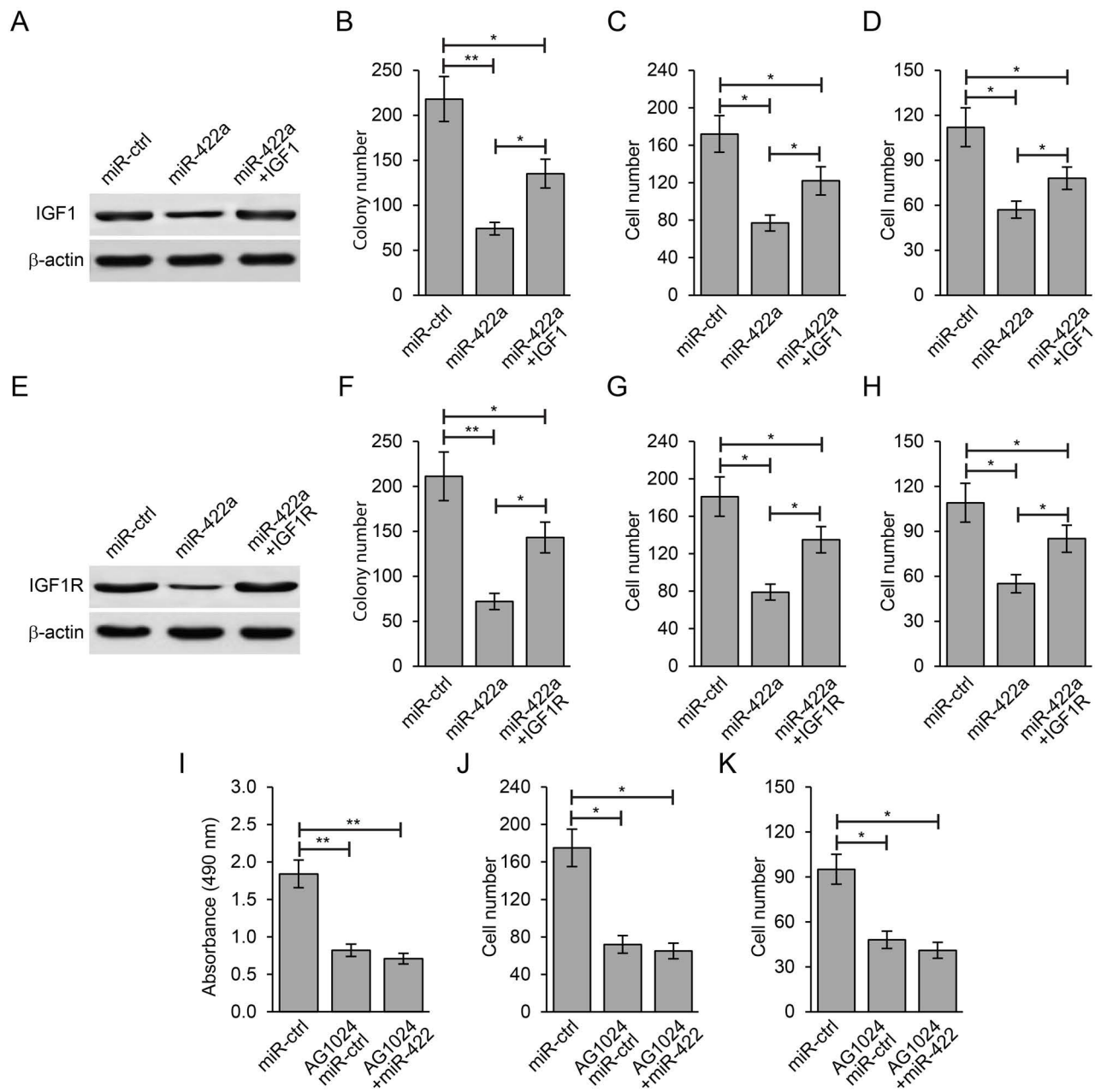


Figure 6. IGF1 or IGF1R partially reverses the effect of miR-422a. (A) IGF1 protein expression was determined by Western blot in U87 cells. (B) Colony formation, (C) cell migration, and (D) cell invasion assays performed in U87 cells transfected with miR-Ctrl, miR-422a, or miR-422a + IGF1. * $p < 0.05$ and ** $p < 0.01$. (E) IGF1R protein expression was determined by Western blot in U87 cells. (F) Colony formation, (G) cell migration, and (H) cell invasion assays performed in U87 cells transfected with miR-Ctrl, miR-422a, or miR-422a + IGF1R. (I) MTT, (J) cell migration, and (K) cell invasion assays performed in U87 cells transfected with miR-Ctrl or miR-422a and treated with or without AG1024 (25 μ M). * $p < 0.05$ and ** $p < 0.01$.

a regulator of cancer metabolism (12). Since advanced therapeutic strategies are increasingly focused on the unique metabolism patterns of cancer cells, IGF1 and IGF1R have become potential targets for cancer therapy (16–19). It was noticed that the proliferation and survival of tumor cells appeared to be dependent on IGF/

IGF1R signaling. Inhibition of the IGF1/IGF1R signaling pathway could inhibit proliferation and promote cell death (15). Consistent with these findings, since IGF1 and IGF1R are negatively regulated by miR-422a, overexpression of miR-422a significantly reduced cell proliferation in glioma. However, since there has been no

report as to how IGF1 and IGF1R affected cell migration and invasion in glioma, the exact role of miR-422a in regulating glioma cell migration and invasion needs to be investigated by future studies.

Significantly, we found that the inhibitory relationship between miR-422a and IGF/IGF1R was not only present in cultured glioma cells but also in glioma tissues from clinical patients. This observation is important, as it is common that the gene expression profiles could be vastly different in *in vivo* tissue samples versus those in *in vitro* cell culture. The inverse correlation as determined by Spearman's rank correlation analysis between the expression levels of miR-422a and both IGF1 and IGF1R provided strong support that the regulation of glioma development by miR-422a was realized via inhibiting the expression of IGF1 and IGF1R. It is important to point out, however, that the genetic relationship between miR-422a and IGF1/IGF1R will still need to be definitively confirmed by rescue experiments in future studies.

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