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Original Article

MiR-422a acts as a tumor suppressor in glioblastoma by targeting PIK3CA

Haiqian Liang[1,2,4*], Renjie Wang[1,2*], Ying Jin[3*], Jianwei Li[1,2], Sai Zhang[1,2]

[1]Institute of Traumatic Brain Injury and Neurology, Pingjin Hospital, Logistics University of Chinese People's Armed Police Forces, Tianjin 300162, China; [2]Department of Neurosurgery, Pingjin Hospital, Logistics University of Chinese People's Armed Police Forces, Tianjin 300162, China; [3]Department of Clinical laboratory, Pingjin Hospital, Logistics University of Chinese People's Armed Police Forces, Tianjin 300162, China; [4]Chinese Glioma Cooperative Group (CGCG), Tianjin 300162, China. [*]Equal contributors.

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Abstract: Although surgical treatment, chemotherapy, and radiotherapy have improved the overall survival rate in glioblastoma multiforme (GBM), further intensive research of GBM's molecular mechanism is still needed. In this study, we observed that miR-422a was downregulated in GBM tissues and cell lines by quantitative real-time polymerase chain reaction (PCR) and primer extension assay. Overexpression of miR-422a significantly reduced the cell proliferation, migration, and invasion of GBM cells. Functional study indicated that miR-422a inhibited cell proliferation, invasion, and migration by targeting PIK3CA, an important member of PI3K/Akt signal pathway. These results demonstrate that the miR-422a/PIK3CA axis may constitute a potential target for GBM therapy.

Keywords: microRNA, miR-422a, PIK3CA, GBM

Introduction

Glioblastoma multiforme (GBM), known also as Grade IV glioma, is the most common and lethal primary brain tumor in adults [1, 2]. GBM is characterized by extensive brain tissue proliferation, invasion, and migration [3, 4]. Although surgical treatment, chemotherapy, and radiotherapy have significantly improved patient survival time, the 5-year survival rate is still at a low level [5]. In recent decades, although increasing evidence has revealed different aspects of GBM's molecular mechanism, which includes proteins and RNAs [6, 7], the detailed molecular signaling pathways in GBM remain to be explored.

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules consisting of 20~22 nucleotides that regulate the expression of special genes through interaction with its 3'UTR [8]. Theoretically, a single miRNA can regulate multiple target genes simultaneously, while a single gene may be regulated by multiple miRNAs.Besides, miRNAs can also build circuits with their target genes [9]. Accumulating evidence has demonstrated that miRNAs play a fundamental role in various cancers, including GBM. For instance, miR-124 acts as a tumor suppressor in glioblastoma via the inhibition of signal transducer and activator of transcription 3 (STAT3) [10]; MiR-519a functions as a tumor suppressor in glioma by targeting the oncogenic STAT3 pathway [11]. More and more miRNAs are passing from laboratory research to clinical use, such as miR-10b [12]. However, the regulation mechanisms of GBM are complicated and we need to further study the role of miRNAs in GBM's progress. Recently, we find the miR-422a was enriched in brain tissues from the open database miRNA map 2.0 (http://mirnamap.mbc.nctu.edu.tw/php/mirna_entry.php?acc=MI0001444). To our knowledge, few studies have examined miR-422a, a brain tissue enriched miRNA, in GBM development. In this study, we discovered that the low level of miR-422a in GBM was associated with the carcinogenesis of GBM via the promotion of PIK3CA protein expression.

Table 1. Sequences used in the construction of plasmids

miR-422a sequence	5. ACUGGACUUAGGGUCAGAAGGC 3'
miR-422a inhibitor (ASO)	5' GCCUUCUGACCCUAAGUCCAGU 3'
PIK3CA-S-Hind III	5' CCCAAGCTTATGCCTCCACGACCATCATC 3'
PIK3CA-AS-KpnI	5' CGGGGTACCGTTCAATGCATGCTGTTTAATTG 3'
PIK3CA-3'UTR-Top	5' AAACTAGCGGCCGCTAGTGCATTTTTATCTATCAGTCCAGAT 3'
PIK3CA-3'UTR-Bot	5' CTAGATCTGGACTGATAGATAAAAATGCACTA GCGGCCGCTAGTTT 3'
PIK3CA-3'UTR mut-Top	5' AAACTAGCGGCCGCTAGTGCATTTTTATCTATCAGACGACAT 3'
PIK3CA-3'UTR mut-Bot	5' CTAGATGTCGTCTGATAGATAAAAATGCACTA GCGGCCGCTAGTTT 3'

Materials and methods

GBM tissues, cell lines, and clinical data

Human GBM and their adjacent normal tissues were obtained from six patients. The tissues used for RNA extraction were flash-frozen in liquid nitrogen and stored at -80°C. Written informed consent was acquired from each patient and this research was approved by the Pingjin Hospital Medical Ethics and Human Clinical Trial Committee. Glioma cell lines (U373, TJ905, U251, and SHG44) were purchased from the American Type Culture Collection and grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were maintained in a humidified atmosphere at 37°C with 5% CO_2. The association between miR-422a expression and survival analysis of GBM patients were performed as for previous studies [13]. Level 3 miRNA isoform expression data based on miR-seq across 510 GBM cases were downloaded from the open-access The Cancer Genome Atlas (TCGA) data; 381 cases were enrolled for overall survival (OS) study. Detailed patient demographics are described in the Supplementary Table 1.

Oligonucleotides, RNA extraction and real-time polymerase chain reaction

All the RNA oligonucleotides were purchased from GenePharme (Shanghai, China). MiRNAs sequences are listed in **Table 1**. The method used for both mRNA and miRNA quantification was as previously described [14]. All the primers used for this study are shown in **Table 1**.

Primer extension to detect mature miRNAs

Primer extensions were performed with 5 mg of total RNA using the AMV PE kit according to the manufacturer's protocol (Promega). Reverse transcription products were separated on 15% TBE-urea polyacrylamide gels and exposed to film.

3.2 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetra-zolium bromide assays

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays were carried out to assess the cell viability. Briefly, U373 and TJ905 cells were seeded at a concentration of 10^4 cells per well in a 96-well plate. miRNA mimic, negative control RNA, miRNA inhibitor, or eukaryotic expression plasmids were transfected into the cells using Lipofectamine 2000 the following day, according to the manufacturer's protocol. The cells were cultured at 37°C in transfection media for 6 hours. The medium was then replaced with complete medium containing MTT (final concentration, 250 μg/mL). The plates were incubated for an additional 12 hours, 24 hours, or 48 hours. The trapped MTT crystals in the cells were solubilized in 200 μL of dimethyl sulfoxide (DMSO) at 37°C for 15 min. The absorbance was determined in a microtiter plate reader (Molecular Devices, Menlo Park, CA) at 570 nm, with 650 nm as the reference wavelength. All experiments were performed in triplicate.

Invasion, migration, and wound healing assays

Cells suspended in serum-free medium (10^5) were placed into the upper chamber of an insert pre-coated with Matrigel (BD Bioscience), while DMEM supplemented with 10% FBS was added to the lower chamber. The cells were allowed to invade for 48 hours. The cells remaining on the upper surface of the membrane were removed, whereas the cells that had invaded through the membrane were stained with 20% methanol and .2% crystal violet, imaged and counted under a microscope (Olympus, Tokyo, Japan). The migration assay

was performed without Matrigel. The cells were seeded in six-well plates and cultured to 100% confluence to perform wound healing assays. A wound was then produced in the cell monolayer using a plastic pipette tip, then the cells were washed in phosphate-buffered saline (PBS) buffer and cultured for another 48 hours. The wound closure rate was observed and described using a percentage.

Colony formation assays

For the colony formation assays, the cells were harvested and seeded at a density of 200 cells per well in 12-well plates and incubated at 37°C and 5% CO_2 in a humidified incubator for 2 weeks. During colony growth, the culture medium was replaced every 3 days. The colony number in each well was counted and calculated.

Flow cytometry analysis

U373 and TJ905 cells were harvested using trypsinization, washed in ice-cold PBS and then fixed in 80% ice-cold ethanol in PBS. Before staining, the cells were pelleted in a chilled centrifuge and re-suspended in cold PBS to a concentration of 1×10^4 cells. Bovine pancreatic RNase (Sigma-Aldrich, USA) was added to a final concentration of 2 mg/ml and the cells were incubated at 37°C for 30 min. To detect the cell cycle distribution, the cells were stained with 20 mg/ml of propidium iodide (Sigma-Aldrich, USA) for 20 min at room temperature. For the cell apoptosis assays, the cells were labeled using the Annexin V-FITC apoptosis detection kit (Invitrogen Corporation, California, USA), as described by the manufacturer. The cell cycle profiles and the cell apoptosis rates were quantified using a flow cytometry kit (Beckman Coulter Corp., CA, and USA).

Xenografted tumor model

Five pairs of BALB/c nude mice were used in this tumor model. In summary, 3×10^6 U373 cells (without transfection miRNA mimics or control miRNA mimics) were suspended in 150 μL of serum-free DMEM for each mouse. The left dorsal flank of each nude mouse was then subcutaneously injected with 3×10^6 U373 cells to establish a xenograft. The tumors formed 1 week later (set as 0 week in this study). Ten tumor-bearing nude mice were randomly divided into two groups (miR-422a and control). The tumor was then injected with a mixture of miR-422a mimics and Lipo-2000 (transfection reagent) or a mixture of control mimics and Lipo-2000. We repeated this injection every 4 days. The animals were sacrificed after 28 days. The nude mice were handled and cared for according to the NIH Committee guidelines for Animal Care and the Use of Laboratory Animals at the Experiment Animal Center of Pingjin Hospital, Tianjin, PR China.

Bioinformatics methods

The miRNA targets predicted by computer-aided algorithms were obtained from miRanda (http://www.microrna.org/microrna/home.do), miRDB (http://mirdb.org/miRDB/), and Targetscan (http://www.targetscan.org).

Western blot

The total protein was extracted from cells using radioimmunoprecipitation (RIPA) lysis buffer supplemented with protease inhibitor cocktail (Roche) and phenylmethylsulfonyl fluoride (PMSF) (Roche). The protein concentration was measured using a bicinchoninic acid protein assay (BCA) (Pierce) and a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, USA). The total protein extracts were separated via 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. The PIK3CA protein was probed with specific antibodies obtained from the Tianjin Saier-Bio Company after the blot was blocked with 5% non-fat milk. The gray intensities of the protein bands were quantified using the ImageJ software (http://rsb.info.nih.gov/ij/). Actin antibody was purchased from Sigma and set as an internal control.

Primer extension to detect mature miRNAs

Primer extensions were performed with 5 mg of total RNA using the AMV PE kit according to the manufacturer's protocol (Promega). The detailed protocol was as described previously [15].

Immunohistochemistry assays

The sections of GBM tumor tissues in the animal assays were mechanically deparaffinized

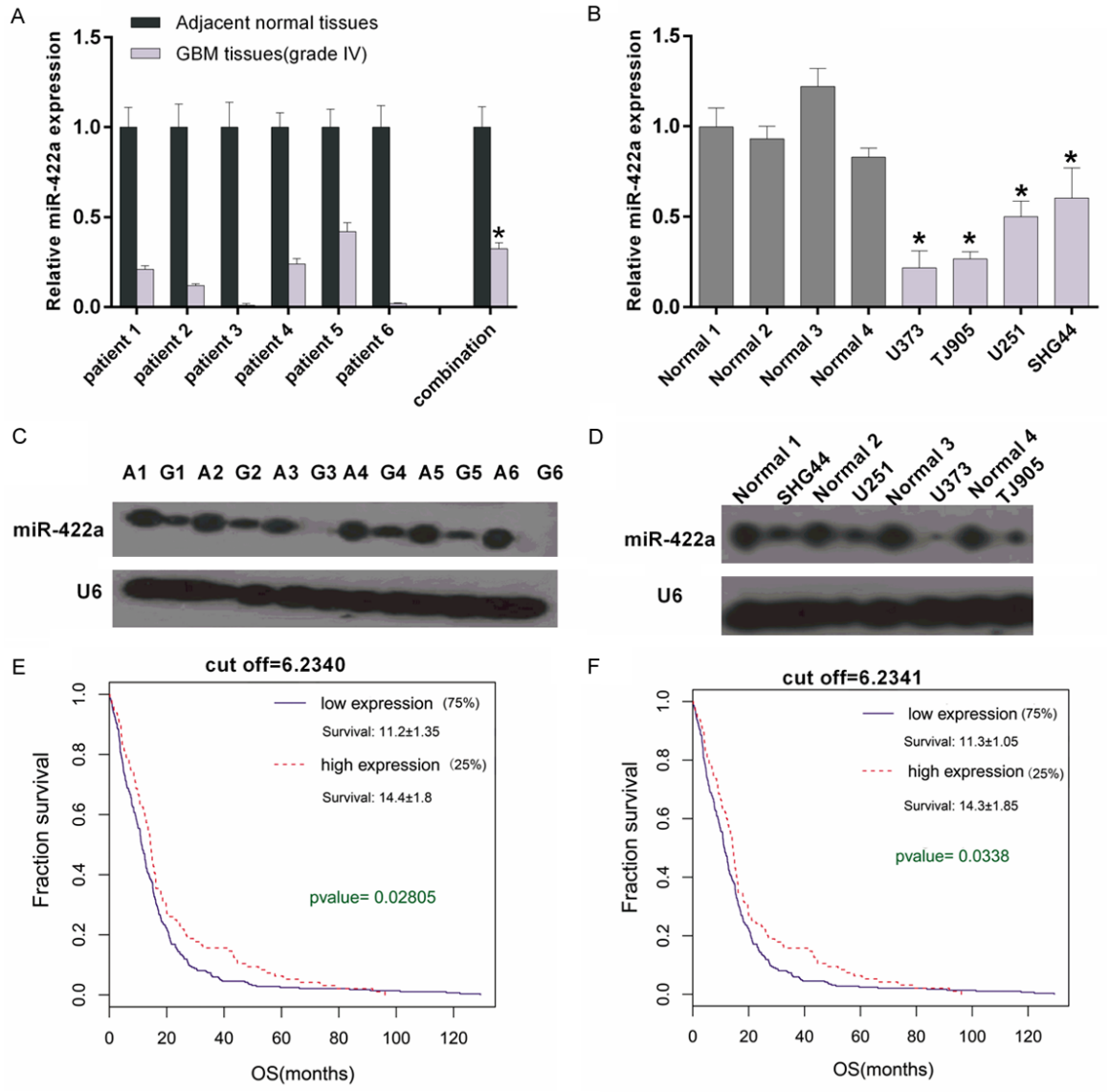


Figure 1. Analysis of miR-422a in glioblastoma multiforme (GBM) tissues and cell lines. A. Relative expression level of mature miR-422a in six pairs of tumor issues and adjacent normal counterparts using real-time reverse transcription polymerase chain reaction (RT-PCR). The lines represent the means of independent experiments in each group. *indicates $P < .05$. B. The relative levels of miR-422a were measured in four normal human astrocytes and four GBM cell lines (U373, TJ905, U251, and SHG44). C, D. The expression of miR-422a was detected by primer extension assay. Six pairs of tumor issues and adjacent normal counterparts were marked G1 (GBM1)-G6 (GBM6), A1 (adjacent normal 1)-A6 (adjacent normal 6). U373, TJ905, U251, and SHG44 were glioblastoma cell lines. 5 mg total RNA was used for each reaction. Primer extension reactions were resolved on 15% TBE-Urea polyacrylamide gels and exposed to film for 50 min. E, F. Overall survival (OS) curves of Kaplan-Meier analysis. Some 381 expression values of miR-422a were sorted by ascending order and OS were analyzed at 75% stratifications of quartiles of miR-422a expression levels. A high level of miR-422a was related to long OS among the TCGA dataset at the indicated cut off (6.2340 and 6.2341). The log-rank test *P* value for the difference between two survival curves for the miR-422a high and low expression GBM patients was indicated.

and incubated in Target Retrieval Solution (Dako, Carpinteria, CA, USA) at 95°C for 40 min. After the endogenous peroxidase activity was blocked with methanol containing 3% hydrogen peroxide (Dako) for 30 min, the tissue sections were incubated with primary antibody to Ki-67 (Tianjin Saierbio company, Tianjin, China) at 4°C overnight. The sections were then

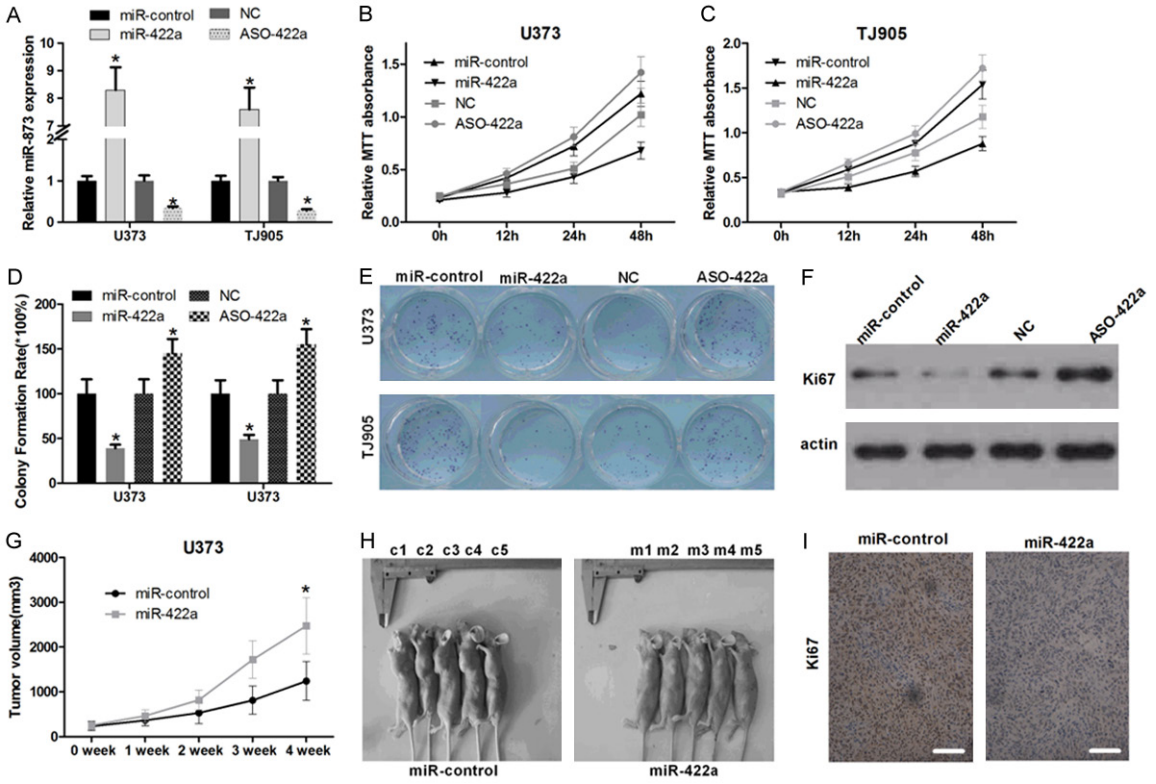


Figure 2. MiR-422a regulates the cell proliferation of GBM *in vitro* and *in vivo*. A. The relative levels of miR-422a in two GBM cell lines, U373 and TJ905, were determined after the cells were transfected with miR-422a mimics, inhibitor, or relative control using real-time RT-PCR. B, C. The relative cell viability in U373 and TJ905 cells was measured after the cells were transfected with miR-422a mimics, inhibitor, or controls using MTT assays. D. The colony formation of miR-422a mimics or inhibitor transfected U373 and TJ905 cells was quantified and compared with that of the GBM cells transfected with control mimics in their respective groups. E. The representative micrographs of the colony formation assays are shown. F, I. The relative Ki-67, a proliferation marker of tumor cells, was determined by western blot or immunohistochemistry test (IHC) in each group. G. The U373 GBM tumor growth *in vivo* was determined as based on the tumor volume, which was calculated as based on weekly measurements after injection. H. A representative image of *in vivo* tumor growth is shown. Error bars represent SD. *means $P < .05$.

incubated in secondary antibody at 37°C for 60 min. Subsequently, the sections were counterstained with hematoxylin for 1 min.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). A student's t-test was used to evaluate significant differences and $P < .05$ was considered statistically significant.

Results

Downregulation of the expression of miR-422a in glioblastoma multiforme (GBM) tissues and cell lines and correlation with patient survival

The MiRNA map 2.0 open database revealed that miR-422a was dominantly expressed in human brain tissues, which imply its important role in the brain (Supplementary Figure 1). To further assess the miR-422a expression in GBM and normal adjacent tissue, we detected its expression in six GBM tumor tissues and their adjacent counterparts by using real-time reverse transcription polymerase chain reaction (RT-PCR) and primer extension assay. As shown in **Figure 1A** and **1C**, the miR-422a expression in malignant tumor tissue was significantly lower than that in non-cancerous tissue. Specifically, the combination expression level of miR-422a in GBM was only 23.6% of adjacent normal tissues. To further confirm this result, we detected the expression level of miR-422a in four normal brain tissues and four GBM cell lines (U373, TJ905, U251, and SHG44) by using real-time RT-PCR and primer extension assay. The results clearly showed that normal brain tissues expressed significantly more miR-

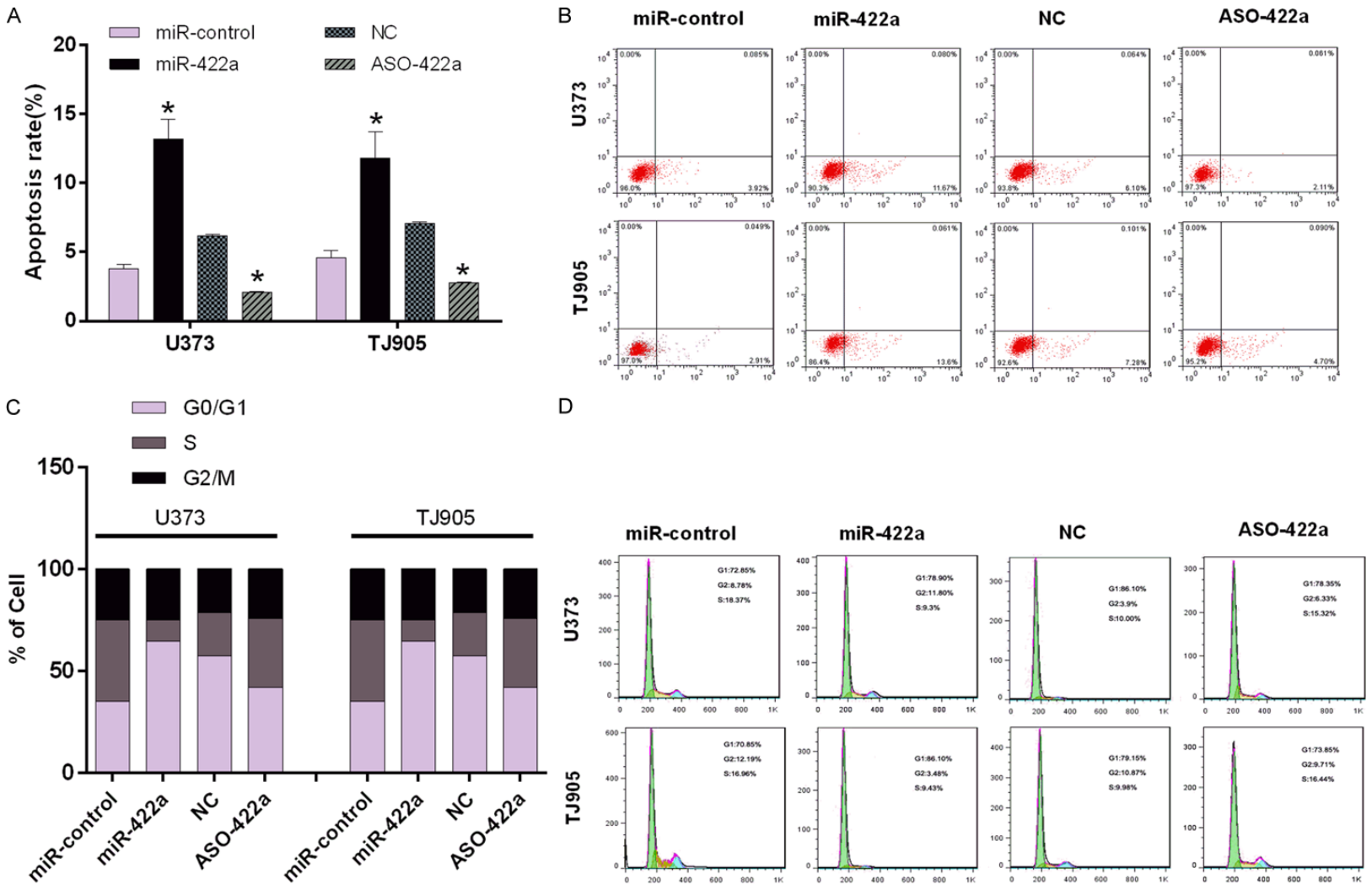


Figure 3. MiR-422a regulation of cell apoptosis and cell cycle of GBM. A, B. The apoptosis rate of the two GBM cell lines transfected with miR-422a mimics or inhibitor were measured and compared with that of the cells transfected with controls. C, D. The cell cycle distributions of the U373 and TJ905 cells transfected with miR-422a mimics, inhibitor, or controls were detected and compared. Error bars represent SD. *means $P < .05$.

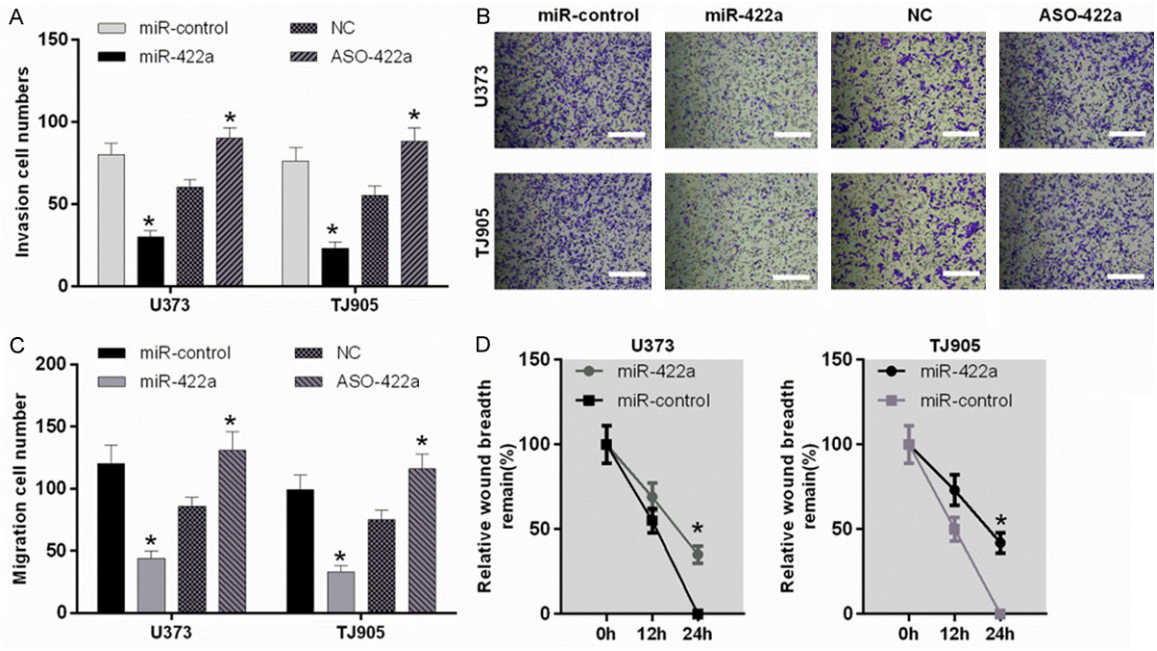


Figure 4. MiR-422a regulates GBM cell migration and invasion *in vitro*. A. The invasion abilities of miR-422a mimics or inhibitor transfected U373 and TJ905 cells were quantified and compared with that of controls in their respective groups. B. The representative micrographs of the invasion of transfected GBM cells are shown. C. The migration of transfected GBM cells were quantified and compared with that of controls in their respective groups. D. The wound healing assay showed that the migration of miR-422a-mimics transfected GBM cells differed from that of cells transfected with miR-control mimics at different time points. The motility is expressed as the percent of migration at the zero-time point. Each bar represents the mean of three independent experiments. Error bars represent SD. *indicates $P < .05$.

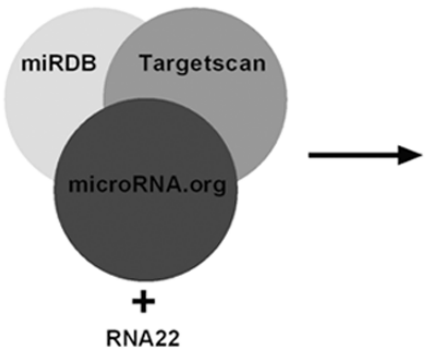
422a than GBM cell lines (**Figure 1B** and **1D**). These data indicated that miR-422a was down-regulated in GBM and may act as a tumor suppressor.

The association between miR-422a expression and post-diagnosis survival was examined in a miRNA expression dataset, The Cancer Genome Atlas (TCGA), consisting of 510 GBM. We excluded 129 cases, which had no accurate time of death, and then investigated 381 cases in GBM patients using the Kaplan-Meier survival analysis. At diverse quartile stratifications, low expression levels of miR-422a were significantly correlated with short OS in comparison to high miR-422a levels. In particular, at the 75% stratification, low expression levels of miR-422a were associated with short OS compared to high levels of miR-422a (median OS: 14.4 vs 11.2 months, cut off: 6.2340, $P = .02850$, **Figure 1E**; median OS: 14.3 vs 11.3 months, cut off: 6.2341, $P = .0338$, **Figure 1F**). These survival data suggested that miR-422a participates in GBM carcinogenesis and might represent a valuable prognostic biomarker for GBM patients.

Effects of ectopic miR-422a and miR-422a blockage on GBM cell proliferation in vivo and in vitro

The levels of miR-422a can be significantly altered in U373 and TJ905 cells after transfection with miR-422a mimics or inhibitor (**Figure 2A**). The effect of the increased or decreased level of miR-422a on U373 and TJ905 cell proliferation was determined using MTT and colony formation assays. The results of the MTT assay demonstrated that miR-422a could regulate the viability of GBM cells, while the results of the colony formation assays suggested that miR-422a could also regulate anchorage-independent growth in GBM cells (**Figure 2B-E**). Furthermore, we analyzed the expression of Ki-67 (a tumor cell proliferation marker) in transfected GBM cells. The results show that the different transfected GBM cells have different Ki-67 expression (**Figure 2F**). To investigate the function of miR-422a in GBM carcinogenesis, we further assessed the influence of miR-422a in GBM cells on the tumor-forming potential *in vivo*. U373 cells were implanted into the left flanks of nude mice by subcutaneous injec-

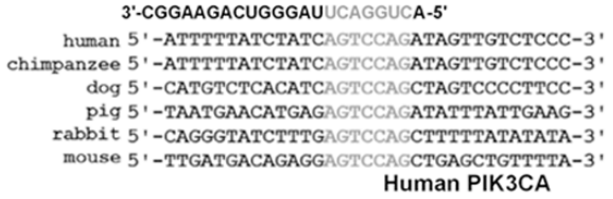
A



Potential targets of miR-422a

Name	ID	Description	Location
PIK3CA	5290	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha [Homo sapiens (human)]	Chromosome 3, NC_000003.12 (179148114..179235137)
PIK3CG	5294	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma [Homo sapiens (human)]	Chromosome 7, NC_000007.14 (106865278..106908978)
PDK1	5163	pyruvate dehydrogenase kinase, isozyme 1 [Homo sapiens (human)]	Chromosome 2, NC_000002.12 (172555373..172625623)
GSK3 β	2932	glycogen synthase kinase 3 beta [Homo sapiens (human)]	Chromosome 3, NC_000003.12 (119821321..120094417, complement)

B



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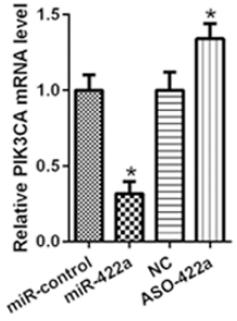

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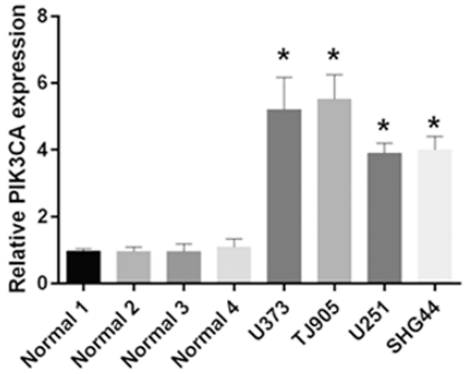

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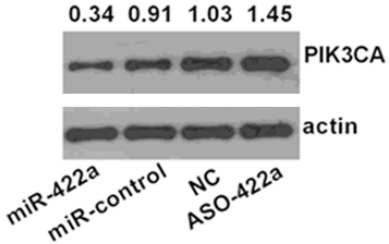

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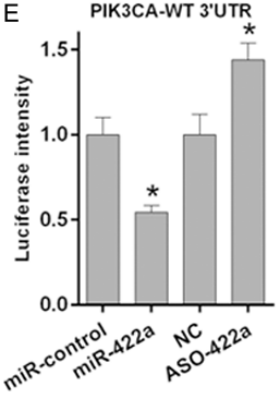

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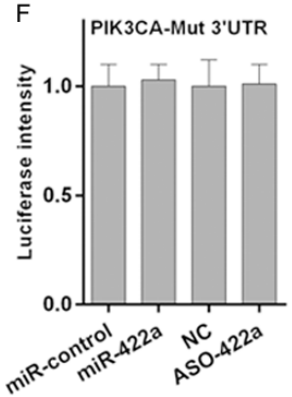

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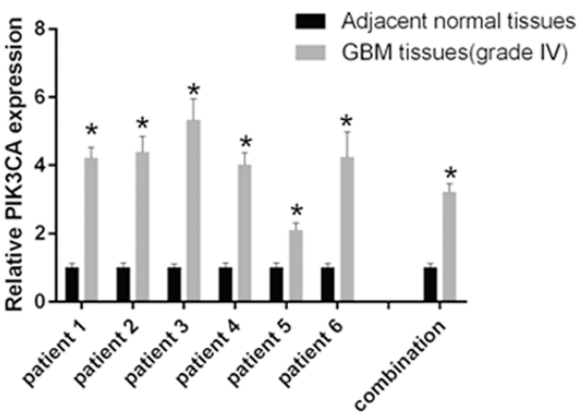

Figure 5. miR-422a directly targeted the PIK3CA gene. A. The potential targeted genes are listed as based on the predictions by the four algorithms. The gene to be further analyzed, PIK3CA, was within the shadow. B. The 3' untranslated region (UTR) fragments of PIK3CA gene are indicated in boxes. The putative binding sites were conserved among species. C, D. Quantative reverse transcription polymerase chain reaction (qRT-PCR) and western blot analyses of the PIK3CA mRNA and protein level were performed following treatment of U373 cells and TJ905 cells with miR-422a, inhibitor, or controls. E, F. The relative firefly activities generated by the luciferase reporter plasmids containing wild-type or mutant PIK3CA 3'UTR in GBM cells with miR-422a mimics, inhibitors, or controls were determined using the dual luciferase assays. G, H. qRT-PCR analyses of the PIK3CA mRNA level were performed in GBM tissues and cell lines. Actin was used as a control. Error bars represent SD. *indicates $P < .05$.

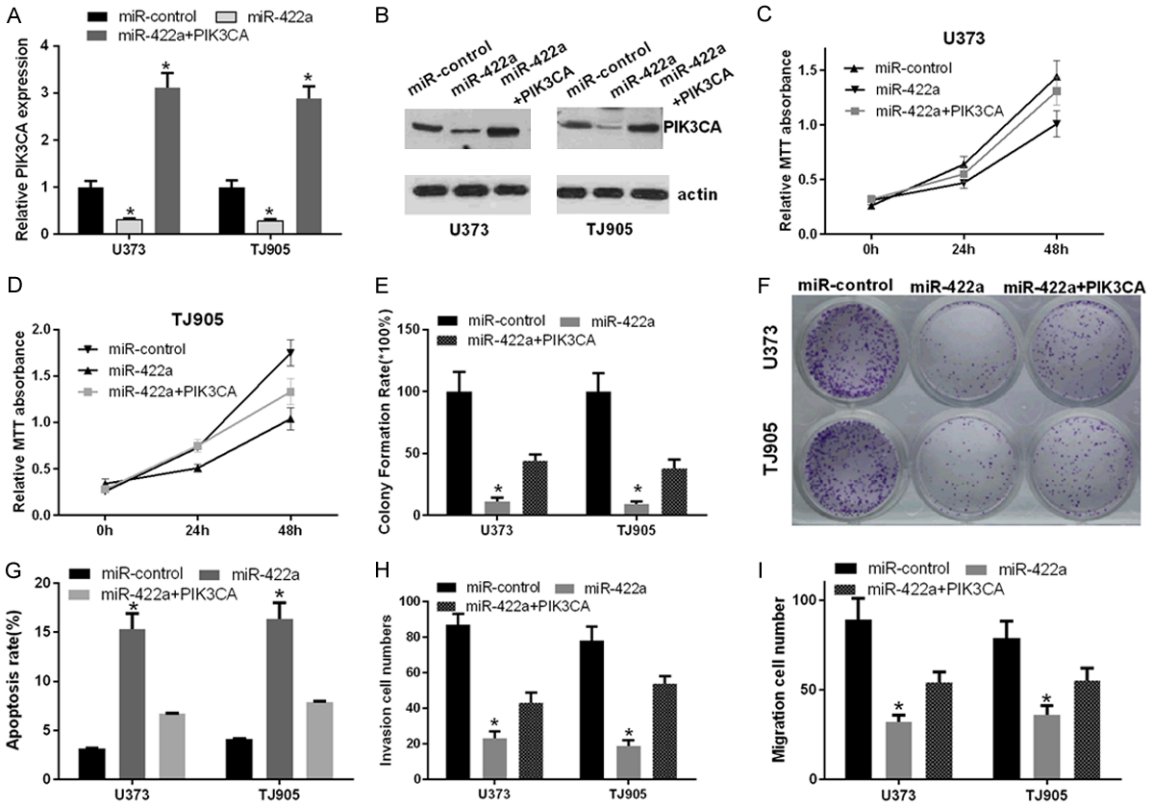

Figure 6. The inhibition of cell growth and invasion caused by miR-422a was reversed by the overexpression of PIK3CA in GBM cells. A, B. The relative expression levels of PIK3CA were detected in the GBM cells transfected with miR-422a or control mimics, and in cells co-transfected with miR-422a and pcMV6.0/PIK3CA using qRT-PCR and western blot. C-F. The cell proliferation ability of GBM cells transfected with miR-422a, control mimics, or miR-422a plus pcMV6/PIK3CA were described by MTT and colony formation assays. G. The cell apoptosis of GBM cells transfected with miR-422a, control, or miR-422a plus pcMV6/PIK3CA are presented. H, I. The invasion and migration abilities of the GBM cells transfected with miR-422a, control, or miR-422a plus pcMV6/PIK3CA are presented using transwell assays. The experiments were performed in triplicate. Error bars represent SD. *indicates $P < .05$.

tion. The mean volumes of xenograft tumors were measured every week. Four weeks after the injection, the xenograft tumors transfected with miR-422a generated smaller tumors than cells transfected with miR-control mimics (n = 5 animals per group, $P < .05$) (**Figure 2G** and **2H**). Furthermore, we detected the Ki-67 expression in two groups of tumors. The results show that the miR-422a group has a lower Ki-67 level by immunohistochemistry test (IHC), which was corrected with *in vitro* assays (**Figure 2I**). Thus, we suggest that the transfection of miR-422a into GBM cells inhibits tumor cell proliferation *in vitro* and *in vivo*.

Mir-422a regulation of GBM cell apoptosis and cell cycle

We measured and compared the apoptosis ratios of U373 and TJ905 cells transfected with

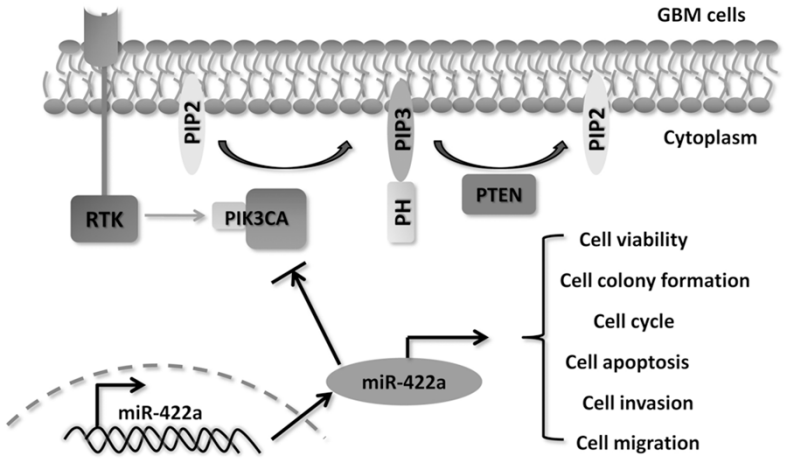

Figure 7. Suggested molecular mechanism of the miR-422a inhibition of GBM proliferation, invasion, and migration by targeting PIK3CA.

miR-422a mimics or inhibitor with the ratios of the corresponding cells with control mimics using flow cytometry. Then, we investigated the number of apoptotic cells labeled with Annexin V or 7AAD, respectively. The results (**Figure 3A** and **3B**) show that the GBM cell apoptosis rate was increased by about 10% with miR-422a mimics treatment, while blockage of miR-422a by miR-422a inhibitor decreased the apoptosis rate by about 5%. Furthermore, analysis of the cell cycle kinetics using flow cytometry indicated that miR-422a significantly induced cell cycle arrests in both U373 and TJ905 cells. The results (**Figure 3C** and **3D**) show that GBM cells treated with miR-422a have decreased S phase populations in both cell lines when compared with the control group. The marked decrease in the S phase fraction suggests that miR-422a may act as an inhibitor by inducing cell apoptosis.

Effects of ectopic miR-422a and miR-422a blockage on GBM cell migration and invasion

Transwell invasion and migration assays were utilized to examine the effect of miR-422a on cell invasion and migration. The invasion level of the U373 and TJ905 cells transfected with miR-422a mimics was significantly lower than that of cells transfected with miR-control mimics (**Figure 4A** and **4B**). Similarly, the migrated potentials of the U373 and TJ905 cells were also decreased after transfection with miR-422a mimics when compared with miR-control mimics (**Figure 4C**). Furthermore, the quantification of wound closure showed that U373 and TJ905 cells transfected with miR-422a mimics had lower migration ability after 24 hours' transfection (**Figure 4D**). Moreover, U373 and TJ905 cells transfected with miR-422a inhibitor showedk the opposite tendency for invasion and migration (**Figure 4A-D**). These data suggest that miR-422a significantly regulates GBM cells' migration and invasion.

PIK3CA characterized as a target of miR-422a

MicroRNAs inhibit gene expression by binding to the mRNA transcript of the target gene to induce its degradation. To identify novel miR-422a target genes, three cited algorithms-miRanda, miRDB, and Targetscan-were used to predict the potential targets of miR-422a. As a result, a potential list of targets was identified. Among these genes, four members of PI3K/Akt signal pathway attracted our attention. Then, we analyzed the mRNA expression level of these four genes in GBM cells treated with miR-422a. PIK3CA mRNA showed the greatest level of reduction compared to the relative control group (data not shown). Besides, PIK3CA is upregulated in many malignancies, which led us to believe that PIK3CA may be a direct target of miR-422a in GBM (**Figure 5A**). The putative binding sites of the miR-422a in the 3'UTR of PIK3CA are described in **Figure 5B**. We conducted luciferase reporter assays to confirm the hypothesis that miR-422a regulates PIK3CA though its 3'UTR. As shown in **Figure 5E**, the luciferase activity generated by the reporter vector with PIK3CA-3'UTR WT decreased by 48% after co-transfection with miR-422a mimics compared with the control group, which increased by 32% after co-transfection with miR-422a inhibitor compared with the control group. However, the mutation of the miR-422a binding sites in the 3'UTR of PIK3CA did not affect the activity generated by the reporter vectors compared to the control groups (**Figure 5F**). These results indicated that miR-422a might suppress the expression of PIK3CA by strongly and directly binding to the putative site in its 3'UTR. The following quantative reverse transcription poly-

merase chain reaction (qRT-PCR) and western blot assays showed that the relative protein level of PIK3CA in U373 and TJ905 was regulated by miR-422a (**Figure 5C** and **5D**). To further confirm this result, we detected the expression level of PIK3CA in GBM tissues and cell lines by using real-time RT-PCR. The results clearly demonstrated that normal brain tissues expressed significantly lower PIK3CA than GBM tissues and cell lines (**Figure 5E** and **5F**), which was reversed with miR-422a. These data indicated that PIK3CA was a directly target of miR-422a.

Upregulation of PIK3CA and reversion of the suppressive effect of miR-422a on the advance of GBM cells

To overexpress PIK3CA in GBM cells, we transfected the cells with pcMV6/PIK3CA vectors. The protein level of PIK3CA increased in U373 and TJ905 cells co-transfected with miR-422a mimics and pcMV6/PIK3CA when compared with the control (**Figure 6A** and **6B**). These results suggested that overexpressing PIK3CA using the pcMV6/PIK3CA vector in GBM cells could partially rescue the downregulation of PIK3CA expression caused by the increased cellular level of miR-422a. The viability of GBM cells transfected with miR-422a mimics and pcMV6/PIK3CA was determined with an MTT assay and colony formation assays. The results indicated that when PIK3CA expression was upregulated, the cell viability inhibition of GBM was rescued (**Figure 6C-F**). Furthermore, PIK3CA overexpression decreased the apoptosis rate of GBM cells, which is induced by miR-422a (**Figure 6G**). The transwell invasion and migration system showed that the percentage of invasive or migrated GBM cells was rescued by co-transfection with miR-422a mimics and pcMV6/PIK3CA (**Figure 6H and 6I**). These data indicated PIK3CA was creating miR-422a-induced GBM cell carcinogenesis alternation (**Figure 7**).

Discussion

GBM is the most cytologically malignant and active primary brain tumor, and novel therapeutic interventions are urgently needed [16]. Extensive genetic and molecular studies, especially comprehensive profiling studies in TCGA, have characterized GBM-related oncogenes and tumor suppressor genes [17, 18]. MiRNAs can act as either oncogenes or tumor suppressor genes based on their copy number changes in GBM development. Recently, miR-422a has been found to play a critical tumor suppressor role in hepatocellular carcinoma tumor growth and metastasis and has been involved in a feedback loop with forkhead box (FOX) families [19]. Previous findings suggested that miR-422a might play a protective role against colorectal cancer, as shown by its decreased expression in colorectal cancer tissue when compared to normal tissue [20]. Noticeably, miR-422a also has been found to suppress tumor cell proliferation by inhibiting related pathways in osteosarcoma [21]. Besides, reports have proposed that miR-422a might provide new biomarkers for personalized treatment strategies in various cancers including colorectal adenocarcinoma [22], hepatocellular carcinoma [23], and pituitary adenomas [24]. One of the most significant discoveries ofthis study is the identification of miR-422a as a key inactivated tumor-suppressing non-protein-coding gene in GBM. Most importantly, it can integrate the regulation of multiple GBM hallmarks by directly targeting the PIK3CA oncogene that is known to be important for GBM. Based on our results from TCGA data samples, we demonstrated that low-level miR-422a expression was correlated with decreased survival in patientswith GBM. Importantly, *in vivo* subcutaneous xenograft tumor growth analysis revealed a significant decrease in tumor growth following treatment with miR-422a, indicating its therapeutic potential for patients with GBM. Although effective delivery of miRNA mimics into the brain, crossing the blood-brain barrier, remains challenging, the inhibitory effect of miR-422a on subcutaneous tumor growth suggests that further efforts toward the development of miR-422a-based therapeutics are fully warranted.

The PI3K/Akt pathway (**Figure 6J**) was a very significant factor for GBM cell proliferation, invasion, migration, apoptosis, and cell cycle as well as for radiation and chemotherapy resistance. PI3Ks have been divided into three classes (I, II, and III). Class I consists of two subclasses, class IA and IB. Class IA contains heterodimers that are composed of a p110 catalytic subunit and a p85 regulatory subunit. The p110 subunit has three isoforms (p110a, p110b, p110g), which are involved in the regu-

lation of cell proliferation, invasion, and migration [25]. For PIK3CA, also known as p110a, it is suggested that only the gene encoding p110a subunit plays a crucial role in tumorigenesis, including GBM [26, 27]. Upregulation of the PIK3CA expression has been reported to constitutively increase PI3K activity in cancer cells. In the present study, we showed for the first time that miR-422a could directly target PIK3CA. That means that the downregulation of miR-422a could lead to the upregulation of PIK3CA expression. Indeed, the introduction of miR-422a may reduce the expression of PIK3CA and subsequently downregulate the downstream targets of p110a, Akt, and pAkt (data not shown), resulting in decreased GBM cell proliferation, invasion, and migration. Our findings highlighted the importance of the miR-422a/PIK3CA axis in the carcinogenesis of GBM cells.

In conclusion, this study's major findings can be summarized as follows: (a) miR-422a was downregulated in GBM tissues and cell lines. (b) A lower level of miR-422a was associated with a lower survive rate in GBM patients in TCGA database. (c) We have shown for the first time that miR-422a directly targets PIK3CA and downregulates the expression of PIK3CA in GBM cells via its 3'UTR. (d) miR-422a markedly regulated the proliferation, invasion, and migration of GBM cell lines and this phenotype was mediated by the miR-422a/PIK3CA axis. These results provide strong evidence that miR-422a acts as a tumor suppressor in GBM development by regulating PIK3CA and may implicate miR-422a as a potential target for GBM therapy.

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Disclosure of conflict of interest

None.

Abbreviations

MiRNA, microRNA; miR-422a, microRNA-422a; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; UTR, untranslated region.

Address correspondence to: Haiqian Liang, Institute of Traumatic Brain Injury and Neurology, Pingjin Hospital, Logistics University of Chinese People's Armed Police Forces, Tianjin 300162, China; Department of Neurosurgery, Pingjin Hospital, Logistics University of Armed Police Forces, Tianjin 300162, China; Chinese Glioma Cooperative Group (CGCG), No220, Chenglin Road, Tianjin 300162, China. Tel: +8602260577125; Fax: +8602260577125; E-mail: lianghaiqian711@163.com; Sai Zhang, Institute of Traumatic Brain Injury and Neurology, Pingjin Hospital, Logistics University of Chinese People's Armed Police Forces, Tianjin 300162, China; Department of Neurosurgery, Pingjin Hospital, Logistics University of Armed Police Forces, No220, Chenglin Road, Tianjin 300162, China. Tel: +86022605771-01; Fax: +8602260577101; E-mail: zhangsai718@vip.126.com

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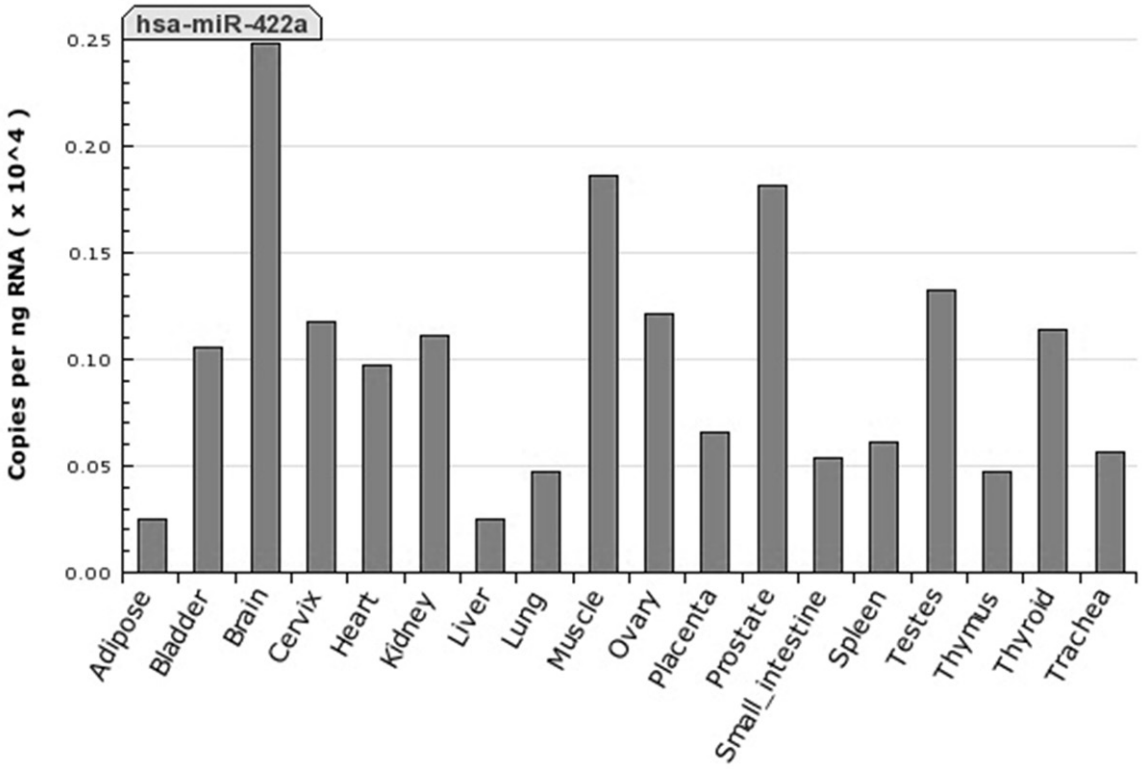

Supplementary Figure 1. The expression of miR-422a in human organs. (Date was download from The MiRNA map 2.0 open database).