Int J Clin Exp Med 2016;9(8):15876-15883
www.ijcem.com /ISSN:1940-5901/IJCEM0029093

Original Article

MicroRNA-421 enhances cell migration and invasion by down-regulating TIMP-2 in glioblastoma

Wei Lu[1*], Ning Zhang[2*], Jing An[3], Ping Zhang[4], Zirun Yang[5]

[1]Department of Neurosurgery, Zibo, China; [2]Department of Internal Neurology, Zibo, China; [3]Department of Radiology, The People's Hospital of LinZi Distric, Zibo, China; [4]Department of Nursing, Zibo Vocational Institute, Zibo, China; [5]Department of Neurosurgery, Zibo No. 1 Municipal Hospital, Zibo, China. [*]Equal contributors.

Received March 24, 2016; Accepted July 6, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: MicroRNA-421 (miR-421) has been identified to play critical roles in various cancers, but the roles of miR-421 in migration and invasion of glioblastoma (GBM) remains unknown. The aim of this study was to investigate biological functions and its molecular mechanisms of miR-421 in GBM cells. Real-time PCR was applied to assess the expression of miR-421. Migration and invasion of GBM cells were analyzed by transwell migration and invasion assays. Luciferase reporter assay was employed to detect cell target genes of miR-421, while and expression of TIMP-2 were verified by western blot analysis. The results showed that miR-421 expression levels were increased in GBM tissues and GBM cell lines compared to normal brain tissues. Then, transwell migration and transwell invasion assays showed that transfection of miR-421 inhibitor significantly inhibited migration and invasion capacity of U87 and U251 cells, while the miR-421 inhibition-induced migratory and invasive abilities of the GBM cell lines were abolished by TIMP-2 downregulation. Further studies indicated that miR-421 negatively regulates TIMP-2 expression via direct binding to putative binding site in the TIMP-2 3' untranslated region. Therefore, we concluded that miR-421 could enhance tumor migration and invasion in GBM by targeting TIMP-2 and may be identified as a potential therapeutic target of GBM.

Keywords: MicroRNA-421, glioblastoma, migration, invasion

Introduction

Glioblastoma (GBM) is the most common malignant neoplasm of central nervous system and its incidence rate tends to be increased in both developed and developing countries [1-3]. Though improvements in available treatments, the poor prognosis of GBM patients has not improved and the median survival is only 12-15 months [4-6]. The outcome of GBM patients mainly resulted from characteristics of migration and invasion. Therefore, to identify the key factors influencing migration and invasion of GBM is urgently needed.

MicroRNAs (miRNAs) are small (approximately 19-24 nucleotides in length), endogenous, non-coding RNAs which regulate gene expression via sequence-specific interactions with the 3' untranslated region (3'-UTR) [7-9]. It is now clear that miRNAs are involved in regulating important cellular functions such as death, invasion, differentiation, stress response, apoptosis, proliferation and development [9]. Moreover, accumulating evidence has showed that miRNAs play critical roles in various cellular processes related to many types of cancers, including GBM [10, 11]. The aberrant expression of microRNA-421 (miR-421) has been reported to be upregulated in certain types of tumor, including liver cancer, gastric cancer as well as pancreatic cancer [12-14]. However, the role of miR-421 in GBM remains enigmatic.

In this study, we examined the expression of miR-421 in GBM samples and GBM cell lines. Compared with normal brain tissue, we found that miR-421 levels were up-regulated in GBM samples and GBM cell lines. In addition, we found that suppression of miR-421 expression with miR-421 inhibitor could suppress migration and invasion ability of GBM cell lines in vitro. Finally, we identified that TIMP-2 is a target gene of miR-421. miR-421 is able to

enhance migration and invasion of GBM cells by paralyzing the function of TIMP-2.

Materials and methods

Patient specimens and cell lines

GBM tissues and normal brain tissues were obtained from patients Department of Neurosurgery at Zibo No 1 municipal hospital. Approval was obtained from the Ethics Committee of the hospital mentioned above. The two human glioma cell lines, U251 and U87, were purchased from Chinese Academy of Sciences Cell Bank. The cells were cultured in DMEM with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Cell transfection

The negative control (NC) miR-421 mimics and mature miR-421 inhibitor were obtained from GenePharma (GenePharma, Shanghai, China). U87 and U251 cells were seeded in 6 cm dishes. Negative control (NC), miR-421 mimics or miR-421 inhibitor was transfected with Lipofectamine 2000 (GenePharma, China) according to the manufacturer's instructions.

Transwell migration and invasion assays

Cell invasion experiments were assayed using 6.5-mm Transwell chambers (8-μm pore size; Costar). The filters were precoated with 1-2 mg/ml matrigel (reconstituted basement membrane; BD Biosciences, Mississauga, Canada). Transfected cells (miR-421 inhibitors or NC) in 100 μl of serum-free medium were seeded to the upper chamber at a density of 5×10^5 cells/ml. Medium supplemented with 10% FBS was added to the lower chamber as the chemoattractant. After 24 h of incubation at 37°C, the cells on the upper side were wiped with a cotton bud. The cells which had invaded into the lower compartment were fixed with methanol, stained with hematoxylin and eosin, and counted in 5 random fields of the insert under a light microscope. The numbers of invaded cells were averaged. Migration assay was carried out as described in the invasion assay but with a shorter incubation period (12 h) and no matrigel coating. These experiments were repeated three times.

Western blotting

The cells were extracted with the RIPA buffer (1 mg/ml PMSF, 1 Mm aprotinin, 1 mg/ml leupeptin, 1 mM EDTA, 150 mM NaCl, 0.25% Na-deoxycholate, 1 mg/ml pepstatin, 1.0% NP-40 and 50 mM Tris-HCl (pH 7.4)) on ice for 15 min. Total proteins were quantified using the BCA method. The equal amounts of protein were separated on SDS-PAGE gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. After blocking in 5% bovine serum albumin for 1 hour at room temperature, membranes were incubated overnight at 4°C with antibody against TIMP-1 (1:1,000; Abcam, UK) or β-actin (1:1,000; Bioss, China). Then, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies (1:2,000, anti-rabbit IgG) for 1 hour at at room temperature. The bands were visualized by enhanced chemiluminescence (ECL) (Thermo Scientific, Rockford, IL). The densitometry analysis was performed using Quality One analysis software (Bio-Rad). These experiments were repeated three times.

Quantitative real-time PCR

Total RNA was extracted from the cell and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). About 1 μg of RNA was reversely transcribed using the miR-421 qPCR Quantitation Kit (Genepharma, China) according to the manufacturer's instructions. The primers of miR-421 and U6 were synthesized from Genepharma (Guangzhou, China) and real-time PCR was carried out on a 7900HT Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems, USA). The thermal-cycling profile: 95°C for 10 minutes, followed by 40 amplification cycles of 95°C for 30 sec and 60°C for 1 minute. The mRNA expression levels of miR-421 were normalized to U6 according to the standard ΔΔCt method. Each assay was carried out in triplicate and all experiments were repeated at least three times.

MicroRNA target prediction and luciferase reporter assay

The following online search databases were used to indentify miRNA-421 potentially binded TIMP-2: miRWalk (http://www.umm.uni-heidel-

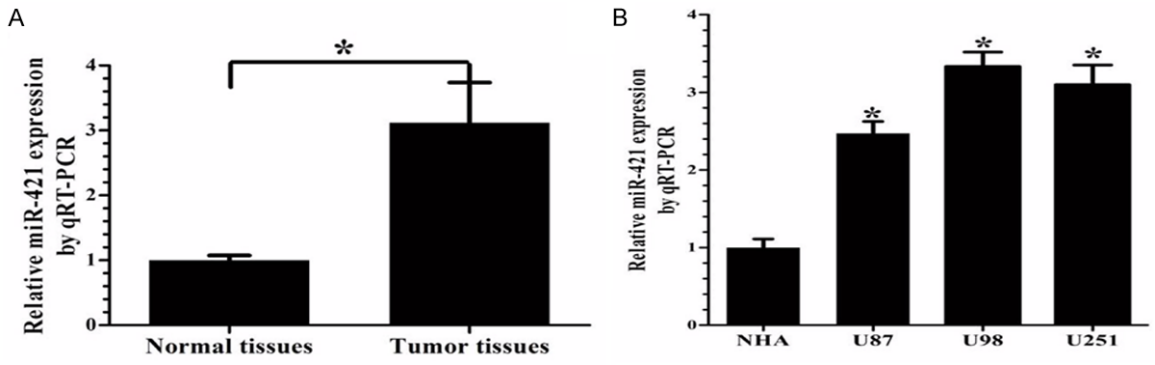

Figure 1. Expression of miR-421 in GBM tissues and GBM cell lines. A. The expression of miR-421 was analyzed by real-time PCR in 31 GBM tissues and 14 normal brain tissues, and the U6 small nuclear RNA was used as an internal control (means ± SD; n=3; *P<0.05). B. The expression of miR-421 was analyzed by real-time PCR in the Normal Human Astrocytes (NHA) and three GBM cell lines (T98G, U87 and U251), and the U6 small nuclear RNA was used as an internal control (means ± SD; n=3; *P<0.05).

berg.de/), Pictar (http://www.pictar.mdc-berlin.de/), TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/). U87 or U251 cells were seeded in a 24-well plate. One day later, cells were co-transfected with luciferase vectors, either the wild-type TIMP-2 3'UTR reporter plasmid or mutated TIMP-2 3'UTR reporter plasmid together with miR-421 inhibitors or miRNA-NC, and the Renilla control. After 48 hours, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA) and normalized to Renilla luciferase activity.

Statistical analysis

The experiments were repeated at least in triplicate. Data were expressed as the means ± SD. SPSS 16.0 statistical software and Student's t-test were used for statistical analysis. A *P* value of <0.05 was considered as statistically significant.

Results

The expression of miR-421 was frequently up-regulated in GBM tissues and GBM cell lines

To evaluate the correlation between miR-421 and GBM, the expression level of miR-421 was examined by quantitative real-time PCR (qRT-PCR) in 31 GBM samples and 14 normal brain tissues. The results showed that miR-421 expression levels were increased in GBM tissues compared to normal brain tissues (P< 0.05) (**Figure 1A**). In addition, miR-421 was also elevated significantly in GBM cell lines U87, U251 and T98, compared with normal brain tissue (**Figure 1B**). These results provided evidence that miR-421 may play some important roles in GBM.

Inhibition of miR-421 suppressed migration and invasion of GBM cell lines in vitro

To investigate whether increased miR-421 in GBM cells affected cell migration and invasion capacity, we used miR-421 inhibitor to perform the loss function analysis. Both transwell migration and transwell invasion assays demonstrated that down-regulation of miR-421 could suppress the migration and invasion capacity of U87 and U251 cells (**Figure 2**, P<0.05). Taken together, these findings suggested that miR-421 was associated with migration and invasion of GBM cells.

TIMP-2 was identified as a direct target of miR-421

To explore the underlying mechanism of miR-421 in GBM, a bioinformatics search was performed in target prediction databases (miRanda, TargetScan, PicTar and miRWalk) to search for potential targets of miR-421. Because miR-421 could regulate GBM invasion, we focused on genes that could influence GBM invasion. All four databases showed that the TIMP-2 mRNA contained miR-421 binding sites (**Figure 3A**). Luciferase reporter assays were then conducted to determine the influence of miR-421 on the expression of TIMP-2. The wild-type TIMP-2 3'UTR reporter plasmid or mutated TIMP-2 3'UTR reporter plasmid was cotransfected into

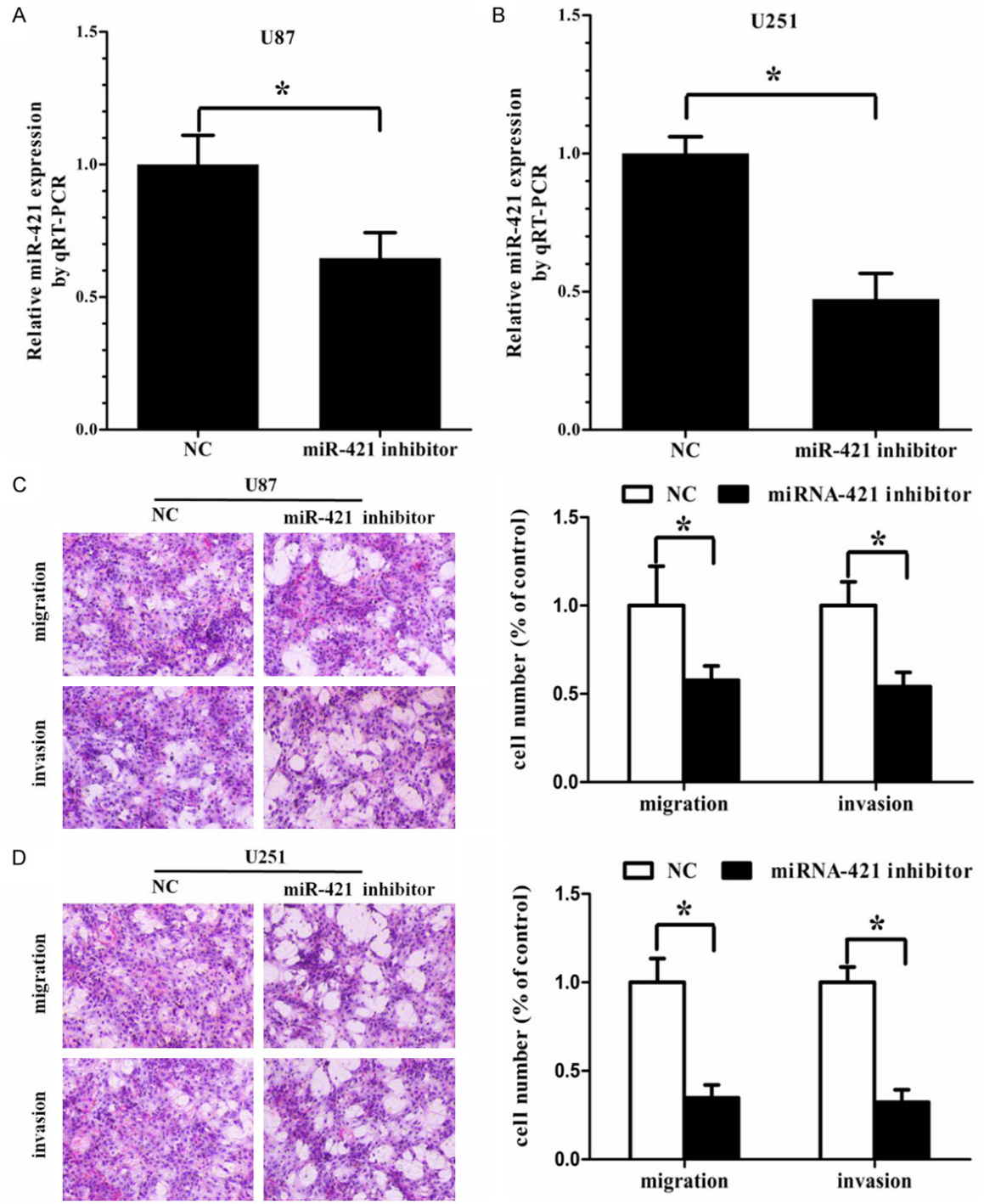

Figure 2. Inhibition of miR-421 suppressed migration and invasion of human GBM cell lines. A and B. The relative expression level of miR-421 in U87 and U251 cells transfected with miR-421 inhibitor was markedly decreased in U87 and U251 cells (means ± SD; n=3; *P<0.05). C and D. Transwell migration and invasion assays using U87 and U251 cells transfected with miR-421 inhibitor or negative control (NC). Representative images are shown on the left, and the quantification of 3 randomly selected fields is shown on the right (means ± SD; n=3; *P<0.05).

U87 and U251 cells with miR-421 mimics or related control. As shown in **Figure 3B**, U87 and U251 cell lines cotransfected with miR-421 mimics and wild-type TIMP-2 3'UTR reporter plasmid showed a significant decrease of reporter activity (P<0.05). Furthermore, trans-

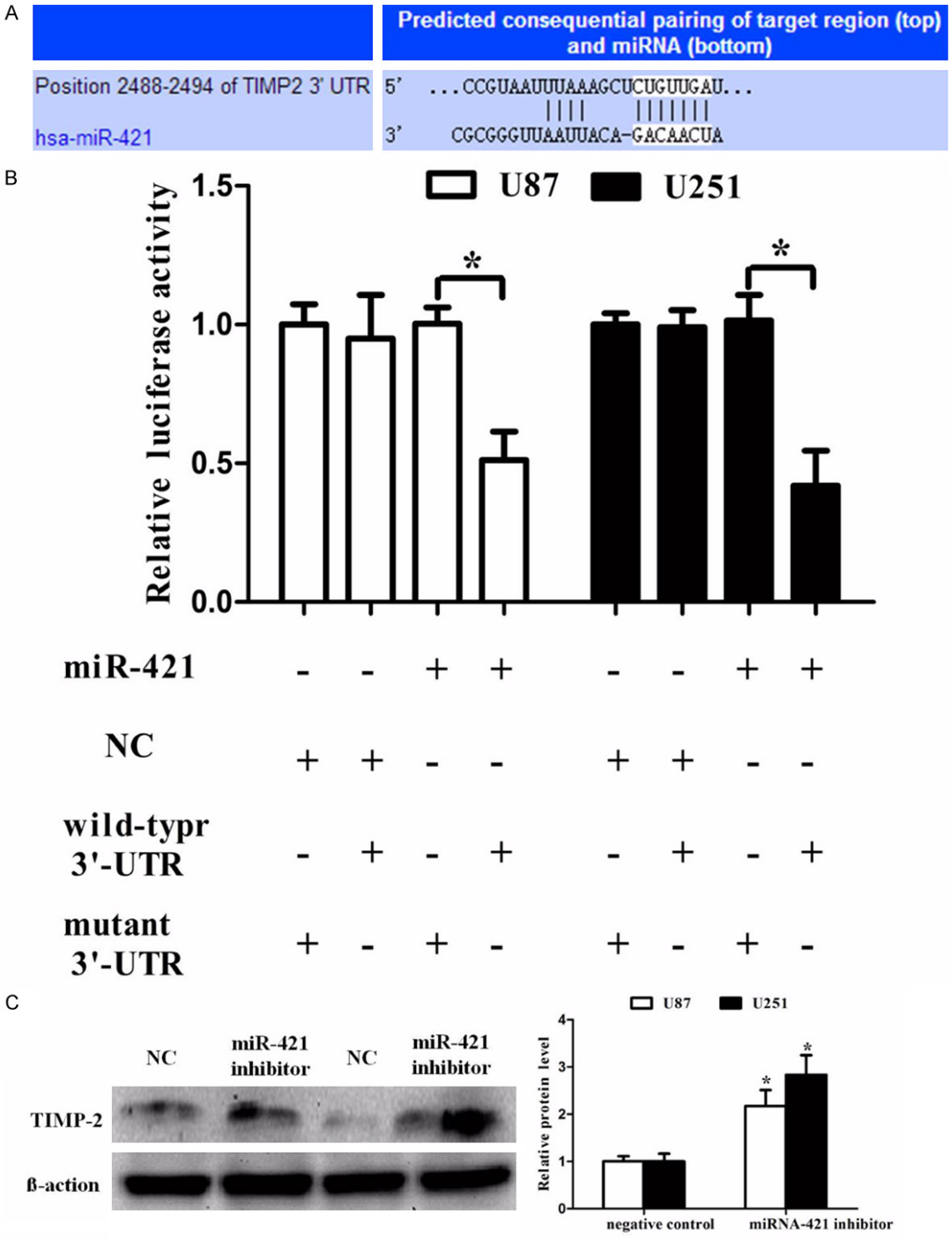

Figure 3. MiR-421 directly targets MMP14. A. Bioinformatics predicted interactions of miR-421 and their binding sites at the 3'UTR of TIMP-2 (TargetScan 6.0). B. The luciferase activity of the wild type TIMP2 3'-UTR (Wt) and mutant TIMP2 3'-UTR (Mut) co-transfected with miR-421 mimics or negative control (NC) was measured. (means ± SD; n=3; *P<0.05). C. The protein level of TIMP-2 in U87 and U251 cells transfected with miR-421 inhibitor or negative control (NC) was determined by Western blot. Representative images of western blotting are shown on the left, and the quantification the bands is shown on the right (means ± SD; n=3; *P<0.05).

fection of miR-421 inhibitor could significantly increased TIMP-2 expression in U87 and U251 cells at the protein levels (**Figure 3C**, *P*<0.05). These results indicated that miR-421 negative-

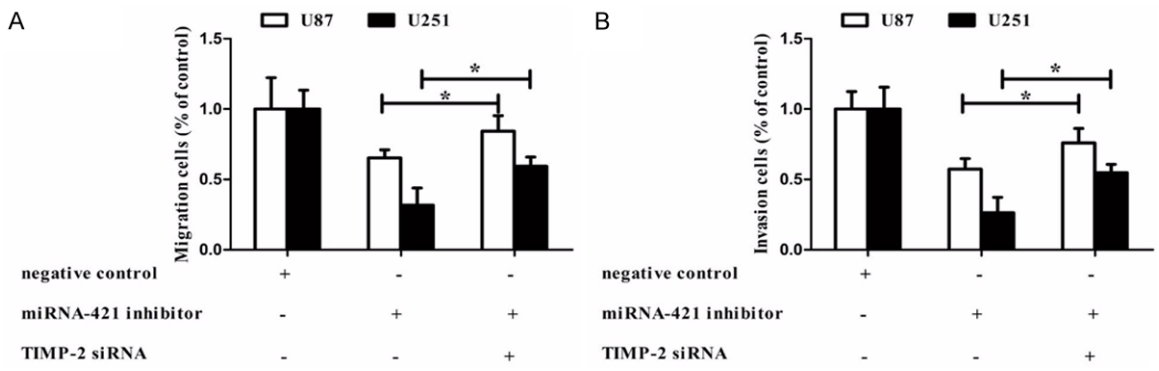

Figure 4. TIMP-2 downregulation was required for miR-421-induced migration and invasion of GBM cells. A. MiR-421-inhibitor-transfected U87 and U251 cells after transfection with TIMP-2-siRNAs promoted the effect on cell migration. The percent migration rate was expressed as a percentage of control (means ± SD; n=3; *P<0.05). B. MiR-421-inhibitor-transfected U87 and U251 cells after transfection with TIMP-2-siRNAs promoted the effect on cell invasion. The percent invasion rate was expressed as a percentage of control (means ± SD; n=3; *P<0.05).

ly regulates TIMP-2 expression via direct binding to putative binding site in the TIMP-2 3'UTR region.

TIMP-2 was involved in miR-421 induced migration and invasion in GBM cells

To investigate whether TIMP-2 was involved in miR-421 induced migration and invasion in GBM cells, we first transfected U251 and U87 cells with miR-421 inhibitor, result in upregulating expression of TIMP-2. Then, we introduced exogenous siRNA against TIMP-2 into the cells mentioned above to assess whether the reduced expression of TIMP-2 mimic the suppressive effect of miR-421. In vitro, migration and invasion assays showed that the miR-421 inhibition-induced migratory and invasive abilities of the GBM cell lines were abolished by TIMP-2 downregulation (**Figure 4**, *P*<0.05). Therefore, our results demonstrated that TIMP-2 was involved in miR-421 regulated migration and invasion of GBM cells.

Discussion

In this study, we for the first time demonstrated a novel molecular mechanism of miR-421 and TIMP-2 in GBM cell migration and invasion. We found that miR-421 was increased in GBM tissues and cell lines. In addition, downregulation of miR-421 significantly suppressed GBM cell migration and invasion via directly targeting TIMP-2. Knockdown of TIMP-2 in cells treated by miR-421 inhibitor elevated the previously abrogated migration and invasion. Therefore, we now reasonably speculated that miR-421 could enhance GBM cell invasion via its target TIMP-2.

miRNA was tiny regulatory molecules and discovered in 1993 [15-17]. In humans, more than 700 miRNAs have been identified, and many of them participate in tumor biological processes such as metabolism, differentiation, cell death as well as proliferation [17, 18]. Recently, emerging evidences have showed that miR-421 functions as a tumor-suppressive miRNA in different tumors [12-14]. For instance, it is identified that microRNA-421 functions as an oncomiR to affect cell proliferation, colony formation and migration in biliary tract cancer by targeting farnesoid X receptor [13]. Ge et al found that miR-421 was upexpressed in gastric cancer tissues and could be considered as a functional marker of circulating tumor cells in gastric cancer patients [12]. However, to our knowledge, no study was conducted to determine the relationship between miR-421 and GBM. In our study, we found the expression of miR-421 was frequently up-regulated in GBM tissues and GBM cell lines compared to normal brain tissues. The inhibition of migration and invasion caused by the suppression of miR-421 expression in the human GBM cell lines (U87 and U251) was also observed. These data provided evidence that miR-421 acted as a tumor suppressor that involved in GBM cell migration and invasion.

miRNAs are believed to function by binding to 3'-UTR of target genes [17]. Previous reports have found some targets of miR-421, including DPC4/Smad4 in pancreatic cancer, casepase-3

in gastric cancer, FOXO4 in nasopharyngeal carcinoma and NR1H4 in biliary tract cancer [12-14, 19]. However, no target of miRNA-421 has been identified in GBM. In this study, our data revealed that miR-421 targets TIMP-2, revealing a potential mechanism involved in development of GBM.

Tissue inhibitors of metalloproteinases (TIMPs), 21 to 29 kDa, are specific inhibitors of matrixes that play a crucial role in regulating the activity the local activities of MMPs in several cancer cells including liver cancer, gastric cancer, breast cancer and so on [20-23]. There are 4 soluble and membrane-anchored members of the TIMP family in humans and TIMP-2 is one of them. The TIMP-2 gene is known to be expressed in normal human tissues, whereas its expression is downregulated in head cancers [22, 23]. Inactivation or silencing of TIMP-2 has been recognized as the main mechanism which promotes the invasive processes of glioma. For example, Parajuli et al found IkappaBalphaM suppressed invasiveness of glioma, with a notable increase in TIMP-2 expression [24]. Zhang et al demonstrated that baicalein could obstruct invasion by influencing expression of TIMP-2 and TIMP-1 [25]. Recently, many studies further to explore the potential regulator of TIMP-2 expression. A research by Valacca C et al found that the expression of TIMP-2 was low in breast adenocarcinoma cells as well as melanoma cells and this minght be regulated by activation of PI3K/AKT signaling pathway [26]. Another article found that TIMP-2, which was a target of miR-221, could suppress the invasion of renal cell carcinoma [27]. However, in this study, we found miR-421 was another potential regulator of TIMP-2. miR-421 inhibition-induced migratory and invasive abilities of the GBM cell lines could be abolished by TIMP-2 downregulation. These data suggested that miR-421 might enhance GBM cell migration and invasion via downregulation of TIMP-2.

In summary, this study provided that miR-421 was upregulated in GBM specimens and cell lines, which played an important role in migration and invasion of GBM. Furthermore, our finding revealed that miR-421 contributed to cell migration and invasion of GBM be mediated via downregulation of TIMP-2, proposing that miR-421 might be identified as a potential therapeutic target of GBM.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zirun Yang, Department of Neurosurgery, Zibo No. 1 Municipal Hospital, Zibo, China. E-mail: zirunyang6666@sina.com

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