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Original Article

A tumor-suppressive microRNA, miRNA-485-5p, inhibits glioma cell proliferation and invasion by down-regulating TPD52L2

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Abstract: Glioblastoma multiforme is the most deadly primary brain tumor and has no effective treatment. Therefore, it is important to identify novel and effective therapies that impede glioma tumorigenesis. MicroRNAs (miRNAs) are helpful analytical biomarkers and may be useful targets for treating multiple human cancers. Previous reports suggest that miRNA-485-5p is dysregulated and contributes to tumorigenesis in some cancer types. Nevertheless, the biological role of miRNA-485-5p in glioma is not well understood. In this study, we demonstrated that miRNA-485-5p expression was reduced in gliomat issues and cell lines. In addition, miRNA-485-5p overexpression inhibited cell proliferation, migration, and invasion in glioma cell lines. Additionally, we identified Tumor Protein D52 Like 2 (TPD52L2) as a direct target of miRNA-485-5p. Moreover, we showed that miRNA-485-5p regulated glioma tumorigenesis by down-regulating TPD52L2 expression in vitro and in vivo. Our results suggest that miRNA-485-5p is a suppressor of glioma tumorigenesis and could serve as a novel candidate for therapeutic applications in glioma treatment.

Keywords: miR-485-5p, glioma, tumorigenesis, proliferation, invasion

Introduction

Malignant brain tumors, primarily gliomas, are the most common types of brain cancer. The World Health Organization (WHO) classifies human gliomas into four grades (grade I-IV) based on their degree of malignancy [1-3]. Among of them, grade IV glioma, which is also recognized as "glioblastoma multiforme (GBM)", is the most deadly primary brain tumor and has no effective treatment [4, 5]. Therefore, therapies that block glioma tumorigenesis are of great need in the field.

Recently, microRNAs (miRNAs) have been identified as promising therapeutic targets in several human cancers [6-8]. miRNAs are short, endogenous, single stranded RNAs that post-transcriptionally inhibit expression of target genes by binding to complementary sequences in the 3'-untranslated regions (3'-UTR) of the target messenger RNAs (mRNAs) [9]. miRNAs control many important cell functions including cell angiogenesis, proliferation, apoptosis, and invasion, and are dysregulated in many human cancers [10-13].

Previous reports have implicated the tumor suppressor miRNA miR-485-5p in the pathogenesis of multiple human cancers. For example, expression of miR-485-5p is significantly down-regulated in gastric cancer tissues compared with normal tissues, and is a novel biomarker for the overall survival of gastric cancer patients [14]. miR-485-5p also inhibits cell proliferation in hepatocellular carcinoma [16], prevents metastasis and EMT of lung adenocarcinoma upon Flot2 targeting [17], and its overexpression suppresses spontaneous metastasis of breast cancer cells in vivo [15]. These previous findings suggest that miR-485-5p might function as a tumor suppressor and its down-regulation may contribute to glioma tumorigenesis. However, the role of miR-485-5p in glioma tumorigenesis has not yet been definitively shown.

In this study, we show that miR-485-5p expression is decreased in both glioma tissues and cell lines. In addition, in glioma tumor cells, miR-485-5p significantly inhibits cell proliferation, migration, and invasion by directly downregulating its target, Tumor Protein D52 Like 2 (TPD52L2). The results from our study confirm that miR-485-5p has tumor suppressor activity in glioma cells and might serve as a novel therapeutic target for glioma.

Materials and methods

Cell lines

The human glioma cancer cell lines U87, U251, U373, T98G, and LN18 were ordered from American Type Culture Collection (ATCC, Manassas, VA, USA). Human embryonic kidney (HEK)-293T cells were purchased from Shanghai Institutes for Biological Sciences Cell Resource Center. All cell lineswere maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and penicillin/streptomycin (100 U/mL). Cells were incubated with 5% CO_2 at 37°C.

Glioma tissues

Fresh tumor tissues were obtained from 36 glioma patients during surgery at the General Hospital of Chinese People's Armed Police Force (2016 to 2017). The selected glioma specimens were immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. This study was approved by the Ethics Committee of the General Hospital of Chinese People's Armed Police Force.

RNA extraction, reverse transcription, and real-time PCR quantification

Total RNA was extracted from glioma cell lines using a mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. To evaluate expression of miR-485-5p in glioma cell lines, cDNA synthesis and subsequent quantitative real-time PCR were performed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and an individual TaqMan MicroRNA assay (Applied Biosystems). To examine the alteration of *TPD-52L2* mRNA expression after transfection of miR-485-5p into U87 and U373 cells, cDNA synthesis was performed using a RNA PCR kit (Takara, Japan), and quantitative real-time PCR was carried out using the SYBR premix Ex TaqII kit (Takara) according to the manufacturer's instructions. *GAPDH* was used as an endogenous control. Real-time PCR was performed using the Applied Biosystems 7500HT Fast Real-Time PCR System, and the threshold cycle (CT) number was automatically determined using the ABI 7500 Real-time PCR System SDS software v2.0.1. All reactions were performed in triplicate. Relative quantification of miR-485-5p and *TPD52L2* expression was calculated using the $2^{-\Delta\Delta}$Ct method.

Lentiviral infection, plasmid construction, and transfection

The mature miR-485-5p sequence was obtained from the miRBase database. The GV217 vector (Ubi-EGFP-MCS) (Genechem, Shanghai, China) was linearized using the restriction enzyme EcoRI (New England Biolabs, Ipswich, MA). A fragment containing themature miR-485-5p sequence was introduced into the GV217 vector's multiple cloningsite. HEK-293T packaging cells were used to generate plasmid-containing viral particles using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For target overexpression, the human TPD52L2 cDNA was cloned into the pcDNA3.1 vector (Clonetech). A scrambled control vector was also constructed. For plasmid transfection, Lipofectamine 2000 transfection reagent (Invitrogen) was used according to the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was evaluated by performing a CCK-8 assay using a cell counting kit (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan). Cells were seeded onto a 96-well plate and cultured at 37°C. Cells were added to each well using 10 μL of the CCK-8 solution. Plates were incubated at 37°C for 2 h, and absorbance at 450 nm was measured. Proliferation rates were determined at days 1, 2, 3, 4 and 5 post-transfection.

Cell migration and invasion assays

Cell migration and invasion assays were performed using 24-well polycarbonate membrane transwell chambers with 8-μm pores (Corning

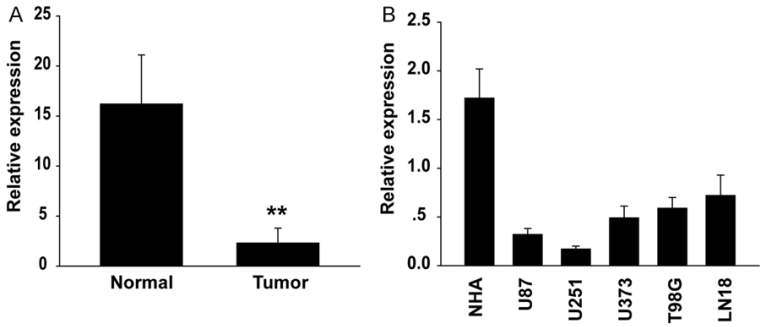

Figure 1. miR-485-5p is expressed at low levels in glioma tissue and cell lines. A. Relative ratios of miR-485-5p expression in 36 glioma tissues compared with 6 normal brain tissues. **, P < 0.01. B. Histograms of the average relative expression of miR-485-5p in a normal human astrocyte (NHA) cell line and glioma cell lines as shown. Data are presented as mean ± SD from three independent experiments. *GAPDH* was used as an internal control for gene expression analysis.

Incorporated, Corning, NY, USA). Cells were plated in serum-free DMEM on the upperside of the membrane with (for the cell invasion assay) or without (for the cell migration assay) Matrigel (BD, Franklin Lakes, NJ, USA). DMEM with 10% FBS was added to the lower chambers. After incubation at 37°C for 16 hours (migration assay) or 24 hours (invasion assay), the nonmigratory or non-invasive cells on the upperside of the membrane were removed. Cells that migrated or invaded to the lower membrane surface were fixed and stained with 0.1% crystal violet. Migration and invasion were quantified by counting cells in 6 random fields.

Protein isolation and western blot

Total proteins were extracted from cell lines, and protein concentrations were determined using a BCA protein assay kit (BeyotimeBiotec, China). Protein samples were fractionated using SDS-PAGE, transferred to PVDF membranes (Millipore, NY, USA), and blocked for 1 hour with 5% non-fat milk. Membranes were then incubated at room temperature with primary antibodies: goatanti-human TPD52L2 (1:1000; Abcam, UK); rabbit anti-human cyclin D1 (1:1000, Cell Signaling Tech, USA); rabbit anti-human cyclin E1 (1:1000, Cell Signaling Tech); rabbit anti-human MMP-2 (1:1000, Cell Signaling Tech); and rabbit anti-human MMP-9 (1:1000, Cell Signaling Tech). Mouse anti-human β-actin antibodies (1:5000, Abcam) were used to detect β-actin as a control. Protein expression levels were detected with ECL detection solution. Full scans of WBs are presented in Supplementary Figure 1.

Dual luciferase reporter assay

HEK-293T cells were plated on 24-well plates and 3'-UTR plasmids were co-transfected. Cell lysates were harvested 48 hours after transfection and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Relative Renilla luciferase activities were normalized to firefly luciferase activities, which are used as internal controls for transfection efficiency.

Intracranial injection

All mouse experiments were approved by the Institutional Animal Care and Use Committee of General Hospital of Chinese People's Armed Police Force. Four-week-old female athymic nude mice were intracranially injected withU87 cells. Ten mice were injected for each group. We used hematoxylin and eosin-stained sections to analyze tumor formation and histological phenotype.

Statistical analysis

All experiments were performed in triplicate and the data are expressed as mean ± standard deviation (SD). The significance of the differences between two groups of data was evaluated using a two-tailed Student's t-test or Mann-Whitney U-test. All statistical analyses were carried out using SPSS software version 19.0 for Windows (SPSS Inc., IL, USA). A *P*-value < 0.05 was considered statistically significant.

Results

miR-485-5p is expressed at low levels in glioma tissues and cell lines

To ascertain the role of miR-485-5p inglioma, we first analyzed miR-485-5p expression levels by qRT-PCR in human glioma tissues and compared it with expression in normal human brain tissues. We found that miR-485-5p expression was markedly decreased in glioma tissues (P < 0.001, **Figure 1A**). qRT-PCR analysis also

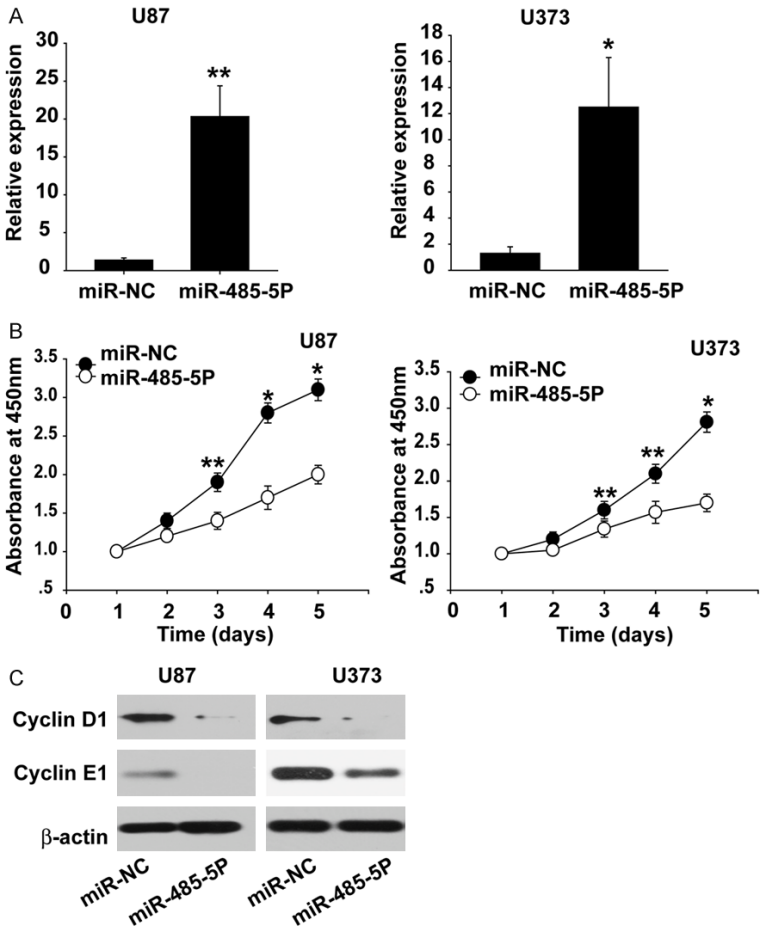

Figure 2. miR-485-5p inhibits glioma cell proliferation. A. Confirmation of miR-485-5p overexpression by qRT-PCR in U87 and U373 glioma cells transfected with miR-485-5p. *GAPDH* was used as an internal control for gene expression analysis. B. Results of a CCK-8 assay to assess the effects of miR-485-5p on cell proliferation in U87 and U373 cells. C. Western blot analysis demonstrating the levels of cell cycle-regulated proteins cyclin D1 and cyclin E1 upon miR-485-5p overexpression in U87 and U373 cells. Data are shown as mean ± SD (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

revealed that compared to its expression in normal human astrocyte cells, miR-485-5p expression was markedly diminished in a panel of glioma cell lines tested, including U87, U251, U373, T98G, and LN18 (**Figure 1B**).

miR-485-5p inhibits glioma cell proliferation

We next overexpressed miR-485-5p in U87 and U373 glioma cell lines and examined the effects on cell proliferation. As shown in **Figure 2A**, using qRT-PCR, we confirmed miR-485-5p overexpression. The CCK-8 proliferation assay showed that exogenous miR-485-5p overexpression significantly suppressed the proliferation of U87 and U373 cells (**Figure 2B**). Additionally, to determine the mechanism of miR-485-5p-mediated inhibition of glioma cell proliferation, we studied expression of the cell cycle-regulated proteins cyclin D1 and cyclin E1 in miR-485-5p-overexpressing U87 and U373 cells. As shown in **Figure 2C**, the levels of these proteins were distinctly reduced in miR-485-5p-overexpressing glioma cells relative to controls. Together, these data demonstrate that miR-485-5p inhibits proliferation through cell cycle arrest in glioma cell lines.

miR-485-5p inhibits the migration and invasion of glioma cells

Next, we evaluated the potential roles of miR-485-5p in glioma cell migration and invasion using transwell migration and invasion assays. As shown in **Figure 3A** and **3B**, miR-485-5p overexpression inhibited both cell migration and invasion in U87 and U373 glioma cell lines (**Figure 3A**, **3B**). To discover the mechanism by which miR-485-5p regulates glioma cell invasion, we measured expression of the invasion-associated molecules MMP-2 and MMP-9. Our findings showed that MMP-2 and MMP-9 protein levels significantly decreased following miR-485-5p overexpression in U87 and U373 cells (**Figure 3C**).

TPD52L2 is a direct target of miR-485-5p

Next, we set out to identify the targets that are directly regulated by miR-485-5p in glioma cells. The putative miR-485-5p target genes were predicted using the Target Scan algorithm and sequence alignment confirmed that that the seed sequence of miR-485-5p is complementary to the 3'UTR of TPD52L2 (**Figure 4A**). We then performed luciferase reporter assays to determine whether TPD52L2 expression was

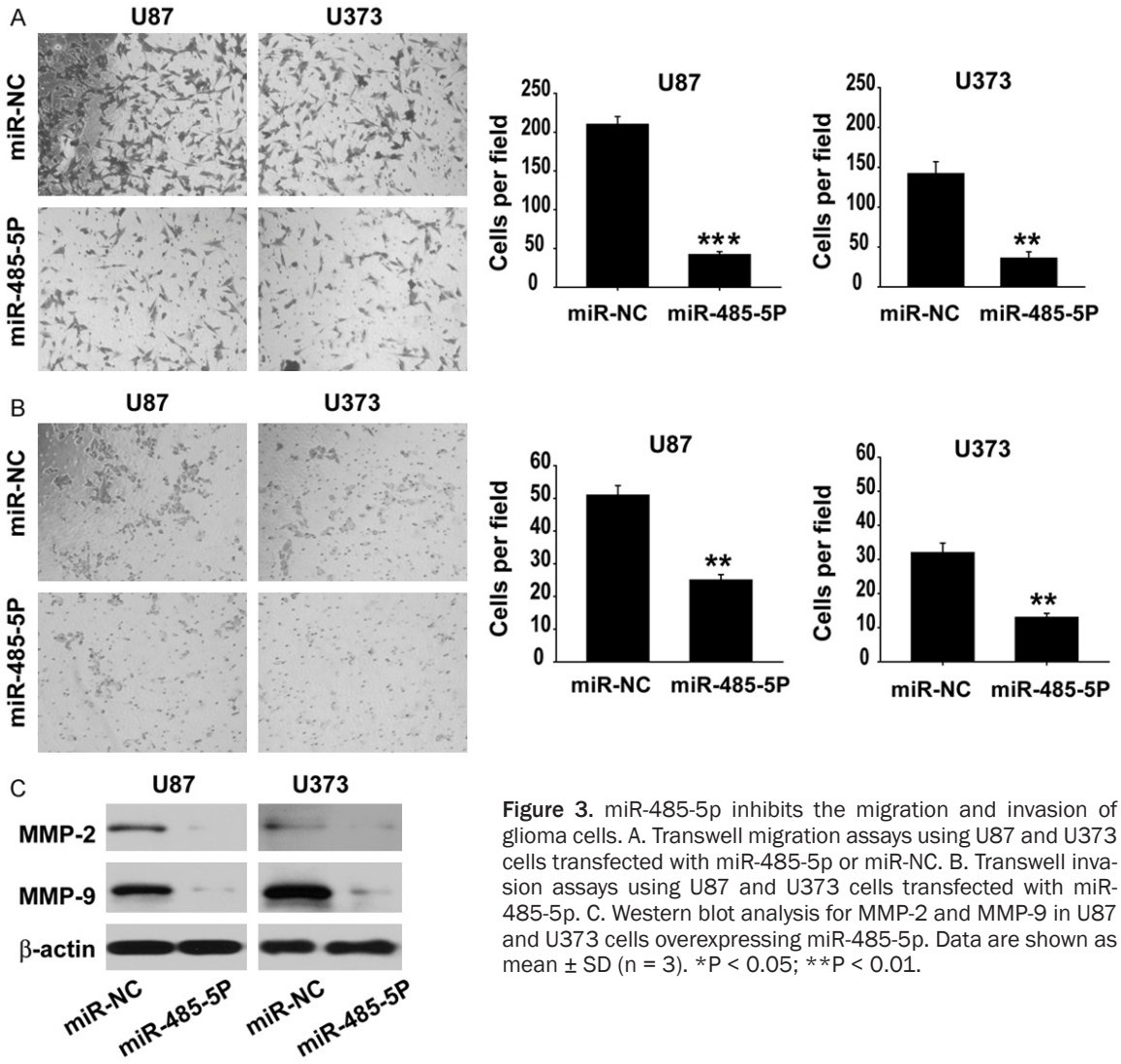

Figure 3. miR-485-5p inhibits the migration and invasion of glioma cells. A. Transwell migration assays using U87 and U373 cells transfected with miR-485-5p or miR-NC. B. Transwell invasion assays using U87 and U373 cells transfected with miR-485-5p. C. Western blot analysis for MMP-2 and MMP-9 in U87 and U373 cells overexpressing miR-485-5p. Data are shown as mean ± SD (n = 3). *P < 0.05; **P < 0.01.

directly regulated by miR-485-5p. As shown in **Figure 4B**, luciferase activity in cells co-transfected with pGL3-TPD52L2 and 3'-UTR-wt/miR-485-5p was lower than that in cells co-transfected with pGL3-TPD52L2 and 3'-UTR-wt/NC. No substantial difference in luciferase activity was observed between cells co-transfected with pGL3-TPD52L2 and 3'-UTR mut/miR-485-5p compared to cells co-transfected with pGL3-TPD52L2 and 3'-UTR-mut/NC.

To further understand the regulation of TPD-52L2 by miR-485-5, we performed qRT-PCR and Western blot analyses to identify how TPD52L2 mRNA and protein expression was altered upon miR-485-5p overexpression. We found that both TPD52L2 mRNA (**Figure 4C**) and protein (**Figure 4D**) expression were significantly reduced following miR-485-5p overexpression in U87 and U373 cells, suggesting that TPD52L2 is negatively regulated by miR-485-5p in glioma cells.

miR-485-5p regulates glioma cell proliferation, migration, and invasion by down-regulating TPD52L2

We have thus far demonstrated that miR-485-5p suppresses glioma cell tumorigenicity and that TPD52L2 is a direct target of miR-485-5p in glioma cells. Our next goal was to determine the role of TPD52L2 in glioma cells using rescue experiments with TPD52L2. We first co-transfected U87 and U373 cells with miR-485-5p and pcDNA3.1-TPD52L2 constructs, or empty vector (pcDNA3.1), and confirmed transfec-

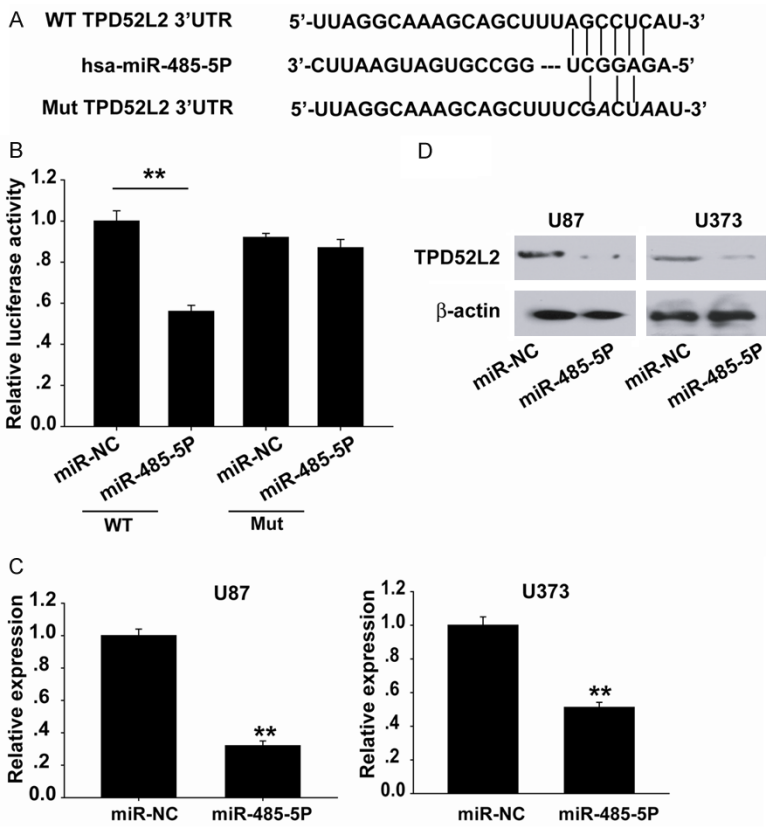

Figure 4. Identification of TPD52L2 as a direct target of miR-485-5p. A. Schematic demonstration of the putative miR-485-5p target site in the 3'-UTR of the *TPD52L2* transcript. B. Luciferase assay results from HEK-293T cells co-transfected with the TPD52L2-3'-UTR-wt reporter plasmids and either miR-485-5p or miR-NC. C. qRT-PCR assay for *TPD52L2* mRNA levels in response to miR-485-5p overexpression in U87 and U373 cells. D. Western blot analysis of TPD52L2 levels following miR-485-5p overexpression in U87 and U373 cells.

tion efficiency by western blot (**Figure 5A**). Next, we performed a CCK-8 assays in U87 and U373 cells, which indicated that TPD52L2 overexpression partly reversed the inhibition of cell proliferation by miR-485-5p (**Figure 5B**). Additionally, TPD52L2 overexpression overcame miR-485-5p-dependent inhibition of cell migration (**Figure 5C**) and invasion (**Figure 5D**). Together, our data show that that TPD52L2 negative regulation by miR-485-5p contributes to inhibition of glioma cell proliferation, migration, and invasion.

*miR-485-5p regulates tumor growth by down-regulating TPD52L2 in vivo (**Figure 6**)*

The relevance of our in vitro findings was further tested in vivo by intracranial injection of U87 stable cells into athymic nude mice. U87 negative control cells formed tumors after injection, however, miR-485-5p overexpression significantly suppressed tumor formation, which was partly reversed by TPD52L2 overexpression (**Figure 6**). Thus, these findings show that miR-485-5p regulates tumor growth by down-regulating TPD52L2 in vivo.

Discussion

Many studies have found that miRNAs can function either as oncogenes or tumor suppressors in human cancer by controlling target gene expression [18-20]. In particular, miR-485-5p plays a tumor suppressive role in multiple human cancers [21-24]. However, the significance and biological function of miR-485-5p in glioma are still unknown.

In this study, we found that miR-485-5p expression was markedly decreased in glioma tissues and cell lines compared with expression in normal brain tissue and astrocyte cells. Next, we explored the effects of miR-485-5p on cell proliferation, migration, and invasion in glioma tumor cells and found that miR-485-5p overexpression significantly subdued these cellular activities. Together, our results suggest that miR-485-5p might adversely regulate glioma tumorigenesis as a tumor suppressor.

To identify the mechanism by which miR-485-5p regulates glioma growth, we predicted its target genes using the TargetScan algorithm and found that miR485-5p negatively regulated the expression of TPD52L2, a target gene identified in glioma cells. In addition, we established that TPD52L2 negative regulation was important for miR-485-5p-mediated growth inhibition in glioma cells. Many reports have indicated that TPD52L2 promotes tumorigenesis in various human cancers. For example, TPD52-L2 depletion inhibits the proliferation of liver

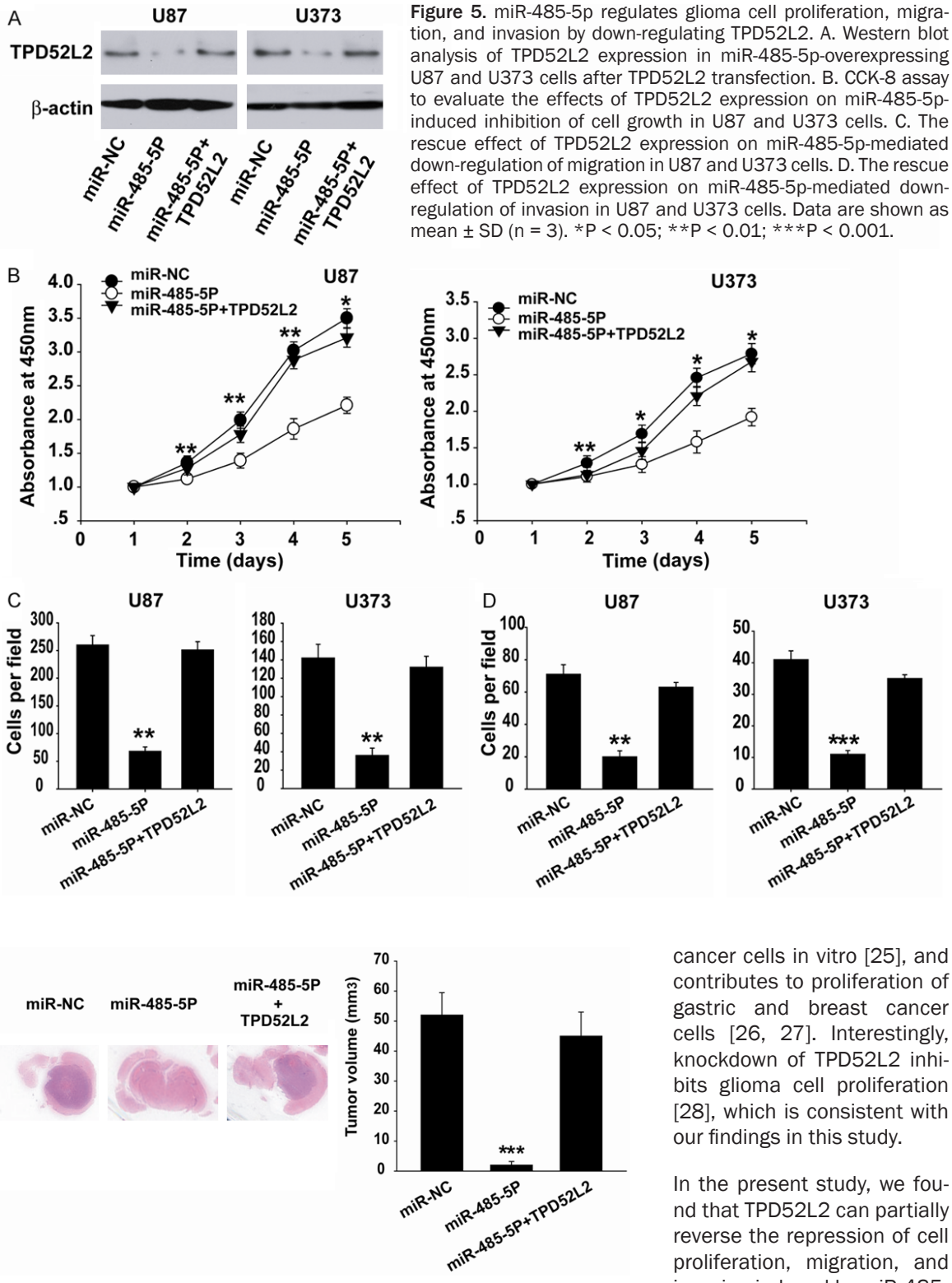

Figure 5. miR-485-5p regulates glioma cell proliferation, migration, and invasion by down-regulating TPD52L2. A. Western blot analysis of TPD52L2 expression in miR-485-5p-overexpressing U87 and U373 cells after TPD52L2 transfection. B. CCK-8 assay to evaluate the effects of TPD52L2 expression on miR-485-5p-induced inhibition of cell growth in U87 and U373 cells. C. The rescue effect of TPD52L2 expression on miR-485-5p-mediated down-regulation of migration in U87 and U373 cells. D. The rescue effect of TPD52L2 expression on miR-485-5p-mediated down-regulation of invasion in U87 and U373 cells. Data are shown as mean ± SD (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 6. miR-485-5p regulates tumor growth by down-regulating TPD52L2 in vivo. U87 stable cells were intracranially injected into athymic nude mice. After 4 weeks, the mice were euthanized and tumor growth was determined. Left panel, H&E-stained coronal brain sections of representative tumor xenografts. Right panel, tumor volume. Data are shown as mean ± SD. ***, P < 0.001.

cancer cells in vitro [25], and contributes to proliferation of gastric and breast cancer cells [26, 27]. Interestingly, knockdown of TPD52L2 inhibits glioma cell proliferation [28], which is consistent with our findings in this study.

In the present study, we found that TPD52L2 can partially reverse the repression of cell proliferation, migration, and invasion induced by miR-485-5p in glioma cells. However, the biological mechanism(s) through which miR-485-5p/TPD52L2 signaling exerts opposing contributions to tumor-

igenesis in other cancers types merits further study. Finally, we demonstrated that miR-485-5p inhibits tumor growth, migration, and invasion in glioma cells by directly regulating expression of TPD52L2. Our study confirms that miR-485-5p acts as a tumor-suppressive miRNA in glioma and suggests that miR-485-5p might serve as a novel therapeutic target for treating these malignant brain tumors.

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Disclosure of conflict of interest

None.

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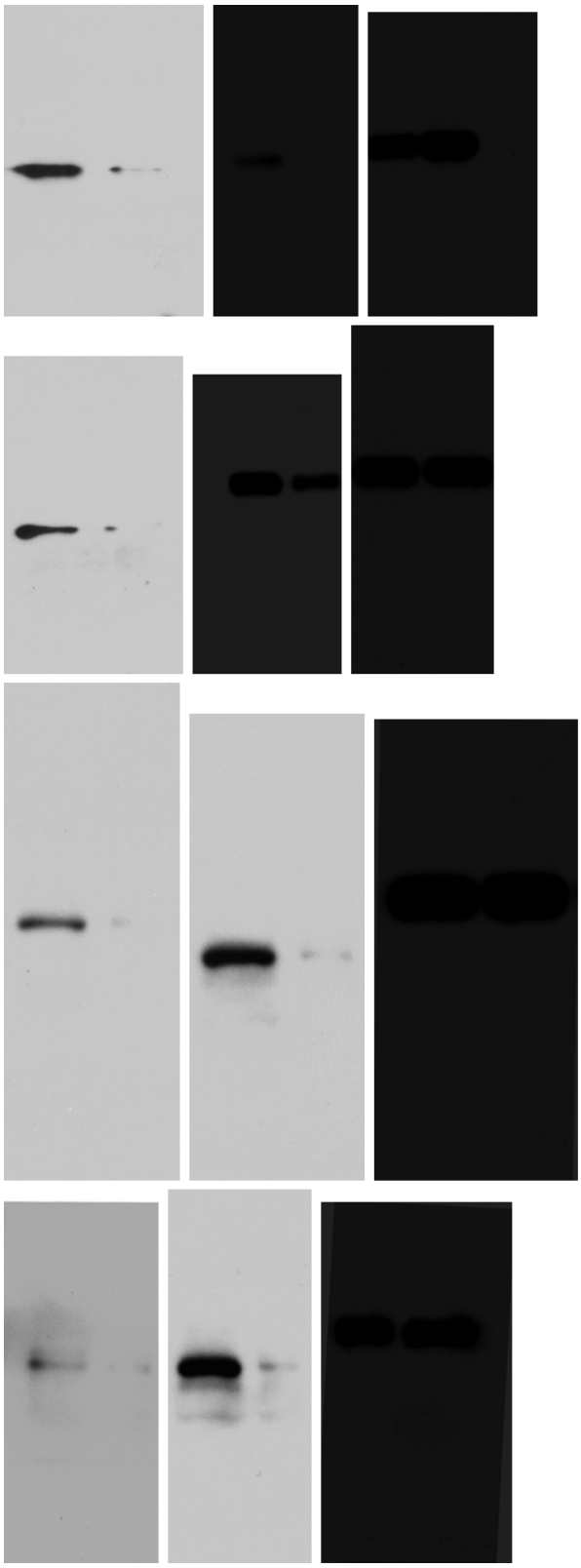

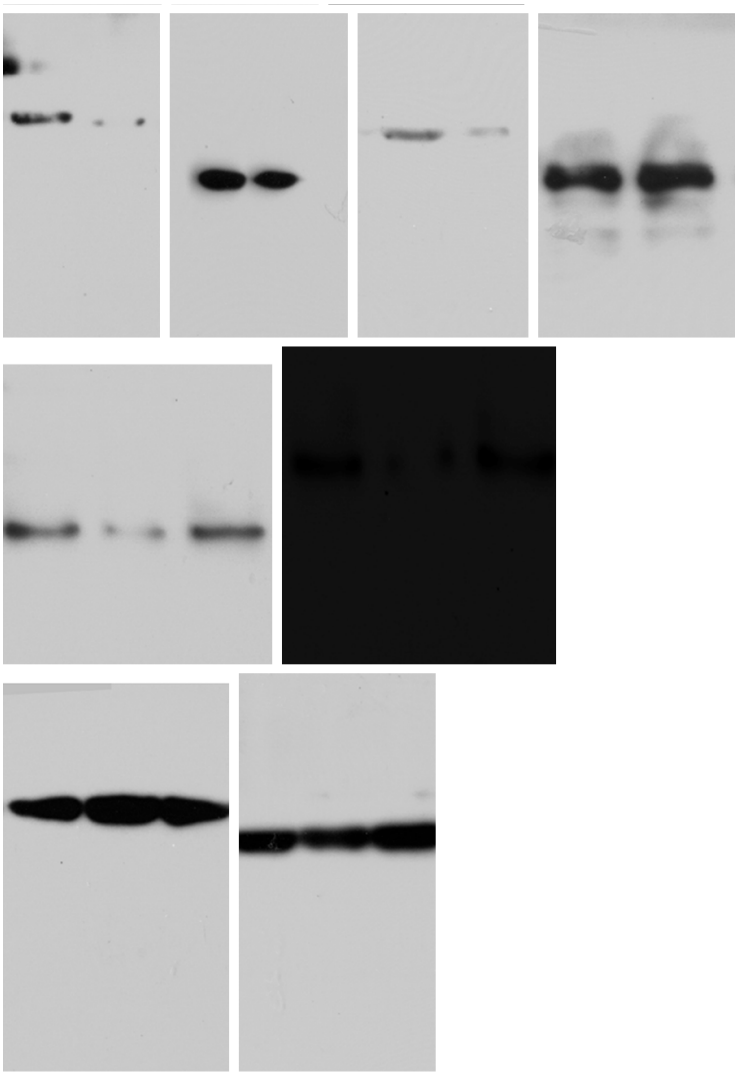

Supplementary Figure 1. Full scans of Western blots.