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Original Article

MicroRNA-520d-5p inhibits human glioma cell proliferation and induces cell cycle arrest by directly targeting PTTG1

Tongle Zhi[1*], Kuan Jiang[1,2*], Xiupeng Xu[1*], Tianfu Yu[1], Weining Wu[1], Er Nie[1], Xu Zhou[1], Xin Jin[1], Junxia Zhang[1], Yingyi Wang[1], Ning Liu[1]

[1]*Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China;* [2]*Department of Neurosurgery, Yixing People's Hospital, Yixing 214200, Jiangsu Province, China.* [*]*Equal contributors.*

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Abstract: Glioma accounts for the majority of primary malignant brain tumors in adults and is highly aggressive. Although various therapeutic approaches have been applied, outcomes of glioma treatment remain poor. Acquiring a better understanding of the pathogenic mechanisms is essential to the design of effective therapeutic strategies. Previous studies have found that miR-520d-5p was negatively correlated with glioma grade, but its role and mechanism in glioma progression remain largely unknown. In the present study, we reported that miR-520d-5p directly targeted the Pituitary Tumor Transforming Gene 1 (PTTG1) and functioned as a tumor-suppressor in glioma. The expression of miR-520d-5p in glioma cells and specimens were detected by Quantitative reverse transcription-PCR and Fluorescence in situ hybridization (FISH). The effects of miR-520d-5p on glioma progression was examined by cell-counting kit 8, colony formation, 5-ethynyl-2-deoxyuridine (EDU) and flow cytometry assays. Using bioinformatics and luciferase reporter assays, we identified PTTG1 as a novel and direct target of miR-520d-3p. A xenograft model was used to study the effect of miR-520d-5p on tumor growth and angiogenesis. We found that miR-520d-5p expression was significantly decreased in glioma cell lines and tissues. Overexpression of miR-520d-5p showed a significant inhibitory effect on cell proliferation and accompanied cell cycle G0/G1 arrest in U87-MG and LN229 glioma cells. PTTG1 was a novel and direct target of miR-520d-5p, and the protein expression of PTTG1 was markedly reduced after overexpression of miR-520d-5p in U87-MG and LN229 cells. Overexpression of PTTG1 reversed the inhibitory effect of miR-520d-5p on glioma cell proliferation. *In vivo* studies confirmed that miR-520d-5p overexpression retarded the growth of U87 xenograft tumors, which was accompanied by reduced expression of PTTG1. In conclusion, these results provide compelling evidence that miR-520d-5p functions as an anti-onco-miRNA, which is important in inhibiting cell proliferation in GBM, and its anti-oncogenic effects are mediated chiefly through direct suppression of PTTG1 expression. Therefore, we suggest that miR-520d-5p is a potential candidate for the prevention of glioblastoma.

Keywords: miR-520d-5p, PTTG1, proliferation, glioma

Introduction

Glioma is the most common cancer in central nervous system, accounting for about 80% of malignant tumors in brain [1]. According to World Health Organization (WHO) classification, Glioma is histologically graded as I-IV [2]. Glioblastoma (GBM), a grade IV glioma, is the most aggressive type with median survival of 12-15 months [3]. Despite the advances in treatment strategies, such as surgical resection, radiation therapy and chemotherapy, the prognosis of glioblastoma remains poor [3, 4]. Recent studies have made a number of important achievements in molecular targeted therapy [5, 6], however, the underlying molecular mechanisms of glioma malignancy remain largely unknown. Therefore, it is essential to reveal the molecular mechanisms of glioma tumorigenesis and to develop effective treatment methods.

MicroRNAs (miRNAs) are a family of small endogenous, non-coding, single RNA molecules that play important roles in gene expression by bind-

ing to the 3' untranslated region (3'-UTRs) of the target gene mRNA, resulting in mRNA cleavage or translation repression [7]. Approximately one third of the human genes might be regulated by miRNAs and each miRNAs could target tens to hundreds of mRNAs [8]. As their target genes include many oncogenes and tumor suppressors, most scientists speculate that miRNAs play vital roles in human cancers [9]. In recent years, an expanding body of evidences has documented that the dysregulation of miRNAs is indeed involved in various physiological and pathological processes, such as cell proliferation, cell cycle progression, migration, metabolism, apoptosis, differentiation, autophagy etc [10, 11]. In glioma, numerous of tumor-promoting [12, 13] and tumor-suppressing miRNAs [14, 15] have been identified. Our preliminary experiments showed that miR-520d-5p was decreased in glioma and its expression was negatively correlated with the pathological grade. However, the role and mechanism of miR-520d-5p in glioma has not been reported so far.

Pituitary tumor-transforming gene (PTTG), was originally identified using a messenger (m) RNA differential display polymerase chain reaction (PCR) technique in mouse pituitary tumor GH4 cell lines [16], Subsequent studies demonstrated its function as a securin, mediating sister chromatid separation during mitosis [17]. The human PTTG family has been found to contain at least three homologous proteins PTTG1, PTTG2 and PTTG3, of which PTTG1 has been studied in detail [18]. Human PTTG1 is located on chromosome 5q33 [19] and its cDNA encodes a protein of 203 amino acids, which is mainly located in the cytoplasm with partial nuclear localization [20]. PTTG1 is a multifunctional protein with roles in the control of mitosis [21], cell transformation [22], DNA repair [23] and fetal development [24]. Most importantly, the expression of PTTG1 in most normal tissues is restricted. Conversely, it is abundantly expressed in a variety of endocrine-related tumors, for instance pituitary [25], breast tumors [26] and thyroid [27], and as well as non-endocrine-related cancers including the digestive [28], respiratory [29] and nervous systems [30]. With respect to patients with glioma, NOBUYUKI GENKAI et al has reported that PTTG1 protein is overexpressed in high-grade astrocytomas compared to low-grade astrocytomas [30], and our experiments also showed that PTTG1 was indeed highly expressed in glioma cells. Thus highlighting the need for further investigation of PTTG expression and function in glioma.

In the present study, we investigated miR-520d-5p expression in both Clinical specimens and from commonly used glioma databases. The result demonstrated that miR-520d-5p was low expressed in glioma, and miR-520d-5p expression correlated negatively with PTTG1 protein levels. Up-regulation of miR-520d-5p leaded to decrease expression of PTTG1. Moreover, we demonstrated that PTTG1 is a direct target of miR-520d-5p. We then sought to understand: (i) what are the roles of miR-520d-5p in tumor growth; (ii) the potential direct target of miR-520d-5p that may be involved in glioma development; (iii) Whether miR-520d-5p overexpression causes cell proliferation inhibition and cell cycle arrest via its direct target; (iv) the role miR-520d-5p has in glioma cell growth in nude mice. Generally speaking, the answers of these questions will provide new insights into the molecular mechanism of glioma development and will help develop a unique miRNA-based therapy for GBM management.

Materials and methods

Ethics statement

The study has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and national and international guidelines. This study was approved by The Research Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, China). Written informed consent was obtained from all patients, and the hospital ethics committee approved the experiments.

Public datasets

Microarray miRNA expression data for 158 gliomas were downloaded from the Chinese Glioma Genome Atlas (CGGA) data portal (http://www.cgga.org.cn.portal.phpg). Whole genome mRNA expression microarray data and clinical information of 158 glioma samples were obtained from Chinese Glioma Genome Atlas (CGGA) database (http://www.cgga.org.cn) and used as discovery set [31]. Three validation sets are The Cancer Genome Atlas database (TCGA, http://tcga-data.nci.nih.gov/), Repository of

Molecular Brain Neoplasia Data (REMBRANDT, http://cainteGrator.nci.nih.gov/rembrandt/) and GSE4290 data (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4290).

Tissue samples and cell lines

Human GBM cell lines U87-MG, LN229, U118, A172, U251 and H4 were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China), and were cultured in Dulbecco's modified Eagle's medium (DMEM, SH30022.01B, Hyclone, UT, USA) supplemented with 10% fetal bovine serum (10082147, Gibco, MD, USA), 100 units of penicillin/mL, and 100 ng of streptomycin/mL. Normal human astrocytes (NHAs) were obtained from Lonza (Basel, Switzerland) and cultured in the provided astrocyte growth media supplemented with rhEGF, insulin, ascorbic acid, GA-1000, L-glutamine and, 5% FBS. All the cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Eight normal brain tissues were collected from patients undergoing internal decompression surgery following severe traumatic brain injury. Thirty one human glioma tissues, including twelve low-grade glioma tissues (grade-I and grade-II) and nineteen high-grade glioma tissues (grade-III and grade-IV) were obtained from the Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University. Glioma specimens were verified and classified by two experienced clinical pathologists according to the WHO standard classification of tumors.

Western blot analysis

Western blot analysis was performed as described previously [32]. Briefly, Cells or tissues were lysed on ice for 30 min in radio immunoprecipitation assay buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 mM pepstatin A. The lysates were centrifuged at 12000 rpm at 4°C for 15 min, the supernatants were collected, and protein concentrations were determined using the bicinchoninic acid assay (KenGEN, Jiangsu, China). Protein extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific, MA, USA) in transfer buffer (20 mM Tris, 150 mM glycine, 20% [volume/volume] methanol). Membranes were blocked with 5% nonfat dried milk for 2 h and incubated with primary antibodies. An electrochemiluminescence detection system (Thermo Fisher Scientific) was used for signal detection. Immunoblot analysis used the following primary antibodies: PTTG1 (ab79546), CD31 (ab28364), Ki67 (ab15580) and Actin (ab8226) were obtained from Abcam (Cambridge, UK). Cyclin E1 (#4129), CDK2 (#2546) and CDC2 (#9116) were purchased from Cell Signaling Technology (Massachusetts, USA).

Oligonucleotides, plasmid construction, and transfection

Hsa-miR-520-5p mimic and hsa-miR-ctrl were chemically synthesized by Ribobio (Guangzhou, China). Small interfering (Si) PTTG1 and control si-non-coding (siNC) oligonucleotides were purchased from GenePharma (Shanghai, China). The PTTG1-targeting sequence of siRNA was 5'-GCCUUACCUAAAGCUACUATT-3' and 5'-UAGUAGCUUUAGGUAAGGCTT-3'. The siRNA negative control sequence was 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'. The PTTG1-overexpression plasmid was generated by inserting the PTTG1-coding region into a pcDNA3.1 vector. The plasmid was sequenced verified by Genepharma. All oligonucleotides and plasmids were transfected into cells using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions.

Lentiviral packaging and establishment of stably transduced cell lines

A lentiviral packaging kit was purchased from Genechem (Shanghai, China). A lentivirus carrying hsa-miR-520d-5p or hsa-miR-negative control (miR-ctrl) was packaged in the human embryonic kidney cell line, 293T, and the virions were collected according to the manufacturer's instructions. Stable cell lines were established by infecting U87-MG cells and LN229 cells with lentiviruses, followed by puromycin selection.

RNA isolation and quantitative real-time PCR (qPCR)

RNA was isolated from harvested cells or human tissues with Trizol reagent according to

the manufacturer's instructions (Life Technologies, CA, USA) following the manufacturer's protocol. A stem-loop–specific primer method was used to measure the expression levels of miR-520d-5p, as described previously [33, 34]. Expression of U6 was used as an endogenous control [35]. The cDNAs were amplified by qRT-PCR using SYBR Premix Ex Taq (Takara) on a 7900HT system, Primers were purchased from Ribobio. Data were analyzed using the 2-ΔΔCt method, and U6 RNA was used as endogenous control.

Cell proliferation assay

For the cell counting kit-8 (CCK-8) assay, stabled transfected U87-MG and LN229 cells were seeded in 96-well plates, and then cells were cultured for 24, 48, 72 and 96 h before performing the CCK-8 assay (Dojindo, Japan). After a 1 h incubation with CCK-8 at 37°C, absorbance (OD value) at a wave length of 450 nm was detected and used for calculating cell viability.

For the colony formation assay, cells were harvested 24 h after transfection and then seeded in a new six-well plate (300 cells/well) and cultured for approximately 2 weeks until colony formation was observed. Colonies were fixed with methanol and stained with 1% crystal violet (Sigma, USA). A colony was considered to be 450 cells. Colony formation rate was used to calculate post-transfection cell survival rate.

For the 5-ethynyl-2-deoxyuridine (EDU) proliferation assay, the Cell-Light EDU imaging detection kit was purchased from Life Technologies (MA, USA). Cells which had been transfected 48 h previously were incubated with 10 μM EDU for 24 h, fixed, permeabilized, and stained with both the Alexa-Fluor 594 reaction cocktail for EDU and Hoechst 33342 for cell nuclei, according to the manufacturer's protocol. Finally, samples were imaged under a fluorescent microscope.

Flow cytometric analysis of the cell cycle

The glioma cells from each group were cultured in six-well plates for 48 h after transfection of lentivirus and/or plasmids, then trypsinized and collected by centrifugation at 1500 r/minute for five minutes. The cells were washed with phosphate buffered saline (PBS), collected by centrifugation and fixed in 70% ethanol overnight. The supernatant was discarded after centrifuging. The cells were washed with PBS, stained using a Cell Cycle Staining Kit (Multi Sciences, Hangzhou, China), and incubated for 30 minutes in the dark before being analyzed by flow cytometry. Ten thousand cells were harvested and analyzed by flow cytometry (Beckman Coulter Gallios, USA).

Dual luciferase reporter assay

Wild-type (WT) and mutated putative miR-520d-5p-binding sites in the PTTG1 3'-untranslated regions (UTRs) were amplified using PCR from human cDNA, inserted into the XbaI site of a pGL3 control vector (Invitrogen). For the reporter assay, cells were cultured in 96-well plates and co-transfected with WT or mutated 3'-UTR luciferase reporters and miR-520d-5p mimics (RiboBio). After 48 h of incubation, luciferase activity was measured with a Dual Luciferase Reporter Assay Kit (Promega, Madison, USA) according to the manufacturer's protocol. Renilla luciferase activity was used as an internal control.

Nude mouse model of intracranial glioma and hematoxylin-eosin staining

Animal experiments were approved by the Animal Management Rule of the Chinese Ministry of Health (documentation 55, 2001) and were in accordance with the approved guidelines and the experimental protocol of Nanjing Medical University. BALB/c-A nude mice at 4 weeks of age were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. Eighteen mice were randomly assigned into two groups and intracranially implanted with 5×10^5 U87-MG cells (pretreated with lentivirus containing the miRNA-520d-5p or negative control sequences) using a stereotactic instrument. The overall survival of the mice was monitored during the experimental period. Paraffin-embedded sections (5 μm) of brain specimens were stained with hematoxylin and eosin (HE) and used for immunohistochemistry. For HE staining, brain tissue sections (5 μm) embedded in paraffin blocks were deparaffinized in xylene and hydrated in alcohol and distilled water. The samples were washed in PBS for 5 min three times each and stained with hematoxylin (USA, Sigma) for 5 min. To observe the clarity of nuclei

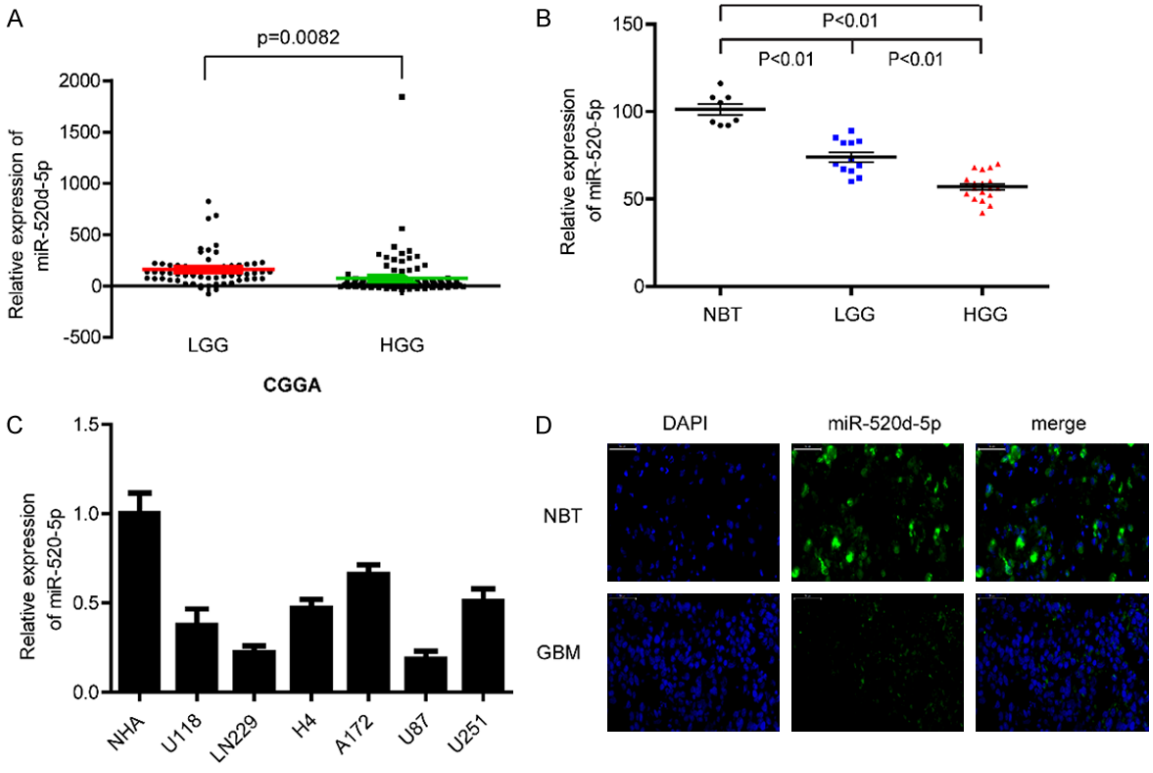

Figure 1. MiR-520d-5p expression correlates negatively with malignant degrees of glioma. A. CGGA database showing reduced miR-520d-5p expression in high-grade glioma tissues compared with that in low-grade glioma tissues. B. The expression of miR-520d-5p in 8 non-cancerous brain tissues, 12 low-grade glioma tissues and 19 high-grade glioma tissues was measured by real-time PCR, miR-520d-5p levels in normal brain tissues were indeed higher than in glioma specimens, and were significantly decreased with ascending pathological grade of tumor. C. The expression of miR-520d-5p in normal human astrocytes (NHAs) and six glioma cell lines (U118, LN229, H4, A172, U87-MG and U251). D. The expression of miR-520d-5p in NBT and GBM specimens was assessed by fluorescence *in situ* hybridization (scale bars, 50 μm).

and cytoplasm under the microscope, sections were stained with eosin (USA, Sigma) for 2 min. After conventional dehydration and sealing, images were observed and collected under a microscope.

Immunohistochemistry (IHC)

The mouse brain was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 3.5-lm-thick sections. The sections were stained with Mayer's hematoxylin and subsequently with eosin (Biogenex Laboratories) or an antibody against PTTG1, CD31 and Ki-67. Brown staining in cells was considered as positive signaling.

Fluorescence in situ hybridization (FISH)

The expression of miR-520-5p in GBM samples and NBTs was detected by FISH. The mature human miR-520-5p sequence is: 3'-CUUUCCCGAAGGGAAACAUC-5'. We used (LNA)-based probes directed against the full length mature miRNA sequence. The 5'-FAM-labelled miR-520-5p probe sequence is: 5'-GAAAGGGCTTCCCTTTGTAG-3', and was purchased from BioSense (Guangzhou, China). The FISH procedure followed the BioSense instructions. Briefly, Fresh tissues were fixed in 4% formaldehyde for 1 h and then dehydrated in 15% sucrose for 8 h. The tissues were fixed in 4% formaldehyde for 10 min, washed three times for 5 min each with PBS (pH 7.4), digested using proteinase K for 2 min, washed again three times for 5 min each using PBS (pH 7.4). After eliminating auto-fluorescence and blocking endogenous biotin, the sections were hy-bridized with the probes overnight. The tissue sections were then washed with warmed in 2 × SSC at 37°C for 10 min, 1 × SSC at 37°C for 10 min, and 0.5 × SSC for 10 min. After incubation in BSA for 30 min at room temperature, tissue sections were treated with

Alexa Fluor 488-avidin (1:400), incubated at room temperature for 50 min, washed three times for 5 min each with PBS. Tissue sections were incubated with the primary antibody overnight and then with the species-specific secondary antibody for 50 min at 4°C. Finally, slides were counterstained with DAPI (Sigma) for 10 min and examined with a Zeiss LSM 700 Meta confocal microscope (Oberkochen, Germany).

Statistical analysis

All experiments were performed three times, and all values are presented as the mean ± standard deviation (SD). One-way ANOVA was used to determine the difference among at least three groups using SPSS v19.0 for Windows. (SPSS, IL, USA). Pearson's correlations analysis and heat map microarray analysis were implemented using Multiple Array Viewer 4.9 software (MEV). KEGG pathway and GO analysis were performed via DAVID (http://david.abcc.ncifcrf.gov/). Kaplan-Meier analysis was used to assess the survival rate of patients and mice. $P<0.05$ indicates a significant difference.

Results

MiR-520d-5p is down-regulated in glioma tissues and cell lines

To investigate miRNA expression in human glioma tissues, the transcription level of miR-520d-5p in Chinese Glioma Genome Atlas (CGGA) databases was analyzed. We found that miR-520d-5p levels in high-grade gliomas (HGG) were significantly decreased compared to low-grade gliomas (LGG) (**Figure 1A**). QRT-PCR was then used to evaluate the expression level of miR-520d-5p in 31 different grades of glioma tissues and in 8 non-cancerous brain tissues, and we found that the expression level of miR-520d-5p was significantly lower in glioma tissues compared to non-cancerous brain tissues, Moreover, the decrease was more pronounced in high-grade gliomas (grade III and IV) compared to low-grade gliomas (grades I and II) (**Figure 1B**). In addition, a panel of glioblastoma cell lines were chosen to evaluate the expression levels of miR-520d-5p, and the results form qRT-PCR assay indicated that miR-520d-5p was also up-regulated in glioblastoma cell lines compared with the normal human astrocyte (NHA) (**Figure 1C**). Then we chose a representative GBM specimen and non-cancerous brain tissue for FISH analysis, and we obtained a consistent result (**Figure 1D**). These data suggested that miR-520d-5p was not only down-regulated in glioma tissues and cell lines, but also negatively correlated with glioma malignancy.

Ectopic expression of miR-520d-5p inhibits cell proliferation of glioma cells

To further investigate the role of miR-520d-5p in the development of glioma, the U87-MG and LN229 GBM cancer cell lines were transfected with miR-ctrl or miR-520d-5p lentivirus. QRT-PCR demonstrated that miR-520d-5p was significantly increased compared to negative control groups (**Figure 2A**). Thereafter, EdU assay was performed to assess the cell proliferation after overexpression of miR-520d-5p. As is shown in **Figure 2B** and **2C**, compared with the control group, EdU positive cells of the miR-520d-5p UP-regulation groups reduced 23% in U87-MG cells. Similarly, the number of EdU positive cells of miR-520d-5p UP-regulation group decreased approximately 26% in LN229 cells. To more objectively evaluate the effect of miR-520d-5p on proliferation, CCK-8 and colony formation assays were also performed for U87-MG and LN229 cells transfected with miR-520d-5p or miR-ctrl. Consistent with the results of the EDU assay, CCK8 and colony formation assays demonstrated that miR-520d-5p significantly inhibited glioma cell proliferation (**Figure 2D-F**). As proliferation is directly connected to cell cycle distribution, we subsequently studied the cell cycle distribution of cells overexpressing miR-520d-5p. Flow cytometry analysis showed that overexpression of miR-520d-5p increased the percentage of G0/G1 phase cells and decreased the percentage of S and G2/M phase cells (**Figure 2G** and **2H**). Whether miR-520d will affect the G1 phase-related proteins? By Western blot we demonstrated that CDK2, CDC2 and cyclin E1 were significantly decreased after miR-520d-5p overexpression (**Figure 3F**). Therefore, these data suggested that miR-520d-5p inhibited glioma cell growth *in vitro*.

PTTG1 is a direct target of miR-520d-5p, and PTTG1 levels are inversely correlated with miR-520d-5p levels in glioma tissues

To investigate the molecular mechanism underlying the miR-520d-5p-induced inhibitory effect

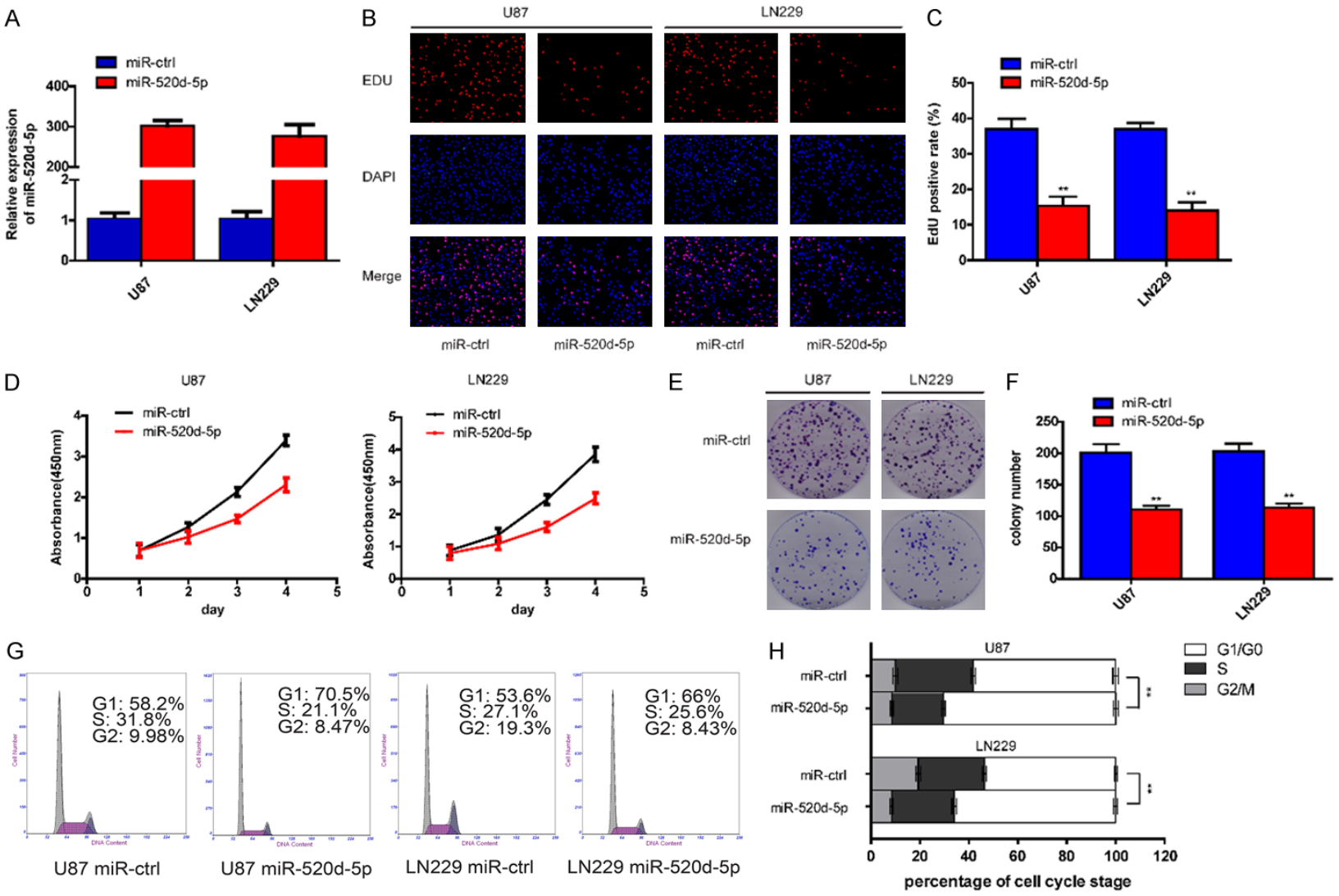

Figure 2. Overexpression of miR-520d-5p inhibits glioma cell proliferation *in vitro*. A. The relative expression level of miR-520d-5p in U87-MG and LN229 cells was analyzed by qRT-PCR after transfection. B, C. EdU assay showed that miR-520-5p up-regulation inhibited cell proliferation both in U87-MG and LN229 cells. Representative images were shown (original magnification, 200 ×). (**P<0.01). D. Overexpression of miR-520-5p inhibited cell proliferation detected by CCK8 assay. Data are presented as the means of triplicate experiments. E, F. Long-term cell viability was evaluated using the colony formation assay. Data are presented as the means of triplicate experiments. G, H. The cell cycle phase of U87-MG and LN229 cells transfected with miR-520d-5p or negative control (miR-ctrl) lentivirus was analyzed by flow cytometry. (**P<0.01).

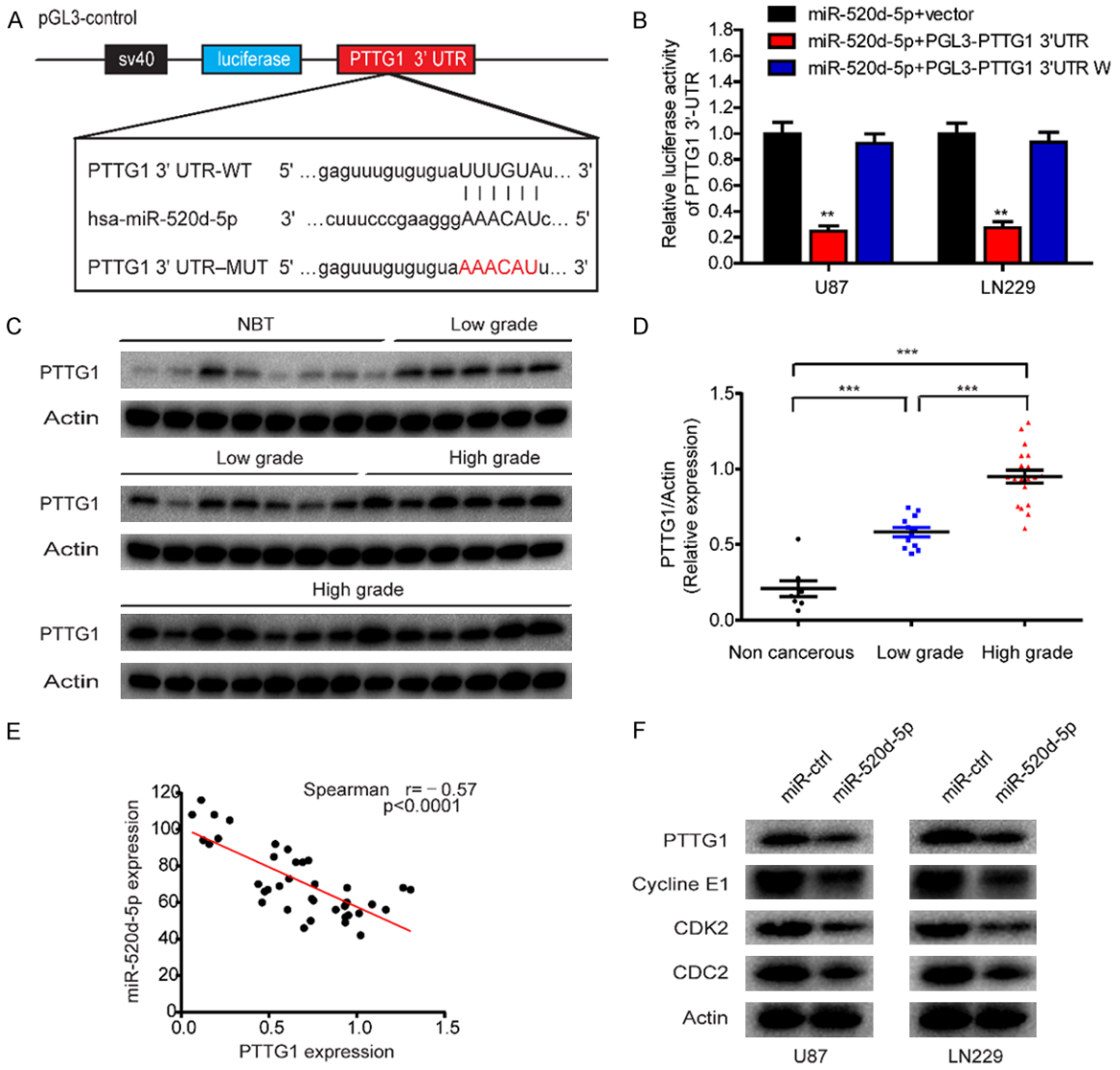

Figure 3. MiR-520d-5p directly targets PTTG1 and negatively regulates cell cycle-related proteins. A. The miR-520d-5p binding sites in the 3'UTR of PTTG1 with the wild type (Wt) and mutated (Mut) sequences highlighted. B. U87-MG and LN229 cells were co-transfected with miR-520d-5p and luciferase reporter constructs containing either pGL3-PTTG1-3'-UTR-WT or pGL3-PTTG1-3'-UTR-Mut. The data shown are representative of three independent experiments. Data shown are mean ± SD of three independent experiments. (**P<0.01). C, D. The expression levels of PTTG1 in NBTS and glioma specimens were determined by western blotting; the fold changes were normalized to β-Actin. The non-neoplastic brain tissues (n=8) were collected from brain trauma surgery. The low-grade (n=12) represents samples derived from grades I and II glioma tissues, whereas high-grade (n=19) represents grades III and IV glioma tissues. Data represent the means ± SD from three independent experiments. (***P<0.001). E. Pearson's correlation analysis of the relative expression levels of miR-520d-5p and the relative protein levels of PTTG1. F. Western blot analysis of CDC2, CDK2 and cyclinE1 in U87-MG and LN229 cells 48 h after transfection. β-Actin served as the loading control.

on glioma cell proliferation and the cell cycle. We used the bioinformatics analytical tools miRNAWalk 2.0 and TargetScan to identify potential targets of miR-520d-5p. Among the putative targets of miR-520d-5p, PTTG1 captured our interest. The putative seed-matched or mutant sequences of miR-520d-5p within the 3'UTR of PTTG1 were shown in **Figure 3A**. Western blot analysis showed that expression of PTTG1 protein was downregulated in miR-520d-5p-transfected cells (**Figure 3F**). We then performed a luciferase reporter assay to determine whether miR-520d-5p could bind directly to the 3'-UTR of PTTG1. U87-MG and LN229

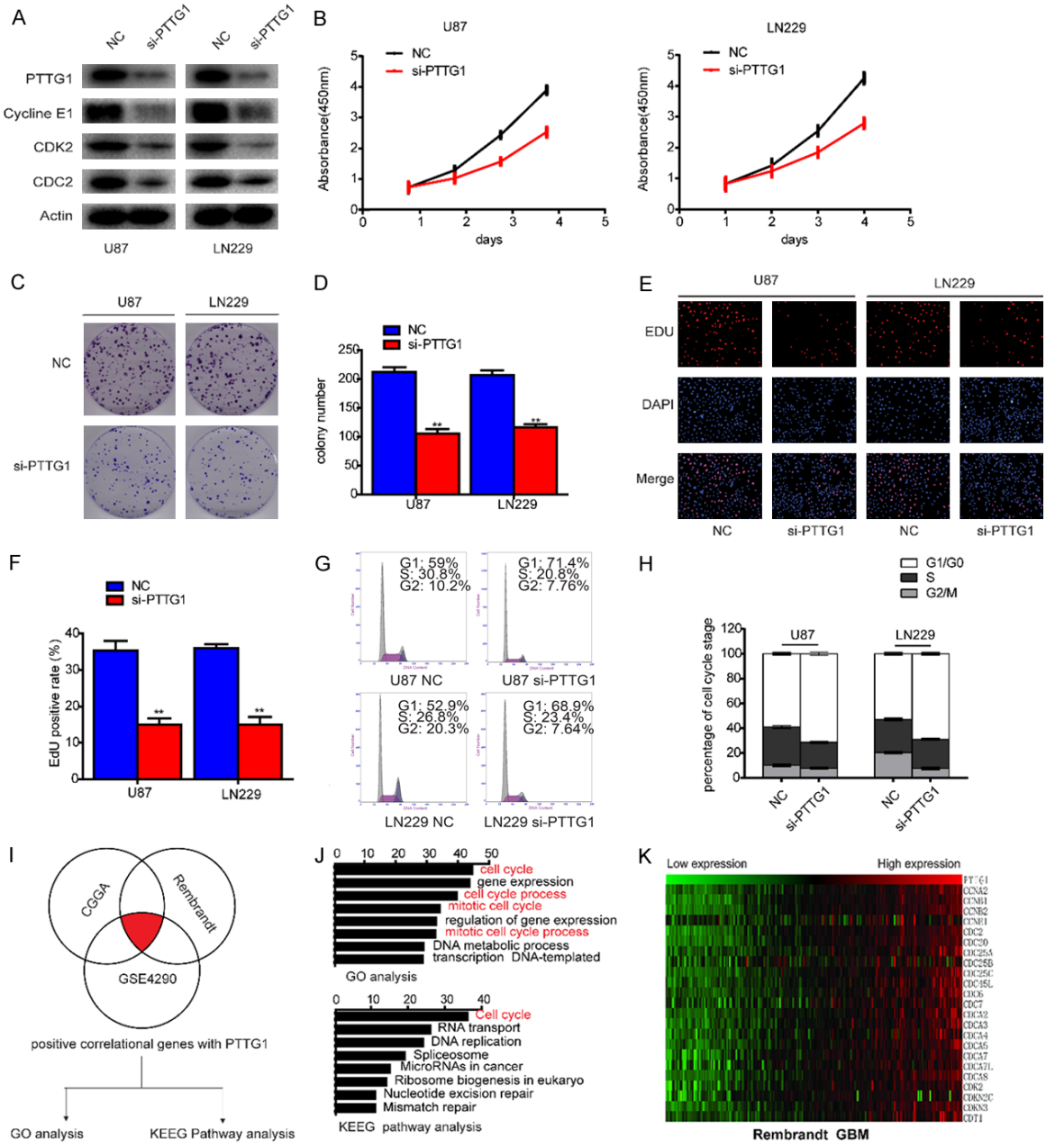

Figure 4. Down-regulation of PTTG1 inhibits glioma cell proliferation *in vitro* and positively-associated genes of PTTG1 were chiefly enriched in cell cycle related pathways. A. Downregulation of PTTG1 in U87-MG and LN229 cells decreased the levels of CDC2, CDK2 as well as cyclinE1. B. Proliferation ability was determined using the CCK-8 assay following culture for 96 h. Data are presented as the means of triplicate experiments. C, D. Long-term cell viability was evaluated using the colony formation assay. Data are presented as the means of triplicate experiments. (**P<0.01). E, F. Proliferating cells were examined using the EDU assay. Representative images are shown (original magnification, 200 ×). (**P<0.01). G, H. The cell cycle phase of U87-MG and LN229 cells transfected with si-PTTG1 or negative control (NC) lentivirus was analyzed by flow cytometry. I, J. PTTG1 positively-associated genes from Correlation analysis performed in the CGGA, Rembrandt, and GSE4290 glioblastoma samples were analyzed with gene ontology analysis and KEEG pathway analysis. Biological processes enrichment results from GO database and KEGG pathways analysis were shown. The orders of different biological processes were based on their enriched number. K. A heat map of relative expression of several PTTG1-associated cell cycle genes in Rembrandt glioblastoma tissues sorted by level of PTTG1 expression (r > 0.4).

cells were co-transfected with vectors carrying wild-type or mutant PTTG1 3'-UTR and miR-520d-5p mimics. Overexpression of miR-520d-5p inhibited Wt PTTG1 reporter activity but not

the activity of the mutated reporter construct in both U87-MG and LN229 cells (**Figure 3B**). To further investigate the correlation between miR-520d-3p and PTTG1 levels, we examined the expression of PTTG1 in 31 glioma specimens and 8 adjacent non-cancerous tissues. We found that PTTG1 was highly expressed in glioma tissue and positively correlated with grade (**Figure 3C** and **3D**). We further assessed the correlation of PTTG1 and miR-520d-5p levels in the same GBM specimens. Pearson's correlation analysis showed that PTTG1 levels in GBM samples were negatively correlated with miR-520d-5p (Pearson's r=-0.57, P< 0.0001). These data suggested that miR-520d-5p directly regulated PTTG1 expression through its binding to the 3'-UTR of PTTG1.

Down-regulation of PTTG1 exhibits a similar effect on the proliferation of glioma cells to that of miR-520d-5p overexpression.

We analyzed the expression level of PTTG1 using the CGGA, TCGA, GSE4290 and Rembrandt databases, and the results showed that the expression of PTTG1 in HGG was significantly up-regulated compared with LGG (Supplementary Figure S1A-D).

In addition, western blot analysis have verified that PTTG1 was up-regulated in HGG compared with LGG. Having verified that PTTG1 is a direct target of miR-520d-5p in human gliomas, we wondered whether PTTG1 is a downstream effecter of miR-520d-5p that mediates its function. To confirm this, endogenous expression of PTTG1 was knockdown in U87-MG and LN229 cells with specific siRNA against PTTG1 (si-PTTG1). Successful transfection was confirmed by Western blot (**Figure 4A**). The effects of PTTG1 knockdown on cell proliferation were evaluated in U87-MG and LN229 cells using CCK-8, colony formation and EDU assays. We found that knockdown of PTTG1 expression dramatically inhibited the proliferation of GBM cells (**Figure 4B-F**). Additionally, Flow cytometry was performed following transfection to assess cell cycle distribution. The results showed that the cell population in the G0/G1 phase was increased but the S and G2/M phase population were decreased after PTTG1 gene silencing compared with the results observed for the si-NC cells (**Figure 4G**, **4H**). These results were consistent with the effect of miR-520d-5p overexpression.

PTTG1-associated genes are mainly involved in the regulation of cell cycle

Pearson correlation analysis was implemented using MEV software to identify target genes that were positively associated with PTTG1 expression in CGGA, Rembrandt and GSE4290 databases (r > 0.4). In total 535 upregulated genes were identified (**Figure 3I**). To clarify the associations between these genes, DAVID Web tool (https://david.ncifcrf.gov/tools.jsp) were used for Gene Oncology enrichment analysis and KEGG pathway analysis. The up-regulated genes were mainly enriched in the terms positive regulation of cell cycle and others in the top 5 GO and KEGG Pathway terms of PTTG1-acossiated genes (**Figure 3J**). Further, when we conducted co-expression analysis of up-regulated genes in the three databases, we found that the expression of 23 cell cycle-related genes positively correlated with that PTTG1 (r > 0.4) (**Figure 3K** and Supplementary Figure S1E and S1F). The in silico prediction of PTTG1 function was in line with tumor-associated phenotypes.

Overexpression of PTTG1 partially attenuates miR-520d-5p inhibited cell growth

As PTTG1 was proved to be not only a direct target of miR-520d-5p but also a carcinogenic effect in gliomas, we further investigated whether the role of miR-520d-5p in glioma was through mediation of PTTG1. PcDNA3.1-PTTG1 plasmid was transfected into miR-520d-5p-overexpressing U87-MG and LN229 cells to reverse the reduced PTTG1 levels. After transfection, western blot analysis was conducted. As indicated in **Figure 5I**, the protein level of PTTG1 was higher in the miR-520d-5p+PTTG1 group, when compared with that in the miR-520d-5p+vector group. To further confirm whether PTTG1 is an important target of miR-520d-5p in cell proliferation, CCK-8, colony formation and EDU assays were performed. The results revealed that restoration of PTTG1 partially rescued miR-520d-5p suppressive functions in U87-MG and LN229 cell proliferation (**Figure 5B-F**, P<0.01). Furthermore, cell cycle distribution analysis detected by flow cytometry revealed that cell population increased in G0/G1 phase after miR-210 expression was abolished by overexpression of PTTG1 (**Figure 5G** and **5H**). Interestingly, Cell cycle related proteins were altered in a similar way to the expression

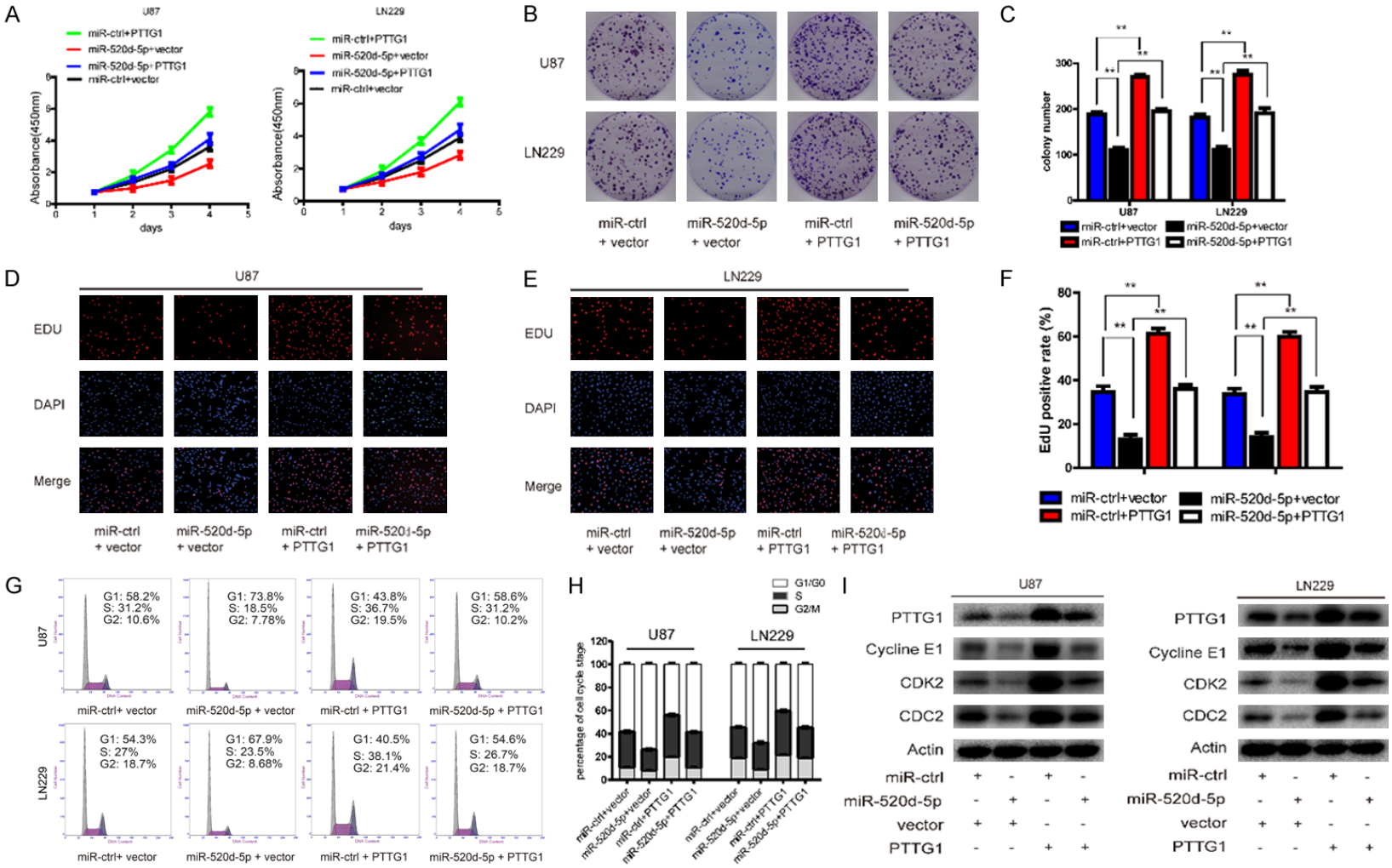

Figure 5. PTTG1 reintroduction reverses the inhibitory effect of miR-520d-5p. A-C. The rescue experiment was performed by introducing pcDNA3.1-PTTG1 or pcDNA3.1 in the presence or absence of ectopic miR-520d-5p or miR-ctrl expression in U87-MG and LN229 cells. Cell viability of glioma cells transfected with pcDNA3.1-PTTG1 and miR-520d-5p separately or together was detected using the CCK-8 and colony formation assays. (**P<0.01). D-F. Cell proliferative potential was evaluated using the EDU assay 48 h after co-transfection. (**P<0.01). G, H. Cell cycle distribution of glioma cells was measured using flow cytometry. I. Western blot analysis of PTTG1, CDC2, CDK2 and cyclinE1 in the indicated cells. β-Actin was used as the loading control.

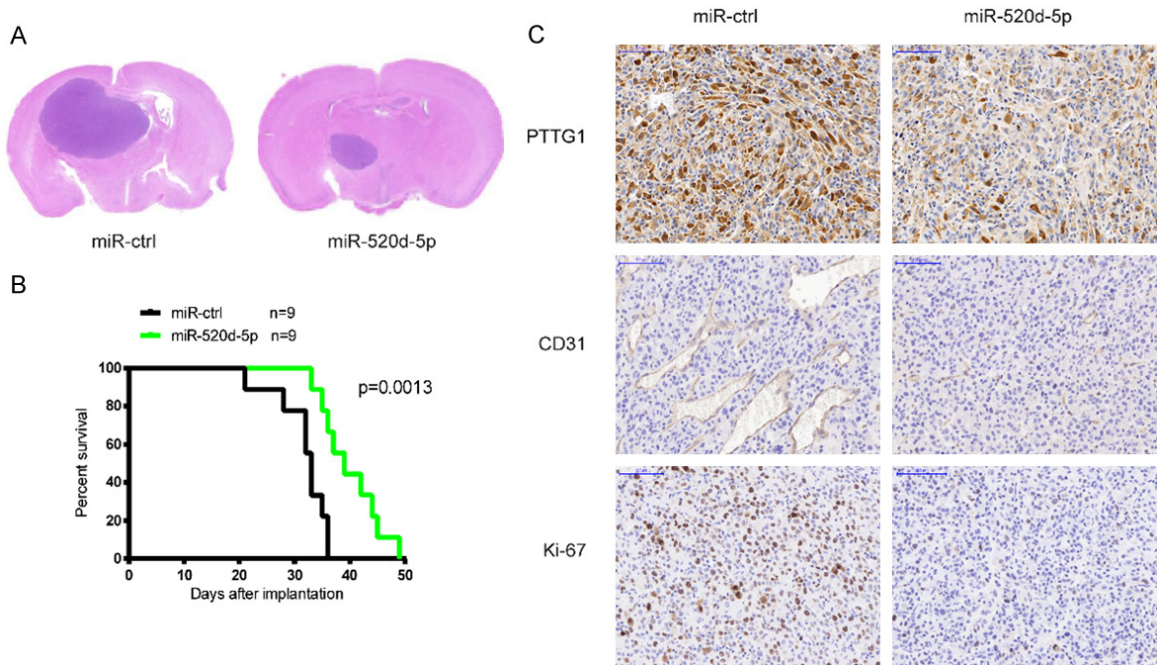

Figure 6. MiR-520d-5p suppresses tumor growth in an intracranial xenograft model. A. U87-MG cells pretreated with a miR-ctrl or miR-520d-5p lentivirus were implanted in the brains of nude mice, and tumor volume in the LV-miRNA-520d-5p group was significantly diminished based upon HE histology. B. Overall survival was determined by Kaplan–Meier survival curves, and a log-rank test was used to assess the statistical significance of the differences. C. After sacrifice, mouse brain tissues were harvested, embedded, and cut into paraffin sections for immunohistochemistry analysis. The expression of PTTG1, CD31 and Ki67 in the LV-miRNA-520d-5p group was significantly reduced.

level of PTTG1; that is, decreased levels of CDK2, CDC2 and cyclin E1 due to miR-520d-5p overexpression could be rescued by up-regulation of PTTG1 (**Figure 5I**). Collectively, these findings suggested that PTTG1 acted as a downstream effector of miR-520d-5p in the regulation of the proliferation of glioma cells *in vitro*.

MiR-520d-5p suppresses glioblastoma xenograft growth in vivo

MiR-520d-5p overexpression inhibit the proliferation of glioma cells *in vitro*. To assess whether miR-520d-5p overexpression reduces glioma growth *in vivo*, the human U87-MG cells stably expressing miR-520d-5p or miR-ctrl were injected into nude brain to form the intracranial xenograft. Hematoxylin and eosin staining showed that intracranial tumor volumes of the miR-520d-5p groups were significantly reduced compared with those of the miR-ctrl groups (**Figure 6A**). In order to analyze the survival of different treatment groups, we generated the Kaplan-Meier survival curve and found that the survival of mice injected with miR-520d-5p expressing U87-MG cells was significantly prolonged (**Figure 6B**). Further, immunohistochemistry (IHC) experiments were performed to examine the levels of Ki67 and CD31, which were commonly used to reflect tumor proliferation and angiogenesis. We found a significant reduction of these two proteins in the miR-520d-5p group (**Figure 6C**). In addition, IHC also revealed decreased expression of PTTG1 in miR-520d-5p group, which was consistent with the vitro results (**Figure 6C**). In conclusion, these data suggested that miR-520d-5p suppressed glioma growth *in vivo* by repressing PTTG1.

Discussion

Dysregulation of microRNAs (miRNAs) is a common feature in human cancers, including glioma. Recently numbers of miRNAs have been identified to function as a tumor suppressor or an oncogene in glioma by regulating their target molecule [36, 37].

For example, miR-506 was reported to be downregulated in glioma tissue and cell lines, and functioned as a novel tumor suppressor to inhibit the proliferation of glioma cells *in vitro*

and *in vivo* by targeting STAT3 [38]. Chen *et al* found that overexpression of miR-19a promoted glioma cell proliferation and invasion by targeting the Ras homolog family member B (RhoB) [39]. MiR-217 was demonstrated to inhibit the proliferation, migration and invasion of glioma cells by repressing Runx2 [40]. The miR-520 family is located on chromosome 19, Keklikoglou I et al reported that microRNA-520 family functions as a tumor suppressor in estrogen receptor negative breast cancer by targeting NF-κB and TGF-β signaling pathways [41]. A recent study showed that miR-520a-3p suppressed breast cancer through CCND1 and CD44 [42]. Moreover, a further study revealed that miR-520b was down-regulated in hepatocellular carcinoma tissue and could inhibit growth of hepatoma cells by targeting MEKK2 and Cyclin D1 [43]. Simultaneously, miR-520c functioned as a tumor suppressor in estrogen-receptor-negative breast cancer by targeting RELA and TGFBRII (transforming growth factor β type II receptor) [44]. However, the report on the role of miR-520d-5p in the development of cancer is indeed relatively few. In our study, we found that the expression of miR-520d-5p in glioma tissues was significantly lower than non-cancerous brain tissue. In addition, the expression level was negatively correlated with glioma grade. The abnormal expression of miRNAs is closely related to malignant biological behaviors, CCK-8, colony formation and EDU measurements showed that the short and long-term proliferative capacity was significantly reduced in miR-520d-5p stably transfected cells. Through flow cytometry assay, we found that overexpression of miR-520d-5p could arrest cells at G1/S phase. As is well known, regulation of the cell cycle is largely dependent on the protein kinase complex, which is composed of various cell cycle proteins (cyclins) and the corresponding cyclin dependent kinases (cyclin-dependent-kinase, CDK). Among them, Cyclin E-CDC2 and Cyclin E-CDK2, the two key kinase complexes, play vital roles in transition from the G0/G1 phase to the S phase [45, 46]. By western bolt, we found that the expression levels of CDK2, CDC2 and cyclin E1 were significantly decreased after overexpression of miR-520d-5p. Therefore, we suggest that miR-520d-5p can arrest the cell cycle in the G0/G1 phase by inhibiting the expression of CDK2, CDC2 and cyclin E1.

Pituitary Tumor Transforming Gene-1 (PTTG1) was originally isolated from a rat pituitary tumor cell line by mRNA differential PCR display [16]. Subsequently, the human homologue of PTTG1 was successfully cloned and proved to be overexpressed in Jurkat cells (human T lymphoma cell lines) as well as from patients with myelodysplastic syndromes [47]. Recent studies have shown that PTTG1 acts as a proto-oncogene and plays a crucial role in promoting cell cycle progression, sustaining chromosomal stability and modulating transformation *in vitro* and tumorigenesis *in vivo* [48]. Many studies have reported the role of PTTG1 in cell proliferation, but in a limited number of cases contrasting results have been reported. For instance, over-expression of PTTG1 was found to promote proliferation in HeLa S3, HEK 293 and NIH3T3 cells [48, 49]. On the other hand, exogenous overexpression of PTTG1 was shown to impair proliferation of the cervix carcinoma cell line HeLa, the lung cancer cell line A549 and the choriocarcinoma cell line JEG-3 [48, 49]. Meanwhile, PTTG1 acted as microRNAs function target to regulate tumor apoptosis, cell proliferation, cell cycle, metastasis [50-55]. Recently it has been published that PTTG1 protein is overexpressed in high-grade astrocytomas compared to low-grade astrocytomas. However the relationships between PTTG1 and the clinical features of glioma have not previously been described. In this study, we showed that PTTG1 protein was overexpressed in glioma tissues and cell lines and its expression was negatively correlated with the WHO grade. Three datasets were overlapped to mine PTTG1-associated biological pathways. Both KEGG and GO analyses indicated that genes that were positively correlated to PTTG1 were strongly associated with the cell cycle pathway. Next, we observed that decreased PTTG1 protein levels inhibited glioma cells proliferation. Then, we verified that knockdown of PTTG1 arrested cell cycle in the G0/G1 phase. By western blot we confirmed that CDK2, CDC2 and cyclin E were indeed reduced after down regulation of PTTG1, which was consistent with the expectation. Moreover, we demonstrated that the PTTG1 oncogene was a functional target of miR-520d-5p *in vitro* and *in vivo*. Firstly, the expression of PTTG1 was significantly decreased in glioma cells stably expressing miR-520d-5p. Secondly, the expression of PTTG1 protein was negatively correlated with miR-520d-5p in

clinical specimens. Thirdly, luciferase reporter assays confirmed that miR-520d-5p can specifically bind to the 3'-UTR region of the PTTG1 transcript. Finally, Reversal experiments confirmed that PTTG1 can reverse the effect of miR-520d-5P on glioma proliferation and cell cycle. Taken together, our results provide the first evidence that miR-520d-5p is significant in suppressing glioma cell growth and cell cycle through inhibition of PTTG1.

In summary, this study investigated the suppressive role of miR-520d-5p in the growth and metastasis of glioma cells, and suggests that miR-520d-5p acts as a novel tumor suppressor miRNA in glioma. MiR-520d-5p has suppressive effects on the proliferation, and cell cycle of glioma cells, partly at least, via targeting PTTG1. Further PTTG1 negatively regulated the cell cycle by effectively inhibiting CDK2, CDC2 and cyclin E1 expression. This newly identified miR-520d-5p/PTTG1 axis provides a new insight into the mechanisms underlying glioma development, and targeting miR-520d-5p/PTTG1 may represent a promising therapeutic strategy for glioma. Nevertheless, we need further studies to determine the exact mechanism of decreased miR-520d-5p expression during the progression of glioma and to further explore other possible targets for miR-520d-5p in glioma. Additionally, a large cohort study, incorporating PTTG1 expression and function should also be investigated.

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Disclosure of conflict of interest

None.

Address correspondence to: Ning Liu and Yingyi Wang, Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. E-mail: liuning0853@126.com (NL); lindsay_wang3431@126.com (YYW)

References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65: 5-29.

[2] Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P and Ellison DW. The 2016 world health organization classification of tumors of the central nervous system: a summary. Acta Neuropathol 2016; 131: 803-820.

[3] Meyer MA. Malignant gliomas in adults. N Engl J Med 2008; 359: 1850; author reply 1850.

[4] Grossman SA, Ye X, Piantadosi S, Desideri S, Nabors LB, Rosenfeld M and Fisher J. Survival of patients with newly diagnosed glioblastoma treated with radiation and temozolomide in research studies in the United States. Clin Cancer Res 2010; 16: 2443-2449.

[5] West ES, Williams VL and Morelli JG. Vemurafenib-induced neutrophilic panniculitis in a child with a brainstem glioma. Pediatr Dermatol 2015; 32: 153-154.

[6] Westphal M, Heese O, Steinbach JP, Schnell O, Schackert G, Mehdorn M, Schulz D, Simon M, Schlegel U, Senft C, Geletneky K, Braun C, Hartung JG, Reuter D, Metz MW, Bach F and Pietsch T. A randomised, open label phase III trial with nimotuzumab, an anti-epidermal growth factor receptor monoclonal antibody in the treatment of newly diagnosed adult glioblastoma. Eur J Cancer 2015; 51: 522-532.

[7] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[8] Lewis BP, Burge CB and Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120: 15-20.

[9] Areeb Z, Stylli SS, Koldej R, Ritchie DS, Siegal T, Morokoff AP, Kaye AH and Luwor RB. MicroRNA as potential biomarkers in glioblastoma. J Neurooncol 2015; 125: 237-248.

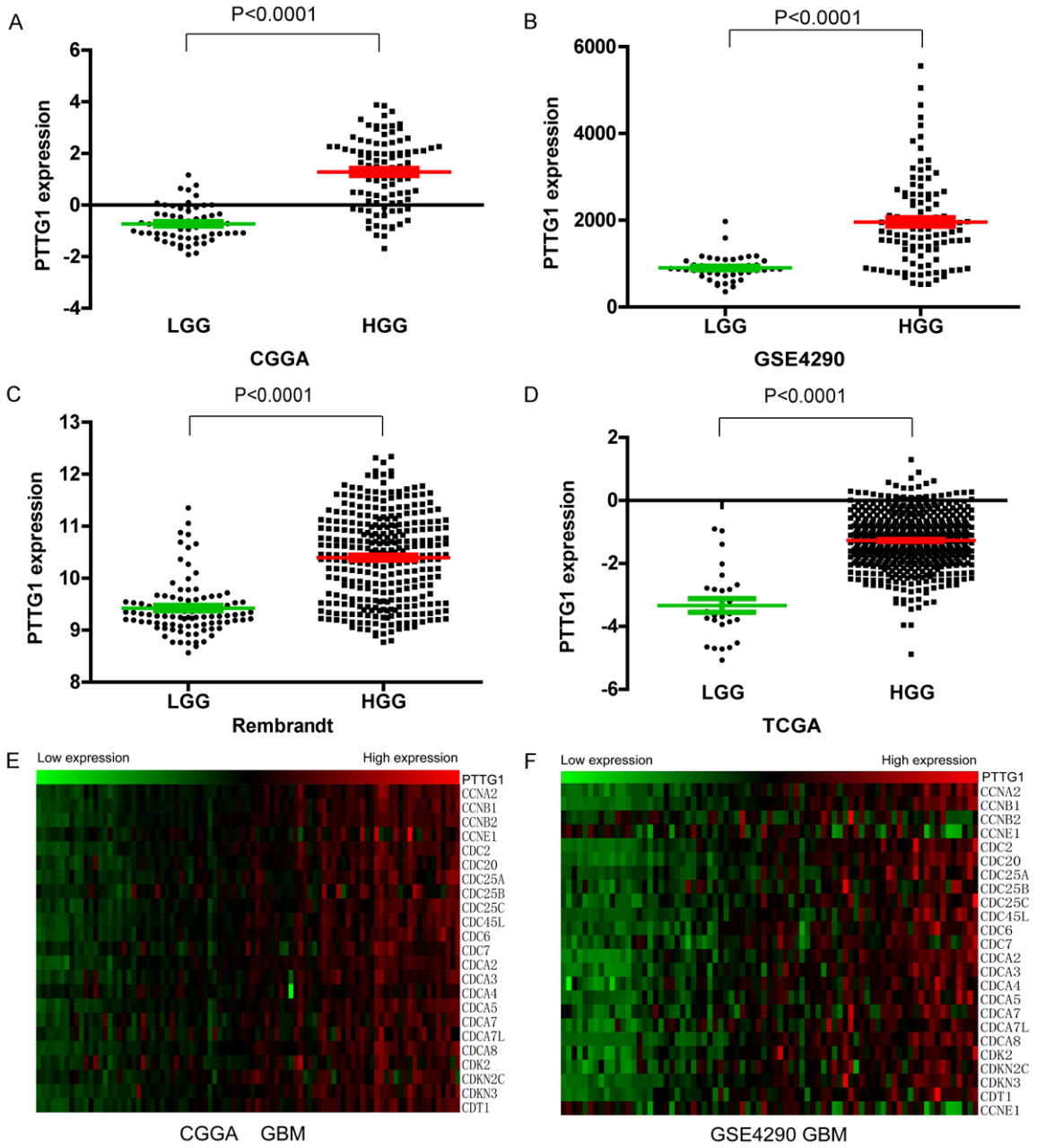
[10] Manikandan J, Aarthi JJ, Kumar SD and Pushparaj PN. Oncomirs: the potential role of non-coding microRNAs in understanding cancer. Bioinformation 2008; 2: 330-334.

[11] Kosaka N, Iguchi H and Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci 2010; 101: 2087-2092.

[12] Kim J, Zhang Y, Skalski M, Hayes J, Kefas B, Schiff D, Purow B, Parsons S, Lawler S and Abounader R. microRNA-148a is a prognostic oncomiR that targets MIG6 and BIM to regu-

late EGFR and apoptosis in glioblastoma. Cancer Res 2014; 74: 1541-1553.
[13] Teplyuk NM, Uhlmann EJ, Wong AH, Karmali P, Basu M, Gabriely G, Jain A, Wang Y, Chiocca EA, Stephens R, Marcusson E, Yi M and Krichevsky AM. MicroRNA-10b inhibition reduces E2F1-mediated transcription and miR-15/16 activity in glioblastoma. Oncotarget 2015; 6: 3770-3783.
[14] Tu Y, Gao X, Li G, Fu H, Cui D, Liu H, Jin W and Zhang Y. MicroRNA-218 inhibits glioma invasion, migration, proliferation, and cancer stem-like cell self-renewal by targeting the polycomb group gene Bmi1. Cancer Res 2013; 73: 6046-6055.
[15] Bier A, Giladi N, Kronfeld N, Lee HK, Cazacu S, Finniss S, Xiang C, Poisson L, deCarvalho AC, Slavin S, Jacoby E, Yalon M, Toren A, Mikkelsen T and Brodie C. MicroRNA-137 is downregulated in glioblastoma and inhibits the stemness of glioma stem cells by targeting RTVP-1. Oncotarget 2013; 4: 665-676.
[16] Pei L and Melmed S. Isolation and characterization of a pituitary tumor-transforming gene (PTTG). Mol Endocrinol 1997; 11: 433-441.
[17] Zou H, McGarry TJ, Bernal T and Kirschner MW. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science 1999; 285: 418-422.
[18] Chen L, Puri R, Lefkowitz EJ and Kakar SS. Identification of the human pituitary tumor transforming gene (hPTTG) family: molecular structure, expression, and chromosomal localization. Gene 2000; 248: 41-50.
[19] Prezant TR, Kadioglu P and Melmed S. An intronless homolog of human proto-oncogene hPTTG is expressed in pituitary tumors: evidence for hPTTG family. J Clin Endocrinol Metab 1999; 84: 1149-1152.
[20] Yu R, Ren SG, Horwitz GA, Wang Z and Melmed S. Pituitary tumor transforming gene (PTTG) regulates placental JEG-3 cell division and survival: evidence from live cell imaging. Mol Endocrinol 2000; 14: 1137-1146.
[21] Zur A and Brandeis M. Securin degradation is mediated by fzy and fzr, and is required for complete chromatid separation but not for cytokinesis. Embo J 2001; 20: 792-801.
[22] Zhang X, Horwitz GA, Prezant TR, Valentini A, Nakashima M, Bronstein MD and Melmed S. Structure, expression, and function of human pituitary tumor-transforming gene (PTTG). Mol Endocrinol 1999; 13: 156-166.
[23] Romero F, Multon MC, Ramos-Morales F, Dominguez A, Bernal JA, Pintor-Toro JA and Tortolero M. Human securin, hPTTG, is associated with Ku heterodimer, the regulatory subunit of the DNA-dependent protein kinase. Nucleic Acids Res 2001; 29: 1300-1307.
[24] Boelaert K, Tannahill LA, Bulmer JN, Kachilele S, Chan SY, Kim D, Gittoes NJ, Franklyn JA, Kilby MD and McCabe CJ. A potential role for PTTG/securin in the developing human fetal brain. Faseb J 2003; 17: 1631-1639.
[25] Chesnokova V, Zonis S, Rubinek T, Yu R, Ben-Shlomo A, Kovacs K, Wawrowsky K and Melmed S. Senescence mediates pituitary hypoplasia and restrains pituitary tumor growth. Cancer Res 2007; 67: 10564-10572.
[26] Solbach C, Roller M, Fellbaum C, Nicoletti M and Kaufmann M. PTTG mRNA expression in primary breast cancer: a prognostic marker for lymph node invasion and tumor recurrence. Breast 2004; 13: 80-81.
[27] Boelaert K, McCabe CJ, Tannahill LA, Gittoes NJ, Holder RL, Watkinson JC, Bradwell AR, Sheppard MC and Franklyn JA. Pituitary tumor transforming gene and fibroblast growth factor-2 expression: potential prognostic indicators in differentiated thyroid cancer. J Clin Endocrinol Metab 2003; 88: 2341-2347.
[28] Heaney AP, Singson R, McCabe CJ, Nelson V, Nakashima M and Melmed S. Expression of pituitary-tumour transforming gene in colorectal tumours. Lancet 2000; 355: 716-719.
[29] Honda S, Hayashi M, Kobayashi Y, Ishikawa Y, Nakagawa K and Tsuchiya E. A role for the pituitary tumor-transforming gene in the genesis and progression of non-small cell lung carcinomas. Anticancer Res 2003; 23: 3775-3782.
[30] Genkai N, Homma J, Sano M, Tanaka R and Yamanaka R. Increased expression of pituitary tumor-transforming gene (PTTG)-1 is correlated with poor prognosis in glioma patients. Oncol Rep 2006; 15: 1569-1574.
[31] Schwartzbaum JA, Fisher JL, Aldape KD and Wrensch M. Epidemiology and molecular pathology of glioma. Nat Clin Pract Neurol 2006; 2: 494-503; quiz 491 p following 516.
[32] Shi Z, Chen Q, Li C, Wang L, Qian X, Jiang C, Liu X, Wang X, Li H, Kang C, Jiang T, Liu LZ, You Y, Liu N and Jiang BH. MiR-124 governs glioma growth and angiogenesis and enhances chemosensitivity by targeting R-Ras and N-Ras. Neuro Oncol 2014; 16: 1341-1353.
[33] Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ and Guegler KJ. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005; 33: e179.
[34] Wang X. A PCR-based platform for microRNA expression profiling studies. Rna 2009; 15: 716-723.
[35] Qian X, Yu J, Yin Y, He J, Wang L, Li Q, Zhang LQ, Li CY, Shi ZM, Xu Q, Li W, Lai LH, Liu LZ and Jiang BH. MicroRNA-143 inhibits tumor growth and angiogenesis and sensitizes chemosensitivity to oxaliplatin in colorectal cancers. Cell Cycle 2013; 12: 1385-1394.

[36] England B, Huang T and Karsy M. Current understanding of the role and targeting of tumor suppressor p53 in glioblastoma multiforme. Tumour Biol 2013; 34: 2063-2074.
[37] Tivnan A and McDonald KL. Current progress for the use of miRNAs in glioblastoma treatment. Mol Neurobiol 2013; 48: 757-768.
[38] Peng T, Zhou L, Zuo L and Luan Y. MiR-506 functions as a tumor suppressor in glioma by targeting STAT3. Oncol Rep 2016; 35: 1057-1064.
[39] Chen Q, Guo W, Zhang Y, Wu Y and Xiang J. MiR-19a promotes cell proliferation and invasion by targeting RhoB in human glioma cells. Neurosci Lett 2016; 628: 161-166.
[40] Zhu Y, Zhao H, Feng L and Xu S. MicroRNA-217 inhibits cell proliferation and invasion by targeting Runx2 in human glioma. Am J Transl Res 2016; 8: 1482-1491.
[41] Keklikoglou I, Koerner C, Schmidt C, Zhang JD, Heckmann D, Shavinskaya A, Allgayer H, Guckel B, Fehm T, Schneeweiss A, Sahin O, Wiemann S and Tschulena U. MicroRNA-520/373 family functions as a tumor suppressor in estrogen receptor negative breast cancer by targeting NF-kappaB and TGF-beta signaling pathways. Oncogene 2012; 31: 4150-4163.
[42] Li J, Wei J, Mei Z, Yin Y, Li Y, Lu M and Jin S. Suppressing role of miR-520a-3p in breast cancer through CCND1 and CD44. Am J Transl Res 2017; 9: 146-154.
[43] Zhang W, Lu Z, Kong G, Gao Y, Wang T, Wang Q, Cai N, Wang H, Liu F, Ye L and Zhang X. Hepatitis B virus X protein accelerates hepatocarcinogenesis with partner survivin through modulating miR-520b and HBXIP. Mol Cancer 2014; 13: 128.
[44] Huang Q, Gumireddy K, Schrier M, le Sage C, Nagel R, Nair S, Egan DA, Li A, Huang G, Klein-Szanto AJ, Gimotty PA, Katsaros D, Coukos G, Zhang L, Pure E and Agami R. The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. Nat Cell Biol 2008; 10: 202-210.
[45] Cheney IW, Neuteboom ST, Vaillancourt MT, Ramachandra M and Bookstein R. Adenovirus-mediated gene transfer of MMAC1/PTEN to glioblastoma cells inhibits S phase entry by the recruitment of p27Kip1 into cyclin E/CDK2 complexes. Cancer Res 1999; 59: 2318-2323.
[46] Kaldis P and Aleem E. Cell cycle sibling rivalry: Cdc2 vs. Cdk2. Cell Cycle 2005; 4: 1491-1494.
[47] Dominguez A, Ramos-Morales F, Romero F, Rios RM, Dreyfus F, Tortolero M and Pintor-Toro JA. hpttg, a human homologue of rat pttg, is overexpressed in hematopoietic neoplasms. Evidence for a transcriptional activation function of hPTTG. Oncogene 1998; 17: 2187-2193.
[48] Vlotides G, Eigler T and Melmed S. Pituitary tumor-transforming gene: physiology and implications for tumorigenesis. Endocr Rev 2007; 28: 165-186.
[49] Panguluri SK, Yeakel C and Kakar SS. PTTG: an important target gene for ovarian cancer therapy. J Ovarian Res 2008; 1: 6.
[50] Su X, Chen J, Ni L, Shi W, Shi J, Liu X, Zhang Y, Gong P, Zhu H and Huang Q. Inhibition of PTTG1 expression by microRNA suppresses proliferation and induces apoptosis of malignant glioma cells. Oncol Lett 2016; 12: 3463-3471.
[51] Liang HQ, Wang RJ, Diao CF, Li JW, Su JL and Zhang S. The PTTG1-targeting miRNAs miR-329, miR-300, miR-381, and miR-655 inhibit pituitary tumor cell tumorigenesis and are involved in a p53/PTTG1 regulation feedback loop. Oncotarget 2015; 6: 29413-29427.
[52] Li L, Han LY, Yu M, Zhou Q, Xu JC and Li P. Pituitary tumor-transforming gene 1 enhances metastases of cervical cancer cells through miR-3666-regulated ZEB1. Tumour Biol 2015; [Epub ahead of print].
[53] Chen B, Hou Z, Li C and Tong Y. MiRNA-494 inhibits metastasis of cervical cancer through Pttg1. Tumour Biol 2015; 36: 7143-7149.
[54] Wang Y, Zang W, Du Y, Ma Y, Li M, Li P, Chen X, Wang T, Dong Z and Zhao G. Mir-655 up-regulation suppresses cell invasion by targeting pituitary tumor-transforming gene-1 in esophageal squamous cell carcinoma. J Transl Med 2013; 11: 301.
[55] Yamanaka S, Campbell NR, An F, Kuo SC, Potter JJ, Mezey E, Maitra A and Selaru FM. Coordinated effects of microRNA-494 induce G(2)/M arrest in human cholangiocarcinoma. Cell Cycle 2012; 11: 2729-2738.



Supplementary Figure 1. A-D. Levels of PTTG1 were analyzed in NBTs and gliomas of CGGA, TCGA, GSE4290 and Rembrandt databases. E, F. Heat maps of relative expression of several PTTG1-associated cell cycle genes in CGGA and GSE4290 glioblastoma tissues sorted by level of PTTG1 expression ($r > 0.4$).