Primate specific miR-663 functions as a tumor suppressor by targeting PIK3CD and predicts the prognosis of human glioblastoma

Yu Shi, Cong Chen, Xia Zhang, Qing Liu, Jin-ling Xu, Hua-rong Zhang, Xiao-hong Yao, Tao Jiang, Zhi-cheng He, Yong Ren, Wei Cui, Chuan Xu, Ling Liu, You-hong Cui, Shi-zhu Yu, Yi-fang Ping, Xi-wu Bian.

Author Affiliations

1 Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, Chongqing 400038, China;
2 Key Laboratory of Tumor Immunopathology of Ministry of Education of China, Third Military Medical University, Chongqing 400038, China;
3 Department of Neuropathology, Tianjin Neurological Institute, Tianjin Medical University General Hospital, Tianjin 300052, China;
4 Department of Neurosurgery, Tiantan Hospital, Capital Medical University, Beijing 100050, China;
5 Department of Health Statistics, College of Preventive Medicine, Third Military Medical University, Chongqing 400038, China

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*Corresponding authors:

Xiu-wu Bian and Yi-fang Ping, Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, Chongqing 400038, China.
Phone: +862368754431; Facsimile: +862368754431; E-mail addresses: bianxiuwu@263.net and pingyifang@126.com

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Translational relevance

Glioblastoma (GBM) is heterogeneous in morphology and varies in outcome. The WHO classification of GBM based on histopathological characteristics is limited in predicting the prognosis of GBM patients. Our present results reveal that miR-663 predicts better prognosis of GBM and suppresses proliferation and invasion of GBM cells by directly targeting PIK3CD. This is the first report that identifies miR-663 as a novel prognostic biomarker and a tumor suppressor of GBM. Thus, our study provides new insights into the diagnostic and prognostic determination as well as therapeutic intervention for GBM based on the functions of miR-663.
Abstract

Purpose: To determine the prognostic significance of miR-663 in glioblastoma (GBM), its effect in tumor progression and the underlying mechanism.

Experimental Design: Specimens from 256 cases of glioma patients, including 239 patients with follow-up information, were used to analyze the association between miR-663 and patients’ prognosis by Kaplan-Meier and multivariate Cox regression analyses. The effects of miR-663 on GBM cell proliferation and invasion were examined both in vitro and in vivo. Bioinformatics prediction and signal network analysis were applied to identify the putative targets of miR-663, which were further verified by luciferase reporter assay, rescue experiments as well as the immunohistochemistry (IHC) and Western blotting examination of downstream effectors. Quantitative reverse transcriptase-PCR (qRT-PCR) and IHC were applied to investigate the clinical association between miR-663 and its target in human GBM specimens.

Results: miR-663 was inversely correlated with glioma grades but positively correlated with patients’ survival. Furthermore, two distinct subgroups of GBM patients with different prognoses were identified based on miR-663 expression in our specimens and that from TCGA database. Overexpression of miR-663 significantly suppressed the proliferation and invasion of GBM cells in vitro and in vivo. Mechanistically, we discovered PIK3CD as a direct target of miR-663 and found that phosphorylated AKT and three key downstream effectors of PIK3CD, i.e., CCND1, MMP2 and MMP7, were down-regulated by miR-663 overexpression. Moreover, PIK3CD was inversely correlated with miR-663 in GBM specimens and predicted poor prognosis of GBM patients.

Conclusion: miR-663 is a novel prognostic biomarker and a potential therapeutic
candidate for GBM.
Introduction

Glioblastoma (GBM) is the most aggressive and lethal malignant brain tumor. Despite efforts have been made to improve therapeutic strategies, the average survival of GBM patients have been improved only slightly in the past decades (1). Although in general, patients with GBM tend to have poor prognoses, significant intra-group variations in their survival have been observed (2). Additionally, traditional histological criteria for GBM diagnosis are not suitable for comprehensive assessment of the status of patients, especially their survival (3). The ineffectiveness to predict patients’ outcomes based on histopathological features suggests the requirement for more precise criteria in GBM subclassification. Recent studies by gene expression profiling and factor analyses have identified subtypes of GBM and a few biomarkers associated with GBM patients’ prognoses (3-5). However, the clinicopathological heterogeneities and prognostic signatures of GBM have not been fully characterized.

Since no ideal protein biomarker has been shown totally effective in predicting GBM patients’ outcomes, some investigators have evaluated the prognostic significance of miRNAs that have recently been recognized as important regulators of cancer biological behavior (6-10). However, the expression pattern and prognostic significance of miRNAs in GBM remain elusive due to the lack of large pools of clinical specimens for screening. Previous studies have shown that miR-663, a member of primate-specific miRNA family, is associated with many important biological processes, including viral infection, inflammatory responses and autoimmune diseases (11-16). However, its role in tumor progression is contradictory. While it acts as an oncogene to promote the malignancy of lung cancer, nasopharyngeal carcinoma and breast cancer (13, 14), miR-663 may also be a
potential tumor suppressor in gastric cancer, colorectal carcinoma, prostate cancer and acute lymphoblastic leukemia (15, 16). Moreover, the prognostic significance of miR-663 in cancer has not been reported.

Correlation of changes in miRNA levels with tumorigenesis and tumor progression remains elusive. However, miRNA expression profiling could be useful in improving the classification of tumors and predicting their behavior (17). In the present study, we identified miR-663 from miRNA profile in human GBM and evaluated its expression and clinical relevance. Its contribution to GBM malignancy and the underlying molecular mechanisms were also investigated. Our data demonstrate that miR-663 has potential values as a prognostic marker and a therapeutic target of GBM.
Materials and methods

Patients and tumor specimens

Surgical specimens were obtained from 256 patients with astrocytic gliomas (Southwest Hospital, Third Military Medical University (TMMU), Chongqing, and Tiantan Hospital, Capital Medical University, Beijing) with written consent. After surgical removal, specimens were immediately fixed in a 4% buffered formaldehyde solution. The formalin-fixed, paraffin-embedded (FFPE) samples were stored at room temperature. Histological diagnoses were independently made by two neuropathologists according to WHO classification of central nervous system tumors (2007). Normal brain tissues adjacent to tumors were used as controls. The clinicopathological features of these patients were summarized in Supplementary Table S1. Two hundred and thirty-nine patients with complete information were followed up after operation until February 1st, 2013, with a median follow-up time of 23.0 months. This study was carried out in accordance with the principles of the Helsinki Declaration and approved by the Ethics Committee of TMMU and Capital Medical University.

An independent cohort of 483 patient specimens from TCGA database (https://tcga-data.nci.nih.gov/tcga) (18) was applied to validate the prognostic value of miR-663 in GBM patients. The mircoarray data of all GBM tissue samples with Level 3 miRNA (Agilent 8 × 15 K Human miRNA-specific Microarray) were used and the results were filtered based on miR-663 expression and clinicopathological features of patients.

Cell culture
Human normal glial cell line, HEB, was generously provided by Professor Guang-mei Yan (Department of Pharmacology, Sun Yat-sen University, Guangzhou) (19, 20). U87-MG was obtained from American Type Culture Collection (ATCC, Manassas, VA). CHG5 (21) and primary GBM cells were established from the tumor specimens of glioma patients (Southwest Hospital, TMMU). HEB, U87-MG and primary GBM cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA). CHG5 was grown in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) with 10% FBS. All the cells were incubated at 37°C in a humidified incubator with 5% CO₂/95% air.

**Real-time RT-PCR**

Total RNA from tumor cells or frozen sections were extracted using RNAiso (Takara, Dalian, China). RNA from FFPE tissues was isolated with a miRNeasy FFPE Kit (Qiagen, Hilden, Germany). For measurement of miR-663, Bulge-Loop™ miRNA qPCR Primer Set (RiboBio, Guangzhou, China), ReverAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) and SYBR® Premix Ex Taq™ II (Takara, Dalian, China) were used. For other genes, PrimeScript™ RT Master Mix (Takara, Dalian, China) and SYBR® Premix Ex Taq™ II were used. Specific primers for amplification were listed in Supplementary Table S2. All reactions were performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad, CA). The results of miRNA and mRNA real-time PCR were normalized using the threshold cycle (Ct) of U6 and GAPDH, respectively.

**Western blotting**

Western blotting was carried out as previously described (22). The primary antibody
used in this study were as follows: anti-PIK3CD (Abnova, Walnut, CA),
anti-phospho-Akt (Ser473), anti-Akt (Cell Signaling, Beverly, MA), anti-MMP-2,
anti-MMP-7, anti-MMP-9 (R&D Systems, Minneapolis, MN), anti-Cyclin D1
(Abcam, Cambridge, UK) and anti-GAPDH (Cell Signaling, Beverly, MA).

Gene cloning and lentiviral vector generation

Pre-miR-663 was cloned from human HEK293T cell genomic DNA using primers
listed in Supplementary Table S2. The expression vector containing PIK3CD coding
sequence (CDS) was obtained from Origene (Rockville, MD). The pre-miR-663 and
PIK3CD-CDS fragments were ligated into the pLOV-CMV-mCherry-EF1a-PuroR
vector or pLOV-CMV-eGFP-EF1a-PuroR vector (Neuron Biotech, Shanghai, China),
respectively. After sequence verification, the mCherry-pre-miR-663 cassette or the
eGFP-PIK3CD cassette, together with psPAX2 and pMD2G constructs (School of
Life Sciences, Lausanne, Switzerland), was transfected into 293FT packaging cells.
Medium was changed after overnight culture and lentiviruses in the supernatant were
collected and filtered through a 0.45-μm filter 48 hrs after transfection.

Lentiviral infection procedures

Cells were infected with concentrated mCherry-pre-miR-663 lentivirus, and/or
eGFP-PIK3CD lentivirus and empty control lentivirus vectors. Cells with stable
integration of pre-miR-663 or PIK3CD were selected and enriched by flow cytometry
(BD FACSaria II, San Jose, CA) to obtain mCherry or eGFP positive cells.

Transfection procedures

miR-663 inhibitor and Negative Control #1 (Ambion, Austin, TX) were transiently
transfected with a final concentrations of 100 nmol/L using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). At 5 hr post-transfection, the medium was replaced by DMEM with 10% FBS. The whole process was conducted according to manufacturer’s guidelines.

6  **Tumor cell proliferation assay**

Tumor cells (500 cells/well for U87-MG and 800 cells/well for primary GBM cells) were seeded into 96-well plates and cultured for 24, 48, 72 and 96 hrs. At the indicated intervals, 20 µl of Cell Counting Kit-8 (Beyotime, Shanghai, China) were added to each well and incubated for 2 hrs at 37°C. Thermo Multiskan Spectrum Reader (Thermo Scientific, Watertown, MA) was used to measure the absorbance at 490 nm.

14  **Tumor cell invasion assay**

Twenty-four-well Millicell® chambers with 8.0 µm pore size (Millipore, Billerica, MA) and BD Matrigel™ (BD Biosciences, Franklin Lakes, NJ) were used in tumor cell invasion assay. Tumor cells were plated in the upper chamber pre-coated with a 5 µl mixture of BD Matrigel™ and DMEM (1:1, v/v). The lower chamber was added with 500 µl of DMEM with 10% FBS. After 24 or 36 hrs of incubation, the Millicell® chambers were removed from the plates and fixed in 4% paraformaldehyde, and then stained with Crystal Violet Staining Solution (Beyotime, Shanghai, China). Non-invasive cells on the upper surface of Millicell® chambers were wiped with a cotton swab. Invaded cell number was manually counted in five randomly selected fields under light microscope at 200×.
Luciferase plasmid construction and reporter assay

The pmiR-REPORT™ Luciferase miRNA Expression Reporter Vector (RiboBio, Guangzhou, China) was used to construct the pmiR-3’UTR-wt vector. The primer pairs for wild and mutant 3’UTR amplification were listed in Supplementary Table S2. U87-MG cells seeded in 96-well plates were co-transfected with 20 ng pmiR-3’UTR DNA and 100 pmol miR-663 precursor and control oligomers (Pre-miR Negative Control #1) (Ambion, Austin, TX). The activities of both renilla and firefly luciferases were measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) on a SpectraMAX Gemini XS Microplate Reader Fluorometer (Molecular Devices, Sunnyvale, CA). The results were presented after normalization with the measured values of firefly luciferase.

Tumor implantation

The animal experiments were approved by the Institutional Animal Care and Use Committee of the Southwest Hospital, TMMU according to the Guide for the Care and Use of Laboratory Animals. Each of 6-weeks-old male SCID mice (Laboratory Animal Center, Southwest Hospital, TMMU) was anesthetized and intracranially injected with $1 \times 10^5$ miR-663 overexpressing cells or control cells. Animals were monitored daily for weight change and survival. The brains were collected until the animals became moribund and then sampled for Harris hematoxylin and alcoholiceosin (H&E) staining and immunohistochemistry.

Immunohistochemistry

The FFPE human gliomas and xenografted tumors were sectioned at 4 μm for immunohistochemical staining. The whole process was conducted using Dako
REAL™ EnVision™ Detection System (DAKO, Carpinteria, CA) according to the manufacturer’s instructions. Primary antibodies included anti-PIK3CD (Sigma-Aldrich, St. Louis, MO), anti-Ki67, anti-vimentin, anti-Cyclin D1 (ZSGB-Bio, Beijing, China), anti-IDH1 R132H, anti-GFAP (Maixin-Bio, Fujian, China), anti-p-AKT (Ser473, Novus Bio, Littleton, CO), anti-MMP-2, anti-MMP-7 and anti-MMP-9 (R&D Systems, Minneapolis, MN). Semi-quantitations of PIK3CD, p-AKT, Cyclin D1, MMP-2, MMP-7 and MMP-9 were independently performed by two neuropathologists according to the staining intensity and the percentage of positive tumor cells as previously described (23). Ki67 proliferation index was determined under 10 randomly selected microscopic fields at 400×.

**Statistical analyses**

All statistical analyses were performed using PASW 18.0 software. Kolmogorov-Smirnov test was used to estimate the normality of distributions. Student’s t-test or one-way ANOVA was conducted for normally distributed data. miR-663 expression in different grade glioma specimens was assessed by Bonferroni-corrected Mann-Whitney U-test. Pearson’s chi-square test was used to determine the correlation between miR-663 expression and clinicopathological features of patients. Patients’ survival was analyzed with Kaplan-Meier method, using the log-rank test for comparison. The statistical software X-tile (24) was used to determine the cutoff in the glioma cohort from our groups and TCGA database. The Cox’s proportional hazard model was used for univariate and multivariate survival analyses. The correlation between the levels of miR-663 and PIK3CD was measured using nonparametric (Spearman’s rho) correlation test and curvilinear regression model. Data were presented as the mean ± SD. Statistic significance was assigned at $P$
< 0.05 (*) or \( P < 0.01 (**). \) All experiments were performed at least three times with triplicate samples.
Results

miR-663 is associated with better prognosis in human glioblastoma

To identify the potential miRNAs in glioma progression, miRNA profiling was conducted in primary GBM tissues and the matched adjacent normal tissues (Supplementary Table S3 and S4). Among the differential expressed miRNAs, we found that miR-663 was downregulated in GBM tissues (Supplementary Table S3). To validate the relationship between miR-663 expression and human glioma malignancy, we applied real-time PCR to quantitatively analyze endogenous miR-663 expression in the established human normal glia cell line (HEB), human glioma cell lines (CHG5 and U87-MG) and primary human glioma cells. We found that CHG5 cells from low-grade human glioma expressed relatively higher level of miR-663 than human GBM (grade IV) cells including U87-MG and primary cells (GBM-1, -2 and -3) (Fig. 1A). We then examined miR-663 expression in the specimens from 256 glioma patients, and found that miR-663 expression was significantly reduced in high-grade glioma as compared to normal human brain ($p < 0.01$) and low-grade glioma ($p < 0.01$) (Fig. 1B and Supplementary Tables S5-S7). Furthermore, miR-663 expression in GBM (grade IV) was lower than that in anaplastic glioma (grade III) ($p < 0.01$) (Fig. 1B). Kaplan-Meier analyses showed that the patients with higher level of miR-663 had longer disease-free survival (DFS) ($p < 0.0001$) and overall survival (OS) ($p < 0.0001$) (Fig. 1C). Significantly, we found that GBM patients could be divided into two subgroups with different outcomes based on miR-663 expression, i.e., the higher expression of miR-663, the better prognosis of patients (DFS: $p < 0.0001$; OS: $p < 0.0001$) (Fig. 1D). The prognostic value of miR-663 in GBM was further verified in 483 GBM patients from TCGA database (Supplementary Fig. S1A). Univariate and multivariate analyses revealed that miR-663 was an independent predictor for DFS.
and OS of GBM patients (Supplementary Table S8 and Table 1). These data indicate
an inverse association of miR-663 expression with glioma malignancy, and reveal that
miR-663 may be a potential biomarker for prognosis in GBM patients.

miR-663 inhibits the proliferation and invasion of GBM cells in vitro and in vivo
To examine the role of miR-663 in glioma malignancy, we used GBM cells with
relatively different levels of miR-663 expression to respectively generate miR-663
overexpressing and knock-down cells (Supplementary Fig. S2A and S2B). We found
that overexpressed miR-663 reduced, whereas downregulated miR-663 promoted,
GBM cell proliferation (Fig. 2A and 2B), without any effect on cell apoptosis
(Supplementary Fig. S2C and S2D). Consistent with the in vitro results, xenografted
tumors formed by miR-663 overexpressing GBM cells showed lower proliferation
capacity as indicated by Ki67 labeling index (Fig. 2C). Ectopic expression of
miR-663 abrogated, whereas suppression of miR-663 enhanced, the migratory and
invasive abilities of GBM cells, as measured by cell scratch and invasion assays (Fig.
2D and 2E; Supplementary Fig. S2E-S2H). Histologically, we found the tumors
formed by miR-663 overexpressing cells might be less invasive compared with those
by control ones (Supplementary Fig. S2I), suggesting that miR-663 overexpression
might abrogate the invasive phenotype of GBM cells. Moreover, the mice bearing
miR-663 overexpressing xenografts survived much longer than those bearing control
ones (Fig. 2F). Our results reveal that miR-663 could attenuate the proliferation and
invasion of GBM cells in vitro and in vivo.

miR-663 directly targets PIK3CD and inhibits its signaling
We next performed bioinformatic analysis to identify the possible targets of miR-663,
and PIK3CD was singled out as a potential one (Supplementary Fig. S3A and S3B, Supplementary Table S9). TargetScan prediction revealed that the 3’UTR of PIK3CD harbored a putative miR-663 binding site (Fig. 3A), which was confirmed by luciferase reporter assay (Fig. 3B), indicating PIK3CD as a direct target by miR-663.

Functionally, PIK3CD overexpression (Fig. 3C) partially compromised the inhibitory effects of miR-663 on GBM proliferation (Fig. 3D) and invasion (Fig. 3E and 3F), while suppression of PIK3CD (Supplementary Fig. S3C) had the opposite effects (Supplementary Fig. S3D-S3F). Our results reveal that the inhibitory effect of miR-663 on glioma is at least in part through targeting PIK3CD.

To further determine the underlying mechanisms that miR-663 suppresses GBM, we focused on PIK3CD and its important downstream effectors to investigate whether they were responsible for the suppressed proliferation and invasion induced by miR-663. As shown in Fig. 4, PIK3CD, CCND1, MMP2 and MMP7 (Fig. 4A) together with their protein abundances (Fig. 4B) were significantly reduced in the miR-663 overexpressing cells as compared to control ones, possibly through the reduction of PIK3CD-mediated AKT phosphorylation (Fig. 4B). Consistently, IHC analysis on the specimens from the intracranially xenografted tumors indicated that the abundances of PIK3CD, p-AKT, CCND1, MMP2 and MMP7 proteins were reduced by miR-663 (Fig. 4C and Supplementary Fig. S4A). The results reveal that targeting PIK3CD by miR-663 suppresses the activation of PI3K-AKT pathway and reduces the expression of genes related to proliferation and invasion, thereby inhibiting GBM progression (Supplementary Fig. S4B).

The reduced miR-663 expression correlates with the high level of PIK3CD in...
human GBM

To investigate the correlation between miR-663 and its target PIK3CD, we detected their expressions in primary human GBM. In comparison with the matched normal brain tissues, miR-663 was reduced while PIK3CD mRNA (Fig. 5A) were increased in the tumor tissues from 35 GBM patients, showing a significantly negative correlation (\( r = -0.773, \ p < 0.01 \)) (Fig. 5B). Consistently, IHC analysis (Fig. 5C) on the specimens from 108 GBM patients revealed an inverse association between miR-663 and PIK3CD (\( p < 0.01 \)) (Fig. 5D). Kaplan-Meier analysis demonstrated that the high level of PIK3CD expression predicted a short-term DFS (\( p = 0.0079 \)) (Fig. 5E) and OS (\( p =0.0376 \)) (Fig. 5F) in GBM patients. Univariate analysis revealed that PIK3CD was a potential predictor for DFS and OS of GBM patients (Supplementary Table S8), further confirming an inverse correlation between miR-663 and its target PIK3CD and their functions on the malignant behaviors of human GBM.
Discussion

miR-663, which is expressed in *Homo sapiens* and *Pan troglodytes*, belongs to the primate-specific miRNAs that possibly attribute to the vertebrates evolution, development and carcinogenesis (24-27). The effect of miR-663 in malignant progression is controversial, since it could act as a tumor promoter or suppressor in an organ-specific fashion (13-16). The clinical relevance of miR-663 in glioma remains unknown. In the present study, we selected miR-663 as our target molecule from miRNA profiling and identified miR-663 as a tumor suppressor to inhibit the proliferation and invasion of GBM, representing the first comprehensive analysis of miR-663 in glioma. Mechanistically, we identified *PIK3CD* as a direct and functional target of miR-663, which facilitated our understanding of the mechanisms underlying GBM progression. Most importantly, we found that miR-663 and *PIK3CD*, not only were correlated with each other, but also predicted the survival of GBM patients, highlighting the potential values of miR-663 and *PIK3CD* as novel prognostic biomarkers in human GBM.

Significant endeavors have been made to demonstrate the altered miRNA expressions and their effects on GBM progression and patients’ survival (28). miRNA-based integrated analyses have provided new perspectives for clinical validation of the association between miRNA expression patterns and tumor grades or specific subtypes, highlighting the potential values of miRNAs in diagnosis and subclassification (29-31). Our present data indicated that miR-663 was differently expressed in 17 grade I glioma, 52 grade II glioma, 62 grade III glioma and 125 GBM (grade IV) specimens, suggesting miR-663 was a potential molecular biomarker in distinguishing tumor grades. In GBM, a subgroup of tumors that expressed a
relatively higher level of miR-663 was correlated with better prognosis, suggesting miR-663 was a specific biomarker for the prognostic-based GBM subclassification. Furthermore, miRNAs stably exist in formalin-fixed and paraffin-embedded (FFPE) samples and can be easily detected by real-time PCR, in situ hybridization or microarrays (32, 33). Thus, miR-663 could be a novel and clinical feasible candidate for miRNA-based diagnosis and subclassification for GBM.

GBM is characterized by rapid growth, relentless invasion and redundant microvessels (1). We identified miR-663 as a tumor suppressor to inhibit the proliferation and invasion of GBM cells thereby suppressing the growth and infiltration of GBM tumors, highlighting the therapeutic potential of miR-663 in GBM treatment. Notably, histopathological features of the xenografted tumors by miR-663 overexpression cells were hardly distinguished from those by control ones, which exhibited the pathological characteristics of GBM, including nuclear atypia, cellular pleomorphism, mitotic activity, microvascular proliferation and necrosis (data not shown). However, xenografted tumors with miR-663 overexpression were much lower in proliferation and weaker in invasion, and animals survived longer than control ones, suggesting that miR-663 could be potentially applied in the treatment of GBM.

PIK3CD, which encodes PI3K subunit p110δ, is primarily expressed in the hematopoietic cells and has recently been discovered to regulate the proliferation and invasion of certain cancer cells (34-39). We identified PIK3CD as a direct target of miR-663 by bioinformatics prediction, signal network analysis, luciferase reporter assay, gain-of-function and rescue experiments. Our results suggested the oncogenic
role of \textit{PIK3CD} in GBM malignant proliferative and invasive behaviors, which were consistent with the previous study showing that the organ-specific \textit{PIK3CD}, but not other isoforms of PI3K subunits, promoted the migration and invasion of GBM cells (36). We further demonstrated that miR-663-induced suppression of \textit{PIK3CD} partially inactivated AKT pathway, which enhanced our comprehension of the molecular mechanism of GBM progression (Supplementary Fig. S4B). However, the tumor-suppressive effects of miR-663 could not be fully compromised by \textit{PIK3CD} overexpression. Besides, the prognostic significance of \textit{PIK3CD} was not as important as that of miR-663, suggesting that other target genes might be involved in the modulation induced by miR-663. Given the therapy-resistance feature of GBM and the drug-resistance properties of some predicted targets of miR-663 (14, 40), the involvement of miR-663 in chemoresistance requires further investigation.

Our study showed that miR-663 was down-regulated in GBM as compared with the matched normal brain tissue, which could be illustrated by two possible mechanisms. Recent studies have shown that transcription factors (TFs), such as ZEB family members and p53, may directly bind to the promoter regions of miRNAs or genes that harbor miRNAs (41, 42). By bioinformatic analysis, we have identified several potential TFs that could bind to pre-miR-663 promoter regions (data not shown). Some of them are the predicted miR-663 targets, which may form a feedback loop between miR-663 and these TFs. Another potential mechanism for miR-663 regulation is epigenetic modification, such as histone acetylation or methylation and DNA methylation (43). In fact, reduction of miR-663 by hypermethylation in its promoter region has been identified in MDA-MB-231 breast cancer cells and K-562 leukemia cells (14, 44). Further studies are underway to examine the methylation
status of miR-663 promoter region in different grade glioma specimens and cell lines.

Several prognostic biomarkers, including IDH mutational status and ATRX expression/mutation, have been established in glioma (45, 46). We investigated IDH1 and ATRX status in TCGA database and stratified the GBM patients into two subgroups by these molecular features (Supplementary Fig. S1B and 1C). Our results showed that miR-663 was a potential prognostic biomarker in IDH1WT subgroup (Supplementary Fig. S1D, left) and IDH1MU subgroup (Supplementary Fig. S1D, right), suggesting that the prognostic value of miR-663 might not rely on IDH1 status. Besides, miR-663 could divide ATRXlow subgroup into two subsets with different prognosis (Supplementary Fig. S1E, left), though its role in the ATRXhigh subgroup was not significant (Supplementary Fig. S1E, right). Further perspective studies are warranted.

In summary, our study revealed that miR-663 inhibited the proliferation and invasion of GBM cells in vitro and in vivo by directly targeting PIK3CD, and predicted better prognosis in human GBM. More importantly, miR-663 might be a novel biomarker for molecular subclassification of GBM and a target for treatment of this lethal disease.
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References


Figure Legends

**Fig. 1.** miR-663 expression correlates with glioma grade and the prognoses of patients.

A, miR-663 expression in normal glial cell line (HEB), glioma cell lines (CHG5 and U87-MG) and primary cells (GBM-1, -2, and -3) assessed by qRT-PCR. The relative expression of miR-663 was normalized against U6 expression, which was employed for the comparison between HEB and other cells. The miR-663/U6 ratio in HEB was arbitrarily set to 1.0. B, Expression of miR-663 in the formalin-fixed, paraffin-embedded (FFPE) glioma tissues from 256 patients assessed by qRT-PCR. The transcript level of miR-663 was normalized against U6. The relative expression of miR-663 in specimens was determined as compared to that in adjacent normal tissues. The average expression of miR-663 in adjacent normal tissues was arbitrarily set to 0 with log 10 fold change. All experiments in A and B were performed at least in triplicate and the data are presented as the mean ± SD. **, $P < 0.01$. C and D, Kaplan-Meier analysis of the correlation between miR-663 and DFS (**left**) or OS (**right**) of all grade glioma patients (C) and glioblastoma (GBM) patients (D). Patients were divided into two groups using X-tile analysis as mentioned in Materials and methods.

**Fig. 2.** miR-663 inhibits the proliferation and invasion of GBM cells *in vitro* and *in vivo*. A and B, Growth curve from the miR-663 up-regulated (A) or down-regulated cells (B) assessed by cell proliferation assay. C, Representative images (**left**) and proliferation index (**right**) in tumors indicated by Ki67 staining. Ki67 positive cells were counted microscopically under 10 high powered fields per group, and the results were presented as the mean number of Ki67 positive cells. Scale bar = 25 μm. D and E, Quantification of invaded tumor cells analyzed by cell invasion assay in the
miR-663 up-regulated (D) or down-regulated cells (E). All experiments in A-E were performed at least in triplicate and the data are presented as the mean ± SD. **, P < 0.01. F, Kaplan-Meier analysis of the survival of SCID mice bearing xenografted tumors formed by miR-663 over-expressing cells and control cells.

**Fig. 3.** miR-663 directly targets PIK3CD. A, The predicted miR-663 binding sites in the PIK3CD-3'-UTR region by Targetscan and the designed mutant sequence (PIK3CD-3'-UTR-mutant). B, Luciferase reporter assay of U87-MG cells transfected with pmiR-PIK3CD-3'-UTR, the corresponding mutant reporter, miR-663 precursor oligomer or control oligomer. C, qRT-PCR analysis of PIK3CD overexpressing efficiency in U87-MG cells as indicated. The transcript levels were normalized against GAPDH expression. D, Growth curves from the indicated cells assessed by cell proliferation assay. a, b, c and d denote statistical significance among different groups. a: (miR-663 vs. miR-Ctrl), P < 0.01; b: (PIK3CD vs. miR-Ctrl), P < 0.01; c: (miR-663+PIK3CD vs. miR-Ctrl), P < 0.01; d: (miR-663 vs. miR-663+PIK3CD), P < 0.01. E and F, Numbers (E) and representative images (F) of indicated invaded cells analyzed by cell invasion assay. Scale bar = 50 μm. Experiments in B-E were performed at least in triplicate and the data are presented as the mean ± SD. **, P < 0.01.

**Fig. 4.** miR-663 overexpression suppresses the activation of AKT pathway. A, qRT-PCR analysis of PIK3CD, CCND1, MMP2, MMP7 and MMP9 in U87-MG with miR-663 overexpressing vector or empty vector. B, Western blot analyses of PIK3CD, phosphorylated AKT, AKT, CCND1, MMP2, MMP7 and MMP9 in the cells as indicated. Expressions of indicated targets were normalized against GAPDH. C,
Quantification of IHC staining of PIK3CD, p-AKT, CCND1, MMP2, MMP7 and MMP9 in the xenograft tumors as indicated. The expressions of targets were scored by staining intensity and area as described in Materials and Methods. All experiments were performed at least in triplicate and the data in A and C are presented as the mean ± SD. *, P < 0.05, **, P < 0.01.

Fig. 5. The relationship between miR-663 and PIK3CD in human GBM specimens. A and B, the expressions of miR-663 and PIK3CD (A) and their correlation (B) in the GBM specimens with matched adjacent normal tissues obtained from 35 patients. The transcript levels of miR-663 and PIK3CD were respectively normalized against U6 and GAPDH, which were employed for the comparison between tumor and adjacent brain tissue. The miR-663/U6 ratio and PIK3CD/GAPDH one in adjacent normal tissues were arbitrarily set to 1.0. Experiments were performed at least in triplicate and the data are presented as the mean ± SD. C, Representative IHC staining of PIK3CD in GBM specimens stratified by miR-663 expression. Scale bar = 25 μm. Case 7 and Case 34 in (A) represented miR-663$^{\text{high}}$ group and miR-663$^{\text{low}}$ group respectively. D, The correlation of miR-663 and PIK3CD protein expression was analyzed by Pearson’s chi-square test. GBM specimens were divided into two groups based on miR-663 or PIK3CD expression using X-tile analysis. **, P < 0.01. E and F, Kaplan-Meier analysis indicated the correlation between PIK3CD and DFS (E) or OS (F) of GBM patients. The patients were divided into two groups based on the conditions mentioned in (D).
Shi et al. Figure 4
Table 1. Multivariate analysis for disease-free survival and overall survival in GBM patients.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Disease-free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95%CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Gender</td>
<td>1.463 (0.893-2.397)</td>
<td>0.131</td>
</tr>
<tr>
<td>Age</td>
<td>0.998 (0.981-1.016)</td>
<td>0.839</td>
</tr>
<tr>
<td>Predominant Side</td>
<td>1.027 (0.651-1.621)</td>
<td>0.909</td>
</tr>
<tr>
<td>Predominant Lobe</td>
<td>0.879 (0.707-1.092)</td>
<td>0.245</td>
</tr>
<tr>
<td>KPS Score</td>
<td>0.997 (0.981-1.014)</td>
<td>0.762</td>
</tr>
<tr>
<td>Extent of surgical resection</td>
<td>1.216 (0.757-1.953)</td>
<td>0.419</td>
</tr>
<tr>
<td>Ki67 Index</td>
<td>1.014 (0.998-1.031)</td>
<td>0.091</td>
</tr>
<tr>
<td>IDH1(R132H) Status</td>
<td>0.285 (0.103-0.794)</td>
<td>0.016</td>
</tr>
<tr>
<td>miR-663</td>
<td>0.833 (0.699-0.992)</td>
<td>0.041</td>
</tr>
<tr>
<td>PIK3CD</td>
<td>1.144 (1.028-1.271)</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Abbreviation: HR, Hazards ratio; CI, Confidence interval; KPS, Karnofsky performance status; IDH1, isocitrate dehydrogenase (IDH) type 1.
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Yu Shi, Cong Chen, Xia Zhang, et al.

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