Primate-Specific miR-663 Functions as a Tumor Suppressor by Targeting PIK3CD and Predicts the Prognosis of Human Glioblastoma

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Abstract

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

Experimental Design: Specimens from 256 cases of patients with glioma, 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 glioblastoma 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 glioblastoma specimens.

Results: miR-663 was inversely correlated with glioma grades but positively correlated with patients' survival. Furthermore, two distinct subgroups of patients with glioblastoma with different prognoses were identified on the basis of miR-663 expression in our specimens and that from The Cancer Genome Atlas (TCGA) database. Overexpression of miR-663 significantly suppressed the proliferation and invasion of glioblastoma 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 downregulated by miR-663 overexpression. Moreover, PIK3CD was inversely correlated with miR-663 in glioblastoma specimens and predicted poor prognosis of patients with glioblastoma.

Conclusion: miR-663 is a novel prognostic biomarker and a potential therapeutic candidate for glioblastoma. Clin Cancer Res; 20(7); 1–11. ©2014 AACR.
Translational Relevance

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

as important regulators of cancer biologic behavior (6–10). However, the expression pattern and prognostic significance of miRNAs in glioblastoma 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 biologic processes, including viral infection, inflammatory responses, and autoimmune diseases (11–16). However, its role in tumor progression is contradictory. Although it acts as an onco gene 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 glioblastoma and evaluated its expression and clinical relevance. Its contribution to glioblastoma 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 glioblastoma.

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 4% buffered formaldehyde solution. The formalin-fixed, paraffin-embedded (FFPE) samples were stored at room temperature. Histologic diagnoses were independently made by two neuropathologists according to the World Health Organization (WHO) classification of central nervous system tumors (2007). Normal brain tissues adjacent to tumors were used as controls. The clinicopathologic features of these patients have been 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 The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/tcga; ref. 18) was applied to validate the prognostic value of miR-663 in patients with glioblastoma. The microarray data of all glioblastoma tissue samples with level 3 miRNA (Agilent 8 × 15K Human miRNA-specific microarray) were used and the results were filtered on the basis of miR-663 expression and clinicopathologic 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, China; refs. 19, 20). U87-MG was obtained from the American Type Culture Collection. CHG5 (21) and primary glioblastoma cells were established from the tumor specimens of patients with glioma (Southwest Hospital, TMMU). HEB, U87-MG and primary glioblastoma cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) containing 10% FBS (Gibco). CHG5 was grown in RPMI-1640 (Sigma-Aldrich) 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 was extracted using RNAiso (Takara). RNA from FFPE tissues was isolated with a miRNAeasy FFPE Kit (Qiagen). For measurement of miR-663, Bulge-Loop miRNA qPCR Primer Set (RiboBio), RevertAid First Strand cDNA Synthesis Kit (Fermentas), and SYBR Premix Ex Taq II (Takara) were used. For other genes, PrimeScript RT Master Mix (Takara) and SYBR Premix Ex Taq II were used. Specific primers for amplification have been listed in Supplementary Table S2. All reactions were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad). 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 antibodies used in this study were as follows: anti-PIK3CD (Abnova), anti-phospho-Akt (Ser473), anti-Akt (Cell Signaling Technology), anti-MMP-2, anti-MMP-7, anti-MMP-9 (R&D Systems), anti-Cyclin D1 (Abcam), and anti-GAPDH (Cell Signaling Technology).
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. 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), 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 hours 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 FACS Aria II) to obtain mCherry- or eGFP-positive cells.

Transfection procedures
miR-663 inhibitor and Negative Control #1 (Ambion) were transiently transfected with a final concentration of 100 nmol/L using Lipofectamine 2000 (Invitrogen). At 5 hours posttransfection, the medium was replaced by DMEM with 10% FBS. The whole process was conducted according to the manufacturer’s guidelines.

Tumor cell proliferation assay
Tumor cells (500 cells per well for U87-MG and 800 cells per well for primary glioblastoma cells) were seeded into 96-well plates and cultured for 24, 48, 72, and 96 hours. At the indicated intervals, 20 μL of Cell Counting Kit-8 (Beyotime) were added to each well and incubated for 2 hours at 37°C. Thermo Multiskan Spectrum Reader (Thermo Scientific) was used to measure the absorbance at 490 nm.

Tumor cell invasion assay
Twenty-four–well Millicell chambers with 8.0-μm pore size (Millipore) and BD Matrigel (BD Biosciences) were used in tumor cell invasion assay. Tumor cells were plated in the upper chamber precoated 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 hours of incubation, the Millicell chambers were removed from the plates and fixed in 4% paraformaldehyde, and then stained with Crystal Violet Staining Solution (Beyotime). Noninvasive 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 pmirR-REPORT Luciferase miRNA Expression Reporter Vector (RiboBio) was used to construct the pmirR-3'-UTR vector. The primer pairs for wild and mutant 3’ untranslated region (3‘-UTR) amplification have been listed in Supplementary Table S2. U87-MG cells seeded in 96-well plates were cotransfected with 20 ng pmirR-3'-UTR DNA and 100 pmol miR-663 precursor and control oligomers (Pre-miR Negative Control #1; Ambion). The activities of both renilla and firefly luciferases were measured using the Dual-Luciferase Reporter Assay System (Promega) on a SpectraMax Gemini XS Microplate Reader Flurometer (Molecular Devices). 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 the 6-week-old male severe combined immunodeficient (SCID) mice (Laboratory Animal Center, Southwest Hospital, TMMU) was anesthetized and intracranially injected with 1 × 10^3 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 alccoholic eosin 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) according to the manufacturer’s instructions. Primary antibodies included anti-PIK3CD (Sigma-Aldrich), anti-Ki67, anti-vimentin, anti-Cyclin D1 (ZSGB-BIO), anti-IDH1 R132H, anti-GFAP (MaiXin-Bio), anti-p-AKT (Ser473; Novus Biologicals), anti-MMP-2, anti-MMP-7, and anti-MMP-9 (R&D Systems). Semiquantifications 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. The Kolmogorov–Smirnov test was used to estimate the normality of distributions. The Student t test or one-way ANOVA was conducted for normally distributed data. miR-663 expression in different grade glioma specimens was assessed by the Bonferroni-corrected Mann–Whitney U test. The Pearson x^2 test was used to determine the correlation between miR-663 expression and clinicopathologic 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 (the Spearman ρ) 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 glioblastoma tissues and the matched adjacent normal tissues (Supplementary Tables S3 and S4). Among the differential expressed miRNAs, we found that miR-663 was downregulated in glioblastoma 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 glioblastoma (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 patients with glioma, and found that miR-663 expression was significantly reduced in high-grade glioblastoma (Fig. 1B). Patients were divided into two groups using X-tile analysis as mentioned in Materials and Methods.

![Figure 1. miR-663 expression correlates with glioma grade and the prognoses of patients.](image-url)

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 used 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 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 with that in adjacent normal tissues. The average expression of miR-663 in adjacent normal tissues was arbitrarily set to 0 with log10 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.
miR-663 Favors Glioblastoma Prognosis

Table 1. Multivariate analysis for DFS and OS in patients with glioblastoma

<table>
<thead>
<tr>
<th>Factors</th>
<th>DFS HR (95% CI)</th>
<th>P</th>
<th>OS HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>1.463 (0.893–2.397)</td>
<td>0.131</td>
<td>1.545 (0.953–2.507)</td>
<td>0.078</td>
</tr>
<tr>
<td>Age</td>
<td>0.998 (0.981–1.016)</td>
<td>0.839</td>
<td>0.996 (0.979–1.013)</td>
<td>0.626</td>
</tr>
<tr>
<td>Predominant side</td>
<td>1.027 (0.651–1.621)</td>
<td>0.909</td>
<td>1.271 (0.806–2.005)</td>
<td>0.302</td>
</tr>
<tr>
<td>Predominant lobe</td>
<td>0.879 (0.707–1.092)</td>
<td>0.245</td>
<td>0.874 (0.708–1.079)</td>
<td>0.21</td>
</tr>
<tr>
<td>KPS score</td>
<td>0.997 (0.981–1.014)</td>
<td>0.762</td>
<td>1.010 (0.992–1.027)</td>
<td>0.273</td>
</tr>
<tr>
<td>Extent of surgical resection</td>
<td>1.216 (0.757–1.953)</td>
<td>0.419</td>
<td>1.088 (0.684–1.730)</td>
<td>0.723</td>
</tr>
<tr>
<td>Ki67 index</td>
<td>1.014 (0.989–1.031)</td>
<td>0.091</td>
<td>1.015 (0.999–1.032)</td>
<td>0.068</td>
</tr>
<tr>
<td>IDH1 (R132H) status</td>
<td>0.285 (0.103–0.794)</td>
<td>0.016</td>
<td>0.253 (0.090–0.707)</td>
<td>0.009</td>
</tr>
<tr>
<td>miR-663</td>
<td>0.833 (0.699–0.992)</td>
<td>0.041</td>
<td>0.828 (0.704–0.975)</td>
<td>0.024</td>
</tr>
<tr>
<td>PIK3CD</td>
<td>1.144 (1.028–1.271)</td>
<td>0.013</td>
<td>1.072 (0.970–1.186)</td>
<td>0.174</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; KPS, Karnofsky performance status.

glioma as compared with 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 glioblastoma (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 patients with glioblastoma could be divided into two subgroups with different outcomes based on miR-663 expression, i.e., higher the expression of miR-663, better the prognosis of patients (DFS: P < 0.0001; OS: P < 0.0001; Fig. 1D). The prognostic value of miR-663 in glioblastoma was further verified in 483 patients with glioblastoma from TCGA database (Supplementary Fig. S1A). Univariate and multivariate analyses revealed that miR-663 was an independent predictor for DFS and OS of patients with glioblastoma (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 patients with glioblastoma.

miR-663 inhibits the proliferation and invasion of glioblastoma cells in vitro and in vivo

To examine the role of miR-663 in glioma malignancy, we used glioblastoma cells with relatively different levels of miR-663 expression to respectively generate miR-663 overexpressing and knockdown cells (Supplementary Fig. S2A and S2B). We found that overexpressed miR-663 reduced, whereas downregulated miR-663 promoted, glioblastoma cell proliferation (Fig. 2A and B), without any effect on cell apoptosis (Supplementary Fig. S2C and S2D). Consistent with the in vitro results, xenografted tumors formed by miR-663–overexpressing glioblastoma 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 glioblastoma cells, as measured by cell scratch and invasion assays (Fig. 2D and E and Supplementary Fig. S2E–S2H). Histologically, we found that 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 glioblastoma 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 glioblastoma 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 glioblastoma proliferation (Fig. 3D) and invasion (Fig. 3E and F), 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 glioblastoma, 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 with control ones, possibly through the reduction of PIK3CD-mediated AKT phosphorylation (Fig. 4B). Consistently, immunohistochemistry (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 the PI3K–AKT pathway and reduces the expression of genes...
related to proliferation and invasion, thereby inhibiting glioblastoma progression (Supplementary Fig. S4B).

**The reduced miR-663 expression correlates with the high level of PIK3CD in human glioblastoma**

To investigate the correlation between miR-663 and its target PIK3CD, we detected their expressions in primary human glioblastoma. In comparison with the matched normal brain tissues, miR-663 was reduced, whereas PIK3CD mRNA (Fig. 5A) was increased in the tumor tissues from 35 patients with glioblastoma, showing a significantly negative correlation ($r = -0.773; P < 0.01$; Fig. 5B). Consistently, IHC analysis (Fig. 5C) on the specimens from 108 patients with glioblastoma 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 patients with glioblastoma. Univariate analysis revealed that PIK3CD was a potential predictor for DFS and OS of patients with glioblastoma (Supplementary Table S8), further confirming an inverse correlation between miR-663 and its target PIK3CD and their functions on the malignant behaviors of human glioblastoma.

**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, because 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 glioblastoma, 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 glioblastoma progression. Most importantly, we found that miR-663 and PIK3CD, not only were correlated with each other, but also predicted the survival of patients with glioblastoma, highlighting the potential values of miR-663 and PIK3CD as novel prognostic biomarkers in human glioblastoma.

Significant endeavors have been made to demonstrate the altered miRNA expressions and their effects on glioblastoma 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 glioblastoma (grade IV) specimens, suggesting that miR-663 was a potential molecular biomarker in distinguishing tumor grades. In glioblastoma, a subgroup of tumors that expressed a relatively higher level of miR-663 was correlated with better prognosis, suggesting that miR-663 was a specific biomarker for the prognostic-based glioblastoma subclassification. Furthermore, miRNAs stably exist in 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 glioblastoma.

Glioblastoma is characterized by rapid growth, relentless invasion, and redundant microvessels (1). We identified PIK3CD as a tumor suppressor to inhibit the proliferation and invasion of glioblastoma cells, thereby suppressing the growth and infiltration of glioblastoma tumors, highlighting the therapeutic potential of miR-663 in glioblastoma treatment. Notably, histopathologic features of the xenografted tumors by miR-663–overexpressing cells were hardly distinguished from those by control ones, which exhibited the pathologic characteristics of glioblastoma, 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 glioblastoma.

PIK3CD, which encodes the phosphoinositide 3-kinase (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 PIK3CD in glioblastoma malignant proliferative and invasive behaviors, which were consistent with the previous study showing that the organ-specific PIK3CD, but not other isoforms of PI3K subunits, promoted the migration and invasion of glioblastoma cells (36). We further demonstrated that miR-663–induced suppression of PIK3CD partially inactivated the AKT pathway, which enhanced our comprehension of the molecular mechanism of glioblastoma progression (Supplementary Fig. S4B). However, the tumor-suppressive effects of miR-663 could not be fully compromised by PIK3CD overexpression. Besides, the prognostic significance of 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 glioblastoma 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 downregulated in glioblastoma as compared with the matched normal brain tissue, which could be illustrated by two possible mechanisms. Recent studies have shown that transcription factors, such as ZEB family members and p53, may directly bind to the promoter regions of miRNAs or genes

![Graph A](image1.png)

![Graph B](image2.png)

![Image C](image3.png)

![Image D](image4.png)

![Image E](image5.png)

![Image F](image6.png)

Figure 5. The relationship between miR-663 and PIK3CD in human glioblastoma specimens. A and B, the expressions of miR-663 and PIK3CD (A) and their correlation (B) in the glioblastoma 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 used 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 glioblastoma specimens stratified by miR-663 expression. Scale bar, 25 μm. Case 7 and case 34 in A represented the miR-663high group and the miR-663low group, respectively. D, The correlation of miR-663 and PIK3CD protein expression was analyzed by the Pearson r² test. Glioblastoma 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 patients with glioblastoma. The patients were divided into two groups based on the conditions mentioned in D.
that harbor miRNAs (41, 42). By bioinformatic analysis, we have identified several potential transcription factors 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 transcription factors. 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 isocitrate dehydrogenase (IDH) mutational status and ATRX expression/mutation, have been established in glioma (45, 46). We investigated IDH1 and ATRX status in the TCGA database and stratified the patients with glioblastoma into two subgroups by these molecular features (Supplementary Fig. S1B and C). Our results showed that miR-663 was a potential prognostic biomarker in IDH1WT subgroup (Supplementary Fig. S1D, left) and IDH1MUT 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), although 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 glioblastoma cells in vitro and in vivo by directly targeting PIK3CD, and predicted better prognosis in human glioblastoma. More importantly, miR-663 might be a novel biomarker for molecular subclassification of glioblastoma and a target for treatment of this lethal disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Shi, X.-W. Bian
Development of methodology: Y. Shi, Q. Liu, J.-L. Xu, Y. Ren
Acquisition of data (provided animals, acquired and managed data, provided facilities, etc.): Y. Shi, C. Chen, Q. Liu, J.-L. Xu, H.-R. Zhang, Y. Ren, C. Xu, X.-W. Bian
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Shi, C. Chen, H.-R. Zhang, L. Liu, X.-W. Bian
Writing, review, and/or revision of the manuscript: Y. Shi, X. Zhang, Y.-F. Ping, X.-W. Bian
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Chen, X. Zhang, X.-H. Yao, T. Jiang, Z.-C. He, W. Cui, Y.-H. Cui, S.-Z. Yu, X.-W. Bian
Study supervision: Y.-F. Ping, X.-W. Bian

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