miR-663 Suppresses Oncogenic Function of CXCR4 in Glioblastoma

Yu Shi1, Cong Chen1, Shi-Zhu Yu2, Qing Liu1, Jun Rao1, Hua-Rong Zhang1, Hua-Liang Xiao1,3, Ti-Wei Fu1, Hua Long1, Zhi-Cheng He1, Kai Zhou1, Xiao-Hong Yao1, You-Hong Cui1, Xia Zhang1, Yi-Fang Ping1, and Xiu-Wu Bian1

Abstract

Purpose: To identify the miRNA regulators of C–X–C motif chemokine receptor 4 (CXCR4) and the underlying mechanism as well as the therapeutic and prognostic values in human glioblastoma (GBM).

Experimental Design: miRNA profile analyses and bioinformatics predictions were used to identify the mediators of CXCR4, which were confirmed by luciferase reporter assay, Western blot assay and immunohistochemistry. The effects of miR-663 on CXCR4-mediated GBM malignancy were investigated by gain-of-function experiments. Orthotopic xenografts derived from constitutive or induced miR-663–expressing GBM cells were used to determine the antitumor effects of miR-663 and CXCR4-specific antagonist AMD3100. Bivariate correlation analyses were used to examine the correlation of miR-663 and CXCR4 levels in glioma. The prognostic values of miR-663 and CXCR4 were examined in 281 cases of astrocytic glioma from our hospital and 476 cases of GBM from The Cancer Genome Atlas database using the multivariate Cox regression analysis and Kaplan–Meier analysis.

Results: miR-663 negatively regulated CXCR4 expression by targeting its coding sequence in GBM and compromised the proliferative and invasive capacities of GBM cells induced by CXCR4 overexpression. Constitutive or induced miR-663 overexpression combined with CXCR4 antagonist AMD3100 suppressed orthotopic GBM growth and prolonged tumor-bearing mice survival. Clinically, miR-663 and CXCR4 were inversely correlated in GBM and composed a valuable biomarker set in predicting the outcomes of GBM patients.

Conclusions: miR-663 negatively regulated CXCR4 to inhibit its oncogenic effect. Combination of miR-663 and CXCR4 can serve as a valuable prognostic biomarker set as well as molecular targets for therapeutic intervention of GBM. Clin Cancer Res; 21(17); 4004–13. ©2015 AACR.

Introduction

Glioma is the most common primary tumor in the central nervous system (1). Grade II glioma tends to progress to higher grade malignancies, including grade III anaplastic glioma and grade IV GBM. GBM is the most lethal and highly recurrent glioma. Currently, therapeutic interventions for GBM are insufficient and partially effective due to limited understandings of the genetic and biologic heterogeneities of the tumor. Although a number of alterations in genome and transcriptome of GBM have been identified (2–4), the molecular mechanisms underlying GBM malignancy remain to be elucidated.

C–X–C motif chemokine receptor 4 (CXCR4) and its ligand CXCL12 have been known to contribute to GBM progression (5–7). The oncogenic role of CXCR4 is further emphasized for its contribution in the transdifferentiation of glioma stem cells (8). However, CXCR4 is also expressed by multiple neural cell lineages (such as cerebellar granule cells, microglial cells, astrocytes, and Purkinje cells) and involved in many important biologic processes. Investigation of tumor-specific regulation of CXCR4 and associated targets is crucially important, with potential clinical implications. We therefore attempted to search for novel molecules that regulate CXCR4 at transcriptional and posttranscriptional levels in glioma, including GBM.

MicroRNAs (miRNAs), which modulate gene expression at the posttranscriptional level, provide potential mechanisms of epigenetic regulation in cancer development (9–11). Recent studies emphasized that manipulation of miRNAs and their oligonucleotide antagonists, including miRNA-based nano-drugs, miRNA mimics, miRNA sponge and miR-mask, represents an attractive approach for antitumor therapy (12, 13). In the GBM model, miRNAs could modulate pivotal protein expressions in the signaling cascades, thus, regulate tumor malignant behaviors (14–16). We propose that by overlapping the datasets of predictive miRNAs targeting CXCR4 and the miRNA profiling between GBM tissues and adjacent normal tissues, we could identify the tumor-specific miRNAs regulating CXCR4 expression in GBMs and offer new miRNA candidates for anti-GBM strategies.
Translational Relevance
Glioblastoma (GBM) is the most malignant and lethal primary brain tumor. To understand the mechanisms underlying GBM malignancy is critical for clinical practice. Our present results identify that miR-663 is an important modulator of CXCR4-mediated GBM malignancy and exerts pronounced antitumor effects in combination with CXCR4-specific antagonist AMD3100. Meanwhile, miR-663 and CXCR4 constitute an intriguing prognostic biomarker set for GBM patients. This is the first report that shows the tumor-suppressive regulation of miR-663 on CXCR4 and their combined values in prognostic determination and therapeutic intervention. Thus, our study contributes to the understanding of GBM progression and identifies potential targets for optimized GBM treatment based on the functions of miR-663 and CXCR4.

Herein, we identified miR-663 as a potential regulator of CXCR4 through human GBM miRNA profiling and bioinformatics analyses. The regulatory mechanism, biologic effects, and combined therapeutic effects of miR-663 and CXCR4 were also determined. We further investigated the expression patterns and prognostic values of these two molecules in glioma specimens from 281 patients. Our results demonstrate that miR-663 suppresses CXCR4 in GBM and these two molecules are promising prognostic biomarkers as well as potential therapeutic targets.

Materials and Methods
Patients and tissue specimens
This study was conducted according to the principles of the Helsinki Declaration. All human specimens used in the experiments were approved by the ethics committees of the Third Military Medical University (TMMU), with written consents from the patients or their guardians. Tumor specimens were surgically removed from 281 astrocytic glioma patients who received treatment in Southwest Hospital of TMMU and histopathologically diagnosed by at least two neuropathologists according to the WHO classification (2007). The clinicopathologic information of the patients was presented in Supplementary Table S1. Follow-ups were performed according to patient survival status until January 30, 2014, with a median follow-up time of 26.0 months.

The clinical information and microarray data of 476 GBM patients were obtained from The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/tcga; ref. 17) to investigate the roles of miR-663 and CXCR4 in prognostic determination. The gene-expression data were derived from Agilent 8K Human miRNA-specific Microarray and Affymetrix U133A platform (TCGA Level 3, public data) and filtered by miR-663 and CXCR4 expression as well as the clinicopathologic features of patients.

Cell culture
The human U87-MG GBM cell line from the ATCC was authenticated by short tandem repeat profiling (Microread Genetics) and passaged for less than 6 months according to the manufacturer’s guideline. Primary GBM-1 and primary GBM-2 cells used in this study were established from the GBM tumor tissues from two patients (Southwest Hospital, TMMU, China). The cells were maintained in DMEM (Gibco) with 10% FBS (Gibco) at 37°C with 5% CO2/95% air.

Tumor cell proliferation and invasion assays
Tumor cell proliferation and invasion assays were performed as previously described (10). Cell Counting Kit-8 (Beyotime) was used to examine the proliferative capacity of GBM cells. Millicell chambers with 8.0-μm pore size (Millipore) and BD Matrigel (BD Biosciences) were used to determine the invasive potential of GBM cells.

Real-time qRT-PCR
Real-time qRT-PCR was performed as previously described (10). Specific primer sets for miR-663 and U6 were purchased from RiboBio. Other PCR primers were listed in Supplementary Table S2. The expression of CXCR4 and miR-663 was normalized by GAPDH and U6, respectively.

Western blot
Western blot analyses were conducted as previously described (18). The primary antibodies were as follows: anti-CXCR4 (Abnova) and anti-GAPDH (Cell Signaling Technology).

Lentiviral vectors production
The CXCR4 cDNA was obtained from Origene and cloned into pLOV-CMV-eGFP-EF1a-PuroR lentiviral vector (Neuron Biotech). The human miR-663–overexpressing CMV-mCherry lentiviral vector was generated as previously described (10). To construct the doxycycline-induced miR-663–expressing vector, the mCherry-pre-miR-663 sequence from human miR-663 overexpressing vector was cloned into the pcW-Cas9 vector (Addgene) with the primers listed in Supplementary Table S2.

Flow cytometry
For enrichment of GBM cells stably overexpressing CXCR4 and/or miR-663, eGFP- and/or mCherry-positive cells were sorted. For CXCR4 labeling, mouse anti-human CXCR4 (R&D Systems) was used as primary antibody. Mouse IgG2B (R&D Systems) was used as isotype control. Goat anti-mouse IgG conjugated with DyLight™ 649 (Jackson ImmunoResearch Laboratories, Inc.) was used as secondary antibody. All analyses were conducted by a BD FACSaria II cell sorter (BD Biosciences) and further processed with FlowJo 7.6.1 software (Treestar).

Luciferase plasmid construction and reporter assay
The coding sequences (CDS) of human CXCR4, generated by PCR amplification from U87-MG, were cloned into pmIR-REPORT Luciferase miRNA Expression Reporter Vector (RiboBio). The corresponding mutant plasmids were generated by substituting miR-663–binding sites of the pmIR-CXCR4-CDS-wt plasmids using overlapping PCR. Primer sequences are listed in Supplementary Table S2. Cotransfection of pmIR-CXCR4-CDS plasmids and miR-663 precursor or control oligomers (Ambion) was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Dual-Luciferase Reporter Assay System (Promega) was used to measure the activities of renilla and firefly luciferases as previously described (10), with the values of firefly luciferase for normalization.
miRNA microarray analysis and bioinformatics prediction

Agilent Human miRNA Microarray (8 × 60 K, v19.0) of GBM tumor tissue and the matched adjacent normal tissue was established and presented in Gene Expression Omnibus (GEO accession: GSE61445). Three different miRNA target prediction databases, Targetscan, mirWalk and RNA22, were used to predict miRNA regulators of CXCR4. Results obtained from these databases were overlapped with the downregulated miRNAs in GBM relative to the adjacent normal tissue from miRNA microarray.

Immunohistochemistry

Immunohistochemical staining and analyses were performed to examine CXCR4 expression in human glioma tissues and tumor xenografts. The procedure was carried out based on the manufacturer’s guidelines of Dako REAL EnVision Detection System (DAKO). Primary CXCR4 antibody was obtained from Abnova. The staining was scored as the proportion of positive tumor cells × the staining intensity by two neuropathologists independently. The proportion score was calculated as follows: 0 (<5% positive tumor cells), 1 (5%–25% positive tumor cells), 2 (26%–50% positive tumor cells), 3 (51%–75% positive tumor cells), 4 (>75% positive tumor cells). The staining intensity was graded as previously described (19). The images were captured using DP72 digital camera (Olympus) connected with BX51 microscope (Olympus).

Orthotopic implantation of GBM cells, AMD3100 treatment, and bioluminescence imaging

miR-663–expressing and control cells with pLVx-eGFP-linker-luciferase lentivirus were orthotopically implanted into the brains of 6-week-old male SCID mice (1 × 10⁵ cells/mouse) (Laboratory Animal Center, Southwest Hospital, TMMU, China). Five days after implantation, mice were i.p. treated with AMD3100 (1.25 mg/kg; Sigma) or 0.01 M PBS twice daily until mice became moribund. To establish the miR-663–inducible expression xenograft model, doxycycline-induced miR-663–expressing cells were intracranially injected into mice (1.5 × 10⁵ cells/mouse). Eight days after injection, mice were administrated with AMD3100 (i.p., 1.25 mg/kg) twice daily and/or doxycycline in drinking water (2 mg/ml; Melonepharma) until mice became moribund. Growing xenografts tumors were detected and quantified by bioluminescence imaging using In Vivo Imaging System (IVIS) Spectrum (PerkinElmer) and Living Image Software for IVIS at days 10 and 20 after AMD3100 treatment. The brains of the sacrificed tumor-bearing mice were sampled for IHC and hematoxylin and eosin (H&E) staining. The animal experiments were approved by the Institutional Animal Care and Use Committee of the Southwest Hospital, TMMU in accordance with the Guide for the Care and Use of Laboratory Animals.

Statistical analyses

PASW Statistics 18 was used for all statistical analyses in this study. The unpaired two-group comparison and multiple comparisons were made with the Student t test or one-way ANOVA, respectively. The Pearson’s χ² test was used to determine the relationship between CXCR4 and miR-663 expression in GBM. Bivariate correlation between miR-663 and CXCR4 mRNA expression was assessed by the Pearson correlation coefficient and linear regression model. Patient survival analyses were carried out using the Kaplan–Meier method, with the log-rank test for comparison.

The cutoff point optimization of patient cohort was calculated with X-tile software (20). Briefly, the expression data of miR-663 and CXCR4 in glioma patients and the related patient survival information, including survival time and censor (alive or dead), were loaded into X-tile as tab-delimited text file. The cohort was then divided into two datasets with the optimal cut points generated by running “Kaplan–Meier” program. Univariate and multivariate survival analyses were performed using the Cox’s proportional hazard model. Data were presented as the mean ± SD. P < 0.05 (*) and P < 0.01 (**) were considered statistically significant. All experiments were conducted independently at least three times.

Results

miR-663 regulates CXCR4 expression by targeting its CDS

To investigate the potential miRNA regulators of CXCR4, we performed bioinformatic analyses and overlapped the predicted regulators with the downregulated miRNAs in GBM tumor tissue from miRNA profiles (Fig. 1A). miR-663 was singled out as a potential regulator of CXCR4, with two binding sites in its CDS (Fig. 1B, CXCR4-CDS-1 and CXCR4-CDS-2, respectively). We then constructed the wild-type reporter (CXCR4-CDS-wt) and the corresponding mutant reporters (CXCR4-mut-1, CXCR4-mut-2 and CXCR4-mut-1,-2) to validate the prediction. miR-663 was effective to reduce the luciferase activity of cells expressing CXCR4-CDS-wt or CXCR4-mut-2 but not cells expressing CXCR4-mut-1 or the double mutant (CXCR4-mut-1,-2) in primary GBM cells (Fig. 1C) and U87-MG (Supplementary Fig. S1). The results suggest that CXCR4-CDS-1 but not CXCR4-CDS-2 is the potential-binding site for miR-663. To address the functional significance of miR-663 binding to CXCR4 mRNA, we overexpressed miR-663 in U87-MG cells and primary GBM cells (Fig. 1D) and found that CXCR4 was reduced at both transcriptional and translational levels (Fig. 1E and F). The results were verified by immunohistochemical analysis of CXCR4 in xenograft tumors formed by miR-663–overexpressing GBM cells (Fig. 1G and H). These results indicate that miR-663 directly binds to the CDS of CXCR4 mRNA to suppress its translation.

miR-663 impairs CXCR4-promoted GBM cell proliferation and invasion

As CXCR4 has been shown to be crucial in GBM progression (6, 7), we investigated whether miR-663 could impair CXCR4–mediated GBM malignancy. Ectopic expression of CXCR4 (Fig. 2A–C) promoted the proliferation (Fig. 2D) and invasion (Fig. 2E and F) of GBM cells, whereas overexpression of miR-663 significantly reduced CXCR4 expression (Fig. 2C) and its associated oncogenic activities (Fig. 2D–F). These data reveal that miR-663 significantly inhibits the effects of CXCR4 on the growth and invasion of GBM cells.

miR-663 combined with CXCR4 antagonist suppresses the growth of GBM xenografts

To investigate the anti-GBM effect of miR-663 in combination with the CXCR4 antagonist AMD3100, we established miR-663–overexpressing GBM xenograft tumor models formed by U87-MG cells (Fig. 3A) and primary GBM-1 cells (Fig. 3B) in SCID mice. Bioluminescence imaging showed that the tumor growth was substantially reduced in mice implanted with miR-663–overexpressing GBM cells or treated with AMD3100 (Fig. 3C and D).
Meanwhile, the combination of miR-663 overexpression and AMD3100 exerted an enhanced tumor-suppressive effect (Fig. 3C and D). Moreover, Kaplan–Meier plots showed that miR-663 overexpression and/or AMD3100 treatment significantly prolonged the survival of tumor-bearing mice (Fig. 3E). These results showed that miR-663 overexpression combined with AMD3100 treatment exhibited additive antitumor efficacy. To determine the therapeutic potential of miR-663, we mimicked selective expression of miR-663 in primary GBM-2 cells transfected with miR-663 mimics or controls oligomer, together with CXCR4-CD5 wild-type reporter (CXCR4-CD5-wt) or the indicated mutant ones. D, level of miR-663 in U87-MG cells and primary GBM cells transfected with miR-663 overexpressing vector or control vector as assessed by qRT-PCR. The expression of miR-663 was normalized against U6. E and F, qRT-PCR (E) and Western blot analysis (F) of CXCR4 expression in the GBM cells. The expression of CXCR4 was normalized against GAPDH. G, H&E (top and middle) and CXCR4 IHC staining images (bottom) of xenograft tumors formed by miR-663-overexpressing cells and control cells; scale bar, 2 mm (top) or 25 μm (middle and bottom). H. IHC analyses of CXCR4 expression in miR-663-overexpressing and control xenografts. The expression of CXCR4 was scored according to the proportion of positive tumor cells and the staining intensity. Data, mean ± SD from three independent experiments; *, P < 0.05; **, P < 0.01; ns, not significant.

Meanwhile, the combination of miR-663 overexpression and AMD3100 exerted an enhanced tumor-suppressive effect (Fig. 3C and D). Moreover, Kaplan–Meier plots showed that miR-663 overexpression and/or AMD3100 treatment significantly prolonged the survival of tumor-bearing mice (Fig. 3E). These results showed that miR-663 overexpression combined with AMD3100 treatment exhibited additive antitumor efficacy. To determine the therapeutic potential of miR-663, we mimicked selective expression of miR-663 in primary GBM-2 cells using doxycycline-inducible gene-expressing model (Fig. 3F). We demonstrated that doxycycline treatment increased the expression of miR-663 in GBM cells (Supplementary Fig. S2) and restrained tumor growth in comparison with the control group (Fig. 3G and H). In particular, the tumor-suppressive effect of miR-663 could be further enhanced with AMD3100 treatment (Fig. 3G and H). Taken together, these results indicate that miR-663 is a potential target for GBM treatment.

CXCR4 expression is inversely correlated with miR-663 expression in human astrocytic glioma specimens

On the basis of the opposing functions of miR-663 and CXCR4 in glioma progression in vitro and in xenograft models, we examined their expression patterns in human astrocytic glioma specimens. The expression of miR-663 was reduced in glioma tissues relative to adjacent brain tissues and negatively correlated with tumor grades (Fig. 4A and Supplementary Fig. S3A). In contrast, CXCR4 expression was increased in glioma tissues and was positively associated with tumor grades (Fig. 4B). Bivariate correlation analysis revealed that miR-663 and CXCR4 mRNA were inversely correlated in all grades of glioma (Fig. 4C). We further analyzed the correlation of miR-663 and CXCR4 expression within each tumor grade and found that miR-663 and CXCR4 mRNA levels were negatively correlated in GBM (grade IV; Fig. 4D), but not in grade II and III tumors (Supplementary Fig. S3B and S3C). Consistently, CXCR4 protein level was negatively associated with miR-663 expression in all grades of glioma (Fig. 4E), including GBM (Fig. 4F and G). Taken together, our data demonstrate that CXCR4 and miR-663 are inversely correlated in astrocytic glioma, particularly in GBM.

miR-663 and CXCR4 compose a prognostic biomarker set for GBM

To determine the combined prognostic value of miR-663 and CXCR4, we stratified patients into four subgroups based on the expression of these two molecules in 281 cases of astrocytic glioma specimens from our hospital and 476 cases of GBM
Upregulated miR-663 reduces the proliferation and invasion of GBM cells promoted by CXCR4 overexpression. A and B, validation of increased CXCR4 expression in U87-MG and primary GBM cells transfected with CXCR4-overexpressing vector or empty vector by qRT-PCR (A) and Western blot analysis (B). C, CXCR4 cell proportions in U87-MG (top) and primary-GBM cells (middle and bottom) measured by flow cytometry. D, growth curves of U87-MG (left) and primary GBM cells (middle and right) transfected with miR-663-overexpressing vector and/or CXCR4-overexpressing vector. The statistical significance among groups: miR-663 versus miR-Ctrl, \( P < 0.01 \); CXCR4-OE versus miR-Ctrl, \( P < 0.01 \); miR-663 + CXCR4-OE versus CXCR4-OE, \( P < 0.01 \); miR-663 + CXCR4-OE versus miR-663, \( P < 0.01 \). E and F, representative images (E) and cartogram (F) of indicated invading cells analyzed by cell invasion assay; scale bar, 100 μm. Data, mean ± SD from three independent experiments; **, \( P < 0.01 \).
from the TCGA database. In all grades of glioma, there was a significant difference in the DFS (disease-free survival) and OS (overall survival) among four subgroups (Fig. 5A and B and Supplementary Table S3). We further analyzed patient survival based on miR-663 and CXCR4 expression within the same grade. Although no differences were found in grade II (Supplementary Fig. S4A and S4B) and grade III glioma (Supplementary Fig. S4C and S4D), GBM patients in CXCR4lowmiR-663low subgroup, CXCR4lowmiR-663high subgroup and CXCR4highmiR-663high subgroup showed prolonged survival in comparison with the CXCR4highmiR-663low patient subgroup (Fig. 5C and D and Supplementary Table S4). These results were confirmed using TCGA database (Fig. 5E and F, Supplementary Fig. S4E–S4H and Supplementary Table S5). We further determined
the prognostic value of CXCR4 and miR-663 using univariate and multivariate analyses, because these analyses could provide a non-random assessment of patient survivals by adjusting for variability of clinicopathologic parameters. The results showed that the combination of CXCR4 and miR-663 was an independent predictor of DFS and OS of patients with GBM, in consideration of patient’s clinical status and other pathologic features (Supplementary Table S6 and Table 1). These data

Figure 4.
The inverse association of miR-663 and CXCR4 in human astrocytic glioma specimens. A and B, the expression of miR-663 (A) or CXCR4 (B) in astrocytic glioma samples with different grades assessed by qRT-PCR. The relative expression of miR-663 and CXCR4 in tumor tissues was determined in comparison with that in the adjacent normal tissues. The miR-663/U6 or CXCR4/GAPDH ratio in adjacent brain tissues was assigned as 1.0. The bounds of boxes correspond to 25th to 75th percentile. Whiskers represent 10th to 90th percentile of the sample population. C and D, correlation of miR-663 and CXCR4 mRNA in all grades (C) and grade IV (D) astrocytic glioma specimens. E and F, miR-663 level was inversely associated with CXCR4 protein level in all grades (E) and grade IV (F) astrocytic glioma specimens. G, representative images of H&E and immunohistochemical staining of CXCR4 showing the inverse correlation between miR-663 and CXCR4 in grade IV GBM specimens; scale bar, 25 μm; **, P < 0.01.
suggest that CXCR4 in combination with miR-663 could serve as a valuable prognostic biomarker set for GBM patients.

Discussion

CXCR4, a G-protein–coupled chemokine receptor, has been shown to be overexpressed in gliomas to trigger diverse pathways that promote tumor progression (6–8). The regulatory mechanisms of CXCR4 in tumor cells are crucial but poorly defined (21). In the present study, we identified miR-663 as an important regulator of CXCR4 in GBM cells. Through binding to the CDS of CXCR4 mRNA, miR-663 posttranscriptionally inhibited CXCR4 and suppressed the aggressiveness of GBM cells. Moreover, miR-663 exhibited combined anti-GBM effect with the CXCR4 antagonist AMD3100, suggesting that miR-663 overexpression in combination with AMD3100 treatment may constitute a novel therapeutic approach to GBM.

miRNAs are closely associated with the tumorigenesis and progression of glioma. The expression patterns of miRNAs in GBM patients were stratified by adjusting for their clinical characteristics and pathologic parameters that have already been definitively established in glioma biology.

Table 1. Multivariate analyses of DFS and OS in patients with GBM

<table>
<thead>
<tr>
<th>Factors</th>
<th>DFS</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Gender</td>
<td>0.759 (0.484–1.188)</td>
<td>0.227</td>
</tr>
<tr>
<td>Age</td>
<td>0.999 (0.981–1.017)</td>
<td>0.905</td>
</tr>
<tr>
<td>Predominant side</td>
<td>0.868 (0.563–1.337)</td>
<td>0.520</td>
</tr>
<tr>
<td>Predominant lobe</td>
<td>0.991 (0.789–1.245)</td>
<td>0.941</td>
</tr>
<tr>
<td>KPS score</td>
<td>1.000 (0.985–1.015)</td>
<td>0.997</td>
</tr>
<tr>
<td>Extent of surgical resection</td>
<td>0.805 (0.502–1.292)</td>
<td>0.370</td>
</tr>
<tr>
<td>IDH1 (R132H) status</td>
<td>0.339 (0.154–0.747)</td>
<td>0.007</td>
</tr>
<tr>
<td>Ki67 labeling index</td>
<td>1.003 (0.987–1.020)</td>
<td>0.694</td>
</tr>
<tr>
<td>miR-663 and CXCR4</td>
<td>0.591 (0.477–0.738)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; DFS, disease-free survival; IDH1, isocitrate dehydrogenase type I; KPS, Karnofsky performance status; OS, overall survival. NOTE: Combination of CXCR4 and miR-663 is an independent predictor of DFS and OS of patients with GBM using multivariate analysis. GBM patients were stratified by adjusting for their clinical characteristics and pathologic parameters that have already been definitively established in glioma biology.
tumors have been proposed for diagnostic or prognostic determination and therapeutic intervention (22–24). Because miRNAs regulate multiple genes, the investigation of miRNA–mRNA interactions is a prerequisite to understand their roles in tumor biology (23). miR-663, which was first identified in glioma by our laboratory, could target PIK3CD and inhibit the proliferation and invasion capacities of glioma cells (10). However, its effect on glioma malignancy could not be fully reversed by reconstitution of PIK3CD in tumor cells, suggesting that other genes may also be regulated by miR-663. In this study, we identified CXCR4 as another novel target of miR-663, which enlarged the scope of miRNA regulation in GBM malignancy. We determined the potential application of miR-663 as a therapeutic agent and evaluated its combined antitumor effect with AMD3100, which provide a rationale for the miRNA-based antitumor strategies and could facilitate the combinatorial therapeutic approaches in GBM treatment. Besides, the inverse correlation between miR-663 and CXCR4 in astrocytic glioma could be applied as a novel biomarker set in prognostic determination.

The binding sites of most miRNAs in their target mRNAs are located in noncoding regions (such as 3’UTR). Recently, accumulating evidence has shown that miRNAs could also modulate targets by binding to their CDS regions (25–27). In this study, we found that miR-663 directly bound to the CDS region of CXCR4 mRNA and suppressed its translation, highlighting the importance of CDS in the posttranscriptional regulation by miRNAs. Further analysis demonstrated that the reduction in CXCR4 protein levels was more prominent than its mRNA. These results indicate that miR-663 may effectively inhibit translation of CXCR4 with less effect on its mRNA stability. Thus, we provide new evidence for the highly debated issues whether miRNAs inhibit gene translation, induce mRNA degradation, or both, when the target binding sequences are harbored in the CDS (27).

Conventional treatments of malignant glioma using radiologic and chemotherapeutic approaches have been improved, but remain inadequate to improve patient’s prognosis. Widely observed dysregulation of miRNAs in tumor progression has prompted us to investigate their functions associated with tumor phenotypes and their possible application in tumor treatment. Moreover, miRNA-based clinical trials are undergoing with promising preliminary results (28–30). Our study provides novel insights into this area of GBM research. Through the doxycycline-controlled miRNA-expressing system, we demonstrated that doxycycline administration increased tumor-derived miR-663 expression to suppress tumor growth, indicating that miR-663 is a potential target for malignant glioma and represents an attractive therapeutic approach for preclinical study. Further studies are worthwhile to determine the value of miR-663 in combination with other conventional treatments such as chemotherapy and radiotherapy.

Recently, combination of agents with different mechanisms of action has been reported to generate a combined or additive tumor inhibition effect, with potential clinical applications (31, 32). The CXCR4 antagonist, AMD3100, which has been applied in regulating hematopoietic stem cell mobilization (33, 34), was recently reported to be effective in suppressing tumor progression (35). Our study showed that the combination of miR-663 overexpression and AMD3100 treatment was more effective than any single treatment to inhibit GBM progression. Given the complex regulatory network in GBM progression, there are several possible explanations for the results. First, miR-663 and AMD3100 target CXCR4 through different mechanisms, that is, miR-663 suppresses CXCR4 protein translation while AMD3100 functions as an antagonist of CXCR4 ligand to block CXCR4 pathway activation. Second, miR-663 may also target other oncogenic molecules (such as PIK3CD) and generate combined tumor-suppressive effects. Our findings, together with previous studies showing the synergic effects of AMD3100 with conventional cytotoxic therapies (36), emphasized the significance of combinatorial therapeutic approach in GBM treatment.

In conclusion, our study enlarged the scope of miRNA regulation in GBM malignancy, which may help to enhance our understanding on GBM tumor biology and to facilitate the development of miRNA-based antitumor strategies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Shi, Y.-F. Ping, X.-W. Bian
Development of methodology: Y. Shi, H. Long, Z.-C. He, Y.-F. Ping, X.-W. Bian
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Shi, Q. Liu, J. Rao, H.-R. Zhang, T.-W. Fu, H. Long, Z.-C. He, X.-W. Bian
Writing, review, and/or revision of the manuscript: Y. Shi, C. Chen, S.-Z. Yu, X. Zhang, Y.-F. Ping, X.-W. Bian
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Chen, S.-Z. Yu, H.-R. Zhang, H.-L. Xiao, T.-W. Fu, H. Long, Z.-C. He, Y.-H. Cui, X.-W. Bian
Study supervision: Y.-F. Ping, X.-W. Bian

Acknowledgments
The authors thank Prof. Ji Ming Wang of Center for Cancer Research, National Cancer Institute at Frederick, for reviewing this article.

Grant Support
This research was supported by the National Natural Science Foundation of China (NSFC, nos. 81230062, 81370071, 61327902, and 81402050) and Programs of Science and Technology Commission Foundation of Tianjin Municipal (12ZCDZNY17400).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 30, 2014; revised May 1, 2015; accepted May 12, 2015; published OnlineFirst May 28, 2015.


miR-663 Suppresses Oncogenic Function of CXCR4 in Glioblastoma

Yu Shi, Cong Chen, Shi-Zhu Yu, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-2807

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/05/30/1078-0432.CCR-14-2807.DC1

Cited articles
This article cites 36 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/21/17/4004.full.html#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.