VHL regulates the effects of miR-23b on glioma survival and invasion via suppression of HIF-1α/VEGF and β-catenin/Tcf-4 signaling

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MicroRNAs (miRNAs) are noncoding RNA molecules composed of approximately 22 nucleotides that regulate the expression of a wide variety of genes through direct interaction with the 3′-untranslated regions of their mRNA. It has been estimated that miRNAs regulate up to one-third of human genes at the posttranscriptional level, suggesting that miRNAs have pivotal roles in physiological and pathological processes. The number of verified miRNAs is increasing rapidly, and the latest version of miRBase has annotated >1500 miRNA sequences in the human genome. The specific roles of most identified miRNA in normal physiology and pathological processes remain to be elucidated. Increasing evidence indicates that the deregulation of miRNAs is involved in a wide range of human cancers, thereby providing new insights into the molecular mechanisms underlying carcinogenesis.

Glioblastoma multiforme (GBM), the highest grade glioma, is the most common malignant primary brain tumor in adults. Despite aggressive treatments, the median survival for patients who receive a diagnosis of GBM has only marginally increased over the past 25 years and remains at approximately 1 year. Although previous studies found that many proteins, including β-catenin, EGFR, K-ras, and Akt, are involved in
the development and progression of glioma, the roles and potential mechanisms of miRNA in glioma development and progression are still largely unknown. Among those miRNAs that have been investigated for the roles in glioma, miR-21, miR-221, and miR-10b have been the best characterized to date.

Here, we demonstrated increased expression of miR-23b in high-grade glioma, compared with low-grade glioma and normal brain tissue. Down-regulation of miR-23b in glioma resulted in enhanced expression of VHL and reduced cell proliferation and invasive activity. Furthermore, we identified that restored expression of VHL counteracted the effects of miR-23b. We also identified that miR-23b functions, at least in part, by regulating β-catenin/Tcf-4 and HIF-1α/VEGF pathway.

Materials and Methods

Tissue Samples and Clinical Data

Informed consent was obtained for the use of human glioma tissue samples from adult patients who received a diagnosis at the General Hospital of Tianjin Medical University. Samples included 12 World Health Organization (WHO) grade I-II tumors, 15 WHO grade III tumors, and 16 WHO grade IV tumors (Table 1). Freshly resected tissue samples were immediately frozen in liquid nitrogen for subsequent total RNA extraction. Five normal adult brain tissue specimens were obtained with informed consent from patients undergoing posttrauma surgery for severe traumatic brain injury (TBI). Fifty paraffin-embedded GBM specimens with clinical data were collected from Tiantan Hospital from January 2006 through June 2006, including 14 grade I-II tumors, 18 grade III tumors, and 18 grade IV tumors (Table 2). This study was approved by the hospital institutional review board, and written informed consent was obtained from all patients. Patients were followed up using clinical and laboratory monitoring on a regular basis starting at definitive diagnosis. Disease-specific survival time was defined as the time from definitive diagnosis to disease-specific death.

Cell Culture and Transfection

Human U87, LN229, SNB19, and LN308 GBM cells and 1 low-grade glioma cell line H4 were obtained from the China Academy Sinica Cell Repository, Shanghai, China. The cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and were incubated at 37°C in a 5% CO2 atmosphere. Cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Plasmids, Oligonucleotides, and Transfection

HA-tagged wild-type VHL in pCDNA3 was provided by Dr. J. Q. Cheng (Molecular Oncology, H. Lee Moffitt Cancer Center). 2’-OMe-oligonucleotides were chemically synthesized and purified using high-performance

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liquid chromatography (GenePharma). The sequences were as follows: 2′-OMe-As-miR-23b (As-miR-23b): 5′-GGUAAUCCCUGGCAAUGUGAU-3′; miR-23b mimics: sense, 5′-AUCACAUUGCCAGGGAUUACC-3′; antisense: 5′-UAAUCCCUGGCAAUGUGAUUU-3′; MiRNA scrambled 5′-UUGUACUACACAAAAGUAUACUG-3′. Cells were transfected with As-miR-23b or miR-23b mimics (200 pmol each) using Lipofectamine 2000 (Invitrogen). Cells transfected with scrambled 2′-OMe oligonucleotides (scramble) were used as control.

Locked Nucleic Acid (LNA)-based In Situ Hybridization for miRNA
To study the spatial and temporal expression of miRNAs with high sensitivity and resolution, miRNA fluorescein in situ hybridization (FISH) was performed according to manufacturer’s protocol.15

Real-Time Polymerase Chain Reaction (PCR)
MiRNA expression was analyzed using the Hairpin-itTM miRNA qPCR Quantitation Kit (GenePharma) and DNA Engine Opticon 2 Two-Color Real-time PCR Detection System (Bio-Rad), according to the manufacturer’s instructions. Cycle threshold (CT value) was acquired using the software provided by the manufacturer. The real-time PCR data were analyzed using the 2^−ΔΔCt method.

Colony Formation Assay
Cells were seeded in 6-well plates (0.5 × 10^3 cells per well) and cultured for 2 weeks. Colonies were fixed with methanol for 10 min and stained with 1% crystal violet (Sigma) for 1 min. Each group was measured in triplicate.

Cell-Cycle Analysis
For cell cycle analysis by flow cytometry, transfected and control cells in the log phase of growth were harvested by trypsinization, washed with PBS, fixed with 75% ethanol overnight at 4°C, and then incubated with RNase at 37°C for 30 min. Nuclei of cells were then stained with propidium iodide for 30 min. Nuclei were examined in a FACS Calibur Flow Cytometer (Becton Dickinson), and DNA histograms were analyzed using Modifit software (Becton Dickinson). Experiments were performed in triplicate.

Apoptosis Assays
Apoptosis was quantified 48 h after transfection, using annexin V labeling and caspase 3/7 activity. For the annexin V assay, an annexin V-FITC–labeled Apoptosis Detection Kit (Abcam) was used according to the manufacturer’s protocol. Caspase 3/7 activity was measured using Caspase-Glo 3/7 reagent (Promega).

In Vitro Invasion Assays
Transwell membranes coated with Matrigel (BD Biosciences) were used to quantify in vitro glioma cell invasion. Transfected cells were plated at 5 × 10^4 per well in the upper chamber in serum-free medium. Medium containing 20% FBS was added to the lower chamber. After 24 h incubation, noninvading cells were removed from the top well with a cotton swab, and bottom cells were fixed with 3% paraformaldehyde, stained with 0.1% crystal violet, and photographed in 3 independent 10× fields for each well. Fold migration was calculated relative to blank control. Data represents mean ± standard error (SE) of 3 independent experiments.

Luciferase Reporter Assay
To evaluate the β-catenin/Tcf-4 transcriptional activity, we used the TOP-FLASH and FOP-FLASH (Upstate) luciferase reporter constructs. TOP-FLASH (with 3 repeats of the Tcf-binding site) or FOP-FLASH (with 3 repeats of a mutated Tcf-binding site) plasmids were transfected into cells treated with miR-23b inhibitor or VHL pcDNA3. In addition, the pGL3-WT-VHL-3′ UTR reporter was created by the ligation of VHL 3′ UTR PCR products into the XbaI site of the pGL3 control vector (Promega). The pGL3-MUT-VHL-3′ UTR-Luc reporter was generated from pGL3-WT-VHL-3′ UTR-Luc by replace the binding site of miR-23b with restriction enzyme cutting site CGGATCCG. For the reporter assay, cells were cultured in 96-well plates and transfected with pGL3-WT-VHL-3′ UTR-Luc or pGL3-MUT-VHL-3′ UTR-Luc, and As-miR-23b. After 48 h incubation, luciferase activity was measured using a dual-luciferase reporter system (Promega). Luciferase activity was measured 48 h after transfection with the Dual-luciferase reporter assay system. The Renilla luciferase activity was used as an internal control.

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Table 2. Clinicopathologic characteristics of patients with high and low expression of VHL from Tiantan Hospital

Chen et al.: Function of miR-23b in human glioma
VEGF Enzyme-Linked Immunosorbent Assay (ELISA)

Twenty-four hours after transfection, cell culture media were collected and quantified for VEGF levels using a VEGF-specific ELISA kit (R&D Systems), according to the manufacturer’s protocol.

Western Blot, Immunohistochemistry (IHC), and Immunofluorescence

Western blot, IHC, and immunofluorescence assays were performed as previously described.12 Immunoblot and IHC assays were performed using antibodies against VHL, β-catenin, HIF-1α, VEGF (1:1000 dilution; Abcam), α-tubulin, fibrillarin, and GAPDH (1:1000 dilution; Santa Cruz). Immunofluorescence assay was performed using antibody against β-catenin (1:1000 dilution; Abcam). IHC scores were performed using a semiquantitative 5-category grading system.16

Nude Mouse Tumor Intracranial Model and As-miR-23b Treatment

LN229 cells that were cotransduced with miR-23b inhibitor/scramble oligonucleotide and luciferase lentivirus were injected into the intracranial of 5-week-old BALB/c-nu mice. After 20 days, tumors were measured by fluorescent images of whole mice using an IVIS Lumina Imaging System (Xenogen) and portions of the tumor tissues were used for quantitative real-time PCR (qRT-PCR) for miR-23b expression and Western blot assay for VHL. Cryosections (4 mm) were stained with hematoxylin and eosin and used for IHC and FISH.

Statistical Analysis

Statistics were performed using the SPSS Graduate Pack, version 11.0, statistical software (SPSS). Descriptive statistics, including mean and SE and 1-way analysis of variance, were used to determine statistically significant differences. Overall survival curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparison. \( P < .05 \) was considered to be statistically significant.

Results

**MiR-23b is Overexpressed in Glioma Samples and Cell Lines**

To investigate whether miR-23b was up-regulated in human gliomas and glioma cell lines as we previously reported, we chose 48 clinical samples. qRT-PCR established that miR-23b was highly expressed in high-grade glioma, compared with low-grade glioma and normal brain tissues (Fig. 1A). Furthermore, qRT-PCR was used to measure miR-23b levels in 5 glioma cell lines (U87, LN229, U251, SNB19, and LN308) and 1 low-grade glioma cell line (H4) (Fig. 1B). Consistent with our previous findings,12 miR-23b overexpression was observed in 5 glioma cells, at levels 7.48–16.04-fold higher than the levels observed in a control H4 cell line.

**Downregulation of miR-23b Inhibits the Tumorigenic Properties of Glioma Cells**

U87 and LN229 cell lines were selected to investigate the function of miR-23b in glioma cell biology. Using sequence-specific antisense knockdown of miR-23b (As-miR-23b), real-time PCR indicated that 89% knockdown of miR-23b was achieved in U87 cells and 79% was achieved in LN229 cells (Fig. 2A; \( P < .05 \)). As-miR-23b decreased proliferation of U87 cells and induced cell arrested in G0/G1 phase (Fig. 2B and C). Similar inhibition of cell proliferation and cell cycle effects were observed in LN229 cells when treated with As-miR-23b. In vitro transwell assays revealed that As-miR-23b treatment reduced the number of invasive U87 and LN229 cells, compared with scramble-treated controls (Fig. 2D). In addition, Annexin V assay revealed that knockdown of miR-23b for 24 h increased U87 cell apoptosis by 16% and LN229 cell apoptosis by 18%, compared with scramble-treated controls (Fig. 2E). Caspase3/7 activity assay confirmed the similar results (Fig. 2F). Together, these data show that the tumorigenic properties of glioma cells, specifically proliferation, migration, and apoptosis, are influenced by miR-23b.

![Fig. 1. Altered expression of miR-23b in glioma and glioma cell lines. (A) MiR-23b expression in glioma and normal brain tissues by qRT-PCR. (B) qRT-PCR analysis shows that LN229, U87 and U251 glioma cells express higher levels of miR-23b, compared with H4 cell line. Data represent mean ± standard deviation of 3 replicates. *\( P < .05 \).](image-url)
VHL is a Direct Target of miR-23b

MiRNA targets search using TargetScan identified that the seed sequence of miR-23b matched the 3′ UTR of the VHL gene (Fig. 3A). To determine whether VHL expression is indeed regulated by miR-23b, we knocked down miR-23b in U87 and LN229 cells and examined VHL expression levels. Western blot analysis showed that VHL expression was upregulated in U87 and LN229 cells after miR-23b knockdown. Similarly, ectopic expression of miR-23b in SNB19 and LN308 cells that contain relatively low expression of miR-23b resulted in down-regulation of VHL (Fig. 3B). To confirm whether VHL was the direct target of miR-23b, we constructed pGL3-WT-VHL-3′UTR and pGL3-MUT-VHL-3′UTR reporter plasmids. Reporter assay revealed that reduced expression of miR-23b triggered a marked increase of pGL3-WT-VHL-3′UTR luciferase activity, whereas up-regulation of miR-23b expression decreased luciferase activity. In contrast, no change of luciferase activity was observed using the mutant pGL3-MUT-VHL-3′UTR-reported plasmid (Fig. 3C and D). These data indicate that miR-23b directly modulates VHL expression by binding to the 3′ UTR.

Restored Expression of VHL Counteracts miR-23b Overexpression

Because VHL is a direct target of miR-23b, we next examined the role of VHL in miR-23b-mediated cell biology. U87 and LN229 cells, which express high level of miR-23b intrinsically, were transfected with a 3′UTR absent plasmid encoding VHL. Both real-time PCR and Western blot assay verified the increased expression of VHL in these cells (Fig. 4A and B). Proliferation, transwell, cell cycle, and apoptosis assay revealed that ectopic expression of VHL counteracted the effects of miR-23b overexpression on cell survival and invasion (Fig. 4C–G). Similarly, we observed that transfection with a 3′UTR absent plasmid encoding VHL counteracted the effects of ectopic expression of miR-23b mimics in SNB19 cells (Fig. 4H–J).
These data indicate that VHL is a critical target of miR-23b involved in multiple cell biology events.

**MiR-23b Abrogation Suppresses β-catenin/Tcf-4 Transcriptional Activity and HIF/VEGF Pathway**

To investigate further downstream effectors of miR-23b, Top/Fop flash luciferase assay was used in cells in which miR-23b was deleted or VHL expression was restored. In U87 and LN229 glioma cells, miR-23b depletion or ectopic expression of VHL reduced Top with no change in Fop flash luciferase activities (Fig. 5A). In addition, Western blot and immunofluorescence assay showed that depletion of miR-23b or ectopic expression of VHL decreased the expression of β-catenin in both the cytoplasm and the nucleus (Fig. 5B and C). Taken together, these data suggest that miR-23b regulates β-catenin/Tcf-4 transcriptional activities via VHL in glioma cells.

Because U87 and LN229 cells displayed reduced invasive capacity after miR-23b depletion, we investigated whether VEGF levels are impacted by miR-23b expression. Indeed, knockdown of miR-23b or ectopic expression of VHL resulted in reduction of VEGF both stimulated by recombinant HIF-1α or not (Fig. 5D). Western blot assay validated that both HIF-1α and VEGF were down-regulated when transfected with As-miR-23b or VHL, suggesting that HIF-1α/VEGF indeed could be a downstream effector of miR-23b (Fig. 5E).

Confirming the essential role of the β-catenin/Tcf-4 and HIF-1α/VEGF pathway as the mediators of miR-23b, qRT-PCR was used. We demonstrated reduced relative expressions of VEGF, MMP2, MMP9, c-Myc, Fra-1, cyclinD1, and STAT3 in cells when miR-23b was inhibited (Fig. 5F). These data suggest critical downstream roles for β-catenin/Tcf-4 and HIF-1α/VEGF pathways in miR-23b–mediated cell biology.

**As-miR-23b Inhibits Tumor Growth In Vivo**

Because downregulation of miR-23b expression inhibits the invasion and proliferation of glioma cells in vitro, we further assessed its effect on tumor growth in vivo. When the mice were intracranially transplanted with LN229 cells that stably express luciferase and miR-23b inhibitors or scramble oligonucleotide, bioluminescence imaging was done for the whole body. As-miR-23b-treated LN229 cells displayed a marked reduction of the tumor (Fig. 6A). FISH and qRT-PCR analysis confirmed that As-miR-23b reduced miR-23b expression in tumor, compared with scramble controls (Fig. 6B and C). Moreover, As-miR-23b treated LN229 tumor cells displayed increased expression of VHL and reduced expression of HIF-1α and β-catenin, as verified by IHC and Western blot assay (Fig. 6B–D). These data indicate
that knockdown of miR-23b expression in vivo functions similarly to that in vitro.

Decreased VHL Expression Correlates with Poor Survival in Human Glioma

To determine whether the inverse relationship between miR-23b and VHL expression was consistent in vitro and in patient samples, we quantified expression levels of VHL and miR-23b in glioma tissue specimens by IHC and FISH assay. High-grade glioma contained comparatively lower VHL expression and higher miR-23b than did low-grade specimen (Fig. 7A). Spearman’s correlation analysis demonstrated that VHL in tumor tissues inversely correlated with miR-23b expression (Fig. 7B). These data suggested that decreased VHL expression might result from miR-23b overexpression in human glioma. Retrospective analysis of the clinical outcome associated with each tissue specimen revealed that reduced immune detection of VHL correlated with poor survival (Fig. 7C). In summary, these data indicate that miR-23b regulation of VHL expression has significant clinical impact on glioma.

Discussion

Previously, we discovered that miR-23b expression is increased in malignant glioma cells, compared with astrocytoma or normal brain tissue. Here, we further confirmed these findings in both primary glioma tissue...
specimen and glioma cell lines. Thus far, there have been no reports investigating the role of miR-23b in glioma. Miska et al., using microarray technology, observed that miR-23b expression is increased during brain development. Because an established correlation exists between deregulated brain development and gliomagenesis, these data support our hypothesis that miR-23b has a significant role in glioma tumorigenesis.

Furthermore, in the present study, we demonstrate that down-regulation of miR-23b impairs glioma cell growth and invasive capacity and induces glioma cell apoptosis. We identify that VHL is a direct and functional target of miR-23b and that miR-23b-mediated down-regulation of VHL results in the oncogenic effects of miR-23b.

GBMs are highly vascularized brain tumors, making them attractive targets for anti-angiogenic therapies. Bevacizumab, a humanized anti-VEGF antibody, has been showing promising results in exploratory phase II trials of recurrent GBM. However, a direct antitumor effect of bevacizumab has remained elusive, whereas tumor invasion may even increase with drug resistance. Enhanced cell invasion has similarly been observed after anti-angiogenic treatment in other tumor models.
In one such report, Keunen et al. demonstrated that bevacizumab significantly decreases intratumoral blood flow and volume; however, an inverse relationship was observed on tumor cell invasion and proliferation. Of note, their results identified that the PI3K/Akt and Wnt signaling pathways were up-regulated after VEGF blockade, suggesting a possible cause for the observed increase in GBM invasion and proliferation. In contrast, we demonstrate that down-regulation of miR-23b not only suppresses the proliferation and invasion of glioma, but also decreases VEGF expression. Furthermore, additional components of the PI3K/Akt and the Wnt signaling pathways, such as EGFR, AKT2, c-Myc, Fra-1, cyclinD1, and STAT3, also display reduced expression after miR-23b deletion. These data may have significant clinical implications, whether knockdown of miR-23b acts as a stand-alone therapeutic method for GBM or as combination therapy with bevacizumab.

We report that VHL is highly expressed in normal brain tissue and astrocytes, whereas its expression is reduced in high-grade glioma. VHL has been described as a tumor suppressor by virtue of its ability to induce the ubiquitination and proteasomal destruction of HIF-1α. In accordance with these findings, our results demonstrate that deletion of miR-23b, or overexpression of VHL, resulted in reduced expression of HIF-1α. Reduced HIF-1α in turn inhibited the expression of VEGF. Because VEGF is an important molecule in neovasculogenesis of tumor endothelium, any change of VEGF expression would be expected to have a significant impact on tumor growth.

In addition, VHL might inhibit tumor growth through other mechanisms. Recently, Chitalia et al. demonstrated that VHL acts through the E3 ubiquitin ligase Jade-1 to target β-catenin for proteasomal degradation. The implication of this finding is that deregulated VHL provides growth stimulatory signals by activating oncogenic β-catenin signaling, while increasing the ability of the tumor to cope with limited oxygen supply by activating HIFs. Conversely, our data indicate that

Fig. 6. Knockdown of miR-23b expression impairs LN229 tumor growth in vivo. (A) Luminescence imaging for As-miR-23b-treated LN229 tumors vs. scramble-treated controls. (B) Representative photomicrographs of tumor sections following FISH analysis for miR-23b and IH analysis for VHL. (C) miR-23b and VHL expression in intracranial graft assessed by real-time PCR and Western blot (D) β-catenin and HIF-1α expression in tumor sections following IH analysis. *P < .05.
knockdown of miR-23b or, alternatively, restored expression of VHL represses the transcriptional activity of β-catenin/Tcf-4. Expression of multiple downstream targets of the β-catenin/Tcf-4 and HIF-1α/VEGF pathway, such as VEGF, STAT3, cyclinD1, c-myc, MMP2, and MMP9, are all reduced in cells in which miR-23b is knocked down or VHL is restored. As such, these findings support the conclusion that miR-23b regulates survival of glioma cells by modulating VHL suppression of the HIF-1α/VEGF pathway and β-catenin/Tcf-4 transcription.

In summary, we demonstrate that down-regulation of miR-23b inhibits survival and invasion of glioma by repressing expression of VHL. Genetic restoration of VHL counteracted the effects of miR-23b overexpression, resulting in the suppression of both HIF/VEGF and β-catenin/Tcf-4 pathways (Fig. 7D). On the basis of these observations, we propose that anti-angiogenic therapy for glioma could benefit from adjuvant miRNA therapy targeting the HIF1α and β-catenin/Tcf-4 pathways.

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