

MicroRNA-588 regulates the invasive, migratory and vasculogenic mimicry-forming abilities of hypoxic glioma cells by targeting ROB01

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Abstract

Background

Glioma is an intracranial malignancy that is the most challenging tumor to treat. The hypoxic microenvironment is an important factor contributing to tumor development, while the migration, invasion and vasculogenic mimicry (VM) of glioma cells are the main causes of the increasing treatment difficulty.

Materials and methods

A microarray was leveraged to identify differentially expressed microRNAs in U251 glioma cells cultured under normoxic and hypoxic conditions. The expression of miR-588 was assessed using quantitative real-time PCR (qRT-PCR). Gain- and loss-of-function studies were used to evaluate the role of miR-588 under hypoxic and normoxic conditions. Cell invasive, migratory, proliferation, as well as VM formation experiments were performed. The relationship between miR-588 and ROBO1 was confirmed using western blot and luciferase reporter assays. Intracranial xenograft tumor mouse models were applied to study the function of miR-588 in vivo.

Results

The expression of miR-588 was obviously upregulated in hypoxic glioma cells in comparison with normoxic glioma cells. miR-588 inhibited the invasive, migratory and VM-forming abilities of glioma cells in vitro and in vivo. Mechanistically, *Roundabout guidance receptor 1 (ROBO1)* is a direct, functionally relevant target of miR-588 in glioma. ROBO1 knockdown suppressed the expression of matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9), thereby inhibiting the invasive, migratory and VM-forming abilities of glioma.

Conclusions

MiR-588 regulated the malignant behaviors of hypoxic glioma cells by targeting ROBO1. miR-588 can be used as a prognostic marker for glioma and has potential implications in glioma gene therapy.

Introduction

Glioma is the most common malignancy tumor in the brain, accounting for 40–50% of all intracranial tumors[1]. The incidence rate of glioma is 3–8/100,000 persons. The biological features of glioma mainly consist of a high fatality rate, high recurrence rate [2], strong invasive ability[3], strong angiogenic ability,[4] and extensive hypoxic state[5].

Solid tumors, such as breast cancer[6], liver cancer[7] and glioma, grow rapidly and correspondingly have an inadequate blood supply. A hypoxic microenvironment is prevalent in these tumors. Tumor hypoxia is directly related to a short survival[8, 9]. To date, numerous studies have confirmed that hypoxia is related to tumor progression, including proliferation[10], invasion[11], migration and angiogenesis[12]. In

addition, glioma has an abundant blood supply. The prospective application of anti-angiogenic drugs to treat glioma has attracted increasing attention[13]. However, according to recent studies, the clinical application of anti-vascular therapies is unsatisfactory due to its short-lived curative effect and poor efficacy[14]. In 1999, the concept of vasculogenic mimicry (VM) was proposed[15, 16]. Other blood supplies exist in tumor tissues in addition to the blood vessels composed of endothelial cells. VM refers to channels that are functionally similar to the normal blood vessels and formed by tumor cells under specific conditions by mimicking the vascular endothelial cells[17, 18]. These channels are connected to the tumor microcirculation, forming a vascular network to provide nutritional support to the tumors. VM occurs in various malignant tumors, including glioma[19]. Moreover, the positive rate of VM is closely implicated in the tumor grade as well as poor prognosis. The hypoxic microenvironment is closely related to VM[20–22].

Despite multiple mechanisms have been proposed to explain hypoxia-induced tumor cell invasion and migration, many researchers are still investigating the roles of microRNAs (miRs) in the tumor hypoxia effect. MiRs are a series of endogenous, single-stranded noncoding RNAs with a length of 20–22 nucleotides. MiRs negatively regulate gene expression at the post-transcriptional level[23, 24]. Previous studies focusing on miRs have revealed key roles in the malignant progression of tumors, such as proliferation, apoptosis, motility and angiogenesis[25–29]. Additionally, miR-588 has been reported to play a tumor suppressive role in lung cancer, breast cancer and other types of cancers [30–32]. In prostate cancer, miR-588 promotes tumor cell proliferation and functions as an oncogene[33]. However, its effects in the development and progression of glioma remain unclear. Our study identified and revealed miR-588 as a novel tumor suppressor and a valuable prognostic marker in glioma. Based on the results from our in vitro experiments, miR-588 is capable of inhibiting invasion, migration and VM in glioma. Animal experiments revealed that miR-588 reduces the invasive and migratory capabilities of glioma cells by targeting hypoxia-induced roundabout guidance receptor 1 (ROBO1) expression. Thus, miR-588 exerts a tumor-suppressing effect on glioma. Our results emphasize the significance of miRs in tumor progression and identify the molecular mechanisms of action of miRs in tumor progression.

Materials And Methods

Cell lines and tissue samples

The human glioma cell lines U251, U87MG as well as A172 were acquired from the Chinese Academy of Sciences Cell Bank. The human glioma sections used in this study were verified in accordance with the World Health Organization tumor classification. Our study was approved by the Institutional Review Board of Shandong University and hospital ethical committee. This study was conducted on the basis of written informed consent obtained from all patients.

Cells culture

Glioma cells were cultured using DMEM medium (Gibco, USA) containing 10% fetal bovine serum (FBS). All cell lines were incubated at 37°C, 5% CO₂ and 5% O₂ for normoxic research. For research under hypoxic conditions, cell culture was performed in modular culture chambers flushed with a gas mixture containing 1% oxygen, 5% carbon dioxide and 94% nitrogen.

Transfection

A mature miR-588 mimic and inhibitor were conceived and synthesized by RiboBio (Guangzhou, China). Transfection was performed with the assistance of lipofectamine 3000 according to the manufacturer's protocol and scrambled oligos were used as negative controls. qRT-PCR was applied to detect the efficiency of transfection and subsequent assays were performed after 48 hours of incubation of glioma cells.

CCK-8 assay

We seeded U251, A172 and U87MG cells in 96-well plates at a density of 2×10^3 cells per well. Cell propagation was assessed at 24, 48, 72, and 96 hours post-transfection using Cell Counting Kit-8 following manufacturer's protocol. The optical density values were recorded and analyzed at 450 nm.

Wound-healing assay

We seeded about 1×10^5 cells in 6-well plates overnight for cells adhesion. Then we performed cell transfection and when cells confluency reached 90 percent, a sterile pipette tip was used to scratched the cell monolayer. Representative images were obtained at 0 h, 12 h, 24 h and 36 h along the scrape lines and the relative scratch widths were recorded for subsequent analysis.

Cell migration and invasion assays

Transwell chambers (8- μ m pore size, Corning) were leveraged to conduct cell migration and invasion assays. In brief, about 5×10^4 transfected cells were seeded in the upper chamber of FBS-free medium. Chambers with or without Matrigel (BD) were used for invasion and migration assays, respectively. The lower chamber was added with medium containing 20% FBS and the numbers of migrating or invading cells were recorded randomly in five views using a microscope.

VM formation assay

Matrigel Basement Membrane Matrix (0.1 ml/well, BD Bioscience) was used for coating the 24-well culture plate. The glioma cells were seeded on Matrigel at a density of 2×10^5 cells/ml and cultured with Serum-free medium for 6 hours. The representative images were collected using an inverted microscope.

Human miRCURY™ LNA array analysis

U87MG cells were cultured under hypoxic or normoxic conditions and total RNA was extracted for microarray analysis using the TRIzol assay. The NanoDrop 1000 was used to evaluate the RNA quantities and miRCURY™ Hy3™/ Hy5™ Power Labeling Kit and hybridized to a miRCURY™ LNA Array (v.18.0) were used to label the RNA samples. The Axon GenePix 4000B microarray scanner was applied for the array scanning and the images were imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Finally, the significant differentially expressed miRNAs among different groups were explored and validated.

Luciferase reporter assay

TargetScan 5.2, miRDB and miRbase were leveraged to predict the potential targets of miR-588. The Reporter constructs containing pGL3-ROBO1 and pGL3-mutROBO1 were designed and constructed by Bio-Asia (Jinan, China). Cells were transfected as described previously, and 48 hours later luciferase assays were performed with a luciferase assay kit according to the manufacturer's protocol.

Real-time quantitative PCR

The TRIzol Reagent was leveraged for RNA extraction in accordance with manufacturer's instruction. Then the extracted RNA was used to perform reverse transcription using a ReverTra Ace qPCR RT Kit. SYBR Premix Ex Taq™ Kit and the primers shown in Figure S1 were applied for real-time PCR. Finally, the absolute expression of RNA was analyzed and calculated by a Roche LightCycler® 2.0 system.

Western blotting

RIPA buffer containing 1% phenylmethylsulfonyl fluoride was used for protein extraction and then loaded onto SDS-polyacrylamide gel for protein separation. The blots were incubated with primary antibodies against ROBO1 (1:1000, Abcam) and others. Then the blots were incubated with secondary antibody at room temperature for 1 h and ECL detection system was used for protein visualization.

Immunohistochemistry staining

4% formaldehyde was used for section fixation, and 1 mM EDTA (pH 8.0) was applied for antigen repair. The sections were incubated with goat serum for 2 hours at room temperature, followed by overnight incubation with primary antibody (Abcam: 1:250 ROBO-1) at 4°C. The sections were then incubated with the secondary antibody and then reacted with diaminobenzidine and counterstained with Mayer hematoxylin.

In vivo experiments

We performed in vivo experiments on mice based on the approval of Institutional Animal Care and Use Committee of Qilu Hospital of Shandong University. Four-week-old male BALB/c nude mice were purchased from SLAC Laboratory Animal Center (Shanghai, China), and mice in similar conditions were randomly divided into different groups in preparation for the establishment of intracranial glioma

xenograft models. Mice were anesthetized with 1% pentobarbital sodium (85.7 mg/Kg) and their heads were fixed on a positioner. U87MG luciferase cells (5×10^5) transfected with lenti-control, lenti-miR-588 or lenti-sh-miR-588 virus were extracted and injected into the skull. The above procedures were performed under aseptic surgical conditions. Tumor growth was monitored by intraperitoneal injection of 150 mg/kg of fluorescein followed by bioluminescence with an IVIS Lumina Series III (PerkinElmer). We euthanized mice when they developed severe neurological symptoms or turned moribund. We strictly implemented the "3R principles of animal experimentation" in our animal experiments.

Statistical analysis

All experiments were conducted three times. SPSS 17.0 and GraphPad Prism software were leveraged for statistical analyses and experimental graphs. Mann-Whitney test was used for comparisons of two groups, while non-parametric Kruskal-Wallis test was used for multiple comparisons. Kaplan-Meier curves and log-rank test were used to visualize and assess survival information. One-way ANOVA and Spearman's correlation test were leveraged to analyze the significant differences, and a $*p < 0.05$, $**p < 0.01$ were regarded as statistically significant.

Results

Experimental analysis of the differential expression of miRs in glioma cell lines cultured under hypoxic and normoxic conditions.

Hypoxia is an important prognostic contributor for short-term survival in glioma patients[34, 35]. High-throughput whole-genome miR screening was conducted in U251 glioma cells cultured under hypoxic and normoxic conditions using miR microarray chips to investigate hypoxia-induced differences in miR expression. The expression of numerous miRs was significantly upregulated under hypoxic conditions (Fig. 1A). The microarray analysis revealed 84 significantly differentially expressed miRs. Among the 84 miRs, miR-210, which showed the greatest upregulation, has been confirmed to be a hypoxia marker. MiR-588 was one of the ten most upregulated miRs. In the present study, 8 miRs that were highly upregulated under hypoxic conditions (including miR-1207 and miR-597) were examined, among which miR-588 significantly inhibited glioma invasion and migration. In addition, we used a volcano plot to analyze the microarray data and confirmed that miR-588 was significantly upregulated under hypoxic conditions (Fig. 1B). We then examined the transfection efficiencies of NC, miR-588 mimics and an miR-588 inhibitor using RT-qPCR. The expression of miR-588 was not affected in cells transfected with NC. In contrast, transfection of the mimics significantly increased the expression of miR-588, whereas transfection of the inhibitor markedly inhibited the expression of miR-588. Subsequently, RT-qPCR was employed again to examine the expression of miR-588 in U251 and U87MG cells cultured under the normoxic condition and different durations of hypoxia. The expression of miR-588 was obviously upregulated under hypoxic conditions compared to normoxic conditions (Fig. 1D). Moreover, miR-588 expression peaked in glioma cells cultured under hypoxic conditions for approximately 24 h.

NC, miR-588 mimics and the miR-588 inhibitor were used in the present study. NC was the negative control RNA. Transfection of mimics leads to overexpression of relevant RNAs. MiR inhibitors were synthetic single-stranded RNA molecules that inhibited the functions of the corresponding miRs through irreversible binding[36, 37]. In the present study, a miR inhibitor was used to block miR-588 function. U87MG cells were stably transfected with lentiviral vectors expressing NC, miR-588 mimics and the miR-588 inhibitor. The Cell Counting Kit-8 (CCK8) assay was conducted to observe the effects of these molecules on cell viability. No statistical differences in viability emerged between glioma cells transfected with NC, miR-588 mimics and miR-588 inhibitors. Thus, stably transfection with 80 nM mimics and inhibitors significantly regulated endogenous miR-588 expression without affecting cell viability.

The expression of miR-588 is implicated in invasive, migratory and VM-forming abilities of hypoxic glioma cells.

The glioma cell lines U87MG, U251 as well as A172 were stably transfected with NC, miR-588 mimics and miR-588 inhibitors in the current study to examine whether the upregulation or downregulation of miR-588 regulated the hypoxia-induced migration, invasion as well as VM formation of glioma cells. Scratch assays were performed using the A172 and U251 cell lines to examine the effects of miR-588 on glioma cell migration (Fig. 2A, 2B). Several interesting phenomena were observed in the present study. First, cell migration was obviously increased under hypoxic culture conditions. This result was consistent with our expectation. Second, miR-588 obviously suppressed the migration of glioma cells and substantially antagonized the hypoxia-induced migration. The miR-588 knock down glioma cells displayed a significantly increase in migration, and hypoxic culture further promoted cell migration. Both the level of miR-588 expression and the oxygen level in the incubator regulated the migration of glioma cells. Moreover, miR-588 and oxygen exerted mutual regulatory effects.

Transwell assays were performed using the U87MG and A172 cell lines to further evaluate the effect of miR-588 on the migration and invasion of glioma cells (Fig. 2C-F). U87MG cells displayed significantly increased migration through the polycarbonate membrane compared to A172 cells. After repeated experiments, U87MG cells were fixed and stained after 6 h of cultivation in the Transwell chambers, whereas A172 cells were fixed and stained after 20 h of cultivation. Nevertheless, similar results were obtained using the two cell lines. In comparison with control group, miR-588 significantly suppressed the ability of glioma cells to migrate through the membrane, whereas the miR-588 inhibitor markedly enhanced the migratory capability in glioma cells. In addition, hypoxia also significantly promoted the transmembrane migration of glioma cells. Subsequently, we examined the invasion in glioma cells using chambers coated with Matrigel and obtained results similar to the migration assays. Thus, miR-588 significantly suppressed the invasion and migration in glioma cells and simultaneously antagonized the promotion of invasion and migration by hypoxia. Knockdown of miR-588 significantly enhanced the invasion and migration in glioma cells.

U87MG and A172 cell lines were cultured in Matrigel to examine the development of VM[20, 38]. During the experiment, glioma cells first aggregated and extended protrusions, which were then connected to

form tubular structures (i.e., VM). The tubular structures collapsed after a certain period. Based on the results, hypoxia accelerated the rate of VM formation and increased the number of tubular structures formed. In comparison with control group, overexpression of miR-588 obviously inhibited the rate of VM formation and decreased the number of tubular structures. In addition, overexpression of miR-588 antagonized the hypoxia-induced VM formation. In contrast, the miR-588 knockdown cells displayed a remarkable increase in VM formation (Fig. 2G, 2H). VM formation was consistent with invasion and migration of the cells. Moreover, miR-588 significantly inhibited the VM-forming ability of glioma cells.

ROBO1 is a key downstream gene in the mechanism by which miR-588 inhibited the invasion, migration and VM-forming abilities of glioma cells.

The key downstream target genes of miR-588 must be identified to clarify the mechanisms by which miR-588 regulates the invasion, migration and VM-forming abilities of glioma cells. We searched for genes that were downstream of miR-588 and associated with invasion and migration of glioma cells using TargetScan, miRDB and miRBase databases. The candidate genes were verified using RT-qPCR. The ROBO1 gene was identified. The three prime untranslated region (3'-UTR) of the ROBO1 gene contains target sites that directly bind miR-588 (Fig. 3A). ROBO1 is a conserved transmembrane receptor protein that is primarily expressed in the nervous system. An analysis of The Cancer Genome Atlas (TCGA) database demonstrated that ROBO1 expression was obviously elevated in glioma tissue in comparison with normal brain, and patients with glioma expressing a high level of ROBO1 experienced significantly shorter survival (Fig. 3C, 3D). Based on these results, ROBO1 promotes the development of gliomas. After transfection with miR-588 mimics and inhibitors for 48 hours, proteins were extracted from glioma cells and Western blot analysis was performed to examine the inhibitory effect of miR-588 at the protein level. Overexpression of miR-588 reduced ROBO1 levels, whereas knockdown of miR-588 increased the levels of the ROBO1 protein (Fig. 3H). Therefore, miR-588 regulated ROBO1 expression and negatively correlated with ROBO1 expression. Luciferase reporter assay was performed for the validation of association between miR-588 and ROBO1. In comparison with control group, luciferase activity was reduced to approximately 61.3% in cells transfected with miR-588, suggesting that the expression of ROBO1 was directly modulated by miR-588 in glioma cells (Fig. 3B).

Western blots also showed that miR-588 overexpression downregulated the expression of matrix metalloproteinase-2 (MMP2) as well as matrix metalloproteinase-9 (MMP9) in U87MG and A172 cell lines (Fig. 3F, 3G). MMP2 is positively correlated with the invasion and migration of glioma cells, while MMP9 is implicated in cell invasion, migration and VM formation. Therefore, the western blot results partially illustrated the mechanisms by which miR-588 inhibited the invasion, migration and VM-forming abilities of glioma cells. No change in the viability of cells stably transfected with the lentiviruses was detected using the CCK8 assay. The stably transfected U87MG cells were then transfected with a small interfering RNA (siRNA) targeting ROBO1. Following siRNA transfection, cellular proteins were extracted. ROBO1 expression was obviously suppressed in cells transfected with the ROBO1 siRNA in comparison with control group, demonstrating that the efficiency of siRNA-mediated knockdown of ROBO1 expression was satisfactory. In addition, the western blot analysis revealed a simultaneous decrease in MMP2 and MMP9

levels after ROBO1 knockdown. The results demonstrated that reduction of ROBO1 expression inhibited the expression of MMP2 and MMP9, thereby affecting the invasive, migratory and VM-forming abilities of glioma cells. The results partially demonstrated the mechanisms by which miR-588 regulated the invasion, migration and VM formation of gliomas.

The invasive, migratory and VM-forming abilities of glioma cells were decreased after ROBO1 knockdown.

U87MG glioma cells stably transfected with NC, miR-588 mimics or the miR-588 inhibitor were further transfected with the ROBO1 siRNA to verify the effects of ROBO1 on glioma cell invasion, migration and VM. After transfection, the cells were subjected to scratch, Transwell and VM assays. Cells displaying normal ROBO1 expression served as the control group. In comparison with control group, the migration of glioma cells in the scratch assay was significantly reduced after ROBO1 knockdown. Cells stably transfected with miR-588 mimics displayed the lowest migration after ROBO1 knockdown. Cells stably transfected with the miR-588 inhibitor in which ROBO1 expression was knocked down exhibited similar migration to NC-transfected cells, which was obviously suppressed in comparison with the control group without normal ROBO1 expression (Fig. 4A).

The similar findings were observed in the subsequent Transwell assay (Fig. 4B, 4C). Normally, knockdown of ROBO1 expression significantly suppressed the migration of glioma cells. In addition, the results from the invasion assay were in accordance with the findings of migration assay. ROBO1 knockdown also inhibited the invasion of glioma cells. Based on these results, ROBO1 significantly increased the invasion and migration of glioma cells, while ROBO1 knockdown enhanced the ability of miR-588 to inhibit glioma cell invasion and migration. ROBO1 served as a downstream target of miR-588. Moreover, miR-588 exerted its cancer-suppressing effect by inhibiting ROBO1 expression.

A VM formation assay was conducted using glioma cells transfected with the ROBO1 siRNA to clarify the effect of ROBO1 in development of VM in glioma cells. Compared with control group, ROBO1 knockdown cells exhibited a delay in VM formation. In addition, knockdown of ROBO1 expression markedly reduced the number of tubular structures formed (Fig. 4D). Thus, the VM-forming capacity of glioma cells was markedly reduced by ROBO1 knockdown. We concluded that ROBO1 also plays a vital function in the formation of VM structures of glioma cells. The above results in accordance with the findings of invasion and migration assays.

ROBO1 expression in glioma tissues positively correlated with VM formation.

Normal brain tissue specimens and specimens of various grades of glioma tissues (WHO grades II-IV) were collected. Subsequently, ROBO1 immunohistochemistry, CD31 immunohistochemistry and periodic acid–Schiff (PAS) staining were performed to examine the differences in ROBO1 expression and VM formation among various grades of glioma tissues and to determine the potential correlation between ROBO1 expression and VM formation. ROBO1 expression gradually increased as the glioma grade

increased (Fig. 5A). At the same time, VM formation increased. Therefore, a positive correlation was identified between ROBO1 expression and VM formation.

Mir-588 Counteracts The Hypoxia-induced Pro-invasive Effects In Vivo

To analyze the impact of miR-588 on hypoxia-induced invasion in vivo, we transplanted lentiviral vector-transfected U87MG cells into the brain of nude mice. We detected and recorded intracranial tumor size by bioluminescence imaging. The results demonstrated that miR-588 knockdown tumors were significantly more aggressive, while miR-588 overexpression strongly reduced the extent of gliomas (Fig. 5B, 5C). Hematoxylin and eosin (HE) staining suggested the extremely aggressive status in association with marked increase in tumor growth of the miR-588 knock down xenografted glioma relative to the NC group (Fig. 5D). Overexpression of miR-588 leads to remarkable inhibition of glioma cell invasion and proliferation. IHC staining demonstrated the negative correlation between the miR-588 and ROBO1 expression. The result revealed that ROBO1 was barely detectable in normal brain, but was potently upregulated in the xenograft tumors with miR-588 knock down (Fig. 5E). Meanwhile, we discovered that MMP2 and MMP9 levels increased in the miR-588 knock down tumors. Therefore, miR-588 deficiency definitely facilitated overall invasive ability of glioma cells in the mouse model. The above results are compatible with the unfavorable prognosis of clinical survival in glioma patients exhibiting low miR-588 levels.

Discussion

Glioma is the most challenging intracranial malignancy, as it is the most common and most difficult tumor to cure[1]. The explanations for the high degree of malignancy of glioma include the hypoxic microenvironment, highly invasive and migratory abilities, abundant blood vessels, VM formation and chemoradiotherapy resistance, which have also been the focus of research in recent years[2–5, 39–41].

The culture of glioma cells under hypoxic conditions leads to specific biological responses, comprising the activation of signaling pathways responsible for regulating propagation, angiogenesis, metastasis and apoptosis[34, 38, 42]. The hypoxic tumor environment is related to a poor prognosis, radiotherapy resistance and chemotherapy resistance[12, 43]. In surgically resected glioma tissues, living cells surround the central necrotic tissues and migrate outward, indicating that hypoxia might regulate this process[35, 44]. According to the results from in vivo and in vitro assays, tumor hypoxia enhances invasion and migration of glioma cells[45]. However, the specific mechanisms remain unclear. A variety of miRs have recently been shown to participate in this process. In the field of glioma research, miR-based treatments have been explored for approximately 10 years and are expected to make a major breakthrough[23, 46]. In recent years, several miRs that are expressed at high levels in glioma (such as miR10b, 21, 210 and 221/222) are markers of a poor prognosis[47–50]. In addition, a number of tumor suppressor miRs have been identified, including miR663, miR128 and etc.[51, 52] In the present study,

miR microarray technology was employed to compare miR expression between glioma cells cultured under normoxic and hypoxic conditions. A number of miRs whose expression was upregulated under hypoxic conditions were identified and considered related to the hypoxic microenvironment of glioma. These miRs were further screened using *in vitro* assays. Notably, miR-588 inhibited the invasion and migration of glioma cells. In some studies, miR-588 functioned as a tumor suppressor in lung cancer, gastric cancer and breast cancer. The mechanisms by which miR-588 suppresses cancer growth include the inhibition of invasion, migration, and angiogenesis and the induction of apoptosis[30–32]. In prostate cancer, miR-588 promotes tumor cell proliferation and functions as an oncogene[33]. The effect of miR-588 in glioma has not been reported previously. Therefore, we decided to conduct an in-depth study of miR-588.

Glioma is a highly vascularized brain tumor. However, the current anti-vascular therapies display a short-lived curative effect and poor efficacy in clinical practice[53, 54]. With the proposition of the concept of VM in 1999, researchers recognized that other blood supplies exist in tumor tissues in addition to the blood vessels composed of endothelial cells[15, 16]. VM refers to channels that are functionally similar to the normal blood vessels formed by tumor cells under specific conditions by mimicking the vascular endothelial cells[55]. These channels are connected to the tumor microcirculation, forming a vascular network to provide nutritional support to the tumors. VM has the following characteristics: (1) the tubular structures are composed of tumor cells rather than endothelial cells and are connected to the tumor microcirculation system, (2) extracellular matrix (ECM) remodeling, and (3) positive PAS staining and negative CD31 staining[21, 56, 57]. VM occurs in a variety of malignant tumors, including glioma, and the positive rate of VM is closely related to the tumor grade and a poor prognosis for patients[20]. Patients with high levels of VM are more prone to tumor metastasis and have a lower survival rate. Therefore, a therapeutic strategy targeting VM may be used as promising anti-tumor vascular therapy. VM formation occurs mainly through glioma stem cells and transforming growth factor beta (TGF β), vascular endothelial growth factor receptor 2 (VEGFR-2)/fetal liver kinase 1 (FLK-1), vascular endothelial cadherin (VE-cadherin), receptor tyrosine kinase (RTK)/phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), MMP-laminin5 γ 2 chain pathways[56, 58]. Both the epithelial-mesenchymal transition (EMT) and hypoxic microenvironment act as a key player in VM formation. In the present study, miR-588 was significantly upregulated under hypoxic conditions, and overexpression of miR-588 suppressed the invasion and migration of glioma cells. Based on these findings, miR-588 is related to VM formation in glioma. A VM formation assay was conducted in the present study using glioma cells and the findings demonstrated that miR-588 inhibited the VM formation in glioma cells. Thus, as a tumor suppressor, miR-588 represents a promising prognostic biomarker for malignant glioma. Therefore, miR-588 is worthy of further investigation.

Few reports have investigated the pathways involved in the mechanisms by which miR-588 suppresses tumor growth. In the recent studies, we attempted to elucidate the implication in miR-588 and hypoxia. Based on findings of our previous study, we proposed a hypothesis that miR-588 antagonized the hypoxia-induced malignant progression of glioma and inhibited the invasion, migration and VM-forming abilities of tumors. Preliminary experimental results showed that the motility and VM-forming ability were

markedly reduced in cells transfected with miR-588 mimics. Knockdown of miR-588 yielded the opposite result. Moreover, the impacts of miR-588 were amplified under hypoxic conditions. The expression of miR-588 was upregulated in response to hypoxia and exerted a cancer-suppressing effect. Therefore, we postulate that miR-588 is associated with the protective compensatory mechanism and plays a key role in suppressing the hypoxia-induced invasion, migration and VM formation of glioma cells. We screened for downstream target genes of miR-588 using three target gene prediction websites[59–61]. The motility-related gene ROBO1 was identified as a potential oncogene target, which was experimentally verified. ROBO1 is a conserved transmembrane receptor protein. It is mainly expressed in nervous system and is implicated in the transmission of biological information[62, 63]. The roles of ROBO1 in tumor development and progression remain unclear. ROBO1 has been reported to function as both an oncogene and a tumor suppressor[64, 65]. An analysis of the TCGA database revealed a significant increase in ROBO1 expression in gliomas compared to normal brain tissues[66]. Moreover, the survival of patients with glioma who expressed ROBO1 at high levels was significantly shortened. The luciferase reporter assay determined a direct target interaction between miR-588 and ROBO1. Western blot analyses indicated that miR-588 simultaneously decreased the expression of ROBO1, MMP2 and MMP9 (markers of invasion, migration as well as VM). Further cell-based experiments revealed a decrease in the invasion, migration and VM-forming abilities of glioma cells after ROBO1 knockdown. In addition, ROBO1 knockdown inhibited the ability of the miR-588 inhibitor to promote invasion and migration. We propose the mechanisms of action of miR-588 listed below. In a hypoxic environment, miR-588 binds directly to the 3'-UTR to downregulate ROBO1 expression. MMP2 and MMP9 levels are also reduced. Consequently, the invasion, migration and VM-forming abilities of the cells are inhibited. Notably, miR-588 is a hypoxia-induced tumor suppressor that is capable of antagonizing the invasion-promoting effect of hypoxia, consistent with our clinical observations. Other molecules regulating ROBO1 expression and target molecules of miR-588 may also play roles in this complex process, requiring further investigation.

Conclusions

In summary, this study described a microRNA that targeted ROBO1 to inhibit the invasion, migration and VM formation in glioma, namely miR-588. We propose that miR-588 represents a prognostic biomarker for glioma and a promising therapeutic target for the treatment of glioma. A limitation of the present study is the relatively small sample size. Besides, the engagement of other important proteins related to invasion, migration and VM formation was not investigated in the present study, including TGF β , Ras-related C3 botulinum toxin substrate 1 (Rac1), cell division cycle 42 (Cdc42) and N-cadherin[21, 56, 67, 68]. Therefore, additional studies are needed to confirm our findings.

Declarations

Data availability

All the data obtained and/or analyzed during the current study were available from the corresponding authors on reasonable request.

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Contributions

GL and HX supervised the project. RY designed the research and performed all experiment. RY and XPS completed the basic experiment part. RY and ZPZ performed statistical analysis. RY, SBW, XG, ZZS, and RRZ helped to revise the manuscript. All authors read and approved the final manuscript.

Ethics declarations

We confirm that the research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All experimental protocols were approved by the Ethics Committee of the Qilu Hospital (Jinan, China) and performed in accordance with the relevant guidelines and regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shandong University (Jinan, China).

Conflict of interest

The authors disclose no potential conflicts of interest.

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Supplementary Figure

Supplementary Figure S1 is not available with this version

Figures

Figure 1

miRNA array analysis of differentially expressed miRNAs in hypoxic glioma cell lines and miR-588 expression levels in human glioma tumors and cell lines. **(A)** miRCURY™ LNA expression array revealed 84 significantly differentially expressed miRNAs (partial data in figure) between normoxic and hypoxic U251 cells. The hypoxic miRNA marker miR-210 and the target miRNA miR-588 are indicated. **(B)** Volcano plots were constructed to screen the differentially expressed miRNAs between the two conditions according to the fold changes and *p*-values. The vertical lines correspond to 2.0-fold changes (either up or down), and the horizontal line represents a *p*-value of 0.05. The red dots in the plot represent the significantly differentially expressed genes, including miR-588. **(C)** miR-588 was among the top ten modulated genes in hypoxic glioma cells, and it was up-regulated (according to the fold change). **(D)** The expression levels of miR-588 in hypoxic U251 and U87MG cells (hypoxia treatment for 0, 12, 24, and 48 h) were assessed by quantitative real-time PCR. ***p* < 0.01, **p* < 0.05 by Kruskal-Wallis one-way ANOVA. **(E)** Cell viability assay of U251 cells transfected with NC, the miR-588 mimics and inhibitor.

Figure 2

The overexpression of miR-588 inhibited and miR-588 knockdown enhanced the invasive, migratory and VM-forming abilities of hypoxic glioma cells. **(A)** and **(I)** Wound-healing assay of U251 cells transfected with miR-588 mimics and inhibitor under normoxia and hypoxia. **(B)** and **(J)** Wound-healing assay of A172 cells transfected with miR-588 mimics and inhibitor under normoxia and hypoxia. **(C)** and **(K)** The regulating effect of the miR-588 mimics and inhibitor on U87MG cell migration under normoxia and hypoxia was examined by Transwell migration assays. **(D)** and **(L)** The regulating effect of the miR-588 mimics and inhibitor on A172 cell migration under normoxia and hypoxia was examined by Transwell migration assays. **(E)** and **(M)** The regulating effect of the miR-588 mimics and inhibitor on U87MG cell invasion under normoxia and hypoxia was examined by Matrigel invasion assays. **(F)** and **(N)** The regulating effect of the miR-588 mimics and inhibitor on A172 cell invasion under normoxia and hypoxia was examined by Matrigel invasion assays. **(G)** and **(O)** The regulating effect of the miR-588 mimics and inhibitor on U87MG cell VM-forming ability under normoxia and hypoxia was examined by VM formation assays. **(H)** and **(P)** The regulating effect of the miR-588 mimics and inhibitor on A172 cell VM-forming ability under normoxia and hypoxia was examined by VM formation assays. At 48h after transfection, a cell suspension was added to a Matrigel-coated well of 96-well tissue culture plate, then take pictures every 2h. ***p* < 0.01, **p* < 0.05 by Kruskal-Wallis one-way ANOVA.

Figure 3

ROBO1 is a key downstream gene of miR-588. **(A)** Sequence of the miR-588 binding site in ROBO1. The miR-588 binding site in the ROBO1 3'-UTR was predicted using Targetscan. **(B)** Results of 3'-UTR luciferase assay. A 3'-UTR vector and miR-588 or miRNA negative control were co-transfected into

U87MG cells. The luciferase activity levels were compared with those of the miRNA negative control-transfected cells, which were normalized to 1. **(C)** Patients with glioma expressing a high level of ROBO1 experienced significantly shorter survival. **(D)** ROBO1 expression was significantly increased in gliomas compared with normal brain tissues. **(E)** Cell viability assay of U87MG cells transfected with NC and ROBO1 siRNA. **(F)** The ROBO1 protein levels in U87MG cell transfected with miR-588. GAPDH was used as a loading control. **(G)** The ROBO1 protein levels in A172 cell transfected with miR-588. **(H)** The ROBO1 protein levels in U87MG cells stably transfected with NC, mimics and inhibitor and transfected with ROBO1 siRNAs. GAPDH was used as a loading control.

Figure 4

The invasive, migratory and VM-forming abilities of glioma cells were decreased after ROBO1 knockdown.

(A) and **(E)** Wound-healing assays of miR-588 inhibitor- and mimics-transfected U87MG cells co-transfected with ROBO1 siRNA. **(B)** and **(F)** Effects of ROBO1 siRNA on miR-588 inhibitor- and mimics-transfected U87MG cells were examined by Transwell migration assays. **(C)** and **(G)** Effects of ROBO1 siRNA on miR-588 inhibitor- and mimics-transfected U87MG cells were examined by Transwell invasion assays. **(D)** and **(H)** Effects of ROBO1 siRNA on miR-588 inhibitor- and mimics-transfected U87MG cells were examined by VM formation assays. $**p < 0.01$, $*p < 0.05$ by Kruskal-Wallis one-way ANOVA.

Figure 5

miR-588 antagonizes hypoxia-induced pro-invasive effects in an orthotopically xenografted glioma mouse model.

(A) ROBO1 expression gradually increased in the glioma compared with normal brain. **(B)** Bioluminescence images of the sizes of the intracranial tumors in the model mice on day 4 and day 15. **(C)** Mice were sacrificed for brain removal 21 days after tumor implantation. **(D)** Hematoxylin and eosin (H&E) staining of orthotopically xenografted glioma. **(E)** Immunohistochemical staining showed ROBO1 increased in xenografted glioma with miR-588 inhibitor transfected, MMP2 and MMP9 raised up also.

Supplementary Files

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