Hypoxic cancer-secreted exosomal miR-182-5p promotes glioblastoma angiogenesis by targeting Kruppel-like factor 2 and 4

miR-182-5p promotes glioblastoma angiogenesis

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Competing interests

The authors declare that they have no competing interests.

Abstract

Glioblastoma (GBM) is the most lethal primary brain tumor and has a complex molecular profile. Hypoxia plays a critical role during tumor progression and in the tumor microenvironment (TME). Exosomes released by tumor cells contain informative nucleic acids, proteins and lipids involved in the interaction between cancer and stromal cells, thus leading to TME remodeling. Accumulating evidence indicates that exosomes play a pivotal role in cell-to-cell communication. However, the mechanism by which hypoxia affects tumor angiogenesis via exosomes derived from tumor cells remains largely unknown. In our study, we found that, compared with the parental cells under normoxic conditions, the GBM cells produced more
exosomes, and miR-182-5p was significantly up-regulated in the exosomes from GBM cells under hypoxic conditions. Exosomal miR-182-5p directly suppressed its targets Kruppel-like factor 2 and 4 (KLF2 and KLF4), leading to the accumulation of vascular endothelial growth factor receptor (VEGFR), thus promoting tumor angiogenesis. Furthermore, exosome-mediated miR-182-5p also inhibited tight junction-related proteins (such as ZO-1, Occludin, and Claudin-5), thus enhancing vascular permeability and tumor transendothelial migration. Knockdown of miR-182-5p reduced angiogenesis and tumor proliferation. Interestingly, we found elevated levels circulating miR-182-5p in patient blood serum and cerebrospinal fluid (CSF) samples, and its expression level was inversely related to the prognosis.

Implications: Overall, our data clarify the diagnostic and prognostic value of tumor-derived exosome-mediated miR-182-5p and reveal the distinctive crosstalk between tumor cells and HUVECs mediated by tumor-derived exosomes that modulate tumor vasculature.

**Keywords**: Glioma, Exosome, miR-182-5p, Angiogenesis, Hypoxia

1. Introduction

Malignant glioma, especially glioblastoma multiforme (GBM), is the most common and most fatal type of brain tumor in humans, partly because of its diffuse invasion of the surrounding normal brain tissues (1-3). Recently, increasing numbers of studies have indicated that the tumor microenvironment (TME) is very important for the sustained survival, growth, invasion and metastasis of tumor cells (4,5). Abnormal tumor vasculature is commonly accepted as the major factor contributing to tumor growth, spread and chemoresistance (6). Increased vascular permeability in the tumor also provides a supportive microenvironment for cancer growth and metastasis by continuously satisfying the nutrition and oxygen demands of the tumor cells, thereby promoting tumor cell dissemination (7-10). Targeting the TME may offer an
important opportunity to intervene in cancer progression (11,12). Hence, identifying novel regulatory molecules for the diagnosis, prognosis, and treatment of cancer is of great value.

Hypoxia is one of the top ten manifestations of cancer. It promotes tumor angiogenesis and progression by altering the TME (13-15). Low oxygen tension (hypoxia) is a common feature in many solid tumors, and tumor hypoxia is closely correlated with increased genetic instability and disease progression (16). Hypoxia also activates hypoxia-inducible factor 1α (HIF-1α) (17), which is basically expressed (18). Under normoxic conditions, HIF-1α is rapidly degraded by the proteasomal system. However, its degradation pathway is blocked under hypoxic conditions, leading to its accumulation in the nucleus (19-21). Increased HIF-1α drives the expression of a broad range of downstream genes, including those involved in glucose-energy metabolism and oxygen homeostasis, thus contributing to cell proliferation, migration and invasion (22). Increased levels of HIF-1α and hypoxia-related proteins are closely related to poor outcomes in glioma patients (23). However, how tumor cells adapt to the hypoxic environment and communicate with the TME during tumor progression remains largely unknown.

Exosomes are small lipid bilayer membrane vesicles (30 to 120 nm in size) that come from the luminal membrane of multi-vesicular bodies (MVBs), which contain lipids, proteins, and numerous nucleic acids, such as DNA, RNA, and miRNAs, and can be continuously released by fusion with the cell membrane (24-26). Recently, a number of studies have reported that cancer cell-secreted exosomes play an important role in the crosstalk between various cell types, such as tumor immune escape, TME remodeling and pre-metastatic niche formation (27-30). Exosomes also provide protection against enzymatic RNase degradation for the substances they contain, thus allowing the substances to safely pass through the vasculature system and extracellular environment (31,32). Subsequently, exosome-mediated rich miRNAs can interact between cells, leading to a range of biological processes (33-36).

Our study was conducted to clarify how tumor-derived exosome-mediated miR-182-5p promotes tumor angiogenesis under hypoxic conditions.
2. Materials and Methods

2.1 Cell lines and cell culture

Normal human astrocyte (HA) cells were cultured in astrocyte medium (Carlsbad, CA, USA), and the other cells (HUVECs, U-251MG, H4, A-172, U-118MG, LN-18, U-87MG (glioblastoma of unknown origin)) were cultivated as described previously (37). Human umbilical vein endothelial cells (HUVECs) and normal human astrocyte (HA) cells were obtained from ScienCell Research Laboratories (San Diego, CA). Human glioma cell lines (U-251MG, H4, A-172, U-118MG, LN-18, and U-87MG (glioblastoma of unknown origin)) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HA cells were cultured in astrocyte medium (Carlsbad, CA, USA), and the other cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan County, KY) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, USA). All cell lines were authenticated every 3 months by short tandem repeat (STR) analysis with the Geneprint 10 System Kit (Promega, Madison, WI, USA) and confirmed to be mycoplasma negative by ERA.

2.2 Patients and sample preparation

Prior approval was obtained from the related ethics committee (S898). All patients signed the consent form on the day of admission. This research was performed in accordance with the Helsinki Declaration and all methods were performed in accordance with the approved guidelines. Details are presented in the Supplementary material.

2.3 Exosome isolation, characterization, and treatment

The exosomes were purified by total exosome isolation reagents (Life Technologies) according to the manufacturer's protocols. See supplementary materials for details.

2.4 RNA interference and plasmids

The protocol and the sequences of the inhibitor and mimics of miR-182-5p are listed in the Supplementary tables 1-2.

2.5 Tube formation assay

This process is similar to the process we described previously. See supplementary
materials for details.

2.6 Cell migration/transendothelial migration/endothelial permeability

All operations are based on the manufacturer's protocols. For more details, see the Supplementary material.

2.7 RT-PCR

The protocol and the sequences of all mentioned primers are listed in the Supplementary tables 3.

2.8 Western blotting

This process is similar to the process we described previously (37). The protocol and all the antibodies are listed in the Supplementary tables 4.

2.9 Dual luciferase reporter assay

All procedures were based on the manufacturer's protocols. For more details, see the Supplementary material.

3.0 Fluorescence in situ hybridization, immunofluorescence and immunohistochemistry

The immunofluorescence (IF) and immunohistochemistry (IHC) assays were performed as previously described (38). The protocol, the miR-182-5p detection probe and all the antibodies are listed in the Supplementary tables 5.

3.1 In vivo Matrigel plug assay

Equal amounts of exosomes derived from U-87MG glioma cells treated with normoxia or hypoxia were added to the miR-182-5p inhibitor or control with Matrigel (BD Biosciences, San José, CA, USA). These mixtures were injected under the mouse skin. Two weeks later, the mouse was euthanized. Prior approval was obtained from the related ethics committee (S2130). For more details, see the Supplementary material.

3.2 Brain orthotopic xenografts

Briefly, the skull of the mouse was fixed in place, an incision was made in the skin, and the injection was positioned. The skull was drilled using a high-speed air-turbine drill. U-87MG-Luc cell suspension with Matrigel (3:1) (BD Biosciences) was injected into the brain parenchyma using a microliter syringe. Then, the bone flap
was returned to its original placement and sealed with histocompatible cyanoacrylate glue, and an indwelling intracranial device was used for continuous administration. Subsequently, the skin on the skull was sutured closed. The tumors were monitored and quantified using a bioluminescence imaging system (PerkinElmer, IVIS Spectrum Imaging System, USA) after two weeks. After confirming that the tumor sizes were similar in each group, the mice received different treatments at random. Equal amounts of exosomes derived from U-87MG glioma cells treated with normoxia or hypoxia were mixed with the miR-182-5p inhibitor or control and delivered five times intratumorally at 3-day intervals. Tumor growth was monitored and quantified using a bioluminescence imaging system. All animal experiments were conducted in accordance with the institutional guidelines. For more details, see the Supplementary material.

3.3 Statistical analyses

This process was similar to the process we described previously. All data are expressed as the mean ± SD from at least three independent assays. Unpaired/paired Student’s t tests (two groups) or 1-way ANOVA + Dunnett’s tests (more than two groups) were adopted to identify statistically significant differences. All statistical analyses were conducted using GraphPad Prism version 7 (GraphPad Inc., CA, USA), and differences were considered statistically significant at p-values < 0.05.

3. Results

3.1 Isolation and features of exosomes shed from GBM cells under hypoxic conditions

U-251MG and U-87MG cells were exposed to 1% oxygen for one day. The expression of HIF-1α in U-251MG and U-87MG cells increased under 1% oxygen conditions compared with the expression in U-251MG and U-87MG cells cultured in normoxic conditions (Fig 1a). Subsequently, we isolated, characterized and quantified exosomes from U-251MG and U-87MG cells under normoxic and hypoxic conditions via transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). Nanovesicles ranging from 30 to 120 nm in size were observed in the exosomes isolated from U-251MG and U-87MG cells exposed to normoxic or
hypoxic conditions (Fig 1b and c). Additionally, Western blotting indicated that the vesicles isolated from U-251MG and U-87MG cells exposed to normoxic or hypoxic conditions were positive for the exosomal markers CD9, CD63, CD81, and TSG101. Compared to normoxic U-251MG and U-87MG cells, the expression levels of all exosomal markers increased in the exosome fractions derived from hypoxic U-251MG and U-87MG cells (Fig 1d). When U-251MG and U-87MG cells were exposed to hypoxic conditions, the number of exosomes also clearly increased by nanoparticle-tracking analysis (Fig 1e). Interestingly, similar results regarding exosome secretion were also repeatedly observed in several glioma cell lines (H4, A-172, U118MG, and LN-18) under hypoxic conditions, regardless of the tumor grade (Fig 1f).

3.2 Exosomes derived from GBM cells promote angiogenesis and increase permeability in HUVECs

Hypoxia is a very common phenomenon in solid tumors, so tumor cells and their surrounding cells are often exposed to an oxygen-deficient environment, especially the surrounding cells that are close to tumor cells. To investigate the effect of tumor cells on endothelial cells, we added exosomes derived from normal HA, U-251MG and U-87MG cells exposed to hypoxic conditions to HUVECs and then observed the biological functions of the HUVECs. Next, PKH67-labeled HUVECs were incubated with Dil-labeled U-251MG and U-87MG exosomes under normoxic and hypoxic conditions. Immunofluorescence analysis demonstrated a colocalization of Dil fluorescence and PKH67 lipid dye in the incubated HUVECs, which indicated that the HUVECs effectively absorbed the exosomes (Fig 2a). Under hypoxic conditions, compared with the exosomes derived from normoxic U-251MG, U-87MG and HA cells, the exosomes derived from hypoxic U-251MG and U-87MG cells facilitated the migration and tube formation of HUVECs (Fig 2b-c and Supplementary Fig 1A-B). Similarly, the exosomes derived from hypoxic U-251MG and U-87MG cells also enhanced the endothelial permeability and transendothelial migration of tumor cells (Fig 2d-e and Supplementary Fig 1C). In addition, compared with the exosomes derived from normal HA and normoxic glioma cell lines, the exosomes derived from
several hypoxic glioma cell lines (H4, A-172, U118MG, and LN-18) also enhanced the migration, tube formation, endothelial permeability and transendothelial migration of tumor cells (Fig 2f-i). These data suggest that the exosomes derived from hypoxic glioma cells increase angiogenesis and destroy the barrier of endothelial cells, thus resulting in tumor cell intravasation and extravasation.

To clarify the role of exosomes in the distant interaction between tumor cells and vascular endothelial cells, we also investigated the proangiogenic effect of exosomes from hypoxic U-251MG and U-87MG cells on HUVECs under normoxic conditions. HUVECs cultivated in normoxia were incubated with the exosomes from HA, U-251MG or U-87MG cells. Similar results were obtained under conditions of hypoxia: compared with HA-derived and normoxic U-251MG-derived and U-87MG-derived exosome-treated HUVECs, the exosomes from hypoxic U-251MG and U-87MG cells increased the migration, tube formation and permeability of HUVECs under normoxic conditions (Supplementary Fig 2A-C). Exosomes derived from U-251MG and U-87MG cells under hypoxic conditions also promoted tumor transendothelial migration compared with exosomes derived from normoxic U-251MG and U-87MG cells (Supplementary Fig 2D). These results demonstrate that hypoxic tumor cells play a role in promoting angiogenesis by secreting exosomes.

3.3 Tumor cells secrete miR-182-5p and are effectively absorbed by HUVECs

To identify the potential exosome-associated miRNAs induced by hypoxia, a miRNA array was performed using GBM primary cells with or without hypoxia. Supplementary Fig 3A shows that the levels of miR-182-5p, -543, -486-5p, -485-3p and -185 increased in primary cells under conditions of hypoxia. Among these molecules, only miR-182-5p levels increased in multiple glioma cell lines, regardless of tumor grade (Fig 3a). In addition, the knockdown of HIF-1α in U-251MG and U-87MG cells failed to prevent the increase in miR-182-5p levels in the exosomes of U-251MG and U-87MG cells under hypoxic conditions (Supplementary Fig 3B-C). Interestingly, miR-182-5p was clearly up-regulated in six glioma cell lines compared with HA cells (Supplementary Fig 3D). Accumulating evidence has shown that exosome-mediated miRNAs play an important role in communication between cells
Therefore, we speculated that exosome-mediated miR-182-5p could play a vital role in the communication between glioma cells and endothelial cells. To investigate this point, miR-182-5p was over-expressed or knocked down in glioma cells (Supplementary Fig 3E).

To further confirm that exosome-mediated miR-182-5p from hypoxic U-251MG and U-87MG cells can be transferred to HUVECs, we measured miR-182-5p levels in HUVECs co-cultured with exosomes derived from U-251MG and U-87MG cells under conditions of normoxia or hypoxia. The level of mature miR-182-5p, but not the level of pri-/pre-miR-182-5p, increased in HUVECs after they were co-cultured with exosomes derived from U-251MG and U-87MG cells under hypoxic conditions (Fig 3b-c). The RT-PCR results indicated that miR-182-5p overexpression or knockdown contributed to the up-regulation or down-regulation of exosomal miR-182-5p, respectively (Supplementary Fig 3F). In addition, the RNA polymerase II inhibitor could not stop the increase in miR-182-5p levels in HUVECs exposed to exosomes derived from U-251MG and U-87MG cells under hypoxic conditions (Fig 3d-e). However, miR-182-5p was not up-regulated in U-251MG cells incubated with HUVECs/miR-182-5p exosomes (Supplementary Fig 3G). Moreover, with Annexin V treatment, which is an inhibitor of exosome internalization (40), U-251MG/miR-182-5p exosomes failed to induce an increase in the level of miR-182-5p in HUVECs (Supplementary Fig 3H). These results indicate that exosome-mediated miR-182-5p from hypoxic U-251MG and U-87MG cells can be effectively absorbed by HUVECs.

3.4 Exosome-mediated-miR-182-5p targets KLF2 and KLF4

To predict how miR-182-5p could regulate vascular permeability and angiogenesis, three independent online databases (TargetScan, miRanda and miRDB) were used to identify the potential downstream targets of miR-182-5p. Overlap analysis of the information from all the databases revealed that KLF2 and KLF4 may be targets of miR-182-5p. Previous studies reported that KLF2 can suppress angiogenesis by stopping the promoter activity of VEGFR2 (41). KLF4 is indispensable for the integrity of the endothelial barrier because it enhances the
promoter activities of tight junction-relevant proteins, such as Claudin-5, Occludin, and ZO-1 (42). To further clarify the functions of KLF2 and KLF4, in vitro tube formation and permeability assays were performed. The results revealed that KLF2 notably suppressed angiogenesis, and KLF4 clearly impeded vascular permeability, which was consistent with the findings of previous reports (Supplementary Fig 4A-F). To confirm whether KLF2 and KLF4 are targets of miR-182-5p, we performed a reporter assay with the luciferase reporter plasmid carrying wild-type (WT) and mutated (MT) KLF2 and KLF4 3′UTRs with the miR-182-5p binding site. MiR-182-5p mimic transfection and hypoxic U-251MG and U-87MG cell-derived exosome treatment decreased the luciferase activities compared with control mimic transfection and normoxic U-251MG and U-87MG cell-derived exosome treatment (Fig 4a-b). In addition, miR-182-5p over-expression reduced the expression levels of KLF2 and KLF4; however, the re-introduction of the plasmid lacking the 3′UTR region rescued their expression levels. miR-182-5p knockdown contributed to the increase in the expression of KLF2 and KLF4. We also tested changes in the genes downstream of KLF2 and KLF4, such as VEGFR2, ZO-1, Occludin, and Claudin-5. miR-182-5p over-expression in HUVECs up-regulated the level of VEGFR2 and down-regulated the levels of ZO-1, Occludin, and Claudin-5, yet the restoration of KLF2 or KLF4 expression abolished these effects (Fig. 4c-d, Supplementary Fig 4G). Furthermore, KLF4 or KLF2 over-expression abrogated miR-182-5p-induced vascular permeability and angiogenesis (Fig. 4e-f). However, the knockdown of miR-182-5p suppressed vascular permeability and tube formation ability (Fig. 4g-h). All these data show that exosome mediated miR-182-5p facilitates vascular permeability and angiogenesis by targeting KLF2 and KLF4.

3.5 Hypoxic GBM-secreted miR-182-5p silences KLF2 and KLF4

Subsequently, we measured the luciferase activities of the KLF2 or KLF4 3′UTRs in HUVECs treated with A-172/miR-182-5p exosomes. As expected, their luciferase activities were attenuated by A-172/miR-182-5p exosomes but not A-172/mock exosomes, demonstrating that they can be silenced by exosome-mediated miR-182-5p from GBM cells (Supplementary Fig 5A-B). In addition,
A-172/miR-182-5p exosomes notably up-regulated the levels of VEGFR2, p-ERK, and p-AKT and down-regulated the expression levels of KLF2, KLF4, ZO-1, Claudin-5 and Occludin. However, the miR-182-5p inhibitor or Annexin V treatment with A-172/miR-182-5p exosomes abolished these effects. Similarly, these effects were also abolished by the ectopic expression of KLF2 or KLF4 in exosome-treated HUVECs (Fig. 5a-c). Conversely, HUVECs treated with U-87MG/zip-miR-182-5p exosomes displayed the opposite results (Supplementary Fig 5C). The in vitro tube formation and permeability assays showed that transfection of A-172/miR-182-5p exosomes with miR-182-5p inhibitor or the pretreatment of A-172/miR-182-5p exosomes with Annexin V attenuated the functions of facilitating angiogenesis and enhancing vascular permeability in HUVECs. However, KLF2 or KLF4 restoration rescued those functions (Fig. 5d-e). Our findings indicate that exosome-mediated miR-182-5p from GBM cells increase angiogenesis and vascular permeability by silencing KLF2 and KLF4.

3.6 Hypoxic GBM-derived exosomal miR-182-5p promotes tumor angiogenesis and growth

To further investigate the effect of exosome mediated miR-182-5p on angiogenesis in vivo, we performed a Matrigel plug angiogenesis assay to quantify the formation of blood vessels by measuring the hemoglobin concentrations in the gel plugs in nude mice. Compared with the gel-only plugs (control), the gel plugs carrying exosomes derived from normoxic U-87MG cells had clearly higher densities of neovessels. Simultaneously, exosomes derived from hypoxic U-87MG cells further enhanced the formation of neovessels (Fig 6a). Interestingly, the miR-182-5p inhibitor notably attenuated the increase in the density of neovessels caused by exosomes derived from hypoxic U-87MG cells (Fig 6b).

To further verify the effect of miR-182-5p in vivo, we injected U-87MG/Luc cells into the brain parenchyma of mice, and the exosomes and the miR-182-5p inhibitor were injected intratumorally. Fig 6c shows that the miR-182-5p inhibitor suppressed hypoxic U-87MG exosome-mediated tumor growth in mice. Additionally, IF showed that the CD31+ (density of blood vessels) in the tumor tissues of mice treated with the
miR-182-5p inhibitor decreased (Fig 6d), and IHC showed the expression levels of KLF2, VEGFR2, KLF4, ZO-1, Occludin, and Claudin-5 (Supplementary Fig 6A). These results show that exosomal miR-182-5p accelerates the increase in endothelial cell density, which facilitates the angiogenic progression of GBM.

3.7 Serum exosomal miR-182-5p is associated with tumor progression

To clarify whether an increased level of exosomal miR-182-5p in the serum and CSF is correlated with tumor progression, circulating exosomes were extracted from the serum and CSF of patients with different grades of glioma and healthy donors. The RT-PCR results showed that compared with healthy donors, glioma patients had up-regulated miR-182-5p from circulating exosomes. Furthermore, the miR-182-5p levels in circulating exosomes from high-grade glioma patients were higher than those from low-grade glioma patients (Fig. 7a). We also tested miR-182-5p levels in circulating exosomes from preoperative and postoperative glioma patients. Interestingly, 84% (21/25) of them displayed a dramatic decrease in miR-182-5p levels in circulating exosomes in the postoperative period (Fig. 7b). Moreover, the miR-182-5p level in circulating exosomes from serum and CSF was positively correlated with the miR-182-5p level in the tumor tissues (Fig. 7c-d), showing that the up-regulation of miR-182-5p in glioma tissues may lead to elevated miR-182-5p levels in circulating exosomes. In addition, FISH and IF results showed that the miR-182-5p levels were negatively correlated with the KLF2 and KLF4 levels (Fig. 7e). Therefore, these results show that the expression level of miR-182-5p in circulating exosomes is positively correlated with the grade of tumor.

4. Discussion

Accumulating evidence has shown that miR-182-5p is a cancer-promoting miRNA that is associated with a poor prognosis in glioma patients (43,44). However, the molecular mechanism by which miR-182-5p regulates glioma progression has not been elucidated. Herein, we demonstrated that hypoxic GBM-secreted miR-182-5p can be delivered to HUVECs by exosomes, damaging the integrity of endothelial barriers and promoting angiogenesis, thus contributing to glioma progression. Interestingly, we also found that the expression levels of miR-182-5p in circulating
exosomes are related to tumor progression. In summary, our data show the function of hypoxic GBM-secreted miR-182-5p in tumor development.

miRNAs effectively inhibit their target gene mRNAs by moving between different cells. For example, exosome-mediated miR-1247-3p from liver cancer cells can diffuse to cancer-associated fibroblasts (CAF) and induce CAF activation, thus promoting liver cancer progression (45). Exosomal miR-19a can diffuse from astrocytes to tumor cells, where it inhibits PTEN expression and promotes tumor outgrowth in the brain (46). Collectively, emerging evidence indicates that the exosome-mediated transfer of miRNAs between tumor cells and stromal cells is vital for tumor progression. FISH showed that the miR-182-5p levels in glioma cells were positively correlated with the levels in endothelial cells (Supplementary Fig 6B), based on the assumption that miR-182-5p can diffuse from glioma cells to endothelial cells via exosomes. Furthermore, we observed the colocalization of Dil-labeled exosome fluorescence and PKH67-labeled HUVECs in the incubated HUVECs. These results indicated that glioma-secreted exosomes can be effectively absorbed by endothelial cells. miR-182-5p has been reported to be involved in tumor proliferation (47-53), invasion (47,49,51), and metastasis (48,54-57). However, the role of miR-182-5p in vascular endothelial cells remains unclear. Cancer-induced vascular permeability and angiogenesis play crucial roles in cancer progression (58).

Hypoxia is a hallmark of cancer and promotes tumor angiogenesis and progression (13-15). HIF-1α is a transcription factor that is a main regulator of the gene expression induced by hypoxia (59). However, our data showed that exosomal miR-182-5p functions independently of HIF-1α in promoting tumor angiogenesis and progression. In addition, our results also showed that serum exosome-mediated miR-182-5p is associated with tumor progression and that the blockade of exosome-mediated miR-182-5p from GBM cells alleviates tumor angiogenesis and tumor burden in mice, indicating that miR-182-5p may be used as a therapeutic target.

Exosome-mediated RNA has already been reported to be a glioma-specific biomarker, similar to EGFR v III, IDH1 and miR-21 (60-62). Exosomal wild-type EGFR expression levels derived from patient CSF have been associated with
Chemotherapy sensitivity in GBM patients because EGFR overexpression exists in 70% of GBM cases (63,64). Interestingly, exosome-mediated EGFR v III RNA has been isolated from the serum of GBM patients, but it is not detected in exosomes derived from healthy individuals. In addition, miR-21 has been detected in the CSF of almost all GBM patients, which indicates that it is a highly sensitive marker for GBM. miR-21 expression also plays a vital role in the TME (65).

KLF2 and KLF4 are members of the KLF subfamily of zinc finger proteins (66,67). Emerging evidence has shown that their ectopic expression has been observed in numerous cancers and that they possess tumor-suppressor features that lead to their inhibitory effects on cell proliferation (68,69). KLF2 suppresses angiogenesis by inhibiting the promoter activity of VEGFR2 (41), while KLF4 maintains the integrity of the endothelial barrier by facilitating the promoter activity of tight junction-related proteins such as Caludin-5, Occludin, and ZO-1 (42). Similarly, our results indicated that the down-regulation of KLF2 and KLF4, which are target genes of miR-182-5p, resulted in vascular endothelial barrier destruction and the promotion of tumor angiogenesis.

In this study, we explored whether exosome-mediated miR-182-5p promotes GBM angiogenesis. An in vivo permeability assay showed that exosome-mediated miR-182-5p derived from hypoxic GBM cells clearly increased vascular permeability. In addition, exosome-mediated miR-182-5p from hypoxic GBM cells suppressed the expression levels of KLF4, Occludin, ZO-1, Claudin-5 and KLF2 and subsequently increased the expression level of VEGFR2. In addition, an increase in tumor burden was observed in the mice pretreated with hypoxic U-87MG/miR-182-5p exosomes. Furthermore, our study also showed that the up-regulation of miR-182-5p in glioma cells facilitates vascular permeability and angiogenesis. Therefore, hypoxic GBM-secreted miR-182-5p contributes to tumor progression by inducing angiogenesis and damaging the tight junctions of vein endothelial cells.

In conclusion, our data showed that the miR-182-5p levels in circulating exosomes from high-grade glioma patients, especially those with GBM, were higher than those in the exosomes from low-grade glioma patients and much lower than...
those in the exosomes from healthy donors. Additionally, a dramatic drop in miR-182-5p levels was found during the postoperative period in most patients. Notably, our data also showed that the level of miR-182-5p in circulating exosomes is positively correlated with the level of miR-182-5p in the tumor tissues. Taken together, our clinical data show that miR-182-5p levels in circulating exosomes could be a potential biomarker for the diagnosis and prognostic prediction of glioma.

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Consent for publication
Not applicable.

Authors’ contributions
N-x Xiong designed the study; J-j Li performed the experiments; J-j Li, H-l Yuan, H Xu and H-y Zhao analyzed the data; and N-x Xiong and J-j Li wrote the manuscript.

References:


Figure Legends:

Figure 1: Isolation and characterization of exosomes shed from GBM cells under hypoxic conditions.
(a) The expression levels of HIF-1α in U-251MG and U-87MG cells under normoxic and hypoxic conditions. (b) Transmission electron microscopy (TEM) and (c) nanoparticle tracking analysis (NTA) of exosomes shed from U-251MG and U-87MG cells under normoxic and hypoxic conditions. Scale bar represents 100 nm. (d) Western blotting analysis of the markers of exosomes and hypoxia increased the production of exosomes in U-251MG and U-87MG (e) and other glioma cell lines by nanoparticle-tracking analysis (f). Glioma cells were cultured under normoxic or hypoxic conditions for one day, and the protein expression levels in U-251MG and U-87MG cells by Western blotting and the quantification of exosomes were measured by nanoparticle-tracking analysis. The exosomes of glioma cells under normoxic or hypoxic conditions were isolated using the Total Exosome Isolation Kit and then visualized by TEM. The means ± standard deviations (SDs) are provided (n=3). *p < 0.05 and **p < 0.01 according to two-tailed Student’s t tests or one-way ANOVA followed by Dunnett’s tests for multiple comparisons.

Figure 2: Exosomes derived from GBM cells promote angiogenesis and increase permeability in HUVECs.
(a) The uptake of U-251MG and U-87MG exosomes in HUVECs. The arrows indicate that the HUVECs absorbed the exosomes. Hypoxic U-251MG and U-87MG exosomes promote the cell migration (b), tube formation (c) and endothelial
permeability (d) of HUVECs, as well as the transendothelial migration of tumor cells (e) under hypoxic conditions. The exosomes derived from several glioma cell lines under hypoxic conditions also promote the cell migration (f), tube formation (g) and endothelial permeability (h) of HUVECs, as well as the transendothelial migration of tumor cells (i) under hypoxic conditions. All the results are shown as the means ± SDs of at least three independent experiments. *p < 0.05 and **p < 0.01 according to two-tailed Student’s t tests or one-way ANOVA followed by Dunnett’s tests for multiple comparisons.

**Figure 3: miR-182-5p is secreted by hypoxic GBM cells and transferred to endothelial cells by exosome secretion.**

(a) The expression level of miR-182-5p in the exosomes of glioma cell lines under normoxic and hypoxic conditions. (b) The expression level of mature or (c) pri-/pre-miR-182-5p in exosome-treated HUVECs. (d) The RNA polymerase II inhibitor failed to change the level of miR-182-5p in HUVECs exposed to hypoxic U-251MG and U-87MG-derived exosomes. All the results are shown as the means ± SDs of at least three independent experiments. *p < 0.05 and **p < 0.01 according to two-tailed Student’s t tests or one-way ANOVA followed by Dunnett’s tests for multiple comparisons. Inh, inhibitor; ns, no significant difference.

**Figure 4: Exosomal miR-182-5p derived from hypoxic GBM cells targets KLF2 and KLF4.**

(a) Luciferase readout from WT or mutant KLF2 or (b) KLF4 3′UTR reporter cotransfected in HUVECs with U-251MG and U-87MG exosomes or miR-182-5p mimics. (c) KLF2, VEGFR2, p-ERK, ERK, p-AKT, and AKT expression levels in miR-182-5p overexpressing or miR-182-5p/KLF2 coexpressing HUVECs by Western blotting. (d) KLF4, ZO-1, Occludin, and Claudin-5 expression levels in miR-182-5p overexpressing or miR-182-5p/KLF4 coexpressing HUVECs by Western blotting. (e) Effects of miR-182-5p and miR-182-5p/KLF4 on the endothelial permeability of HUVEC monolayers via an in vitro permeability assay. (f) Effects of miR-182-5p and miR-182-5p/KLF2 on the tube formation ability of HUVECs via tube formation assay. Scale bars: 100 μm. (g) Effect of miR-182-5p knockdown on the endothelial permeability.
permeability of HUVEC monolayers via an in vitro permeability assay. (h) Effect of miR-182-5p knockdown on the tube formation ability of HUVECs via a tube formation assay. All the results are shown as the means ± SDs of at least three independent experiments. *p < 0.05 and **p < 0.01 according to two-tailed Student’s t tests or one-way ANOVA followed by Dunnett’s tests for multiple comparisons.

**Figure 5: Hypoxic GBM-secreted miR-182-5p silenced KLF2 and KLF4 in HUVECs.**

comparisons.

**Figure 6: Hypoxic GBM-derived exosomal miR-182-5p promotes tumor angiogenesis and growth in vivo.**

(a) Hemoglobin content in the Matrigel plugs and hypoxic U-87MG-derived exosomes increased angiogenesis. (b) Hemoglobin content in the Matrigel plugs; administration of the miR-182-5p inhibitor decreased the angiogenesis induced by hypoxic U-87MG exosomes in U-87MG-bearing mice. (c) Administration of the miR-182-5p inhibitor decreased the tumor growth induced by hypoxic U-87MG exosomes in U-87MG-bearing mice; histogram analysis of luminescence representing the size of the tumors measured (right panels); n = 5. (d) Representative images (left panels) of IF staining with CD31 and histogram analysis of the expression level of CD31 (right panels). All the results are shown as the means ± SDs of at least three independent experiments. *p < 0.05 and **p < 0.01 according to two-tailed Student’s t tests or one-way ANOVA followed by Dunnett’s tests for multiple comparisons.

**Figure 7: Serum exosomal miR-182-5p is associated with tumor progression.**

(a) RT-PCR analysis of miR-182-5p levels in circulating exosomes from healthy donors or glioma patients (10 healthy donors, 24 low-grade glioma (LGG) patients, 30 high-grade glioma (HGG) patients). (b) Fold-change in miR-182-5p in circulating exosomes from 25 glioma patients after surgical resection of glioma tissues. The expression level of miR-182-5p in circulating exosomes from glioma patients was normalized to 1 in the postoperation period. (c) Spearman correlation analysis between miR-182-5p levels in glioma tissues and miR-182-5p levels in the circulating exosomes in the serum from glioma patients. (d) Spearman correlation analysis between miR-182-5p levels in glioma tissues and miR-182-5p levels in circulating exosomes of CSF from glioma patients. (e) Spearman correlation analysis between miR-182-5p expression and KLF2 and KLF4 expression in glioma tissues. Scale bars: 50 µm. *p < 0.05, **p < 0.01 and ***p < 0.001 according to Spearman’s test, two-tailed Student’s t tests or one-way ANOVA followed by Dunnett’s tests for multiple comparisons.
Figure 3
Figure 7

(a) Relative expression level of miR-182-5p (log2 fold changes) in Normal, LGG, and HGG.

(b) Fold changes of miR-182-5p (post operate/before operate).

(c) Relative expression of miR-182-5p in serum versus glioma tissues.

(d) Relative expression of miR-182-5p in CSF versus glioma tissues.

(e) Immunohistochemical analysis of KLF2 and KLF4 in NBT, LGG, and HGG.

<table>
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<th>KLF4</th>
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