Abstract. The present study was the first to examine the effect of microRNA-Let-7f (miR-Let-7f) inhibiting vasculogenic mimicry (VM) of human glioma cells. The postoperative survival time was significantly poor in VM-positive glioma patients compared with those without VM. Thus, it is reasonable to postulate that miR-Let-7f functions as a potent tumor suppressor by inhibiting glioma VM. However, the molecular mechanisms involved remain poorly clarified. Our preliminary studies revealed that miR-Let-7f suppressed VM by disturbing periostin (POSTN)-induced migration of glioma cells. Our results clearly demonstrated that inhibiting the pro-migratory function of POSTN by the overexpression of miR-Let-7f significantly reduced the formation of VM. Our findings suggest that miR-Let-7f may serve as a potential complementary therapeutic target in the anti-angiogenesis treatment of gliomas via suppressing VM.

Introduction

Glioma, which is the most malignant tumor type, accounts for more than 70% of all brain tumors (1). The most common subtype is glioblastoma multiforme (GBM), with age-adjusted incidence rate ranging from 0.59 to 3.69/100,000 persons (2). The biological properties of GBM primarily include high mortality and recurrence rates, uncontrollable invasiveness, strong angiogenesis (3) and widespread hypoxia (4). Tumor angiogenesis is an independent prognostic factor associated with poor survival (4). In fact, there is increasing evidence that hypoxia activates angiogenesis (5), metastasis (6) and many other cellular processes in tumors. Although multitude of mechanisms have been proposed to elucidate the hypoxia-induced angiogenesis of tumor cells, more research is warranted to determine the role of a new way of angiogenesis in mediating the effects of hypoxia.

Vasculogenic mimicry (VM) is a new tumor vascular pattern different from angiogenesis (7). It describes a specific capacity of aggressive tumor cells to form vessel-like networks without vascular endothelial cells that provide adequate blood supply for tumor growth (7,8). A variety of molecular mechanisms and signal pathways participate in VM induction (8). Additionally, tumor stem cells are also shown to be implicated in VM formation (9). As a unique vessel formation manner, VM is associated with tumor invasion and poor patient prognosis in various tumors (10). Due to its important effects on tumor progression, VM-related target molecules and strategies are being studied for anticancer treatment (8). However, the specific molecular mechanisms of VM in glioma are still unclear.

miRNAs are a class of endogenous small non-coding RNAs that have been identified as negative regulators of gene expression at the post-transcriptional level (11,12). These small molecules are incorporated into the RNA-induced silencing complex and bind to the seed sequence in the 3'-untranslated regions (3'-UTRs) of their target mRNAs to silence gene translation via mRNA degradation, translational repression and/or miRNA-mediated mRNA decay (12). Therefore, additional research is warranted to determine the important roles of numerous miRNAs in diverse tumor-related cellular processes, such as proliferation (13), angiogenesis (14) and metastasis (15). In gliomas, various tumor-promoting and tumor-suppressing miRNAs (16-19) have been identified. However, there are only few miRNAs targets discovered regulating tumor VM (20-22). Our observation suggest that miRNAs may be important for the tumor VM and provide new insights into understanding the molecular mechanism underlying tumor progression.

The value and novelty of the present study is to indicate, for the first time in human glioma that microRNA-Let-7f...
(miR-Let-7f) is a VM-negative regulator by targeting the perivascular proteins (POSTN)–induced migration. Firstly, we demonstrated VM as an indicator of poor prognosis by testing its positive rate in human glioma tumor samples. Then, we investigated the expression of miR-Let-7f in human glioma tumor samples, and we found that miR-Let-7f functions as a clear VM suppressor. Further study using the glioma cell line A172 revealed that miR-Let-7f knockdown drastically enhanced the VM forming capacity of glioma cells and the miR-Let-7f overexpressed glioma cells almost completely lost the VM forming ability. Using specific small interfering RNA (siRNA), POSTN was considered as the potential intermediary anti-VM molecule of miR-Let-7f. These findings suggest an unexpected fundamental tumor-suppressive role for miR-Let-7f in glioma due to its anti-VM effect.

Materials and methods

Tissue samples and cell culture. The human glioma cell line A172 was purchased from the Chinese Academy of Sciences Cell Bank. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C with 5% CO2 in a humidified chamber. Hypoxic conditions were induced by incubating the cells in a modular incubator chamber with a gas mixture containing 1% O2, 5% CO2, and 94% N2 at 37°C. Forty-five human glioma tissues including 4 World Health Organization (WHO) grade I tumors, 8 grade II tumors, 13 grade III tumors and 20 grade IV tumors, and 2 normal brain tissues of decompression operation were obtained from the Department of Neurosurgery of Qilu Hospital of Shandong University. The glioma specimens were verified and classified according to the WHO classification standard of tumors by two experienced clinical pathologists. The present study was approved by the Institutional Review Board of Shandong University. Written informed consent was obtained from all patients, and the hospital Ethics Committee approved the experiments.

Cell transfection. Mature miR-Let-7f mimics, the scrambled mimic control, the miR-Let-7f inhibitor, the scrambled inhibitor control, the miR-584-5p mimic and POSTN siRNA were designed and synthesized by RiboBio. Cell transfections and co-transfections were performed using Lipofectamine 2000 and 2x Lipofectamine TM 2000 reagents (Invitrogen). In total, 5x10⁴ transfected cells in FBS-free medium were seeded in the upper chamber. Medium containing 10% FBS was added to the lower chamber. After 12 h, the cells that did not migrate or invade were removed using cotton buds. The cells migrating on the lower surface were fixed and stained with crystal violet.

RNA extraction and real-time quantitative PCR. Total RNA was extracted from the tissue samples using TRIzol. Then, total RNA (50 ng) was reverse-transcribed with miR-Let-7f stem-loop RT primers or with the U6 RT primers using a ReverTra Ace qPCR RT kit to generate cDNA. Real-time PCR was performed using a SYBR Premix Ex Taq™ kit with miR-Let-7f or U6 PCR primers. The reactions were performed using a LightCycler 2.0 instrument. U6 expression levels were calculated as concentration ratios using a Roche LightCycler® 2.0 system.

Immunohistochemistry staining. Paraffin-embedded human glioma tissue samples were sectioned and dewaxed. Endogenous peroxidase activity was quenched by incubating the slides in methanol containing 3% hydrogen peroxide for 30 min, after which the sections were incubated for 2 h at room temperature with normal goat serum and subsequently incubated at 4°C overnight with primary antibody (1:300 CD34; Abcam). Next, the sections were incubated with horseradish peroxidase-linked second antibody, followed by reaction with diaminobenzidine and counterstaining with PAS staining kit and then Mayer's hematoxylin.

VM analysis. VM formation was evaluated using a commercial Matrigel matrix (BD Biosciences, France). A172 glioma cells were digested and resuspended at 5x10⁴ cells/ml in DMEM containing 1% FBS. Wells of 96-well tissue culture plates were coated with Matrigel (50 µl/well; BD Biosciences) which was allowed to polymerase at 37°C for 30 min. The glioma cell suspension was then plated at 100 µl/well onto the surface of Matrigel and incubated at 37°C. Cells were photographed using an Olympus inverted microscope.

Statistical analysis. All experiments were performed three times. The statistical analysis and experimental graphs were generated using SPSS 17.0 and GraphPad Prism software. Descriptive statistics including the means ± SD, Mann-Whitney test, Kaplan-Meier plots, log-rank tests were used to analyze the significant differences. p<0.05 and p<0.01 were considered to indicate a statistically significant result.

Results

VM positively correlates with the WHO grades of human glioma tissues. To examine whether the VM correlates with
As shown in fig. 1A, the VM was not found in the normal brain tissues by immunochemical staining (Table I). In 45 human glioma specimens with different grades and the WHO grades of human glioma, we assessed the VM (Table I).

<table>
<thead>
<tr>
<th>Pathological type</th>
<th>No. of patients</th>
<th>N (%)</th>
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<tbody>
<tr>
<td>Astrocytoma</td>
<td>6</td>
<td>12.77</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>10</td>
<td>21.28</td>
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<tr>
<td>Pilocytic astrocytoma</td>
<td>2</td>
<td>4.26</td>
</tr>
<tr>
<td>Oligodendrogloma</td>
<td>4</td>
<td>8.51</td>
</tr>
<tr>
<td>Anaplastic oligodendrogloma</td>
<td>3</td>
<td>6.38</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>20</td>
<td>42.56</td>
</tr>
<tr>
<td>Normal brain tissues</td>
<td>2</td>
<td>4.26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>WHO tumor grade at diagnosis</th>
<th>No. of patients</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4</td>
<td>8.89</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>17.78</td>
</tr>
<tr>
<td>III</td>
<td>13</td>
<td>28.89</td>
</tr>
<tr>
<td>IV</td>
<td>20</td>
<td>44.44</td>
</tr>
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WHO, World Health Organization.

In the present study, we investigated the time-dependent effects of miR-Let-7f in A172 glioma cells. First, we observed that miR-Let-7f knockdown significantly promoted the VM formation and overexpression of miR-Let-7f completely blocked it (Fig. 2C). It suggests that miR-Let-7f may hinder the VM formation capacity of glioma cells by anti-migratory effects.

Overexpression of miR-Let-7f paralyses hypoxia-induced VM formation. Several studies have demonstrated the hypoxia-induced angiogenesis and VM, and the pro-migratory effects of hypoxia. In this regard, we supposed that miR-Let-7f was involved in the regulation of hypoxia-induced VM formation. After 6 h hypoxia treatment, A172 glioma cells revealed an excessively enhanced VM formation (Fig. 4B, upper).

To understand the mechanisms implicated in the VM suppressive role under hypoxia condition of miR-Let-7f, we investigated the time-dependent effects of miR-Let-7f knockdown and overexpression in A172 glioma cells. First, we observed a significant VM suppression effect of miR-Let-7f mimics, particularly even under hypoxic conditions in A172 cells. While miR-Let-7f inhibitor significantly promoted A172 glioma cell VM under normoxic and hypoxia conditions (Fig. 4). Notably, the VM had a dynamic process with increased observation time. The undisturbed VM formation initiated at 4 h, peaked at 6 h and vanished after 12 h in normoxia control A172 cells. In addition, miR-Let-7f inhibitor further enhanced the VM structures, particularly revealing the effect of hypoxia. In contrast, miR-Let-7f mimics completely blocked the VM formation throughout the experiment whether with hypoxia or not.

Taken together, our results clearly demonstrated that miR-Let-7f knockdown markedly promoted the VM formation capacity of human glioma cells and aggravate the hypoxia-induced VM promoting effects. While miR-Let-7f overexpression antagonized the VM formation, particularly the hypoxia-induced VM on human glioma cells.
miR-Let-7f inhibited glioma migratory ability by directly targeting POSTN (23). It suggested that miR-Let-7f may block the VM forming capacity of glioma cells by regulating the POSTN-dependent migration. To investigate whether miR-Let-7f and POSTN are linked, we utilized a POSTN siRNA. siRNA transfected A172 glioma cells almost lost the migratory and VM forming ability as miR-Let-7f overexpressed cells (Fig. 5, middle), and the VM promoting effect of miR-
Figure 3. miR-Let-7f inhibits the migratory and VM forming capacities of human glioma cells. The pro-migratory effect of the miR-Let-7f inhibitor and anti-migratory effect of the miR-Let-7f mimics was examined by wound healing assay (A) Transwell migration assays (B) on A172 cell migration. At 48 h after transfection, a cell suspension was added to the upper chamber of an uncoated Transwell membrane insert, and the lower chamber was filled with media. The cells were cultured under normoxia conditions for 12 h. Then, migratory cells were stained, and the average number of cells was counted in triplicate. (C) Effect of miR-Let-7f mimic and inhibitor transfection on A172 cell VM formation under normoxic conditions. After the cells were transfected and incubated, they were transferred into wells of 96-well tissue culture plates coated with Matrigel in the Materials and methods.

Figure 4. Overexpression of miR-Let-7f paralyses hypoxia-induced VM formation in human A172 glioma cells. The VM suppression effect of miR-Let-7f mimics, particularly even under hypoxic conditions in A172 cells. While miR-Let-7f inhibitor significantly promoted A172 glioma cell VM under normoxic and hypoxia conditions. The undisturbed VM formation was initiated at 4 h, peaked at 6 h and vanished after 12 h in normoxia control A172 cells. In addition, miR-Let-7f inhibitor further enhanced the VM structures, particularly revealing the effect of hypoxia. In contrast, miR-Let-7f mimics completely blocked the VM formation throughout the experiment whether with hypoxia or not.
numerous highly expressed miRNA predictors (13,29-31) for glioma. Vascularization is crucial for the growth of glioma cells has been reported (35), but its mechanisms are still unclear. The detailed VM phenomenon in non-GSC (CSCs) were identified as a VM-initiating cells in many types of glioma. However, the pathways implicated in the tumor suppressive role of miR-Let-7f are poorly defined. Our results suggest that miR-Let-7f may suppress VM by inhibiting the migration capacity of glioma cells. Based on our results and previous reports, we validated the role of potential target POSTN in VM using the specific siRNA. This inhibitor prevented the VM promoting effect of the miR-Let-7f inhibitor as expected. These results demonstrated that POSTN is a direct target of miR-Let-7f in VM forming regulation process.

In summary, the mechanism by which miR-Let-7f functions is summarized as follows. First, miR-Let-7f downregulates POSTN directly and inhibits POSTN-mediated migration. Consequently, this decreased cell motility significantly induced the glioma cell VM forming failure. Ultimately, reduced blood supply improved the prognosis of glioma patients. However, a limitation of the present study is the small number of samples, and the involvement of other key invasion-associated proteins such as Rac1, Cdc42 (39) or MMPs (40) were not investigated. Therefore, additional studies will be required to substantiate this mechanism.

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References


