Abstract. Glioma is the most frequent primary malignant tumor of the human brain. Recently, great progress has been made in the combined therapy of glioma. However, the clinical effects of these treatments and prognosis for patients with glioma remains poor. MicroRNAs (miRNAs) have been demonstrated to play important roles in the initiation and progression of various types of human cancers, also including glioma. The present study investigated the expression patterns of microRNA-140 (miR-140) in glioma, and the roles of miR-140 in glioma cell proliferation, migration and invasion. The results showed that miR-140 was significantly downregulated in glioma tissues and cell lines, and low expression levels of miR-140 were correlated with World Health Organization (WHO) grade and Karnofsky performance score (KPS) of glioma patients. Restoration of miR-140 obviously suppressed glioma cell proliferation, migration and invasion. The results showed that miR-140 was significantly downregulated in glioma tissues and cell lines, and low expression levels of miR-140 were correlated with World Health Organization (WHO) grade and Karnofsky performance score (KPS) of glioma patients. Restoration of miR-140 obviously suppressed glioma cell proliferation, migration and invasion. In addition, a disintegrin and metalloproteinase 9 (ADAM9) was identified as a novel direct target gene of miR-140 in glioma. Furthermore, knockdown of ADAM9 simulated the tumor suppressor functions of miR-140, while overexpression of ADAM9 abrogated these suppressive effects induced by miR-140 in glioma cells. In conclusion, the present study demonstrated the expression and clinical roles of miR-140 in glioma and suggested that miR-140 inhibited proliferation, migration and invasion of glioma cells, partially at least via suppressing ADAM9 expression. Therefore, miR-140 may be a novel candidate target for the development of therapeutic strategies for patients with glioma.

Introduction

Glioma is the most frequent primary malignant tumor of the human brain, accounting for 35.12-61.10% of intracranial tumors (1). According to the 2007 World Health Organization (WHO) classification, glioma can be divided into three major histologic groups: well-differentiated low-grade diffuse astrocytoma, anaplastic astrocytoma and glioblastoma multiforme (2). To date, the underlying molecular mechanism for pathogenesis of gliomas remains elusive, and is possible to be closely associated with many factors, including tumor origin, genetic factors, biochemical environment, ionizing radiation, nitroso compounds, air pollution, bad living habits and infection (3). Currently, the mainly therapeutic treatment options for patients with glioma include surgery resection, radiotherapy and chemotherapy (4,5). Although great progress has been made in the combined therapy, the clinical effects of these treatments and prognosis for patients with glioma remains poor (6). This occurs mainly due to the characteristic rapidly growth, diffuse invasion and unclear pathogenesis of this disease (7). Therefore, great effort is required to fully understand the pathogenesis of glioma and identify novel efficiency therapeutic targets for future targeted strategy.

MicroRNAs (miRNAs) belong to a large family of ~22-25 nucleotides in length, non-protein-coding and single strand RNA molecules that are expressed in the vast majority of eukaryotes (8). They negatively regulate gene expression through binding to the 3'-untranslated regions (3'UTRs) of their target mRNAs, resulting in translational repression and/or mRNAs degradation (9). Generally, one gene can be regulated by multiple miRNAs, and one certain miRNA regulated multiple target genes, which results in the establishment of complex regulatory feedback loops (10,11). miRNAs, as important regulators, have been reported to play important roles in a great deal of biological processes, including cell proliferation, cell cycle, apoptosis, differentiation, metabolism, migration, invasion and metastasis (12-14). The abnormal expression of miRNAs have been found in many diseases, particularly in human cancers (15). Increasing studies have demonstrated that the deregulation of miRNAs is involved in carcinogenesis and progression of glioma (16-18). miRNAs can function as tumor suppressors or oncogenes in glioma initiation and tumor development. For example, miR-519a, a tumor-suppressor miRNA, represses glioma cell proliferation,
migration and invasion through targeting the oncogenic STAT3 pathway (19). miR-183 functions as an oncogene in glioma progression via blockade of NEFL (20). These findings suggested that miRNAs potentially serve as effective biomarkers to improve diagnostic and prognostic accuracy, or as therapeutic targets for novel treatment strategies against glioma.

In the present study, we found lower levels of miR-140 in glioma tissues and cell lines. In addition, low miR-140 expression levels were correlated with WHO grade and Karnofsky performance score (KPS) of glioma patients. Subsequently, the functional roles of miR-140 in glioma cells were also investigated. Moreover, ADAM9 was validated as a novel direct target gene of miR-140. Our results illustrated the expression pattern and roles of miR-140 in regulating the proliferation, migration and invasion of glioma cells, and suggested a potential therapeutic target for patients with glioma.

Materials and methods

Human samples and cell lines. The present study was approved by the Ethics Committee of the Affiliated Hospital of Weifang Medical University, and each patient had written informed consent. Ninety-two glioma and 12 normal brain tissues were obtained from patients undergoing surgery at Affiliated Hospital of Weifang Medical University. None of the patients received preoperative treatment, including radiation or chemotherapy. All fresh tissues were immediately snap-frozen and stored at -80˚C. The clinical characteristics of the glioma patients are listed in Table I.

Cell lines and cell culture. Human glioma cell lines (U87, U251, U373, U118, A172 and LN18) and normal human glial cell line (HEB) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (all from Gibco, Grand Island, NY, USA) in a humidified atmosphere of CO₂/air (5%/95%) at 37˚C.

Cell transfection. miR-140 mimics, miRNA mimics negative control (NC), ADAM9 siRNA and negative control siRNA (NC siRNA) were synthesized and purified by GenePharma (Shanghai, China). ADAM9 overexpressed plasmid (pCDNA3.1-ADAM9) and blank vector pCDNA3.1 were obtained from the Chinese Academy of Sciences (Changchun, China). Cells were seeded into 6-well plates. After incubation overnight, cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

RNA isolation and qRT-PCR. The total RNA from the tissues and cell lines was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The concentration and purity of total RNA was measured using a NanoDrop™ ND-1000 spectrophotometer. For miR-140 expression, cDNA was generated using stem-loop RT (Fermentas, Glen Burnie, MD, USA) followed by real-time PCR analysis (Takara, Dalian, China) according to the manufacturer's instructions. For ADAM9 mRNA expression, RT-PCR kit (Invitrogen) was used to perform reverse transcription. Real-time PCR was performed using SYBR-Green PCR Master Mix (Invitrogen) following a standard quantitative PCR procedure. U6 and GADPH was used as internal standard to normalize the expression of miR-140 and ADAM9 mRNA, respectively. Primers are shown in Table II.

**Table I.** Association of miR-140 expression with clinicopathological characteristics of human gliomas.

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>Case no.</th>
<th>miR-410 expression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Male</td>
<td>53</td>
<td>35</td>
<td>18</td>
</tr>
<tr>
<td>Female</td>
<td>39</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;55</td>
<td>36</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>≥55</td>
<td>56</td>
<td>37</td>
<td>19</td>
</tr>
<tr>
<td>Extension of resection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subtotal</td>
<td>31</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
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<td>40</td>
<td>21</td>
</tr>
<tr>
<td>KPS scores</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥80</td>
<td>40</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>&lt;80</td>
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<td>42</td>
<td>10</td>
</tr>
<tr>
<td>WHO grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>36</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>III</td>
<td>56</td>
<td>43</td>
<td>13</td>
</tr>
</tbody>
</table>

WHO, World Health Organization; KPS, Karnofsky performance score.

**Table II.** Real-time PCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-140</td>
<td>Forward GAGTGTCAGTGGTTTTACCCT</td>
</tr>
<tr>
<td>Reverse GCGAGGTCGGAGCAGTATTCA</td>
<td></td>
</tr>
<tr>
<td>U6</td>
<td>Forward CTGCCTCCGCGACACAGCA</td>
</tr>
<tr>
<td>Reverse AACGCTTCACGAATTTGCT</td>
<td></td>
</tr>
<tr>
<td>ADAM9</td>
<td>Forward GCTAGGTTGGACTGGAGATTTGG</td>
</tr>
<tr>
<td>Reverse TTATTACCACAGGGAGGAGCAG</td>
<td></td>
</tr>
<tr>
<td>GADPH</td>
<td>Forward GCACCGTCAAAAGCAGTGACACGAC</td>
</tr>
<tr>
<td>Reverse TGAGTAGGACGCCAGTGGA</td>
<td></td>
</tr>
</tbody>
</table>

**MTT assay.** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) assay was performed to evaluate the glioma cell proliferation. Transfected cells were harvested and seeded into 96-well
plates (3x10^3 cells/well) in regular culture medium. MTT assay was performed every 24 h until 96 h after plating. In each time point, 20 µl MTT solution (5 mg/ml) was added to each well. After incubation for an additional 4 h, the culture medium containing MTT solution was removed and 200 µl dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured at 490 nm wavelength using a microplate reader (Bio-Rad, Richmond, CA, USA). Each sample was performed in triplicate.

Transwell migration and invasion assays. Transwell migration and invasion assays were performed to determine glioma cell migration and invasion abilities using Transwell chamber (8-µm pores; Millipore, Billerica, MA, USA). For Transwell migration assay, transfected cells were collected and seeded in the upper chamber (3x10^4 cells/chamber) in FBS-free medium. The lower chamber was filled with 500 µl DMEM containing 20% FBS. After 24 h of incubation, the upper surface of the membrane was carefully wiped with a cotton tip and cells attached to the lower surface were fixed with 100% methanol, stained with 0.5% crystal violet and washed with phosphate-buffered saline (PBS) (Gibco). Cell number was counted in five random areas of each Transwell chamber. Transwell invasion assay was performed similarly to the Transwell migration assay except that Transwell chambers were coated with Matrigel (BD Biosciences, San Jose, CA, USA).

Bioinformatics analysis. The target gene information of miR-140 was analyzed using miRanda (www.microrna.org) and TargetScan (www.TargetScan.org/).

Luciferase report assay. The pmirGLO-ADAM9-3'UTR Wt and pmirGLO-ADAM9-3'UTR Mut luciferase reporter vectors were synthesized and purified by GenePharma. For luciferase reporter assay, HEK293T cells were transfected with pmirGLO-ADAM9-3'UTR Wt or pmirGLO-ADAM9-3'UTR Mut, followed by transfection with miR-140 mimics or NC in 24-well plates. Then, 48 h after transfection, cells were harvested, and the activities of firefly and Renilla luciferase were determined using the Dual-Luciferase Reporter Assay system (Promega, Manheim, Germany), according to the manufacturer's protocol. The Renilla luciferase activities were normalized to the firefly luciferase activities for each individual analysis.

Western blotting. Transfected cells were harvested at 72 h after transfection, and the total protein was extracted using the RIPA lysis buffer (SolarBio, Beijing, China), supplemented with protease inhibitor and phosphorylated proteinase inhibitor (both from Sigma). Equal amounts of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Millipore). After blocking with 5% skimmed milk in TBS/0.1% Tween (TBST), the membranes were incubated with primary antibodies, mouse anti-human monoclonal ADAM9 (sc-377233) and anti-human monoclonal GADPH antibodies (sc-166574) (both from Santa Cruz Biotechnology, Santa Cruz CA, USA) at 4˚C overnight. Subsequently, the membranes were washed with TBST, and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The signals on the membranes were detected by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA). The integrated density of the band was quantified by Quantity One software (Bio-Rad).

Statistical analysis. Data are expressed as mean ± SD. Statistical analyses were performed using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to be statistically significant.

Results

miR-140 is downregulated in glioma tissues and cell lines. To explore whether miR-140 was differentially expressed in glioma, its expression levels were measured in glioma and normal brain tissues. The results showed that miR-140 was significantly downregulated in glioma tissues compared with the normal brain tissues (Fig. 1A; P<0.05). The expression levels of miR-140 were also determined in six glioma cell lines (U87, U251, U373, U118, A172 and LN18) and in the normal human glial cell line (HEB). Compared with HEB, miR-140 expression levels were reduced in six glioma...
cell lines, particularly in U251 and A172 (Fig. 1B, P<0.05). Accordingly, U251 and A172 cells were used in the subsequent experiments.

Association of miR-140 expression with clinicopathological characteristics of human gliomas. We then explored whether the expression levels of miR-140 were correlated with clinicopathological characteristics of gliomas. As shown in Table I, a significant relationship was observed between miR-140 expression and WHO grade (P=0.002) and KPS scores (P=0.024) of glioma patients. No statistically significant association of miR-140 expression with other clinicopathological factors,
including gender, age and extension of resection was found (both P>0.05; Table I).

**Upregulation of miR-140 inhibits proliferation, migration and invasion of glioma cells.** To further investigate the roles of miR-140 in glioma cells, U251 and A172 cells were transfected with miR-140 mimics or NC. Using qRT-PCR, it was observed that miR-140 expression was markedly increased in miR-140 mimic-transfected U251 and A172 cells compared with NC groups (Fig. 2A; P<0.05).

MTT, Transwell migration and invasion assays, were performed to evaluate the effects of miR-140 on growth and metastasis of glioma cells. The results revealed that restoration of miR-140 expression repressed the growth (Fig. 2B; P<0.05), migratory (Fig. 2C; P<0.05) and invasive (Fig. 2C; P<0.05) capacities of U251 and A172 cells. These results indicated that miR-140 functions as a negative regulator of glioma cell growth and metastasis.

**ADAM9 is a direct target of miR-140 in glioma.** To explore the mechanisms of miR-140-induced cell proliferation, migration and invasion inhibition, we searched for the direct target genes of miR-140. miRanda and TargetScan were used to investigate the direct targets of miR-140. Base-pairing complementation indicated that the 3'UTR of ADAM9 contains a putative binding region of pmirGLO-ADAM9-3'UTR Wt, but not pmirGLO-ADAM9-3'UTR Mut in HEK293T cells. miR-140 overexpression repressed ADAM9 expression in U251 and A172 cells at (C) mRNA and (D) protein levels; *P<0.05 compared with respective control.

![Figure 3. ADAM9 is a direct target gene of miR-140 in glioma.](image-url)
Figure 4. Knockdown of ADAM9 simulates the tumor suppressor functions of miR-140 in glioma cells. (A) qRT-PCR analysis of ADAM9 expression in U251 and A172 cells transfected with ADAM9 siRNA or NC siRNA. (B) Western blot analysis of ADAM9 expression in U251 and A172 cells transfected with ADAM9 siRNA or NC siRNA. MTT, Transwell migration and invasion assays showed that ADAM9 siRNA inhibited U251 and A172 cell (C) proliferation, (D) migration and invasion; *P<0.05 compared with respective control.
Knockdown of ADAM9 simulates the tumor suppressor functions of miR-140 in glioma cells. ADAM9 was validated as a direct target gene of miR-140 in glioma. Therefore, we wondered whether knockdown of SOX9 could simulate the tumor suppressor functions of miR-140 in glioma cells. After transfection with ADAM9 siRNA or NC siRNA, qRT-PCR and western blotting were performed. The results showed that ADAM9 was significantly downregulated in U251 and A172 cells transfected with ADAM9 siRNA at mRNA (Fig. 4A; P<0.05) and protein (Fig. 4B; P<0.05) levels.

Following, MTT, Transwell migration and invasion assays, we showed that ADAM9 siRNA inhibited U251 and A172 cell proliferation (Fig. 4C; P<0.05), migration and invasion (Fig. 4D; P<0.05), which were similar with those induced by miR-140 overexpression in U251 and A172 cells. These results suggested that ADAM9 contributed to the tumor suppressor functions of miR-140 in glioma.

Overexpression of ADAM9 partially rescues miR-140-mediated suppressive functions in glioma cells. To determine whether ADAM9 overexpression could reverse the tumor-suppressor functions of miR-140 in glioma, a series of rescue experiments were performed. After transfection with pCDNA3.1-ADAM9, high expression levels of ADAM9 were determined by qRT-PCR (Fig. 5A; P<0.05) and western blotting (Fig. 5B; P<0.05). Subsequently, MTT, Transwell migration and invasion assays were performed. The results revealed that restoration of ADAM9 partially rescued miR-140-mediated suppressive functions in U251 and A172 cell proliferation (Fig. 5C; P<0.05), migration and invasion (Fig. 5D; P<0.05). These results further indicated that ADAM9 was a direct target gene of miR-140 and that miR-140 inhibited glioma cell proliferation, migration and invasion, at least in part through negatively regulation of ADAM9 expression.

Discussion

Glioma is among the most aggressive and lethal neurological human cancers (21). Currently, the clinical therapeutic effects of gliomas treated with modern microsurgery, radiotherapy, chemotheraphy and other comprehensive therapeutic treatments are not satisfactory (22). Hence, elucidation of the molecular mechanisms underlying initiation and progression of glioma is crucial for investigating effective therapeutic strategies for patients with glioma. Recently, a great deal of miRNAs have been demonstrated to play important roles in glioma occurrence and development (23-25). Moreover, the abnormal expression of miRNAs contributes to the malignant biological behavior of glioma, such as proliferation, cell cycle, invasion, migration, metastasis, apoptosis inhibition, and chemotherapy resistance (26-28). Therefore, the investigations of miRNAs and mRNAs targeted by those miRNAs are likely to provide not only new insight into understanding the development of the glioma, but also potential therapeutic strategies.

In the present study, we focused on investigating the expression levels of miR-140 in glioma, correlation between miR-140 expression and clinicopathological characteristics, and roles of miR-140 in glioma progression. The main findings of the present study were as follows: first, miR-140 was downregulated in glioma tissues and cell lines compared with that in normal brain tissues and the normal human glial cell line, respectively. Second, the low levels of miR-140 were correlated with WHO grade and KPS scores of gliomas. Third, restoration of miR-140 significantly repressed glioma cell proliferation, migration and invasion in vitro. Fourth, ADAM9 was identified as a direct target gene of miR-140 in glioma. miR-140 inhibited glioma cell proliferation, migration and invasion, at least in part through negative regulation of ADAM9 expression.

Numerous studies have reported that miR-140 was frequently deregulated in various types of cancers. Reduced expression levels of miR-140 has been demonstrated in pancreatic duct adenocarcinoma (29), lung (30,31), colorectal (32), ovarian (33), esophageal (34) and tongue cancer (35). However, miR-140 also has been found upregulated in spinal chordoma (36) and breast cancer (37). These studies suggested that expression of miR-140 has tissue specificity. miR-140 expression was demonstrated to be correlated with clinicopathological characteristics of cancers. For example, in spinal chordoma, miR-140 expression levels were positively associated with surrounding muscle invasion. The Kaplan-Meier survival analysis revealed that the patients with high miR-140 expression had a significantly worse recurrence-free survival than those with a low expression. In addition, univariate and multivariate analyses for recurrence-free survival showed that miR-140 expression was an independent prognostic factor for patients with spinal chordoma (36). Gullu et al reported that miR-140 expression levels were correlated with metastasis of breast cancer patients (37). In hepatocellular carcinoma, its expression levels were correlated with multiple nodules, vein invasion, capsular formation and differentiation, as well as overall and disease-free survival (38). These findings indicated that miR-140 could be a diagnostic and prognostic biomarker for human cancers.

Changes in miR-140 expression have been verified to contribute to the initiation and progression of cancers. Yuan et al showed that miR-140 inhibited non-small cell lung cancer cell proliferation, migration and invasion in vitro. Moreover, miR-140 overexpression suppressed non-small cell lung cancer growth and metastasis in vivo (39). Liang et al found that ectopic of miR-140 expression decreased pancreatic duct adenocarcinoma cell growth and invasion (29). Zhang et al demonstrated that miR-140 acted as a tumor suppressor in colorectal cancer by inhibiting cell proliferation, migration and invasion (32). In ovarian cancer, enforced miR-140 expression repressed cell proliferation and enhanced cells apoptosis (33).

In esophageal cancer, miR-140 underexpression induced epithelial-mesenchymal transition and promoted cell invasion (34). In hepatocellular carcinoma, overexpression of miR-140 suppressed cells growth and metastasis (38). These studies indicated that miR-140 acted as a tumor-suppressor, and restoration of miR-140 expression may be a potential therapeutic strategy for cancer treatment in the future.

Thus far, several target genes of miR-140 have been validated, including iASPP (29), ATP6AP2 (30), ATP8A1 (31), VEGFA (32), PDGFA (33), Slug (34) and IGF-1R (39). However, no target of miR-140 has been identified in glioma. The present study verified that the roles of miR-140 on glioma cells were possibly via negative regulation the expression
Figure 5. Restoration of ADAM9 rescues miR-140-mediated changes in proliferation, migration and invasion of glioma cells. (A) qRT-PCR analysis of ADAM9 expression in U251 and A172 cells transfected with pCDNA3.1-ADAM9 or blank vector pCDNA3.1. (B) Western blot analysis of ADAM9 expression in U251 and A172 cells transfected with pCDNA3.1-ADAM9 or blank vector pCDNA3.1. Restoration of ADAM9 partially rescued miR-140-mediated suppressive functions in U251 and A172 cells (C) proliferation, (D) migration and invasion; *P<0.05 compared with respective control.
of its novel identified target, ADAM9. A disintegrin and metalloproteinase (ADAMs) are members of the metzincin superfamily of matrix metalloproteinases (40). ADAM9, a membrane of ADAMs, contains of an N-terminal prodomain followed by a metalloprotease and a disintegrin domains, and cysteine-rich region, an epidermal growth factor repeat, a transmembrane domain, and a cytoplasmic tail with potential SH3 ligand domains (41,42). Accumulated evidence has been reported on the upregulation of ADAM9 in human cancers, such as renal cell (43), prostate (44) and breast cancer (45), hepatocellular carcinoma (46), and pancreatic cancer (47). In glioma, ADAM9 is also upregulated and plays a major role in glioma invasion and migration (48,49). Therefore, ADAM9 is considered a valuable therapeutic target for human cancers. In the present study, we found that miR-140 targeted ADAM9 to inhibit glioma cell proliferation, migration and invasion. miR-140/ADAM9 based targeted therapy may be a promising therapeutic method for gliomas.

Taken together, our results suggested that miR-140 expression was frequently downregulated in glioma tissues and cell lines, and that low expression levels of miR-140 correlated with WHO grade and KPS scores. In addition, we demonstrated that miR-140 acted as a tumor suppressor in glioma, by inhibiting cell proliferation, migration and invasion. Furthermore, ADAM9 was identified as a novel target gene of miR-140 in glioma. miR-140 may be a potential therapeutic target for drug development in treating gliomas.

References


