miR-96 inhibits EMT by targeting AEG-1 in glioblastoma cancer cells

SHIYU FENG1*, JIE YAO2*, ZHIBIN ZHANG1, YANYANG ZHANG1, ZHIYUAN ZHANG1, JIALIN LIU1, WENLONG TAN2, CAIHONG SUN1, LING CHEN1 and XINGUANG YU1

Departments of 1Neurosurgery and 2Oncology, Chinese People’s Liberation Army General Hospital, Beijing 100036, P.R. China

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Abstract. The induction of epithelial to mesenchymal transition (EMT) is important for carcinogenesis and cancer progression. Previous studies have estimated that microRNA (miRNA/miR) expression is associated with EMT via the regulation of the expression of target genes. miR-96 has been reported to exhibit a correlation with the EMT process. However, the functional role of miR-96 and its mechanism in glioblastoma multiforme (GBM) remains to be completely elucidated. The objective of the present study was to investigate the functional role and mechanism of miR-96 in the migration and invasion, in addition to proliferation, apoptosis and cell cycle distribution, of GBM. In the present study, the results suggested that the introduction of miR-96 significantly inhibited the migration and invasion, in addition to proliferation and cell cycle progression, of GBM cells and promoted their apoptosis in vitro, leading to the hypothesis that miR-96 may be a potential tumor suppressor. It was subsequently confirmed that astrocyte elevated gene-1 (AEG-1) was a direct target gene of miR-96, using a luciferase assay and reverse transcription-quantitative polymerase chain reaction analysis, in addition to western blotting. miR-96 was observed to downregulate the expression of AEG-1 at the mRNA and protein levels. Notably, AEG-1 may suppress EMT by increasing the expression levels of E-cadherin, an epithelial marker, and decreasing the expression levels of vimentin, a mesenchymal marker. Therefore, it was concluded that miR-96 may impede the EMT process by downregulating AEG-1 in GBM. Additionally, it was observed that inhibition of AEG-1 led to a similar effect compared with overexpression of miR-96 in GBM. In conclusion, the results of the present study demonstrated that miR-96 may act as a tumor suppressor by regulating EMT via targeting of AEG-1, suggesting that miR-96 may be a potential biomarker and anticancer therapeutic target for GBM in the future.

Introduction

Glioblastoma multiforme (GBM), also termed glioblastoma, is a type of brain tumor derived from star-shaped glial cells termed astrocytes, which support and protect neural tissues in brain and spinal cord (1-3). It is well-acknowledged that GBM is one of the most lethal and difficult to treat types of human cancer due to its histological heterogeneity, aggressive invasion and poor response to treatment (4). Despite advances in research into the molecular mechanisms underlying GBM occurrence and development, treatment and prognosis remain poor and require further investigation.

Previous studies have demonstrated that the induction of epithelial to mesenchymal transition (EMT) is of importance for carcinogenesis and cancer progression (5,6). EMT is an important process at the cellular level, wherein epithelial cells lose their polarity and are converted to more motile and invasive mesenchymal phenotypes, thereby resulting in tumor progression (7). Evidence has suggested that the expression of microRNAs (miRNAs/miRs) may be associated with EMT via the regulation of certain target genes (8,9). miRNAs, a class of small non-coding RNAs (20-22 nt in length) may bind to the 3'-untranslated region (3'-UTR) of their target genes, thereby regulating gene expression. Previous studies have demonstrated that miRNAs are involved in the regulation of the expression of genes associated with development, differentiation, proliferation and apoptosis (10,11). Notably, miR-96, miR-182 and miR-183 are known to be associated with EMT (12). In particular, it has been reported decreased expression of miR-96-5p may be associated with poor clinical outcomes for patients with colorectal cancer (13), which provides a basis for further investigation.

Astrocyte elevated gene-1 (AEG-1), also known termed metadherin, was first identified to be a human immunodeficiency virus-1- and tumor necrosis factor-α-inducible late response gene in human fetal astrocytes (14). Recent findings have suggested that AEG-1 may serve a dominant role in the development and progression of multiple types of cancer,

Correspondence to: Dr Xinguang Yu or Dr Ling Chen, Department of Neurosurgery, Chinese People’s Liberation Army General Hospital, 28 Fuxing Road, Beijing 100036, P.R. China
E-mail: yuxinguang1682@163.com
E-mail: chenling16913@163.com

*Contributed equally

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including glioma (15), pancreatic ductal adenocarcinoma (16), cervical carcinoma (17), colorectal cancer (18) and hepatocellular carcinoma (19). In addition, AEG-1 activates various signaling factors involved in mediating EMT, including sonic hedgehog protein (20), transforming growth factor-β (21), neurogenic locus notch homolog protein 1 (22) and protein Wnt (23). For GBM, according to a previous bioinformatics study, AEG-1 is an underlying target gene for miR-96 (Feng et al, unpublished data). However, the functional role of miR-96 and its mechanism in GBM remain poorly understood. Therefore, the identification of novel miR-96 targets may provide novel insights into the molecular mechanism underlying the miR-96-induced suppression of tumorigenic properties in cancer cells.

The present study investigated the functional role and mechanism of miR-96 in the migration and invasion, proliferation, apoptosis and cell cycle progression of GBM. In vitro approaches were used to explore whether AEG-1 may be a direct target gene of miR-96, and whether miR-96 serves a role in EMT by regulating AEG-1.

Materials and methods

Cell culture. The human glioma cell line U251 was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 mg/ml), in a humidified atmosphere containing 5% (v/v) CO₂ at 37°C, for 18 h prior to transfection.

Transfection. The human glioma cells were seeded in different culture plates (6, 24, 48 or 96-well) and cultured for 18 h prior to transfection. Transfection was performed in U251 cells using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA with no homology to any human genomic sequence was regarded as negative control (miR-NC). The miRNA mimics and small interfering (si)RNA sequences were designed and synthesized by View Solid Biotech Co., Ltd. (Beijing, China). Three siRNAs (si-AEG1-744, si-AEG1-1432 and si-AEG1-1883) were used in the present study. All forward and reverse sequences are listed in Table I.

Cell proliferation assays. The proliferative abilities of transfected cells were assessed using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), following the manufacturer's protocol. U251 cells were seeded at a density of 3,000 cells/well in 96-well culture plates and cultured at 37°C for 18 h prior to transfection. Subsequently, U251 cells were transfected with miR-96 mimics and the corresponding miR-NC, in addition to si-AEG1 and si-NC. Cells were harvested 48 h post-transfection. Lipofectamine RNAiMAX was added at a density of 1.5 µl/well and either miR-96 mimic or si-AEG1 was then added (15 µM/well). Cells were harvested 48 h post-transfection. Following the manufacturer's protocol, 2x10⁴ cells with 100 µl serum-free DMEM (Thermo Fisher Scientific, Inc.) were seeded into the upper chamber of the Transwell plates (Costar; Corning Incorporated, Corning, NY, USA) for the migration assays, while cells with 100 µl serum-free DMEM were plated into the upper chamber of an insert coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) for the invasion assays. The lower chambers were filled with 600 µl DMEM containing 10% FBS. Following 48 h of incubation, the cells remaining on the upper membrane were removed with cotton swabs, whereas those that had migrated or invaded through the membrane were fixed in 4% polyformaldehyde and stained with 0.1% crystal violet for 20 min at 4°C. The number of cells was calculated to photograph five random fields/fields using the fluorescence inversion microscope system (Nikon Corporation, Tokyo, Japan) at a magnification of x200. All experiments were performed at least three times independently.

Cell cycle and apoptosis assays. U251 cells were seeded in six-well culture plates at a density of 5x10⁴ cells/well and cultured for 18 h prior to transfection. Subsequently, U251 cells were transfected with miR-96 mimics and the corresponding miR-NC, in addition to si-AEG1 and si-NC. Cells were harvested 48 h post-transfection. Lipofectamine RNAiMAX was added at a density of 7.5 µl/well and either miR-96 mimic or si-AEG1 was then added (75 µM/well). For the cell cycle assay, cells were stained with propidium iodide (PI) using a Cell Cycle kit (BD Biosciences), according to the manufacturer's protocol. The cell cycle distribution was analyzed using flow cytometry (BD Biosciences). For the cellular apoptosis assay, cells were double-stained with Annexin V and PI using Roche Annexin V/PI kits (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. The apoptotic cells were detected using a flow cytometer (BD FACSCalibur; BD Biosciences) and analyzed using BD CellQuest Pro software (version 5.1; BD Biosciences). All experiments were performed three times, independently.

Plasmid construction. A fragment of the AEG-1 3'-UTR (AEG1-U1) and a mutated 3'-UTR of AEG-1 (AEG1-U1-Mut) that contained the putative miR-96 binding sites were prepared for constructing the reporter plasmids. In addition, DNA fragments were cloned downstream of the luciferase gene in the pGL3-REPORT luciferase vector (Promega Corporation, Madison, WI, USA). Primers used for the constructions are listed in Table I. All the constructions were confirmed via sequencing (BGI, Shenzhen, China).
Luciferase assays. For the luciferase assays, cells were seeded into 48-well plates at a density of 5x10^4 cells/well for 24 h and co-transfected with the experimental group (100 ng AEG1-U1, 100 ng hsa-mir-96-5p mimics and 5 ng Renilla) or the control group (100 ng AEG1-U1, 100 ng hsa-mir-96-5p mimics empty vectors and 5 ng Renilla). Transfection was performed using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Cells were harvested at 48 h and lysed using lysis buffer (Promega Corporation). The luciferase reporter gene assay was implemented using a dual-luciferase reporter assay system (Promega Corporation), according to the manufacturer’s instructions. Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. All experiments were performed at least three times.

Western blot analysis. The human glioma U251 cells were lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc.) in the presence of protease inhibitors at 4°C for 1 h. A total of 20 µg of protein was loaded per lane. The protein lysates were separated by electrophoresing on 10% SDS-PAGE gels, and the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Prior to incubation, the PVDF membranes were blocked with 5% non-fat dried milk at room temperature for 1 h. Subsequently, the PVDF membranes were washed three times with TBS-Tween 20 (25 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 0.1% Tween 20) following incubation with the following primary antibodies at 4°C overnight: AEG-1 (cat. no. ab32081; 1:500; Abcam, Cambridge, UK) tubulin (cat. no. ab6046; 1:5,000; Abcam) β-actin (cat. no. ab8229;
miR-96 inhibits migration, invasion, proliferation and cell cycle progression in U251 cells, and promotes their apoptosis.

In order to evaluate the effect of miR-96 on the migration, invasion, proliferation and cell cycle of U251 cells, miR-96 mimics or miR-NC were transfected into U251 cells in vitro. As presented in Fig. 1A and B, it was observed that miR-96 notably decreased the migratory and invasive abilities of U251 cells in vitro, demonstrated by the Transwell assay (P<0.01). In addition, the CCK-8 assay demonstrated that the proliferative ability of U251 cells transfected with miR-96 mimic was decreased compared with U251 cells transfected with miR-NC. The results demonstrated that, post-transfection, miR-96 markedly decreased the proliferation of U251 cells, particularly at 4-6 days post-transfection (Fig. 1C). It may be concluded from Fig. 1E (P<0.05) that miR-96 markedly suppressed the cycle progression of U251 cells and led to cell cycle arrest at G1 phase, as demonstrated by the flow cytometry analysis.

AEG-1 acts as a target for miR-96 in GBM cells. Putative target genes of miR-96 in human cells were screened using the tools miRNA.org, TargetScan version 6.2 and RNA22, aiming to detect the molecular inhibition mechanisms of miR-96 in the metastatic progression of GBM cells (Feng et al, unpublished data). AEG-1 was identified to be a target gene of miR-96; AEG-1 expression in GBM was increased compared with the other predicted candidates. In addition, AEG-1 expression was observed to increase as the tumor grade of GBM increases. As presented in Fig. 2A, there exists one predicted binding site in miR-96 which corresponds to the 3'-UTR of AEG-1, which was inserted into the luciferase reporter gene plasmid pGL3-3'-UTR following mutation to AEG1-U1. Subsequently, AEG1-U1 and AEG1-U1-mut were respectively co-transfected with the reporter gene plasmid has-mir-96-5p into U251 cells. The luciferase reporter assay demonstrated that miR-96 significantly decreased the luciferase activity of the co-transfected U251 cells (P<0.05) compared with NC (Fig. 2B), indicating that the 1809-1831 gene sequence of AEG-1 is the target of has-mir-96-5p. The luciferase activity of has-mir-96-5p co-transfected with AEG1-U1-mut was almost stable (Fig. 2C).

miR-96 downregulates the expression of AEG-1 to inhibit EMT in GBM. In order to analyze the association between miR-96 and AEG-1, U251 cells were transfected with miR-96 mimics or miR-NC, and analyzed using western blotting and RT-qPCR analysis. Following transfection, the amount of AEG-1 mRNA was significantly decreased compared with control mRNA as demonstrated by RT-qPCR analysis (Fig. 3A). Western blot analysis revealed that the expression of AEG-1 protein was significantly downregulated in U251 cells transfected with miR-96, compared with miR-NC (Fig. 3B; P<0.05). These results suggested that miR-96 may negatively regulate the expression of AEG-1 at the mRNA and protein levels. Notably, previous research has demonstrated that miR-96 can enhance EMT in tumor cells (25). Therefore, U251 cells were transfected with si-AEG1 or si-NC to detect...
the expression levels of E-cadherin and vimentin, in order to further identify whether AEG-1 serves a role in EMT.

As depicted in Fig. 3C, when compared with corresponding si-NC, the expression of vimentin decreased, while that of
E-cadherin markedly increased, in U251 cells transfected with si-AEG1. The results suggested that AEG-1 may inhibit the EMT process. In conclusion, miR-96 may downregulate the expression of AEG-1 to inhibit EMT in GBM.

Inhibition of AEG-1 exerted a similar impact to that of miR-96 overexpression. Aiming to investigate the impact of AEG-1 on U251 cell migration, invasion, proliferation, apoptosis and cell cycle progression, we transfected si-AEG1 or si-NC into U251 cells, respectively. As presented in Fig. 4A, RT-qPCR analysis and western blotting were performed to confirm that si-AEG1 was able to significantly attenuate the mRNA and protein expression levels of AEG-1 in U251 cells (P<0.01). The results suggested that the siRNA efficiency of AEG-1 was most significant at the 744 and 1883 sites in U251 cells. Therefore, these two siRNAs were combined in the following experiments to obtain the best interfering effect (Fig. 4A). In addition, the inhibition of AEG-1 exerted a similar impact to that of miR-96 overexpression in the GBM cells, markedly repressing the cell migration, invasion, proliferation and cell cycle progression of U251 cells and promoting their apoptosis in vitro (Fig. 4B-F).

Discussion

miRNAs serve important roles in regulating gene expression, inhibiting the translation of target genes by recognizing binding sites in the mRNA 3'-UTR. It has previously been reported that aberrantly-expressed miRNAs were associated with cell cycle distribution, cellular migration and invasion, cellular apoptosis and other processes in a variety of types of tumor (10,11). miRNA may be regarded as a novel prognostic marker in tumors. It was reported that miR-19a promoted cell proliferation and invasion by targeting RhoB in human glioma cells (26). An additional report demonstrated that microRNA-198 expression was decreased and exhibited prognostic value in human glioma (27). Notably, the effect of decreased expression of miR-96-5p on poor survival in colorectal cancer patients was investigated (13). Similarly, it
Figure 4. Inhibition of AEG-1 exerts a similar effect to that of miR-96 overexpression. (A) Reverse transcription-quantitative polymerase chain reaction and western blot analyses confirmed that si-AEG1 was able to significantly inhibit AEG-1 expression at the mRNA and protein levels in U251 cells, and that the siRNA efficiency of AEG-1 was most marked at the 744 and 1883 sites in U251 cells. AEG-1 repressed the (B) cell migration, (C) invasion, (D) proliferation, (E) apoptosis and (F) cell cycle progression of U251 cells in vitro. *P<0.05; **P<0.01 vs. NC group. siRNA, small interfering RNA; NC, negative control; AEG-1, astrocyte elevated gene-1; miR, microRNA; OD, optical density; FITC, fluorescein isothiocyanate; PI, propidium iodide.
has been reported that increased expression of miR-96 was may be associated with advanced stages of colorectal adenocarcinoma, and it may predict an increased risk of disease recurrence and poor overall survival (28). These previous results notwithstanding, the functional role of miR-96 and its mechanism in tumorigenesis remain largely unknown. In the present study, the functions of miR-96 in the metabolism of GBM cells were analyzed.

In the present study, it was observed that miR-96 expression was associated with various biological processes, including cell migration, invasion, proliferation and the cell cycle. Therefore, it was hypothesized that miR-96 may be a type of miRNA that acts as a tumor suppressor in human malignancies and serves a regulatory role in the occurrence and developmental processes of human tumors. In order to investigate the functional mechanisms of miR-96, its target genes were screened using target gene prediction software, since the biological functions of miRNAs depend on their downstream target genes. Out of all predicted candidates, AEG-1 was highlighted due to the fact that it serves important diagnostic and prognostic roles in multiple malignant tumors. Subsequently, a luciferase assay was performed to confirm that AEG-1 was a direct target gene of miR-96. AEG-1 has been proven to exert functions in the development of various types of cancer, including liver cancer (19,29), breast cancer (30), colorectal cancer (18,31), lung cancer (32) and ovarian cancer (33). It has been reported that C-C motif chemokine 3/C-C chemokine receptor type 5-induced EMT may be regulated by AEG-1 via extracellular signal-regulated kinase 1/2 and RAC-α serine/threonine protein kinase signaling in cardiac myxoma (34). In addition, a study analyzed the role of miR-302c-3p in suppressing the invasion and proliferation of glioma cells by downregulating AEG-1 expression (15). The present study suggested that miR-96 is likely to repress the EMT process by down-regulating the mRNA and protein expression levels of AEG-1. In addition, it was observed that the inhibition of AEG-1 and overexpression of miR-96 exerted similar effects on GBM cell migration, invasion, proliferation and cell cycle progression. The results of the present study suggested that miR-96 is likely to regulate the expression of AEG-1 to suppress EMT, leading to an inhibition of GBM cell migration, invasion, proliferation and cycle progression, and a promotion of apoptosis.

In conclusion, miR-96 may be a novel tumor-suppressing miRNA. miR-96 may suppress EMT by downregulating AEG-1, resulting in an inhibition of the metabolism of GBM cells in vitro. The results of the present study provide a novel insight into the occurrence and development of GBM. In addition, miR-96 may be a potential therapeutic target for the treatment of glioma.

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