Astrocyte elevated gene-1 serves as a target of miR542 to promote glioblastoma proliferation and invasion

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Abstract

Background: Epithelial to mesenchymal transition (EMT) is strongly linked with tumor invasion and metastasis, which performs a vital role in carcinogenesis and cancer progression. Emerging evidence suggests that microRNAs (miRNAs) expression are closely associated to EMT by regulating targeted genes. MiR542 has been found to be involved in the EMT program and bound up with various cancers. However, the functions of miR542 and its underlying mechanism in glioblastoma multiforme (GBM) remain largely unknown. In the current study, we investigated the effect of astrocyte elevated gene-1 (*AEG-1*) on U251 cells aggressiveness, proliferation, apoptosis, and cell cycle.

Methods: The screening of targeted miRNAs was performed, as well as the functional roles and mechanisms of miR542 were explored.

Results: MiR542 was selected as the target because of the most significantly differential expression and this high level of expression negatively correlated with cell migration and proliferation, which suggested that miR542 could be a novel tumor suppressor. Moreover, we confirmed that *AEG-1* was a direct targeted gene of miR542 by luciferase activity assay, reverse transcription-polymerase chain reaction, and immunoblotting analysis. Furthermore, miR542 suppressed the expression of *AEG-1*, which upgraded the level of E-cadherin and degraded Vimentin expression contributing to retraining EMT.

Conclusion: The *in vitro* findings demonstrated that miR542 inhibited the migration and proliferation of U251 cells and suppressed EMT through targeting *AEG-1*, indicating that miR542 may be a potential anti-cancer target for GBM.

Keywords: Glioblastoma; MiR542; *AEG-1*; Epithelial-mesenchymal transition

Introduction

Glioma is the most common malignant neoplasm originating from central nervous system, accounting for about 40% to 60% of intracranial tumors.[1] Glioblastoma multiforme (GBM), accounting for about half of glioma, is a multigene-related disease and caused by abnormal regulation of gene networks which maintains stable cellular normality and differentiation.[2,3] Recently, a comprehensive therapeutic strategy for GBM is popular combining tumor resection with post-operative chemoradiotherapy. However, the high recurrence rate and unsatisfactory conventional chemotherapy are still important issues to perplex clinicians.[4]

Epithelial to mesenchymal transition (EMT) refers to the phenomenon that epithelial cells transit into mesenchymal cells in special physiological and pathological conditions.[5] Accumulated researches have shown that EMT is closely related to tumor invasion and metastasis.[6,7] The microRNA (miRNA) has been reported to produce the effects on EMT process by regulating targeted genes.[8,9] MiRNAs are a class of small, 20 to 22 nucleotides (nt) non-coding RNAs, which can regulate the expression of different genes by binding to the specific 3'-untranslated region (3'-UTR).[10] They are implicated widely in the regulation of gene expression for development, differentiation, and apoptosis.[11,12] Notably, the miR590, miR182, and miR183 are well-known to contribute to EMT.[13] Furthermore, recent studies have shown that miR542 not only functioned importantly in the development of human neuroblastoma and colon cancer,[14,15] but also in the suppression of astrocytoma.[16]

Astrocyte elevated gene-1 (*AEG-1*), also known as the metadherin, was identified as a human immunodeficiency virus-1- and tumor necrosis factor-α-inducible late

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000001072

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Chinese Medical Journal 2020;133(20)

Received: 11-07-2020 **Edited by:** Yan-Jie Yin and Xiu-Yuan Hao

response gene in human fetal astrocytes for the first time.[17] *AEG-1* functions as a key regulator to promote tumor invasion and metastasis and inhibit apoptosis by activating a wide array of pathways, such as WNT, TGF-β, and Notch signaling.[18,19] Recent studies have indicated that *AEG-1* plays a dominant role in the development and progression of various cancers, including glioma.[20] Based on our previous study,[21] we found that *AEG-1* was a potential targeted gene for miR542 in GBM. However, the functional role of miR542 and its underlying mechanisms in GBM remain largely unknown. Therefore, the identification of miR542 targets will provide new insights into the molecular mechanism regarding the miR542-induced suppression of tumorigenic properties in cancer cells.

In the current study, we investigated the effects of *AEG-1* on U251 cell aggressiveness, proliferation, apoptosis, and cell cycle. The screening of targeted miRNAs was performed and whether *AEG-1* functioned as a direct targeted gene of miR542 was explored via *in vitro* approaches. Our findings demonstrated that miR542 inhibited the migration and proliferation of U251 cells and suppressed EMT through targeting *AEG-1*.

Materials and Methods

Cell culture

The human GBM cell line U251 was acquired from the American Tissue Culture Collection. Before transfection, cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified atmosphere of 95% (v/v) air and 5% CO_2 at 37°C for 18 h. All procedures were approved by the Research Ethics Committee of our institution.

Transfection

The U251 cells were cultured in six-well plates with a confluence of 5×10^5 cells/well for 18 h before transfection. MiRNA mimics and siRNA sequences were designed and synthesized by View Solid Biotech (Beijing, China). The forward and reverse sequences were designed in our previous work.[21] MiR-NC (a type of RNA with no homology to any human genomic sequence) and candidate targeted miRNAs (miR128, miR520c, and miR542) were transfected into U251 cells with Lipofectamine RNAi-MAX (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Cell proliferation assays

Cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) was used to evaluate the viability of cells following the manufacturer's recommendation. U251 cells were seeded at 3000 cells/well in 96-well culture plates and cultured for 18 h. Then part of cells was transfected with miR128, miR520c, miR542 mimics, and the corresponding negative control (miR-NC). Others were transfected with siRNA targeting *AEG-1* (si-AEG1) and the corresponding negative control siRNA (si-NC). After 48-h transfection, culture medium of each well was replaced with a DMEM medium containing 20% CCK-8 solution. Each group was performed in triplicate. Following 4 days of post-transfection, the optical density was calculated at a wave length of 450 nm using enzyme linked immunosorbent assay (PerkinElmer, Waltham, MA, USA) at regular intervals. The growth curve was portrayed on the basis of the calculated number of viable cells.

Cell migration and invasion assays

Cells were collected after 48-h transfection. Migration assays were conducted by plating 2×10^4 cells with 100 μL serum-free medium into the upper chamber of Transwell system (Costar, Corning Corp, NY, USA). While invasion assays were performed by implanting cells with 100 uL serum-free medium into the upper chamber of the insert coated with Matrigel (BD Bioscience, San Jose, CA, USA). Transwell assays were performed strictly following the manufacturer's protocols. The lower chambers were filled with 600 μL complete medium containing 10% FBS. The cells plated in the upper chambers migrated to the lower chambers over time. After 48 h of incubation, cells remaining on the upper membrane were revoked with cotton swabs, whereas those that had migrated through the membranes were fixed in 4% polyformaldehydel and stained with 0.1% crystal violet. Finally, the number of cells was measured by photographing five random high-power microscopic fields per filter. Each experiment was conducted at least three times, independently.

Cell cycle and apoptosis assays

U251 cells were plated in six-well culture plates and cultured for 18 h before transfection with si-AEG1 plasmids and the corresponding si-NC. Then cells were harvested after 48 h. For the cell cycle analysis, cells were stained by propidiumiodide (PI) using Cell cycle kit according to the manufacturer's protocol prepared for analyzing the cell cycle on the basis of flow cytometer (FCM, BD, San Jose, CA, USA) detection. For the apoptosis assay, cells were double-stained by Annexin-V and PI using Roche kits according to the manufacturer's protocol prepared for analyzing the apoptotic proportion on the basis of FCM detection. Each experiment was repeated three times, independently.

Luciferase assays

For the luciferase assays, cells were seeded into 48-well plates and cultured for 24 h before co-transfection. Then 100 ng AEG1-U1 (Luciferase reporter gene plasmid), and 100 ng hsa-mir-542-3p mimics or 100 ng hsa-mir-542-3p mimics empty vectors were co-transfected into those cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Following the co-transfection for 48 h, cells were harvested and lysed using the lysis buffer (Promega, Madison, WI, USA). Luciferase reporter gene assay was carried out by means of the dual-luciferase reporter assay system (Promega) based on the manufacturer's instructions.[22] Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. The experiments were repeated at least three times.

Western blot analysis

The U251 cell lysates were prepared using M-PER Mammalian Protein Extraction Reagent (Thermo, Canoga Park, CA, USA) supplemented with protease inhibitors at 4°C. One hour later, the protein concentrations were separated by electrophoresing in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Subsequently, the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). After 1 h of blockage with 5% non-fat dry milk at room temperature, the PVDF membranes covered with separated proteins and primary antibodies were incubated at 4°C overnight. Then, the peroxidase-conjugated goat-anti-rabbit secondary antibody was used to go on incubating at room temperature for 2 h after the PVDF membranes were washed thrice with tris buffered saline with tween (25 mmol/L Tris-HCl, 0.2 mol/L NaCl, 0.1% Tween 20, pH 8.0). Finally, the blots were detected in chemiluminescence (Thermo), and protein levels were determined by normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

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-542 binding sites were prepared for constructing reporter plasmids consisting of the 3'-UTR regions of *AEG-1*. DNA fragments were cloned into the downstream of the luciferase gene in the pGL3-REPORT luciferase vector (Promega, Madison, WI, USA). All the constructions were confirmed by sequencing.

RT-PCR

Total RNA was extracted from U251 cells using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then RNA was reverse-transcribed into complementary DNA (cDNA) using reverse transcription system (TAKARA, Osaka, Japan) following the manufacturer's instructions. RT-PCR was conducted using a standard qPCR kit (TransGen Biotech, Beijing, China) protocol on an RT-PCR System (ABI, CA, USA). The reaction volume was 10 μL and the mixture contained 5 μL qPCR kit Premix Ex Taq, 1 μL cDNA, 0.2 μL (10 μmol/L), and 20 μL double distilled H_2O. The reaction went on as follows: 94°C for 1 min, 40 cycles of 94°C for 5 s, and 60°C for 15 s, 72°C for 31 s. The values were normalized by internal control products of GAPDH. All reactions were performed in triplicate.

Statistical analysis

The statistical analysis was performed utilizing SPSS 19.0 (IBM Corp, Somers, NY, USA) and Origin 9.0 (OriginLab Corp, Northampton, MA, USA). Data were presented as the mean ± standard deviation, which were analyzed by Student's *t* test and Mann-Whitney test. The unpaired *t* test was used for comparison between two groups, and comparison of mean values between multiple groups was evaluated by one-way Analysis of Variance followed by Student-Newman-Keuls *post hoc* test. Each reported experiment was repeated at least three times. Statistical significance was analyzed using the two-tailed *t*-test for independent groups, and all tests were determined on a statistically significant result.

Results

The effect of AEG-1 on U251 cells aggressiveness, proliferation, apoptosis, and cell cycle

si-AEG1 or the corresponding negative control si-NC was respectively transfected into U251 cells to investigate the effect of *AEG-1* on U251 cells migration, invasion, proliferation, apoptosis, and cell cycle. As shown in Figure 1A, RT-PCR and Western blot assays confirmed that si-AEG1 could significantly suppress *AEG-1* expression at the mRNA and protein levels in U251 cells ($P < 0.01$), which indicated that siRNA interference efficiency of *AEG-1* was extremely obvious at the 744 and 1883 sites. Therefore, it prompted us to mix two siRNAs in the following experiments to obtain the best interference effects [Figure 1A]. Furthermore, the transwell assay showed that *AEG-1* deficient cells presented the low aggressive ability [Figure 1B and 1C]. With transfection of si-AEG1, the proliferation of U251 cells was dramatically inhibited and the cells were mostly interdicted at G1/S phases [Figure 1D and 1E]. The apoptosis was significantly promoted [Figure 1F] suggesting that AEG1 suppressed the malignant phenotypes.

Screening miRNAs and their functional investigation

MiRNA expression profiles were formed by deep sequencing technology from collected GBM samples to screen the three most differentially expressed miRNAs. With the goal of screening miRNA, cell migration and proliferation assays were conducted by transfecting candidate miRNA mimics or miR-NC into U251 cells. As shown in Figure 2A and 2B, transwell analysis showed that miR542 lessened the migration abilities of U251 cells more obviously than the others *in vitro* ($P < 0.01$). CCK-8 assay proved that all candidate miRNAs markedly suppressed the proliferation of U251 cells [Figure 2C]. Comprehensively, miR542 was selected as our target miRNA.

AEG-1 as a targeted gene of miR542 in U251 cells

Putative targeted genes of miR542 in human cancer cells were predicted via the tools including *miRNA.org*, *TargetScan version_6.2* (http://www.targetscan.org), and *RNA22* (https://cm.jefferson.edu/rna22) in order to investigate the molecular mechanism of miR542. *AEG-1* as one of the predicted candidates was selected as the focus in the current study because of its highest expression in GBM and its positive correlation with tumor grades. As described in Figure 3A, miR542 contained one predicted binding site in the 3'-UTR of *AEG-1* gene, which was structured into luciferase reporter gene plasmid PGL3-3' UTR after being mutated as *AEG1-U1*. Subsequently, AEG1-U1 and AEG1-U1-mut were co-transfected with the mimics of reporter gene plasmid hsa-miR-542-3p into U251 cells, respectively. The former luciferase reporter assay proved that miR542 markedly decreased the luciferase activity of the co-transfected U251 cells ($P < 0.05$) compared with the

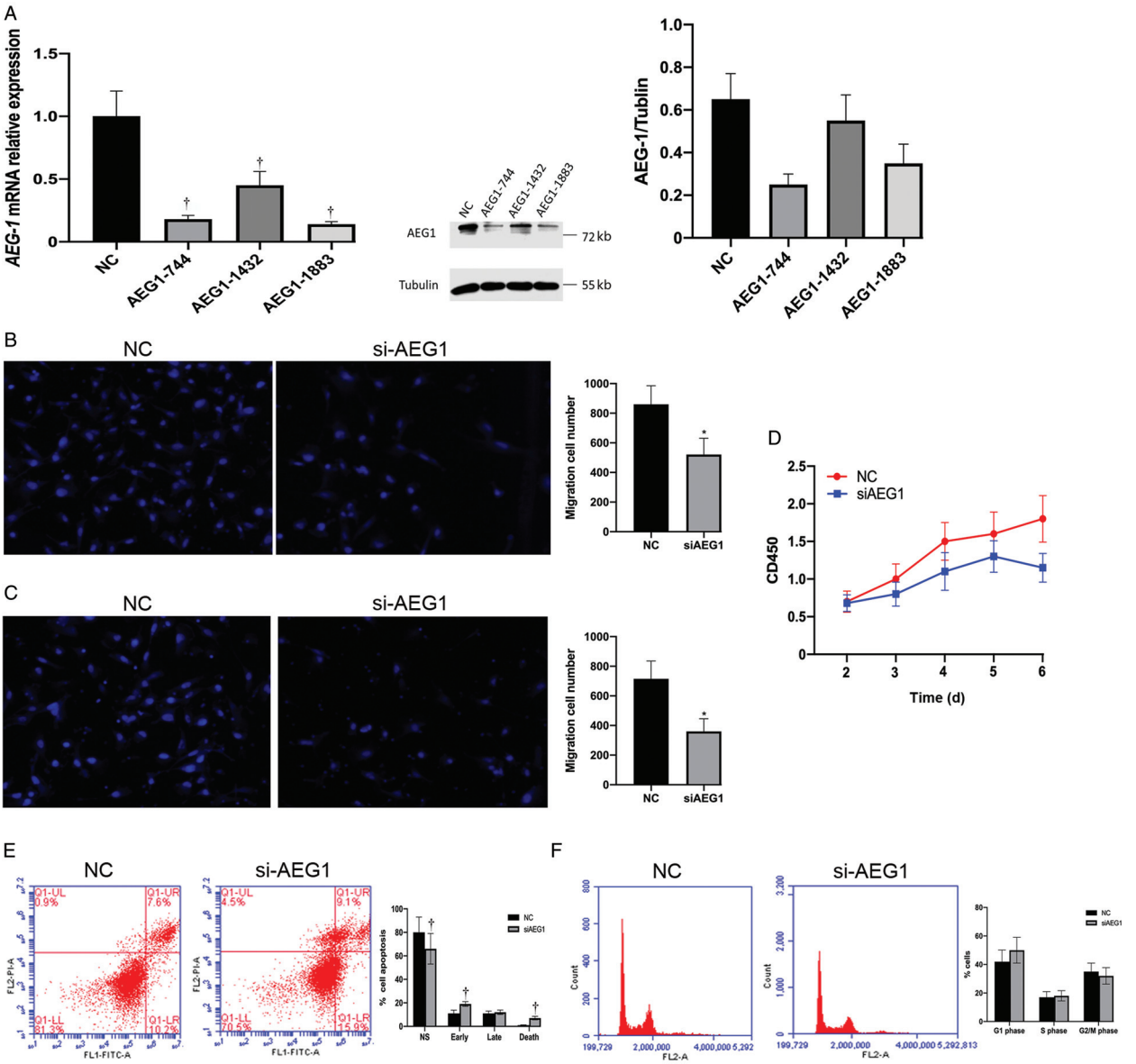

Figure 1: The effect of *AEG-1* on U251 cells migration, invasion, proliferation, apoptosis, and cell cycle. RT-PCR and Western blot assay confirmed that si-AEG1 could significantly suppress *AEG-1* expression at the mRNA and protein levels in U251 cells ($^{\dagger}P < 0.01$), which suggested that siRNA interference efficiency of *AEG-1* was extremely obvious at 744 and 1883 sites in U251 cells (A). *AEG-1* significantly repressed the cell migration (B), invasion (C), proliferation (D), apoptosis (E), and cell cycle (F) of U251 cells *in vitro*. B, C: immunofluorescence staining, original magnification ×200. $^{*}P < 0.05$; $^{\dagger}P < 0.01$ *vs.* the si-NC group; NC, negative control. *AEG-1*: Astrocyte elevated gene-1; mRNA: Messenger RNA.

control group [Figure 3B], suggesting that 2468–2490 gene sequence of *AEG-1* functioned as a targeted spot of hsa-mir-542–3p. However, the latter's luciferase activity remained almost unchanged, further emphasizing on that point of view [Figure 3C].

EMT inhibited by miR542 via down-regulating the expression of AEG-1

MiR542 and miR-NC were transfected into U251 cells to explore the relationship between miR542 and *AEG-1* via the immunoblotting and RT-PCR analysis. U251 cells transfected with miR542 displayed the lower expression of *AEG-1* at both protein and mRNA levels than those transfected with miR-NC [Figure 4A and 4B]. The above-mentioned statements indicated that miR542 negatively regulated the expression of *AEG-1* in U251 cells. Moreover, to investigate whether miR542 produced an effect on EMT process in U251 cells, we transfected U251 cells with si*AEG-1* or si-NC plasmids to detect the expression levels of E-cadherin and Vimentin. As shown in Figure 4C, compared with the corresponding si-NC, Vimentin expression in U251 cells transfected with si*AEG-1* was declined while E-cadherin expression was upgraded, which suggested that *AEG-1* efficiently restrained the EMT process. Taken together, miR542 suppressed the EMT process of U251 cells via down-regulating the *AEG-1* expression.

Discussion

In the recent years, miRNAs have been reported to play the crucial roles in regulating cancer cell cycle, migration and invasion, apoptosis, and other processes.[12] For example, miR451 inhibited glioma cell proliferation and invasion by

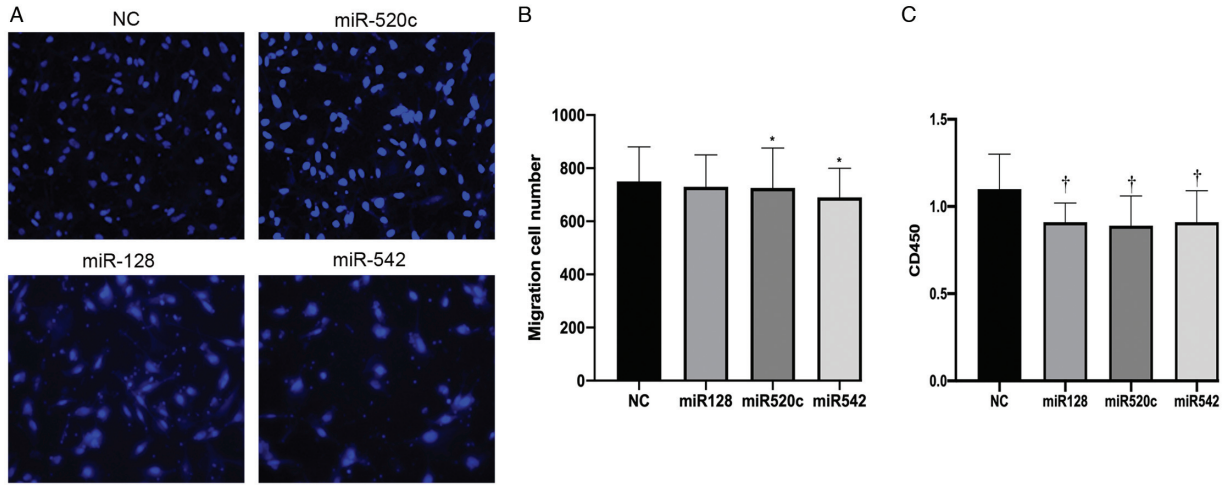

Figure 2: Screening miRNAs and their functional research. Transwell assay was used to evaluate the migration (A and B) abilities of U251 cells transfected with candidate miRNAs mimic or miR-NC. A: immunofluorescence staining, original magnification ×100. Cell counting kit-8 (CCK-8) assay was applied for detecting the proliferation (C) abilities. $^{*}P < 0.05$, $^{\dagger}P < 0.01$ *vs.* the miR-NC group; NC, microRNA-negative control. miRNAs: MicroRNAs.

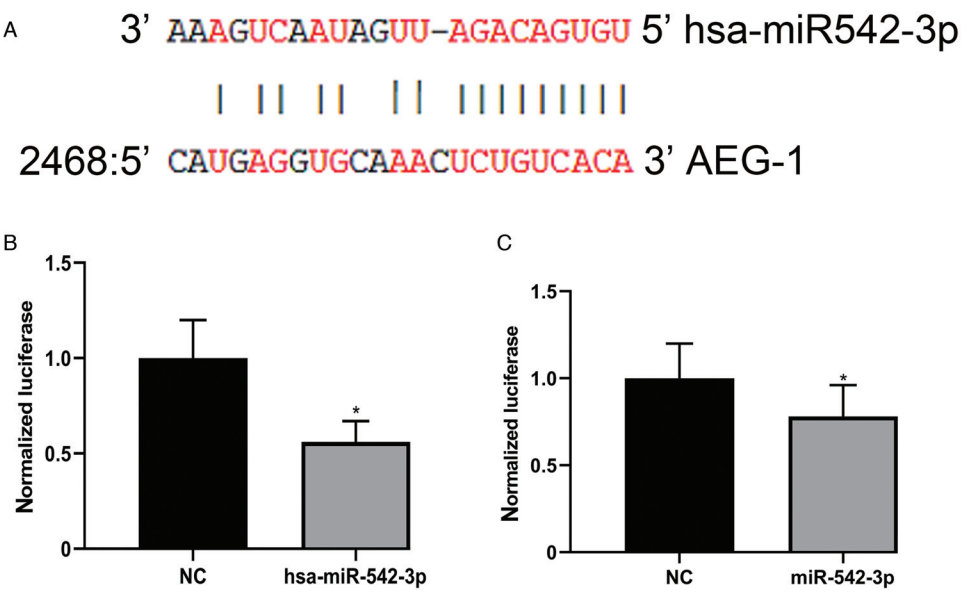

Figure 3: *AEG-1* as a target gene of miR542. Schematic illustration of the *AEG-1* 3'-UTR with one putative binding site for miR542 (A). Luciferase activity assay of AEG1-U1 proved that miR542 markedly decreased the luciferase activity of the co-transfected U251 cell line paralleling with NC, suggesting that 2468-2490 gene sequence of *AEG-1* was confirmed as a target spot of hsa-mir-542-3p (B). AEG1-U1-mut's luciferase activity remained almost unchanged (C). $^{*}P < 0.05$ *vs.* the miR-NC group. NC, microRNA-negative control. 3'-UTR: 3'-Untranslated region; *AEG-1*: Astrocyte elevated gene-1; miRNAs: MicroRNAs.

downregulating the glucose transporter.[23] Another report has demonstrated that the decreased expression of miR198 was considered as a vital prognostic indicator for glioma progression.[23] MiRNAs function as tumor promoters or suppressors in the progression of inducing targeted mRNA degradation or inhibiting the translation to regulate gene expression at post-transcriptional level. A variety of miRNAs are involved in multiple disorders, such as miR542, miRNA200 family, miRNA103/107, and so on. The previous study reported that miRNA200c could significantly suppress tumor EMT process through upregulating the E-cadherin expression.[24] What's more, miR542 has been illustrated to be not only closely associated to the development of neuroblastoma and colon cancer,[14,15] also in the restraining of human astrocytoma progression.[16] In spite of this wealthy data, the functional role of miR542 and its mechanisms in tumor genesis remain largely unknown. In the current study, we revealed the functions of miR542 in metabolism of U251 cells by analyzing the phenotypic data after transfection.

In this study, we investigated the effect of *AEG-1* on the malignant phenotype of U251 cells. Deep sequencing technology from GBM samples contribute to screening the most differentially expressed miRNAs. Afterward, further study was conducted to obtain the most significant down-regulation expression of miRNA in GBM. We found that miR542 expression was strongly involved in various biological processes, including cell migration and proliferation. Therefore, we deduced that miR542 might act as a

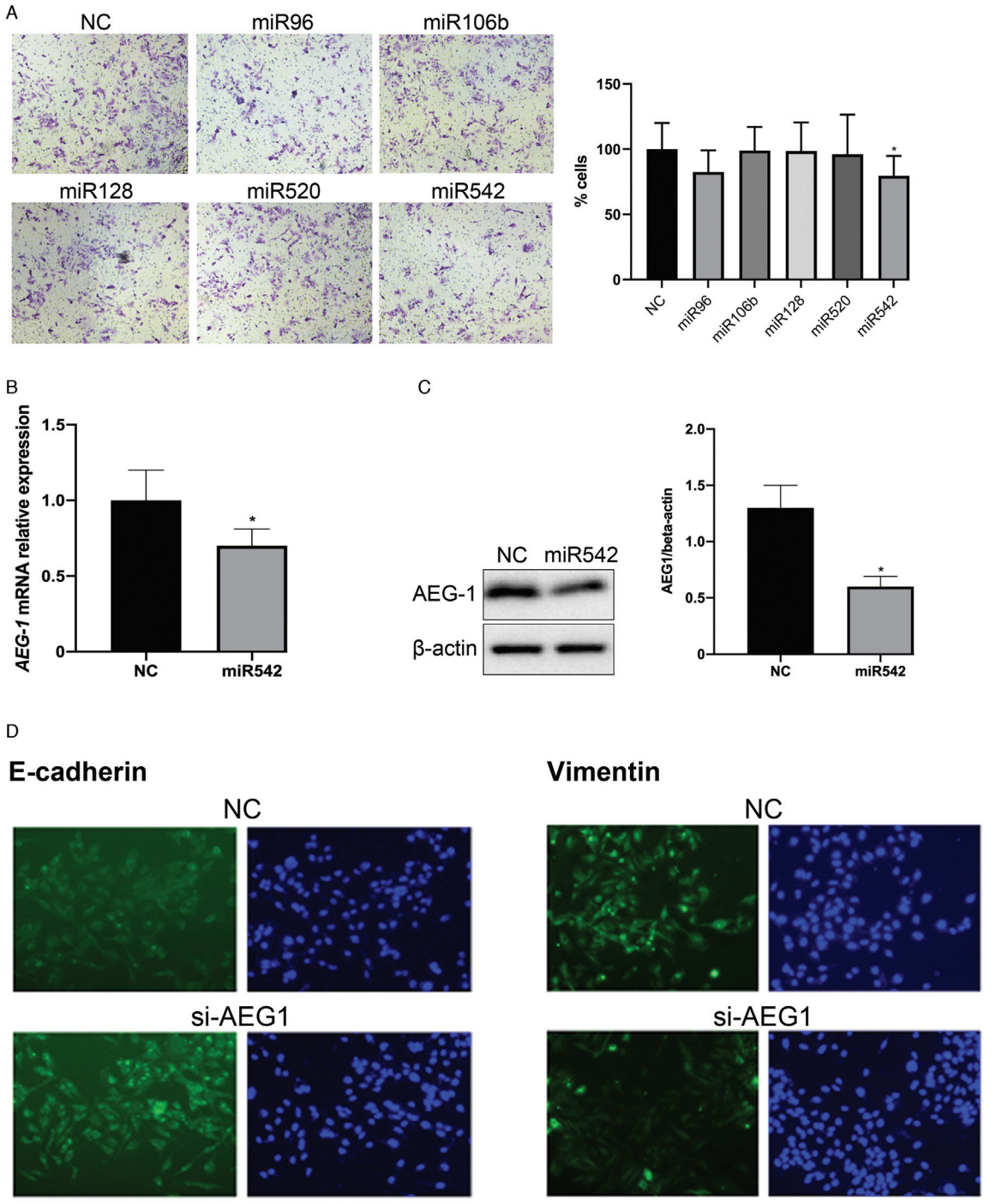

Figure 4: EMT suppressed by miR542 via down-regulating the expression of *AEG-1* in U251 cells. The migration of U251 cells transfected with siNC, miR96, miR106b, miR128, miR520, and miR542 was evaluated by Transwell chamber assay (A). U251 cells transfected with miR-542 displayed less expressions of *AEG-1* both at mRNA (B) and protein levels (C). Compared with corresponding si-NC, Vimentin expression in U251 cells transfected with si-AEG1 was declined while E-cadherin expression was upgraded (D). D: immunofluorescence staining, original magnification ×100. $^{*}P < 0.05$ *vs.* the miR-NC group; NC, microRNA-negative control. *AEG-1*: Astrocyte elevated gene-1; EMT: Epithelial to mesenchymal transition; miRNAs: MicroRNAs.

tumor suppressor in human malignancies and play a regulatory role in the occurrence and development of cancers. Aiming at exploring the functional mechanism of miR542, we screened its targeted genes by the internet opening tools since the biological functions of miRNAs depended on its downstream targeted genes. *AEG-1* was one of the predicted candidates and selected as our research focus for the reason of its highest expression in GBM and the association with tumor grades. In fact, *AEG-1* has been proved to exert significant functions in the development of various cancers, including glioma. On the other hand, it is reported that *AEG-1*-activated autophagy enhances human malignant glioma susceptibility to TGF-β1-triggered EMT process.[25] What's more important, some previous studies have illustrated that miR542 correlated positively with EMT process.[20,26] Our study also suggested that

miR542 repressed the EMT program by down-regulating *AEG-1* at mRNA and protein levels in U251 cells. Taken together, these results demonstrate that miR542 regulates *AEG-1* expression to suppress EMT program, leading to an inhibition of U251 cell aggressiveness and proliferation.

Conclusions

MiR542 as a tumor suppressor inhibits EMT process by down-regulating *AEG-1*, which provides a new insight into U251 cell aggressiveness and proliferation and a potential therapeutic target in the treatment of glioma.

Acknowledgements

The authors acknowledge Drs. Li J (Translational Medical Center, The Medical Center of Chinese PLA General Hospital) and Jiang J (Clinical Data and Specimen Repositories, Chinese PLA General Hospital) for providing technical assistances. The authors thank Dr. Jin W (Department of Pathology, The first Medical Center of Chinese PLA General Hospital) for interpretation of pathological data.

Funding

This work was supported by the Medical Big Data Research Program of Chinese PLA General Hospital (No. 2018MBD-20) and the Clinical Research Support Program of Chinese PLA General Hospital (No. 2016FCCXYY2010).

Conflicts of interest

None.

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How to cite this article: Li C, Liu HL, Zhou YM, Shi YC, Zhang ZB, Chen L, Feng SY. Astrocyte elevated gene-1 serves as a target of miR542 to promote glioblastoma proliferation and invasion. Chin Med J 2020;133:2437–2443. doi: 10.1097/CM9.0000000000001072