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Original Article

miR-876-5p exerts tumor suppressor function by targeting TWIST1 and regulating the epithelial-mesenchymal transition in glioblastoma

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Abstract: Objective: Glioblastoma (GBM) remains one of the most lethal types of cancer, and is the most common brain tumor in adults. The purpose of this study was to determine the role of miR-876-5p in regulation of epithelial mesenchymal transition (EMT) and metastasis of GBM cells. Materials and methods: TWIST1 and miR-876-5p expression was analyzed by real-time PCR and Western blot assays. We confirmed the association between miR-876-5p and TWIST1 by dual luciferase reporter assay. The roles of the miR-876-5p/TWIST1 pathway in migration and invasion were examined *in vitro*. The effects of miR-876-5p on EMT-related molecules E-cadherin, N-cadherin and vimentin were evaluated by real-time PCR and Western blot. Results: miR-876-5p expression levels were decreased while TWIST1 expression levels were increased in detected GBM tissue samples and all of the GBM cell lines. In addition, ectopic expression of miR-876-5p suppressed and miR-876-5p-in promoted EMT, migration, and invasion in T98G cells. Bioinformatics coupled with luciferase and Western blot assays also revealed that miR-876-5p inhibited expression of TWIST1, one of the master regulators of tumor metastasis. Conclusions: This study first indicates that miR-876-5p functions as a suppressor in regulating of GBM EMT by targeting TWSIT1, and it promise as a therapeutic target and prognostic marker for metastatic GBM.

Keywords: miR-876-5p, glioma, invasion, TWIST1

Introduction

Glioma is the most frequent primary tumor of the brain and is generally classified into four grades based on histology. High-grade gliomas are the most common primary brain tumors in adults, and their malignant nature ranks them highly regarding cause of cancer death [1]. Grade 4 gliomas, glioblastoma (GBM), are highly malignant, often associated with strong microvascular proliferation and necrosis and display strong infiltrating properties. The prognosis for a newly diagnosed glioblastoma patient is grim, with a median survival of 12-14 months despite tumor resection and chemotherapy [2].

MicroRNAs (miRNAs) are an abundant family of small RNAs (~22 nucleotides) that fine-tune the expression of genes implicated in fundamental biological processes such as differentiation, proliferation and apoptosis [3, 4]. miRNAs regulate the expression of multiple targets by binding to the 3'-untranslated regions of target mRNAs to promote mRNA degradation at a post-transcriptional level or by inhibiting the initiation of translation [5]. Recent advances have revealed that aberrant miRNA expression is implicated in pathogenesis of a variety of tumors and would be tested as potential biomarkers [6-9].

In order to find miRNAs that participate in the tumorigenesis of glioma, a genome wide survey for microRNA expression was performed and identified miR-876-5p reduced in human glioblastomas samples. The effect of miR-876-5p on glioma has not been reported so far. Thus, whether miR-876-5p contributed to the metastatic behavior of glioma cells was investigated.

Tumor cell migration and invasion of surrounding tissue are important characteristics of GBM.

The basic helix-loop-helix (bHLH) family of proteins regulates normal development and differentiation by forming DNA-binding heterodimers composed of tissue-specific class B) and ubiquitously expressed (class A) proteins that direct cell-specific gene expression [10, 11]. Both *ex vivo* and *in vivo* validation studies indicate that the class B bHLH protein, TWIST, is a biomarker that can be used to confirm or detect the presence and grade of gliomas, particularly high-grade gliomas in humans and this biomarker may play an important diagnostic role in addition to currently used markers for gliomas [12]. The results presented strongly suggest that the associative analysis method was able to accurately identify TWIST as a glioma-associated biomarker, possibly due to increased angiogenesis. Furthermore, the regulatory relationship between miR-876-5p and TWIST1 was established. Evidence that miR-876-5p can impede migration and invasion of T98G cells indicates that it may be a possible therapeutic target of GBM.

Materials and methods

Tissue collection

All human tissue samples of normal brain and glioma were obtained from the Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University. For the use of clinical specimens for research purposes, informed consent and approval were obtained.

Cell lines and cell culture

Human Normal human astrocytes NHA and glioblastoma cell lines T98G, LN18, U87MG, and U251MG were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, MD, USA) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) in a humidified atmosphere of CO_2/air (5%/95%) at 37°C (Thermo Fisher Scientific, Nepean, Canada).

Western blot analysis and antibodies

Total cell extracts were obtained by lysing the cells in RIPA buffer and boiled for 5 minutes. Protein concentration was measured by the Bradford assay (Bio-Rad, Hercules, CA, USA). Cellular proteins were extracted and separated in 4-10% Tris glycine/SDS-polyacrylamide gels and electrotransferred to ECL nitrocellulose membranes (#IPFL00010, Millipore). The membranes were blocked with 5% nonfat milk and incubated with specific antibodies. The β-actin protein was used as the endogenous control. Antibodies against the following proteins were purchased from Abcam: TWIST1 (1:1000), TWIST2 (1:800), Vimentin (1:1000). The following antibodies were purchased from Santa Cruz Biotechnology: E-cadherin (1:400), N-cadherin (1:500), β-actin (1:1000). Immunocomplexes were visualized by ECL (Pharmacia-Amersham, Freiburg, Germany). The blots were quantified by image J software.

Transfection and luciferase assay

The 3' untranslated region (UTR) of TWIST1 (containing their potential binding sites) and mutated controls were purchased from GENEWIZ (Suzhou, China) and inserted into the pMIR-GLO Luciferase vector (Promega, USA). miRNA mimics were then transfected into T98G cells containing wild-type or mutant 3'UTR pMIR-GLO plasmids. A total of 5×10^5 cells were seeded in per well of six-well plate. After 24 hours, 10 μL miRNA mimics and 4 μg constructed plasmids, 185 μL DMEM and 5 μL lipofectamine 2000 reagent (Thermo Fisher, USA) were mixed. After cell lysis the luciferase activity was measured using Luciferase Reporter Assay System (Promega Corporation Madison, WI, USA) according to manufacturer's protocol.

Quantitative real-time PCR assay

Total RNA was extracted from the cells with Trizol reagent (Invitrogen) according to the manufacturer's instruction. The concentration and purity of the RNA samples were determined at 260 nm. First-strand cDNA was generated through reverse transcription via Revert Aid Reverse Transcriptase (Thermo Scientific Life Technologies, NY, USA). Primer sequences used in the experiments were as follows: TWIST1 forward: 5'-CCAGGTACATCGACTTCCTCTA-3', Reverse: 5'-CCA TCCTCCAGACCGAGAA-3'; E-cadherin forward: 5'-CTGCTGCAGGTCTCCTCTTG-3', Reverse: 5'-TGTCGACCGGTGCAATCTTC-3'; N-cadherin forward: 5'-ACAGTGGCCACCTACAAAGG-3', Reverse: 5'-CCGAGATGGGGTTGATAATG-3'; Vimentin forward: 5'-AAGGCGAGGAGAGCAGGATT-3', Reverse: 5'-GGTCATCGTGATGCTGAGAAG-3'; β-actin forward: 5'-CATGTACGTTGCTATCCAGGC-3', Reverse: 5'-CTCCTTAATG TCACGCACGAT-3'.

The expression levels of miRNA were detected by hydrolysis Probes miRNA assays (Applied Biosystems) and normalized to U6 small nuclear RNA. The $2^{-\Delta\Delta Ct}$ method was adopted and applied to calculate the relative quantities of subject genes. qRT-PCR was performed according to MIQE guideline standards. All reactions were performed in triplicate.

Motility and invasion assay

For *in vitro* cell motility and invasion assay, transwell plates and cell culture inserts (BD Biosciences, San Jose, CA) were used. For the coating of invasion assay, matrigel (BD Biosciences, San Jose, CA) was diluted to 0.3 mg/ml concentration with coating buffer (0.01 M Tris, 0.7% NaCl, pH 8.0) and 100 µl matrigel was coated onto upper compartment of cell culture insert. After incubation for 1 hour at 37°C, the cell culture insert was ready for seeding. After transfection of miR-876-5p and miR-876-5p-in, T98G cells were appropriately (5×10^4 cell/well for motility assay, 1×10^5 cell/well for invasion assay) seeded into the cell culture insert with serum-free media and 5% fetal bovine serum was used as a chemoattractant. After 4 hours (motility) or 12 hours (invasion) of incubation at 37°C, migrated or invaded cells were stained using Diff-Quik staining kit (Sysmex, Japan). The images of cells were photographed with Axiovert 200 inverted microscope (Zeiss, Germany) at × 200 magnification and the cell number was counted in three random fields of view.

Statistical analysis

Each experiment was performed at least three times, on independent passages, usually in triplicates. Data were analyzed by Analysis of Variance (ANOVA) with post-test (Dunnett: compare all columns vs. control columns) using GraphPad Prism 5 software as indicated and are presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

Expression levels of miR-876-5p in glioma cell lines and tissues

In a genome wide survey for microRNA expression, previously our work identified several microRNAs that were reduced in human glioblastomas samples (unpublished results), including miR-876-5p. To validate the result from our deep sequencing experiments, miR-876-5p expression in various human glioma cell lines was identified and total RNA was isolated from four human glioma cell lines T98G, U251MG, LN18 and T98G as well as from primary normal human astrocytes (NHA). Quantitative real time PCR (qPCR) was used to determine the mRNA level of miR-876-5p. miR-876-5p was much lower in GBM cell lines as compared with NHA cells (**Figure 1A**). In addition, the expression of miR-876-5p in 20 human glioblastomas samples and 5 non-neoplastic brain specimens. miR-876-5p expression was down-regulated in all glioblastomas samples when compared to the non-neoplastic brain specimens (**Figure 1B**). Next, the TWIST1 protein level in NHA or cell lines representing GBM was analyzed and found to have a much higher reduction in NHA (**Figure 1C**). Taken together, these data suggest that miR-876-5p is down-regulated in GBM, which may contribute to GBM pathogenesis.

miR-876-5p inhibits the motility and invasion of glioma cells and regulates EMT related gene expression

To elucidate the role of miR-876-5p downregulation in the development and progression of glioma, the T98G cell line was selected from among the four cell lines tested, to conduct further functional studies. miR-876-5p-mimic and miR-876-5p-inhibitor respectively were generated and transfection with miR-876-5p-mimic and miR-876-5p-inhibitor and negative control microRNA was performed to reveal the gain-of-function effect or lose-of-function effect on the migration and invasion of the GBM cell lines. T98G cells exhibited high transfection efficiency (**Figure 2A**). As demonstrated by Transwell migration and invasion assays, over-expression of miR-876-5p significantly reduced the invasiveness and migration of T98G cell, whereas miR-876-5p-inhibitor significantly promoted the numbers of migrated and invaded cells in the same cells (**Figure 2B-D**).

Since EMT is closely related to cancer cell metastasis ability, we next examined EMT markers in control-, miR-876-5p-, or miR-876-5p-in-transfected GBM cells. Real-time PCR and Western blot analysis were performed for the EMT regulatory mRNA and proteins in glioma cancer cells. Notably, N-cadherin and vimentin, hallmarks of EMT, were dramatically

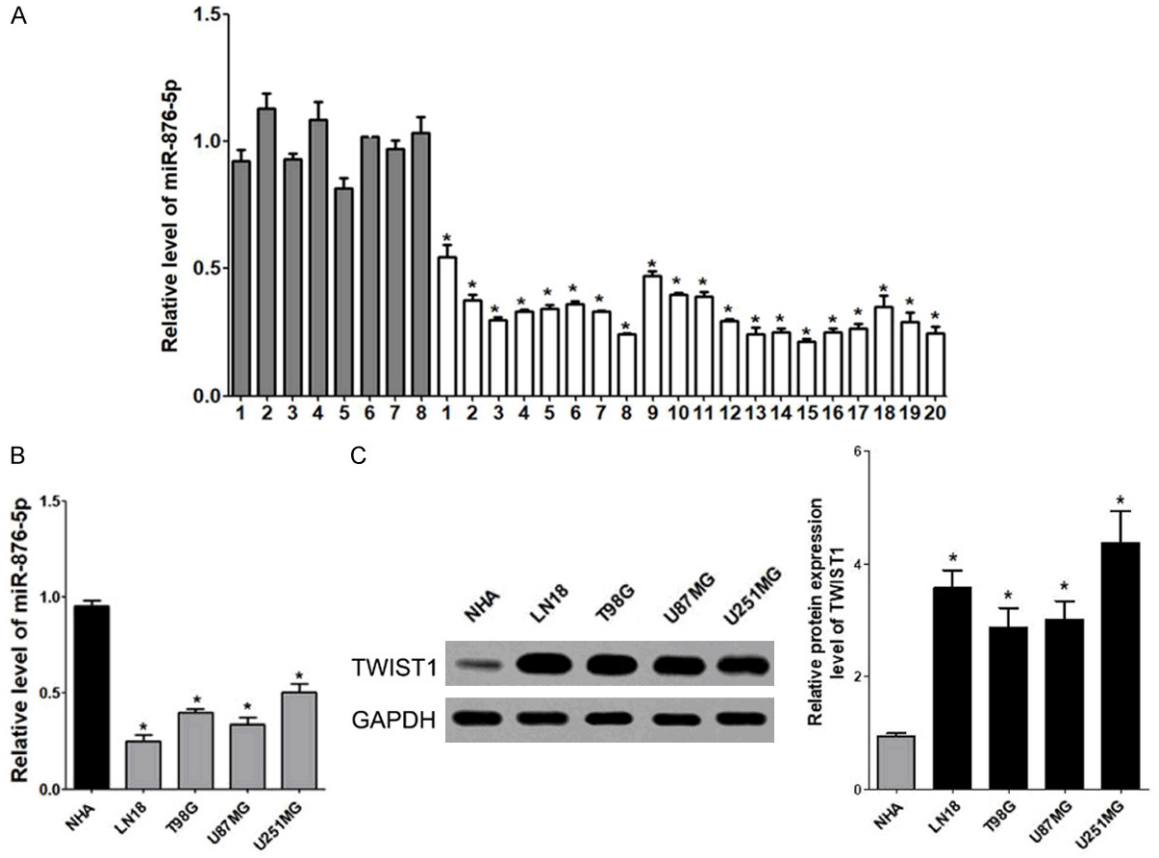

Figure 1. Reduced levels of miR-876-5p expression in glioma cell lines and glioma tissues. A. Real-time PCR analysis of miR-876-5p expression in primary normal human astrocytes (NHA) and various tumor cell lines. Data are represented as mean ± SEM. n = 3, **P* < 0.05 by ANOVA. B. Real-time PCR analysis of miR-876-5p expression in 5 non-neoplastic brain specimens and 20 human glioma tissues. The average miR-876-5p expression was normalized by U6 expression. Data are represented as mean ± SEM. n = 3, **P* < 0.05 by ANOVA. C. Western blot analysis of TWIST1 in primary normal human astrocytes (NHA) and glioma cell lines, GAPDH served as the loading control. Data are represented as mean ± SEM. n = 3, **P* < 0.05 by ANOVA.

decreased in miR-876-5p mimic transfectants, whereas E-cadherin, an epithelial markers, was increased in T98G cells. In contrast, miR-876-5p-inhibitor had the opposite effects (**Figure 2E** and **2F**). Taken together, these results indicate that anti-metastatic potential could be attributed to the selective regulation of EMT proteins in glioma cancer cells.

miR-876-5p directly targets TWIST1

Since miRNAs mainly function through inhibiting their target mRNAs by binding to the 3'UTR, the putative target genes of miR-876-5p were searched in an online miRNA target prediction database (microRNA.org), and TWIST1 was found to be a potential target of miR-876-5p. The conserved target genes TWIST1, which have been identified as invasion associated gene, were selected for subsequent investigation (**Figure 3A**). To further confirm that TWIST1 is a direct target of miR-876-5p, a dual-luciferase reporter system was used with co-transfection of miR-876-5p and a luciferase reporter plasmid containing a wild-type or mutant 3'UTR of human TWIST1 Luciferase activity was significantly inhibited by miR-876-5p, but increased by miR-876-5p-in co-transfected with wild-type TWIST1 3'UTR, and miR-876-5p and miR-876-5p-in failed to inhibit the expression of luciferase constructs with mutated target sites, suggesting that miR-876-5p directly targets the 3'UTR of TWIST1 (**Figure 3B**). In addition, a change in mRNA and protein expression levels of TWIST1 in response to miR-876-5p over-expression or inhibition was verified by RT-PCR and Western blot in T98G cells (**Figure 3C** and **3D**), confirming that miR-876-5p negatively regulates TWIST1 expression by directly targeting their 3'UTR regions.

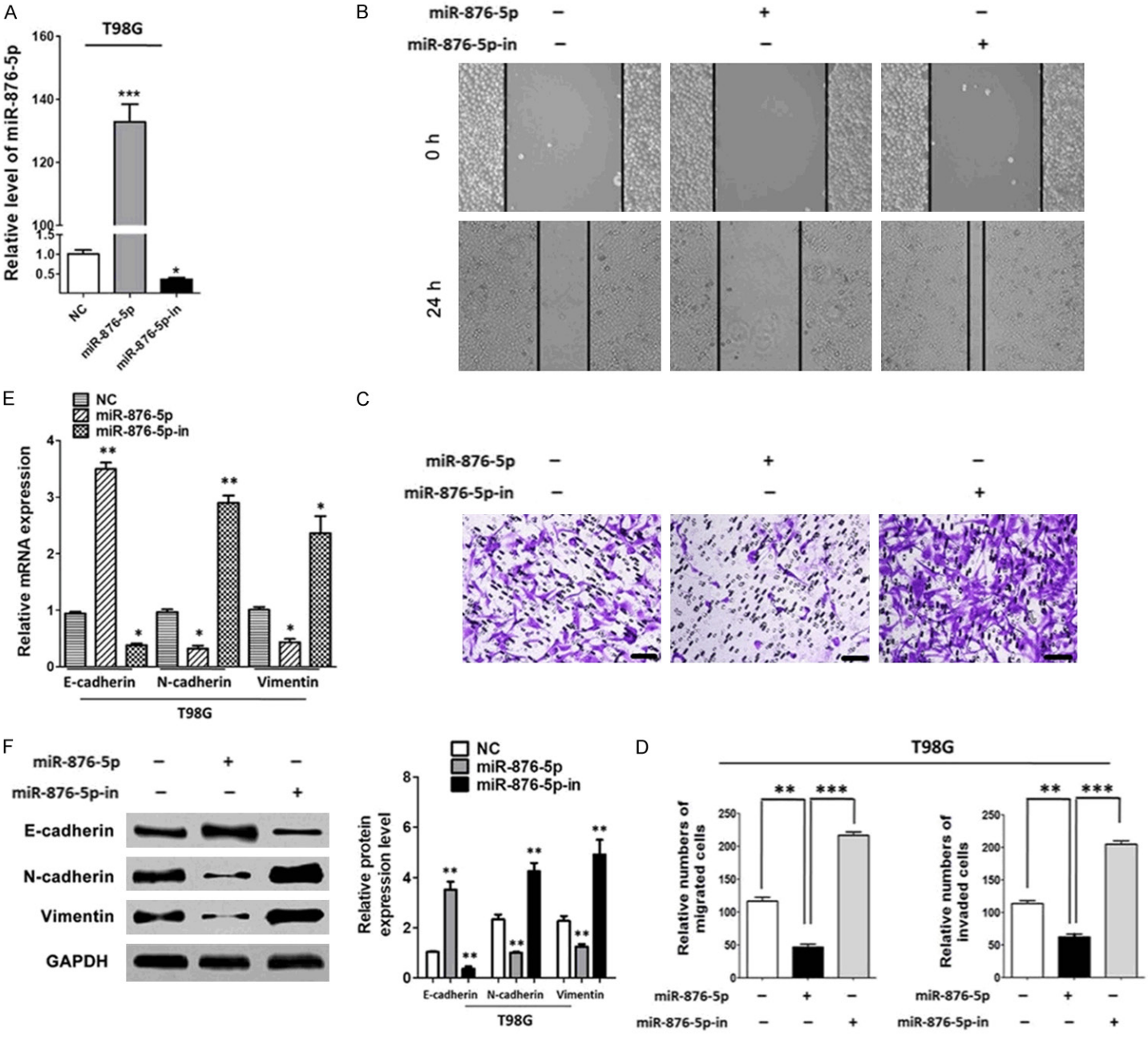
A
T98G
Relative level of miR-876-5p
NC
miR-876-5p
miR-876-5p-in
B
miR-876-5p
miR-876-5p-in
0 h
24 h
C
D
T98G
Relative numbers of migrated cells
Relative numbers of invaded cells
E
NC
miR-876-5p
miR-876-5p-in
Relative mRNA expression
E-cadherin
N-cadherin
Vimentin
T98G
F
miR-876-5p
miR-876-5p-in
E-cadherin
N-cadherin
Vimentin
GAPDH
Relative protein expression level

Figure 2. miR-876-5p regulates cell metastasis ability and EMT marker expression. (A) miR-876-5p levels in T98G cells transfected with control miRNA, miR-876-5p, or miR-876-5p-in. Real-time PCR analysis of miR-876-5p levels in transfected T98G cells. Data are represented as mean ± SEM. n = 3, $*P < 0.05$, $***P < 0.001$ by ANOVA. Motility (B) and invasion (C) assays of T98G cells transfected with miRNA, miR-876-5p, or miR-876-5p-in. The cell number of migrated cells were counted in six randomly selected fields and presented in bar graph. Invasion assay was performed with transwell-inserts coated with matrigel. Images were taken with invert microscope. Bar, 50 μm. (D) Data are represented as mean ± SEM. n = 3, $**P < 0.01$, $***P < 0.001$ by ANOVA. (E) Real-time PCR analysis of E-cadherin, N-cadherin, and Vimentin in T98G cells, in response to miR-876-5p over-expression and inhibition. Data are represented as mean ± SEM. n = 3, $*P < 0.05$, $**P < 0.01$ by ANOVA. (F) Western blot analysis of E-cadherin, N-cadherin and Vimentin in T98G cells, in response to miR-876-5p over-expression and inhibition. GAPDH was used as loading control. Data are represented as mean ± SEM. n = 3, $**P < 0.01$ by ANOVA.

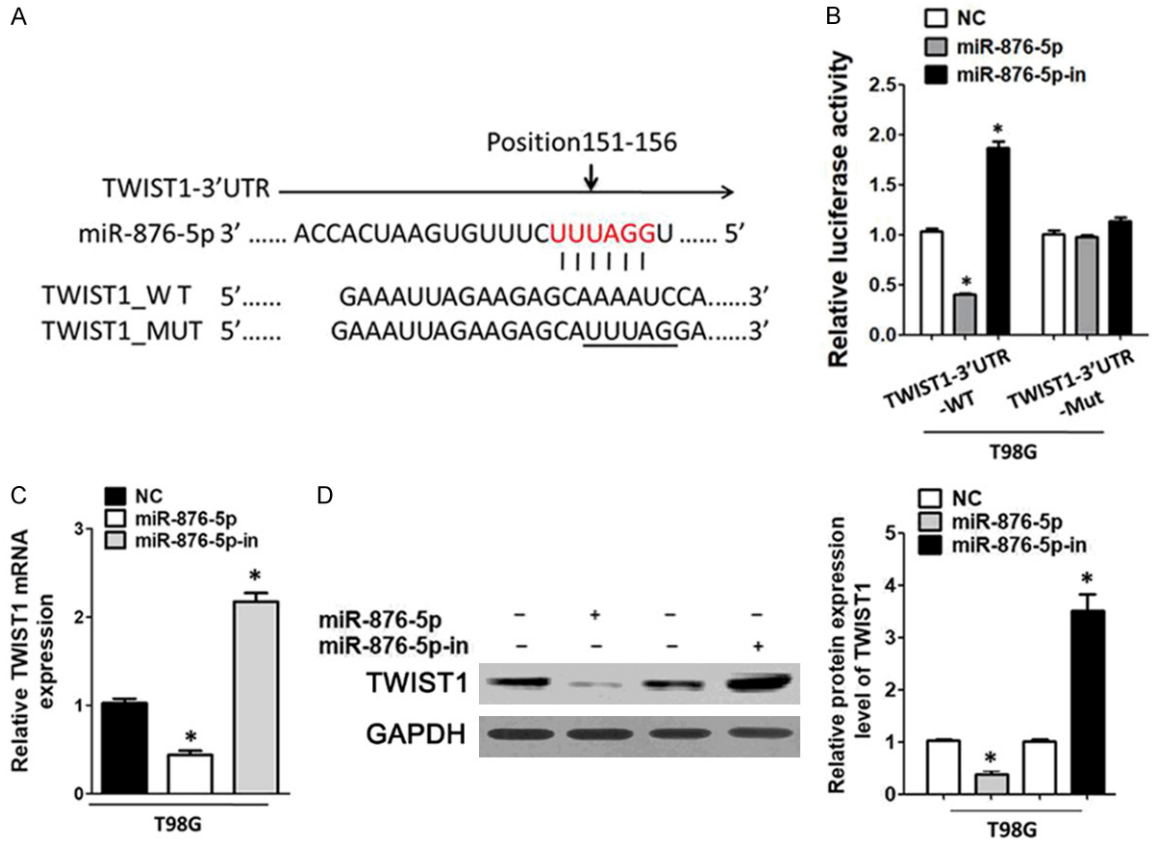

Figure 3. miR-876-5p regulates TWIST1 expression by binding 3'UTR in GBM. A. Schematic representation of TWIST1 3'UTRs showing putative miR-876-5p target site. B. Luciferase activity of T98G cells transfected with luciferase reporter plasmids carrying a wild-type or mutant 3'UTR of TWIST1, in response to miR-876-5p over-expression or inhibition. Data are represented as mean ± SEM. n = 3, $*P < 0.05$ by ANOVA. C. mRNA levels of TWIST1 examined by Real-time PCR in T98G cells transfected with miR-876-5p, miR-876-5p-in, or control miRNA. Data are represented as mean ± SEM. n = 3, $*P < 0.05$ by ANOVA. D. TWIST1 protein levels were analyzed by Western blot in T98G cells transfected with miR-876-5p, miR-876-5p-in, or control miRNA. GAPDH was used as loading control. Data are represented as mean ± SEM. n = 3, $*P < 0.05$ by ANOVA.

miR-876-5p regulates cell migration and invasion through targeting TWIST1 in GBM

Down-regulation of TWIST1 by miR-876-5p in GBM prompted us to investigate whether miR-876-5p affects GBM cell invasion and migration via TWIST1. T98G cells were co-transfected with miR-876-5p and either pMIR-GLO-TWIST1 (without 3'UTR region) or empty pMIR-GLO vector. Western blot analysis was used to validate the TWIST1 expression in the rescue experiment (**Figure 4A**). The cells were then subjected to transwell assays to identify whether TWIST1 rescues miR-876-5p-induced suppressed invasion and migration of GBM cells. These data show that TWIST1 partially recovered GBM cell invasion and migration profiles (**Figure 4B** and **4C**). These results confirm that

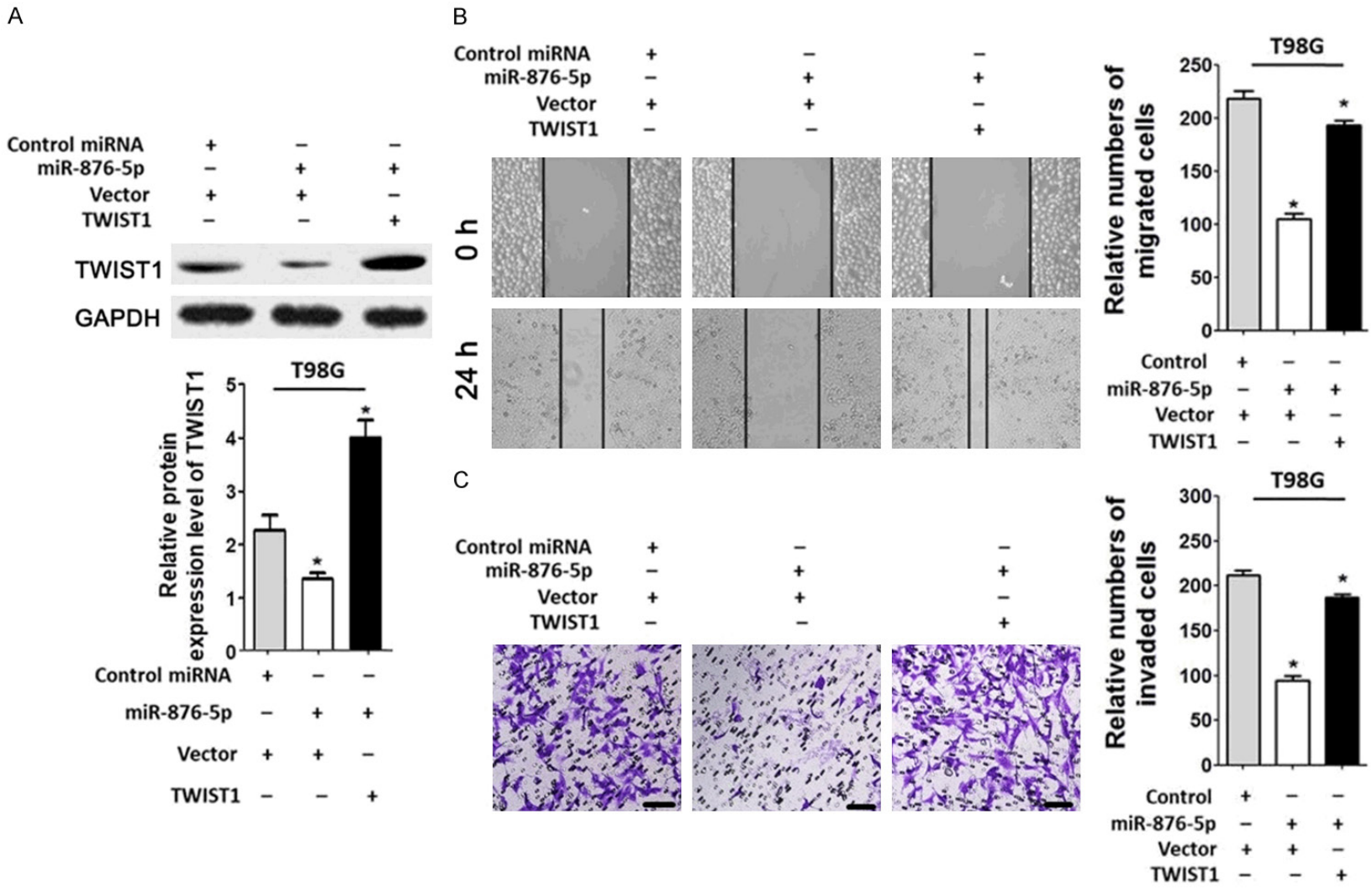

Figure 4. The role of TWIST1 in miR-876-5p-regulated cell migration and invasion. (A) T98G cells were co-transfected with control miRNA or miR-876-5p mimics and with either TWIST1 3'UTR deleted plasmid or empty vector. Ectopic expression of TWIST1 was confirmed by Western blot assays. GAPDH was used as a loading control. Data are represented as mean ± SEM. n = 3, **P* < 0.05 by ANOVA. Motility (B) and invasion (C) assay of T98G cells co-transfected with control miRNA or miR-876-5p mimics and with either TWIST1 3'UTR deleted plasmid or empty vector. The cell number of migrated cells were counted in six randomly selected fields and presented in bar graph. Invasion assay was performed with transwell-inserts coated with matrigel. Images were taken with invert microscope. Bar, 50 μm. Data are represented as mean ± SEM. n = 3, **P* < 0.05 by ANOVA.

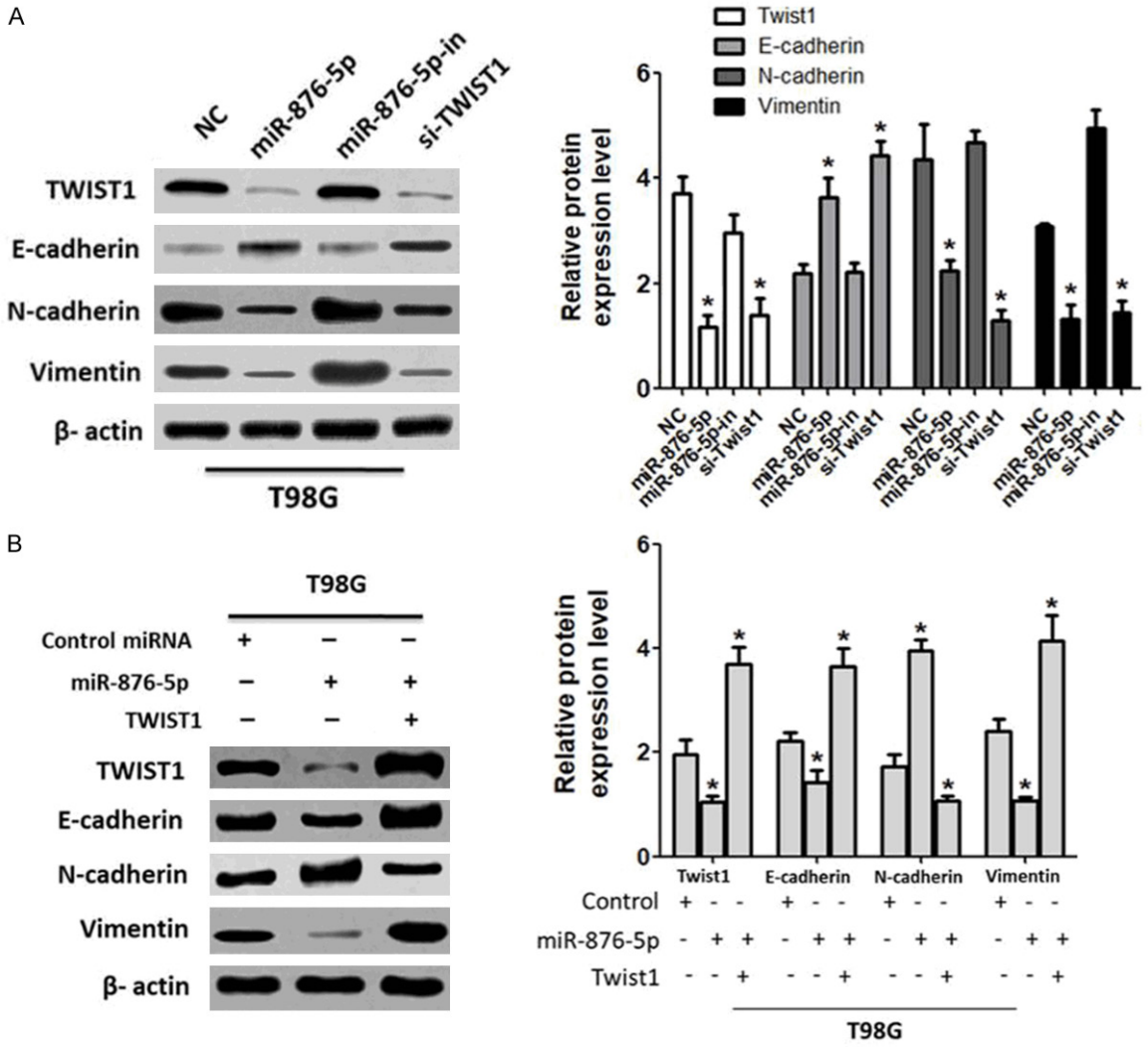

Figure 5. miR-876-5p regulating epithelial-mesenchymal transition related molecules in glioma through targeting TWIST1 3'UTR. A. T98G cells were transfected with miR-876-5p mimics or inhibitor. si-TWIST1 were used for knockdown of miR-876-5p target genes, respectively. The protein expression levels of EMT-related molecules were analyzed by immunoblotting. NC represents negative control miRNA. Data are represented as mean ± SEM. n = 3, $*P < 0.05$ by ANOVA. B. Co-transfection of miR-876-5p with 3'UTR-deleted TWSIT1 plasmid rescued the expressions of EMT-related molecules. Expression levels were analyzed by immunoblotting. Data are represented as mean ± SEM. n = 3, $*P < 0.05$ by ANOVA.

TWIST1 is regulated by miR-876-5p and miR-876-5p down-regulation, and may participate in GBM carcinogenesis and progression through potentiation of TWIST1 expression.

TWIST1 regulates epithelial-mesenchymal transition are functionally related to the effects of miR-876-5p

miR-876-5p exerts tumor suppressor function by regulating cell migration and invasion in GBM. Therefore, some EMT regulators such as E-cadherin, N-cadherin and vimentin, have been implicated in the control of the epithelial-mesenchymal transition in mammals. Interestingly, the protein level of N-cadherin and vimentin, hallmarks of EMT, was downregulated in T89G glioma cells transfected with miR-876-5p-mimic, which is similar as transfected the si-TWIST1, but increased in the T89G glioma cells transfected with miR-876-5p-inhibitor, compared with control cells (**Figure 5A**). On the other hand, the expression of E-cadherin was increased in miR-876-5p overexpressing cells and inhibited in the miR-876-5p inhibited cells. Co-transfection of miR-876-5p with 3'UTR-

deleted TWIST1 rescued the expressions of N-cadherin and vimentin while downregulating the E-cadherin expression (**Figure 5B**).

Discussion

It is well known that miRNAs play important roles in tumorigenesis of various human cancers, including glioma cancer [13-15]. Here, it was found that miR-876-5p was down-regulated in glioma cells and glioma tissues, compared with that in nonneoplastic brain specimens and primary normal human astrocytes (NHA). Moreover, restoration of miR-876-5p suppressed invasion and migration of glioma cancer cells, while miR-876-5p inhibition had the opposite effect, suggesting a fundamental role of miR-876-5p as a tumor suppressor in glioma cancer.

Recently, a cluster of miRNAs determining the regulation of TWIST1 expression has been noticed. For example, miR-151-3p, miR-610, and miR-186 which were located on separated miRNA clusters can cooperate to inhibit TWIST1 translation [16-18]. It has been shown that the low expression of miR-151-3p in human breast cancer specimens is significantly correlated with high levels of TWIST1 protein. The suppressive effect of miR-151-3p on the glioma may result from inhibition of TWIST1 via post-transcriptional regulation [18]. Ectopic expression of miR-610 suppressed the osteosarcoma cell proliferation, cell cycle, invasion and increased the sensitivity of osteosarcoma cells to cisplatin through targeting the Twist1 expression [17]. Previous study shown that miR-186 affects the proliferation, invasion and migration of human gastric cancer by inhibition of Twist1, and could be a tumor suppressor in GC development [16]. miR-876-5p is a novel miRNA which were only found involved in GBM tumorigenesis and development in the present study. The molecular mechanisms underlying miR-876-5p-mediated biological behaviors are still unclear. To comprehensively understand the effect of miR-876-5p on glioma cancer cells, we performed in vitro motility and invasion assays to identify the migration and invasion ability of T98G cells. These results show that over-expression of miR-876-5p in glioma cells decreases invasion and migration, whereas miR-876-5p-inhibitor significantly promoted the numbers of migrated and invaded cells in the same cells. Furthermore, we confirmed that miR-876-5p negatively regulates TWIST1 expression by directly targeting their 3'UTR regions.

EMT has a pivotal role in the initiation of metastasis, a process in which epithelial cells lose adhesion and cytoskeletal components concomitant with a gain of mesenchymal components and the initiation of a migratory phenotype [19, 20]. Because the effect of miR-876-5p on cell migration and gene expression regulation, we detected the change of EMT markers in T98G cells transfected with miR-876-5p and miR-876-5p-in. Ectopic expression of miR-876-5p significantly increased E-cadherin expression. Expression of epithelial markers increased, while the expression of mesenchymal markers decreased in U87 cells with miR-517c mimics transfection [21]. Accordingly, expression of N-cadherin and Vimentin was reduced when miR-876-5p was over-expressed in T98G cells. Glioma stem cells (GSC) and epithelial-mesenchymal transition (EMT) are strongly associated with therapy resistance and tumor recurrence [22, 23]. Consistently, our results supported that miR-876-5p may reverse EMT process to inhibit cell migration.

Conclusion

In summary, the analysis revealed that restoring miR-876-5p expression attenuated protein level of TWIST1 by binding the 3'UTR, and inhibited cell migration, invasion and regulates EMT related gene expression in glioma. Targeting to the miR-876-5p/TWIST1 interaction or rescuing miR-876-5p expression may be a new therapeutic application to treat glioma patients in the future.

Disclosure of conflict of interest

None.

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