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Original Article

MiR-186 acts as a tumor suppressor by targeting TWIST1/2 and regulating the epithelial-mesenchymal transition in glioblastoma multiforme

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Abstract: Glioblastoma 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-186 in regulation of epithelial mesenchymal transition (EMT) and metastasis of GBM cells. We showed that miR-186 expression levels were decreased while TWIST1 and TWIST2 expression levels were increased in detected GBM tissue samples and all of the GBM cell lines. In addition, ectopic expression of miR-186 suppressed and miR-186-in promoted EMT, migration, and invasion in U87MG and LN18 cells. Bioinformatics coupled with luciferase and Western blot assays also revealed that miR-186 inhibited expression of TWIST1 and TWIST2, which are master regulators of tumor metastasis. Our study first indicates that miR-186 functions as a suppressor in regulating of GBM EMT by targeting TWSIT1 and TWIST2, and it promises as a therapeutic target and prognostic marker for metastatic GBM.

Keywords: miR-186, Glioma, epithelial-mesenchymal transition (EMT), TWIST1/2

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. Recent advances have revealed that aberrant miRNA expression was implicated in pathogenesis of a variety of tumors and would be tested as potential biomarkers [5-8].

In order to find miRNAs that participate in the tumorigenesis of glioma, we performed a genome wide survey for microRNA expression and identified miR-186 reduced in human glioblastomas samples. miR-186 is frequently downregulated in various human tumors and appears to play an important role in the antineoplastic process as indicated by its association with low invasion and metastatic potential [9-11]. However, the effect of miR-186 on glioma has not been reported. Thus, we investigated whether miR-186 was contributed to the metastatic behavior of glioma cells.

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 [12, 13]. 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 [14]. Our results presented strongly suggested that the associative analysis method used in this study was able to accurately identify TWIST as a glioma-associated biomarker, possibly due to increased angiogenesis.

In the present study, we confirmed the regulatory relationship between miR-186, a known tumor suppressive miRNA, and the oncogene, TWIST1 and TWIST2. We provided evidences that miR-186 could impede migration and invasion of U87MG and LN18 cells, so it may be a possible therapeutic target of GBM.

Materials and methods

Patients

All human tissue samples of normal brain and glioma were obtained from the Department of Neurosurgery, Huai'an First People's Hospital (Jiangsu, China). For the use of clinical specimens for research purposes, informed consent and approval were obtained from the Qi Lu Hospital Command of PLA.

Cell lines and cell culture

Human glioblastoma cell lines U87MG, LN18 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 min. 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, TWIST2, vimentin. The following antibodies were purchased from Santa Cruz Biotechnology: E-cadherin, N-cadherin, β-actin. Immunocomplexes were visualized by ECL (Pharmacia-Amersham, Freiburg, Germany).

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 RevertAidTM Reverse Transcriptase (Thermo ScientificTM Life Technologies, NY, USA). Primer sequences used in the experiments were as follows: TWIST1 forward 5'-CCAGGTACATCGACTTCCTCTA-3', reverse 5'-CCATCCTCCAGACCGAGAA-3'; TWIST2 forward 5'-GCAAGATCCAGACG CTCAA-3', reverse 5'-GCTGCAGCTGGTCATCTTAT -3'; E-cadherin forward 5'-CTGCTGC AGGTCTCCTCTTG-3', reverse 5'-TGTCGACCGGTGCAATCTTC-3'; N-cadherin forward 5'-ACAGTGGCCACCTACAAAGG-3', reverse 5'-CCGA GATGGGGTTGATAATG-3'; Vimentin forward 5'-AAGGCGAGGAGAGCAGGATT-3', reverse 5'-GGTCATCGTGATGCTGA GAAG-3'; β-actin forward 5'-CATGTACGTTGCTA TCCAGGC-3', reverse 5'-CTCCTTAATGTCACGCACGAT-3'.

The expression levels of miRNA were detected by hydrolysis Probes miRNA assays (Applied Biosystems) and normalized to U6 small nuclear RNA. The 2-ΔΔCt 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

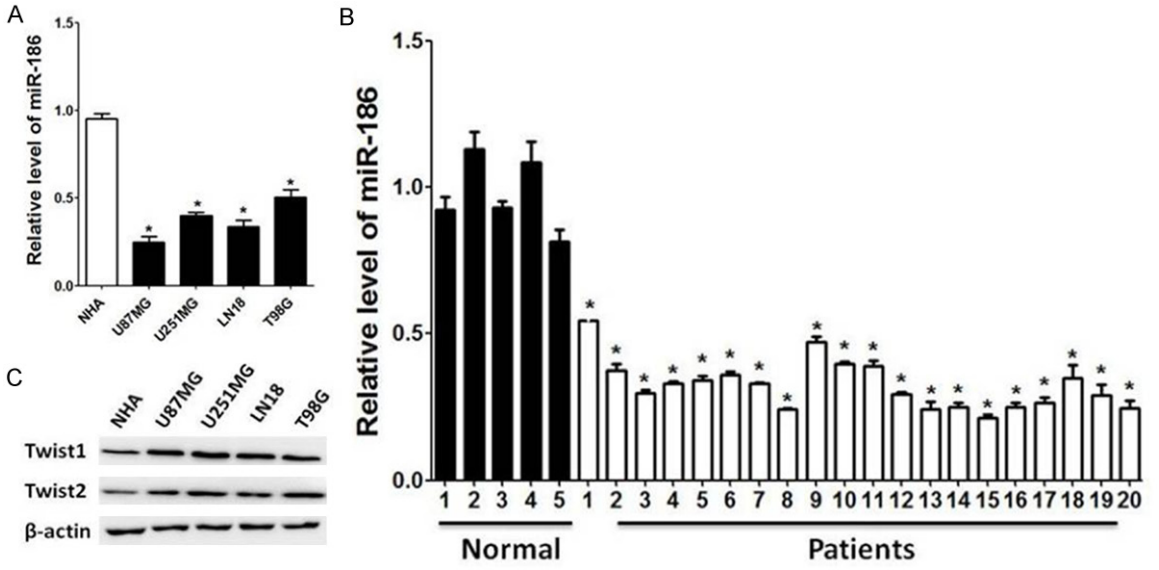

Figure 1. Reduced levels of miR-186 expression in glioma cell lines and glioma tissues. A. Real-time PCR analysis of miR-186 expression in primary normal human astrocytes (NHA) and various tumor cell lines. B. Real-time PCR analysis of miR-186 expression in 5 nonneoplastic brain specimens and 20 human glioma tissues. The average miR-186 expression was normalized by U6 expression. Each bar represents the mean of three independent experiments. *P<0.05. C. Western blotting analysis of TWIST1 and TWIST2 in primary normal human astrocytes (NHA) and glioma cell lines, β-actin served as the loading control.

Tris, 0.7% NaCl, pH 8.0) and 100 μl Matrigel was coated onto upper compartment of cell culture insert. After incubation for 1 h at 37°C, the cell culture insert was ready for seeding. After transfection of miR-186 and miR-186-in, U87MG and LN18 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 h (motility) or 12 h (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 3 times, on independent passages, usually in triplicates. Data were analyzed by Newman-Keuls test using Statistica software as indicated and are presented as mean ± SEM. P<0.05 was considered statistically significant. Results of time lapse microscopy experiments were analyzed with Wilcoxon test in R software.

Results

Expression levels of miR-186 in glioma cell lines and tissues

In a genome wide survey for microRNA expression, we have previously identified several microRNAs were reduced in human glioblastomas samples (unpublished results), including miR-186. To validate the result from our deep sequencing experiments, we examined miR-186 expression in various human glioma cell lines, total RNA was isolated from four human glioma cell lines U87MG, 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-186. We found miR-186 was much lower in GBM cell lines as compared with NHA cells (**Figure 1A**). In addition, we examined the expression of miR-186 in 20 human glioblastomas samples and 5 nonneoplastic brain specimens. We found that miR-186 expression was down-regulated in all glioblastomas samples when compared to the nonneoplastic brain specimens (**Figure 1B**). Although it has been reported that class B bHLH protein, TWIST, could be used as a putative glioma-associated marker, we validated the TWIST1 and TWIST2 protein level in NHA or cell lines representing

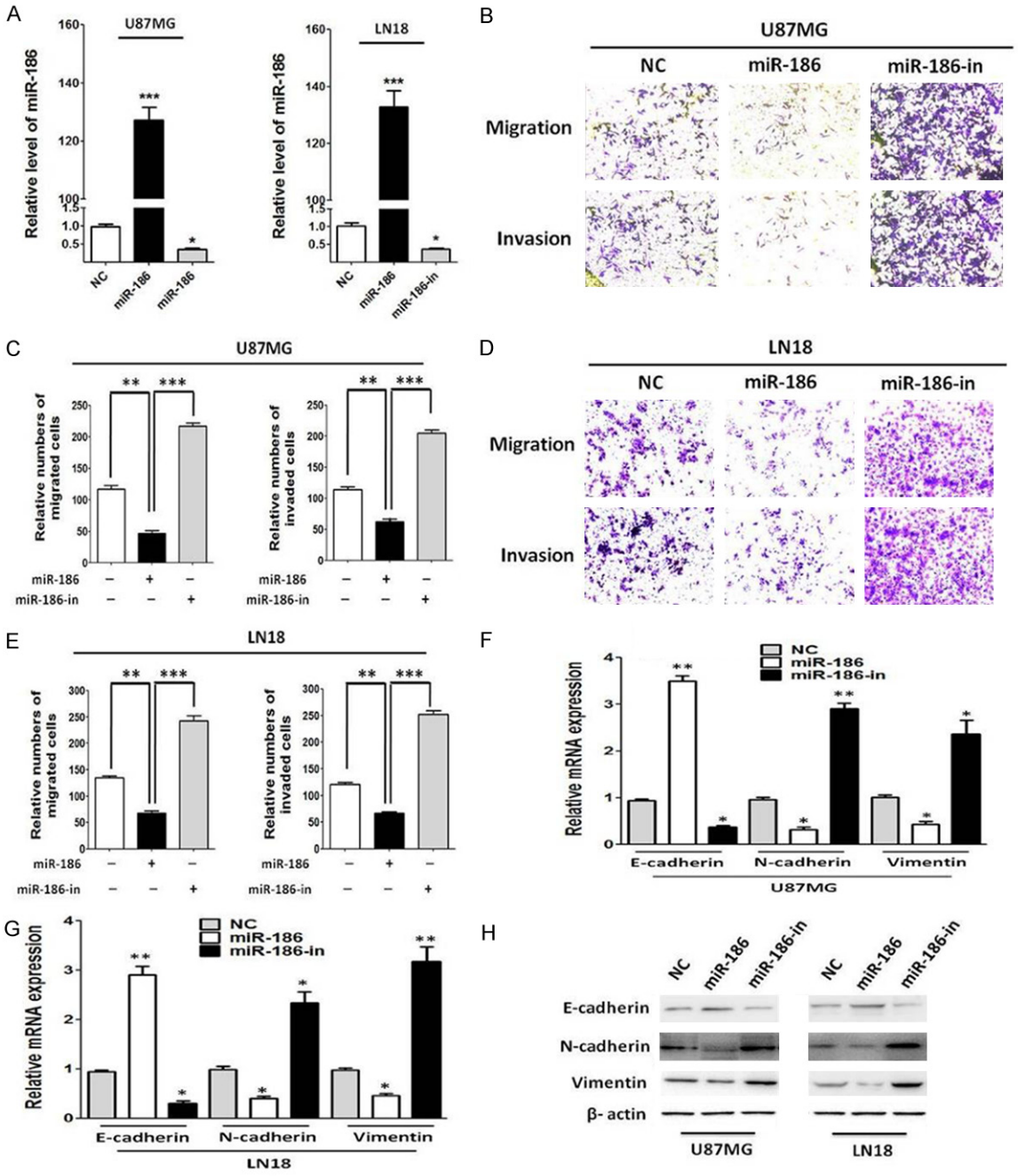

Figure 2. miR-186 regulates cell metastasis ability and EMT marker expression. A. miR-186 levels in U87MG and LN18 cells transfected with control miRNA, miR-186, or miR-186-in. B and D. Motility and invasion assay of U87MG and LN18 cells transfected with miRNA, miR-186, or miR-186-in. Invasion assay was performed with transwell-inserts coated with Matrigel. Images were taken with invert microscope (magnification, ×100). C and E. The cell number of migrated cells were counted in randomly selected fields and presented in bar graph (means ± SD; *P<0.05; **P<0.005; ***P<0.001, Student's t test). F and G. Real-time PCR analysis of E-cadherin, N-cadherin, and Vimentin in U87MG and LN18 cells, in response to miR-186 over-expression and inhibition. *P<0.05, **P<0.01 when compared to control miRNA group. H. Western blot analysis of E-cadherin, N-cadherin and Vimentin in U87MG and LN18 cells, in response to miR-186 over-expression and inhibition. β-actin was used as loading control.

GBM and found much higher reduction in NHA (**Figure 1C**). Taken together, these data suggest that miR-186 is down-regulated in GBM, which may contribute to GBM pathogenesis.

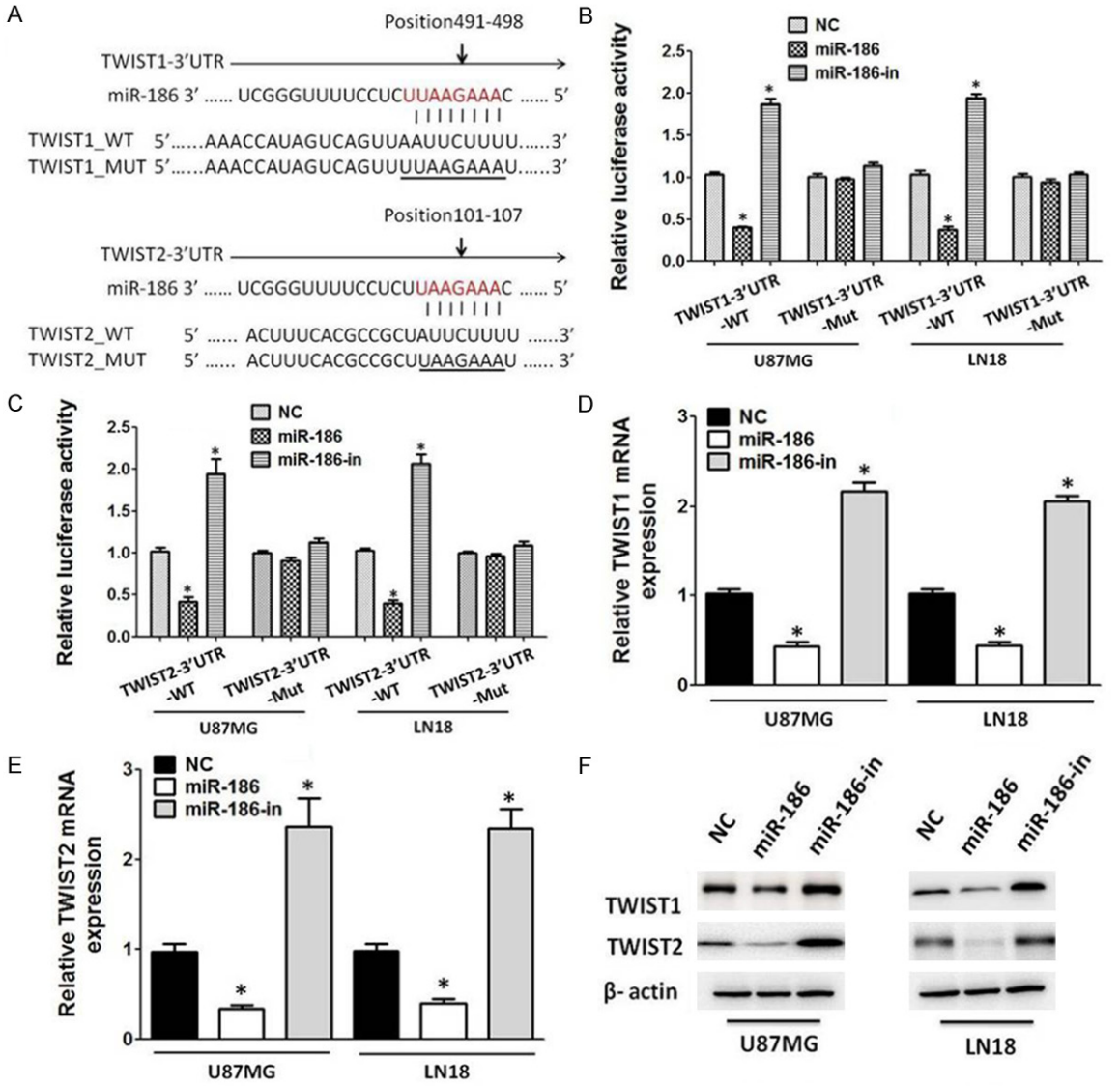

Figure 3. miR-186 regulates TWIST1 and TWIST2 expression by binding 3'-UTR in GBM. (A) Schematic representation of TWIST1 and TWIST2 3' -UTRs showing putative miR-186 target site. (B) Luciferase activity of U87MG or LN18 cells transfected with plasmids carrying a wild-type or mutant 3'UTR of TWIST1, in response to miR-186 over-expression or inhibition. (C) Luciferase activity of U87MG or LN18 cells transfected with plasmids carrying a wild-type or mutant 3'UTR of TWIST2, in response to miR-186 over-expression or inhibition. (D) mRNA levels of TWIST1 (D) and TWIST2. (E) Examined by Real-time PCR in U87MG or LN18 cells transfected with miR-186, miR-186-in, or control miRNA. (F) TWIST1 and TWIST2 protein levels were analyzed by Western blot in U87MG or LN18 cells transfected with miR-186, miR-186-in, or control miRNA. β-actin was used as loading control. *P<0.05 compared with the control miRNA group.

miR-186 inhibits the motility and invasion of glioma cells and regulates EMT related gene expression

To elucidate the role of miR-186 downregulation in the development and progression of glioma, we selected the U87MG and LN18 cell lines among the four cell lines that we tested, to conduct further functional studies. We generated miR-186-mimic and miR-186-inhibitor respectively. Transfected with miR-186-mimic and miR-186-inhibitor and negative control microRNA, we tried to reveal the gain-of-function effect or lose-of-function effect on the migration and invasion of the GBM cell lines. We found that both of U87MG and LN18 cells exhibited high transfection efficiency (**Figure 2A**). As demonstrated by Transwell invasion

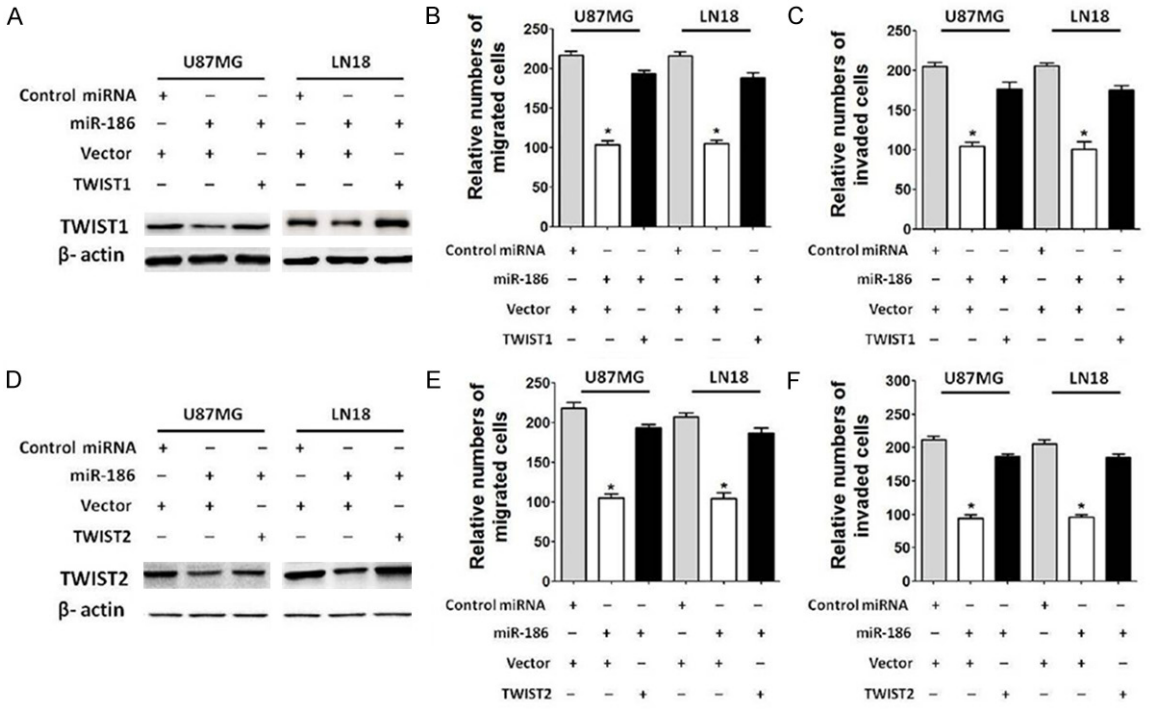

Figure 4. The role of TWIST1 and TWIST2 in miR-186-regulated cell migration and invasion. A. U87MG or LN18 cells were co-transfected with control miRNA or miR-186 mimics and with either TWIST1 3'-UTR deleted plasmid or empty vector. Ectopic expression of TWIST1 was confirmed by Western blotting assays. β-actin was used as a loading control. B and C. Motility and invasion assay of U87MG or LN18 cells co-transfected with control miRNA or miR-186 mimics and with either TWIST1 3'-UTR deleted plasmid or empty vector. Invasion assay was performed with transwell-inserts coated with Matrigel. Images were taken with invert microscope (magnification, ×100). D. U87MG or LN18 cells were co-transfected with control miRNA or miR-186 mimics and with either TWIST2 3'-UTR deleted plasmid or empty vector. Ectopic expression of TWIST2 was confirmed by Western blotting assays. β-actin was used as a loading control. E and F. Motility and invasion assay of U87MG or LN18 cells co-transfected with control miRNA or miR-186 mimics and with either TWIST2 3'-UTR deleted plasmid or empty vector. Invasion assay was performed with transwell-inserts coated with Matrigel. Images were taken with invert microscope (magnification, ×100).

and migration assays, over-expression of miR-186 significantly reduced the invasiveness and migration of U87MG and LN18 cells, whereas miR-186-inhibitor significantly promoted the numbers of migrated and invaded cells in the same cells (**Figure 2B-E**).

Since EMT is closely related to cancer cell metastasis ability, we next examined EMT markers in control-, miR-186-, or miR-186-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 decreased in miR-186 mimics transfectants, whereas E-cadherin, an epithelial marker, was increased in both U87MG and LN18 cells. In contrast, miR-186-inhibitor had the opposite effects (**Figure 2F-H**). Taken together, these results indicate that anti-metastatic potential could be attributed to the selective regulation of EMT proteins in glioma cancer cells.

miR-186 directly targets TWIST1 and TWIST2

Since miRNAs mainly function through inhibiting their target mRNAs by binding to the 3'UTR, we searched the putative target genes of miR-186 in online miRNA target prediction databases (Targetscan and microRNA.org), and found that TWIST1 and TWIST2 are potential targets of miR-186. The conserved target genes TWIST1 and TWIST2, which have been identified as invasion associated genes, were selected for subsequent investigation (**Figure 3A**). To further confirm that TWIST1 and TWIST2 are the direct targets of miR-186, a dual-luciferase reporter system was used with co-transfection of miR-186 and a luciferase reporter plasmid

containing a wild-type or mutant 3'UTR of human TWIST1 and TWIST2. Luciferase activity was significantly inhibited by miR-186, but increased by miR-186-in co-transfected with wild-type TWIST1 or TWIST2 3'UTR, and miR-186 and miR-186-in failed to inhibit the expression of luciferase constructs with mutated target sites, suggesting that miR-186 directly targets the 3'UTR of TWIST1 or TWIST2 (**Figure 3B**, **3C**). In addition, a change in mRNA and protein expression levels of TWIST1 and TWIST2 in response to miR-186 over-expression or inhibition was verified by RT-PCR and Western blotting in U87MG and LN18 cells (**Figure 3D-F**), confirming that miR-186 negatively regulates TWIST1 and TWIST2 expression by directly targeting their 3'UTR regions.

miR-186 regulates cell migration and invasion through targeting TWIST1 and TWIST2 in GBM

Down-regulation of TWIST1 and TWIST2 by miR-186 in GBM prompted us to investigate whether miR-186 affects GBM cell invasion and migration via TWIST1 and TWIST2. U87MG and LN18 cells were co-transfected with miR-186 and either pMIR-GLOTM -TWIST1 (without 3'-UTR region) or empty pMIR-GLOTM 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-186-induced suppressed invasion and migration of GBM cells. Our data shows that TWIST1 partially recovered GBM cell invasion and migration profiles (**Figure 4B**, **4C**). Meanwhile, similar to the experiments in TWIST1, we downregulated and rescued TWIST2 expression (**Figure 4D**) and, consistent with TWIST1 findings, TWIST2 also rescued cell migration and invasion that was inhibited by miR-186 (**Figure 4E**, **4F**). These results confirm that TWIST1 and TWIST2 are regulated by miR-186 and miR-186 down-regulation, and may participate in GBM carcinogenesis and progression through potentiating of TWIST1 and TWIST2 expression.

Discussion

It is well known that miRNAs play vital role in various biological processes, including proliferation, cellular differentiation, signal transduction and carcinogenesis [15-17]. Here, we found that miR-186 was down-regulated in glioma cells and glioma tissues, compared with that in nonneoplastic brain specimens and primary normal human astrocytes (NHA). In addition, we demonstrated that restoration of miR-186 suppressed the invasion and migration of glioma cancer cells, while miR-186 inhibition had the opposite effect, suggesting a fundamental role of miR-186 as a tumor suppressor in glioma cancer.

Epithelial-mesenchymal transition (EMT), which enables epithelial cells to acquire invasive mesenchymal phenotype, is attracting increasing attention as an important mechanism for the initial step of metastasis [18, 19]. Because the effect of miR-186 on cell migration and gene expression regulation, we detected the change of EMT markers in U87MG and LN18 cells transfected with miR-186 and miR-186-in. ectopic expression of miR-186 significantly increased E-cadherin expression. Accordingly, expression of N-cadherin and Vimentin was reduced when miR-186 was over-expressed in U87MG and LN18 cells. The results supported that miR-186 may reverse EMT process to inhibit cell migration.

Homodimeric and heterodimeric basic helix-loop-helix (bHLH) transcription factors function as master regulators of lineage specification and differentiation. Among these, TWIST1 and TWIST2, and inhibitor of differentiation proteins have key roles in EMT progression [12, 20, 21]. In the present study, TWIST1 and TWIST2 were predicted as the target of miR-186 by Targetscan and miRanda. Through dual-luciferase assays, we confirmed the TWIST1 and TWIST2 3'UTR as direct target of miR-186. The role of TWIST in promoting EMT processes has been widely reported [22, 23]. In our current study, we found re-expression of TWIST1 or TWIST2 could partially rescue the miR-186-induced inhibition of cell migration and invasion, suggesting that TWIST1 and TWIST2 act as functionally relevant targets of miR-186 in glioma cancer. Taken together with previous studies, it seems that miR-186 serves as a pivotal mediator in regulation of GBM metastasis.

Meanwhile, our data showed that miR-186 exerts tumor suppressor function by regulating epithelial-mesenchymal transition in GBM. Given the previous reports, we examined some EMT regulator molecules which are reported to be important for regulating the epithelial-mes-

enchymal transition [19, 24, 25]. Our results showed that the important molecules in the regulation at epithelial-mesenchymal transition, N-cadherin and Vimentin, was reduced in U89MG and LN18 glioma cells transfected with miR-186-mimic, which is similar as transfected the si-TWIST1 and si-TWIST2, but increased in the cells transfected with miR-186-inhibitor, compared with control cells. As TWIST1 and TWIST2 play an essential role in EMT as well as tumor metastasis, our data establish a mechanistic link between miR-186, TWIST1/2, EMT and tumor metastasis.

In conclusion, we found, for the first time, that miR-186 directly regulated TWIST1 and TWIST2 expression in glioma cells. Our data provide evidence that down-regulation of miR-186 is involved in metastatic events, which is consistent with the function of miR-186 in modulating EMT. These results reveal a previously unknown function of miR-186 to prevent metastasis by suppressing EMT. Targeting to the miR-186/TWIST1/2 interaction or rescuing miR-186 expression may be a new therapeutic application to treat glioma patients in the future.

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Disclosure of conflict of interest

None.

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