Genome-wide identification of epithelial-mesenchymal transition-associated microRNAs reveals novel targets for glioblastoma therapy

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Abstract. MicroRNAs (miRNA) regulate a number of cellular processes. Recent studies have indicated that these molecules function in the epithelial-mesenchymal transition (EMT). However, the crucial systematic role of EMT and miRNAs together in glioblastoma (GBM) remains poorly understood. The present study demonstrated that EMT was closely associated with malignant progression and clinical outcome using three independent glioma databases (GSE16011, Rembrandt and The Cancer Genome Atlas). Furthermore, integrated analysis of miRNAs and mRNA profiling in 491 GBM samples revealed an EMT biological process associated with an miRNA profile (19 positively and 18 negatively correlated miRNAs). Among these miRNAs, miR-95 and miR-223 indicated a high level of functional validation, reflecting their positive correlation with EMT. Additionally, the upregulation of miR-95, which was negatively correlated with EMT, inhibited cellular invasion in glioma U251 and LN229 cells and decreased the expression of the mesenchymal marker N-catenin, whereas an miRNA positively correlated with EMT, miR-223, exhibited the opposite effect. Therefore, the results of the present study could further enhance the current understanding of the functions of miRNAs in GBM, indicating that the EMT-specific miRNA signature may represent a novel target for GBM therapy.

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Introduction

Glioma is the most common type of brain tumor in children and adults (1). Stage IV glioblastoma (GBM), graded according to the World Health Organization tumor classification system, as having a duration of 12-15 months (2). The 5-year survival rate of patients with stage IV GBM is <5%, despite the administration of chemotherapy, radiotherapy, surgical resection and other intensive treatment modalities. As a result, it is necessary to develop novel efficacious therapies to support the continuous improvement of the prognosis of patients with GBM (3).

MicroRNAs (miRNAs/miRs) are non-coding, small, endogenous RNAs that regulate the expression of a number of genes by specific antisense complementarity with target mRNAs. These molecules can act as oncogenes and tumor suppressors (4). Previous studies have indicated that miRNAs regulate a number of different biological processes, including the invasion, apoptosis, proliferation and differentiation of cells (1,5-7). Recent studies have identified and implicated the miRNAs involved in the progression of various cancer types as novel targets for anticancer therapies (4-7).

The epithelial-mesenchymal-transition (EMT) involves the transdifferentiation of epithelial cells into mesenchymal cells. This process has been implicated in the progression of cancer, including metastasis and invasion (8,9). The present study demonstrated that EMT is closely associated with malignant progression and clinical outcome in patients with glioma, and identified the EMT biological processes associated with the miRNA profile of GBM, which may provide potential novel targets for GBM therapy.

Materials and methods

Microarray data and bioinformatics analysis. Microarray data from the GSE16011 and Rembrandt datasets were collected (10,11). The Rembrandt dataset (Affymetrix GeneChip Human Genome U133 Plus 2.0 Array) and the CEL files for GSE16011 were used, and the data were separately merged using Matlab software R2012a (Mathworks, Inc., Natick, MA, USA). The expression data were normalized according to the robust multi-array average normalization. The array data from The Cancer Genome Atlas (TCGA) GBM

Agilent miRNAs (gene expression level 3) and HG-U133A gene expression mRNAs (gene expression level 3) were downloaded from TCGA Data Portal (12).

Gene expression signatures were used in the present study to define the process in which epithelial cells transition to the mesenchymal cells, based on a meta-analysis of gene expression studies (GES) (13). A total of 130 downregulated or upregulated genes from the EMT-core-gene list, with >10 GES (EMT_up and EMT_down genes) were used in the present study. EMT_up and EMT_down gene set enrichment scores in the gene expression microarray were assessed using gene set variation analysis (GSVA) (14).

Oligonucleotide transfection and cell culture. Human glioma U251 and LN229 cell lines were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) culture medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). Cells were maintained at 37˚C and 5% CO_2. The cells were regularly passaged at 2-3 day intervals. Cells at passages 2-4 were used in the present study.

The miR-95 and miR-223 mimic (5'-UCAAUAAAUGUC UGUUGAAUU-3' for miR-95 and 5'-CGUGUAUUUGAC AAGCUGAGUU-3' for miR-223) and control (5'-UUCUCC GAACGUGUCACGUTT-3') were synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China). Oligonucleotide transfection was performed using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in U251 and LN229 cells at 70-90% confluence. On the basis of the manufacturer's instructions, the transfection complexes were prepared and subsequently added to the glioma cells to obtain a 10-nmol/l final oligonucleotide concentration. The transfection medium was replaced at 8 h post-transfection.

Invasion assays. Cell invasion assays were performed using Transwell membranes coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). According to the manufacturer's instructions, 500 μl DMEM without serum was used to prehydrate the 24-well invasion chambers (8.0 μm; BD Biosciences) for 2 h at 37˚C with 5% CO_2. In DMEM without serum, the cells were seeded at a density of $5x10^4$ cells/well in the upper chamber. The lower chamber was filled with 500 μl 20% FBS as a chemo-attractant. The non-migrating cells were removed from the top well using a cotton swab following incubation for 24 h. The cells in the bottom chamber were then fixed using 75% alcohol (37˚C for 5 min) and subsequently stained the cells with 0.1% crystal violet (37˚C for 1 min). The cells in each field of view migrating towards the bottom side across the filter were counted at a magnification of x100 (light microscope) to quantify glioma cell migration. A total five fields of view were counted in this experiment. Three independent experiments were conducted.

Reverse transcription quantitative PCR (RT-qPCR). RNA was extracted from U251 and LN229 glioma cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was converted into a cDNA template using PrimeScript™ RT reagent kit (Takara Bio, Inc., Otsu, Japan). N-cadherin mRNA level was analyzed by RT-qPCR on the ABI 7300 HT Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR® PrimeScript™ RT-PCR kit (Takara Bio, Inc.). N-cadherin were amplified with the primer: 5'-GGTGGAGGA GAAGAAGACCAG-3' (Sense) and 5'-GGCATCAGGCTC CACAGT-3' (Antisense). The amplification of β-actin with primer: 5'-AAGACCTGTACGCCAACACAGT-3' (Sense) and 5-AGAAGCATTTGCGGTGGACGAT-3' (Antisense) was taken as an internal control. Relative gene expression was calculated via the $2^{-\Delta\Delta Cq}$ method (15).

Statistical analysis. Significant differences were calculated using one-way analysis of variance followed by the Student-Newman-Keuls method for multi-group comparisons; Student's t-test was used to perform two-group comparisons. Data are presented as the mean ± standard deviation. SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis, other than Pearson's correlation analysis, which was conducted using Matlab, and the log-rank test of Kaplan-survival curves for survival analysis, which was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

EMT is closely associated with malignant progression and clinical outcomes in glioma. In the present study, the association of EMT with malignant progression and clinical outcome was investigated. The EMT_up and EMT_down gene set enrichment scores in the gene expression microarray were assessed using GSVA. As depicted in Fig. 1, the EMT_up geneset enrichment score was significantly upregulated in gliomas, compared with non-tumor tissues, and the increasing expression of EMT_up geneset enrichment score was significantly associated with the grade of glioma malignancy in the GSE16011 and Rembrandt datasets. In TCGA, the EMT_up gene set enrichment score was also increased in GBMs, compared with non-tumor tissues. Furthermore, the EMT_up geneset enrichment score could predict the clinical consequences in patients with low-grade gliomas, GBMs and anaplastic gliomas (Fig. 2).

Identification of EMT biological process associated with miRNA profiles. To analyze aberrant gene expression during EMT, the paired profiling data of miRNAs and mRNA profiling (level 3) were downloaded from TCGA. A total of 491 TCGA GBM samples were examined in the present study. Matlab software was used to calculate the Pearson's correlation to determine the association between the miRNAs and the EMT_up gene set enrichment score. miRNAs exhibited a high correlation with the EMT_up gene set enrichment score ($P<0.01$, $r<-0.3$ and $r>0.3$ correlated with EMT_up gene set enrichment score, Table I and Fig. 3), and were considered as the EMT-specific miRNA signature. As depicted in Table I and Fig. 3, the EMT-specific miRNA signature included 18 and 19 miRNAs negatively and positively correlated with EMT_up gene set enrichment scores, respectively. In the present study,

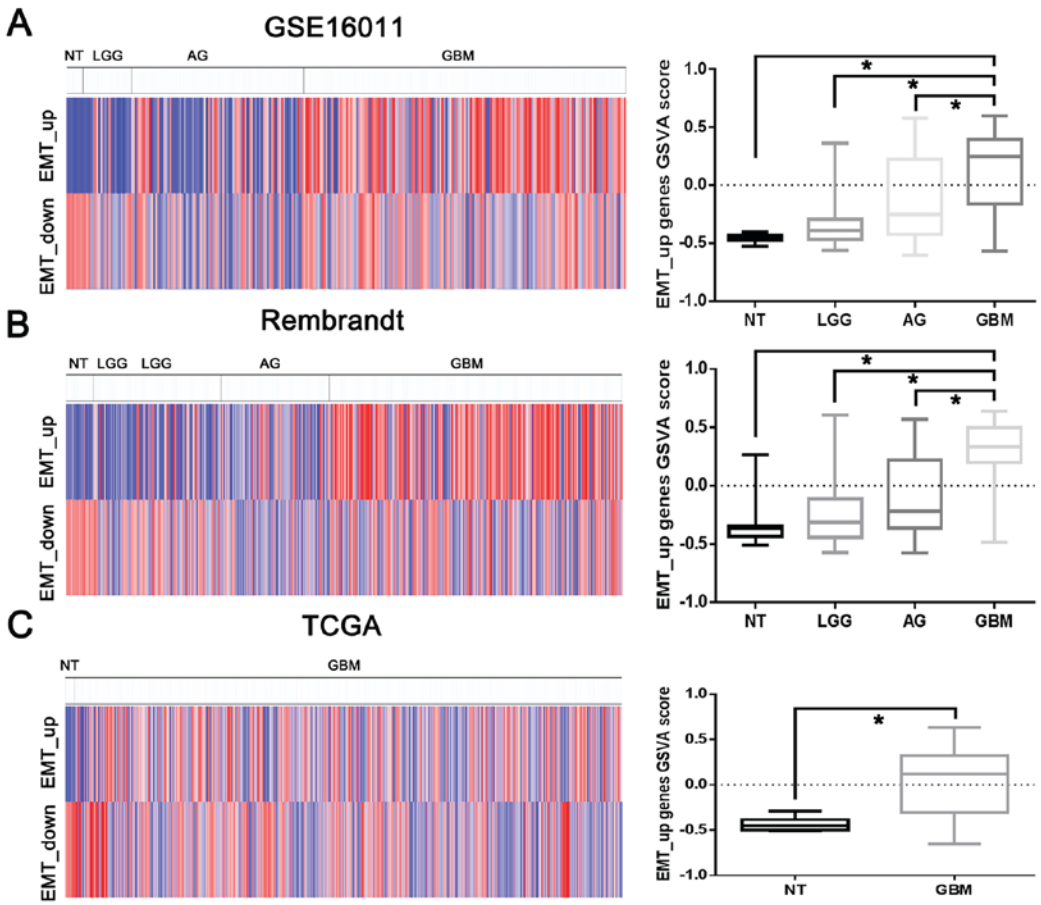

Figure 1. EMT biological process is involved in the malignant progression of glioma. The increasing EMT_up geneset enrichment score is significantly associated with the grade of glioma malignancy in (A) GSE16011 and (B) Rembrandt datasets (*P<0.05). (C) EMT_up geneset enrichment score is also increased in TCGA GBMs, compared with NT tissues. *P<0.05. TCGA, The Cancer Genome Atlas; NT, non-tumor; EMT, epithelial-mesenchymal transition; GBM, glioblastoma; GSVA, Gene Set Variation Analysis; LGG, low-grade gliomas; AG, anaplastic gliomas.

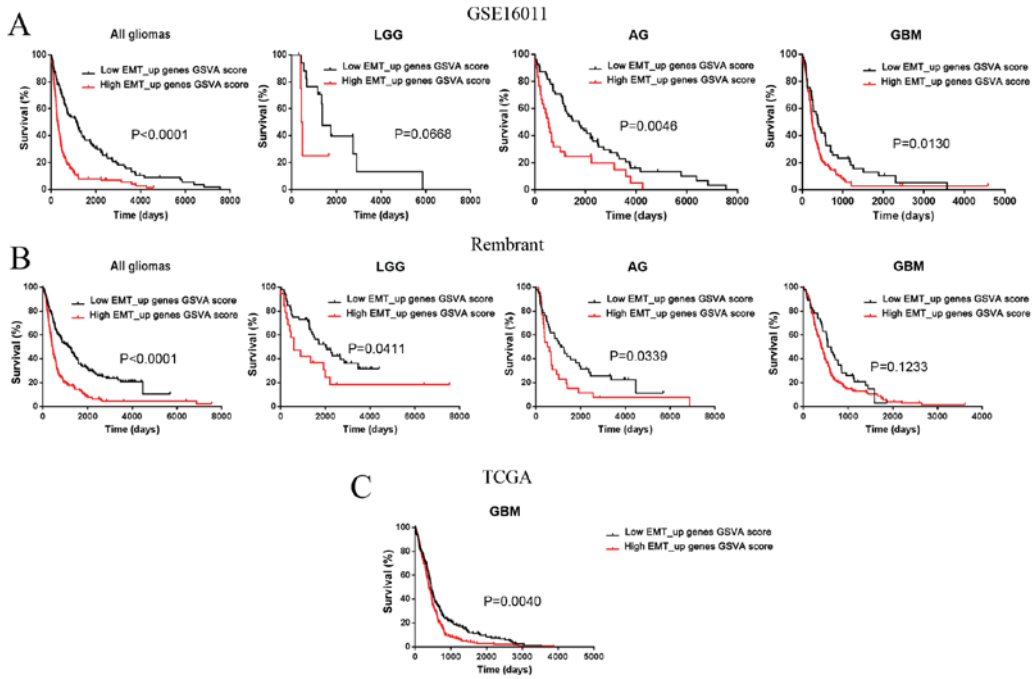

Figure 2. EMT biological process predicting the clinical outcome in patients with malignant glioma, regardless of the grades of glioma malignancy in three independent glioma datasets. Kaplan-Meier analysis of overall survival rates in the (A) GSE16011, (B) Rembrandt and (C) TCGA datasets. TCGA, The Cancer Genome Atlas; EMT, epithelial-mesenchymal transition; GBM, glioblastoma; GSVA, Gene Set Variation Analysis; LLG, low-grade gliomas; AG, anaplastic gliomas.

Table I. Specific miRNA signature of the epithelial-mesenchymal transition biological process.

Negatively correlated miRNAs			Positively correlated miRNAs		
ID	R-value	P-value	ID	R-value	P-value
miR-128b	-0.4205	1.85×10^{-22}	miR-21	0.4774	2.53×10^{-29}
miR-95	-0.4185	3.09×10^{-22}	miR-223	0.4715	1.53×10^{-28}
miR-128a	-0.4167	4.78×10^{-22}	miR-155	0.4384	1.78×10^{-24}
miR-9	-0.3815	1.85×10^{-18}	miR-222	0.4046	9.10×10^{-21}
miR-340	-0.3596	1.96×10^{-16}	miR-34a	0.3978	4.62×10^{-20}
miR-9*	-0.3581	2.67×10^{-16}	miR-199a	0.3773	4.71×10^{-18}
miR-101	-0.3555	4.47×10^{-16}	miR-22	0.3679	3.47×10^{-17}
miR-301	-0.3534	6.93×10^{-16}	miR-214	0.3599	1.86×10^{-16}
miR-488	-0.3523	8.54×10^{-16}	miR-210	0.3441	4.30×10^{-15}
miR-181c	-0.3495	1.48×10^{-15}	miR-199a*	0.3400	9.44×10^{-15}
miR-33	-0.3431	5.20×10^{-15}	miR-142-5p	0.3356	2.17×10^{-14}
miR-181d	-0.3351	2.37×10^{-14}	miR-221	0.3284	8.22×10^{-14}
miR-598	-0.3320	4.22×10^{-14}	miR-422b	0.3279	9.07×10^{-14}
miR-181a	-0.3177	5.63×10^{-13}	miR-34b	0.3271	1.04×10^{-13}
miR-181a*	-0.3166	6.82×10^{-13}	miR-23a	0.3201	3.65×10^{-13}
miR-769-5p	-0.3068	3.68×10^{-12}	miR-27a	0.3192	4.30×10^{-13}
miR-129	-0.3021	8.08×10^{-12}	miR-142-3p	0.3181	5.23×10^{-13}
miR-153	-0.3009	9.83×10^{-12}	miR-630	0.3090	2.53×10^{-12}
			miR-513	0.3011	9.46×10^{-12}

miRNA/miR, microRNA.

several well-characterized tumorigenesis-associated miRNAs, including miR-21-, miR-181- and miR-128-family members, were detected. During EMT, these molecules exhibited the same, decreased or increased expression. Furthermore, a number of miRNAs with currently unknown functions, including miR-95, miR-155 and miR-223, exhibited similar characteristics. Of these molecules, two miRNAs, miR-223 and miR-95, exhibited a high positive and negative correlation with EMT, respectively, and were selected for functional validation. The upregulation of miR-95 was demonstrated to inhibit cellular invasion in U251 and LN229 glioma cells and reduce the expression of the mesenchymal marker N-catenin, whereas miR-223 was demonstrated to have the opposite effect (Fig. 4).

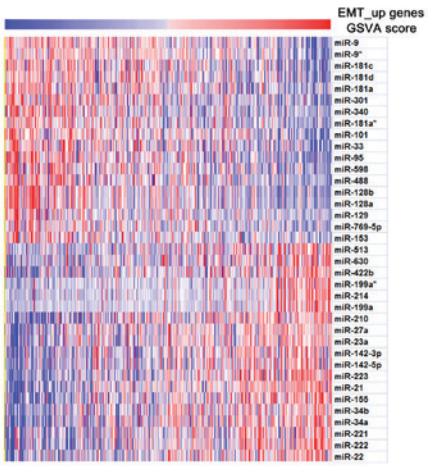

Figure 3. Identification of the EMT expression profile of specific miRNAs in GBM. Heatmap depicting the EMT expression profile of specific miRNAs (19 positively correlated and 18 negatively correlated miRNAs, with EMT_up geneset enrichment scores of r>0.3 and r<-0.3, respectively, P<0.01) in 491 GBM samples, sorted in order of EMT_up geneset enrichment score. miRNA/miR, microRNA; EMT, epithelial-mesenchymal transition; GSVA, Gene Set Variation Analysis; GBM, glioblastoma.

Discussion

miRNAs exhibit different expression signatures that exert an influence on the behaviors of a number of cancer cell types (16). EMT involves the conversion of cells to a mesenchymal phenotype from an epithelial phenotype, which leads to increased chemo-resistance and high cell mobility and therefore represents a notable event during the dissemination and progression of cancer (13). However, the role of EMT and the patterns of miRNA expression involved in the EMT of gliomas have not sufficiently been investigated to date. The present study observed that EMT was positively associated with malignant progression and clinical outcome in three independent glioma datasets, GSE16011, Rembrandt and TCGA. Furthermore, integrated analysis of miRNAs and the profiling of mRNAs 491 GBM samples were performed, which revealed that the EMT-associated miRNA profile may provide potential novel targets for GBM therapy.

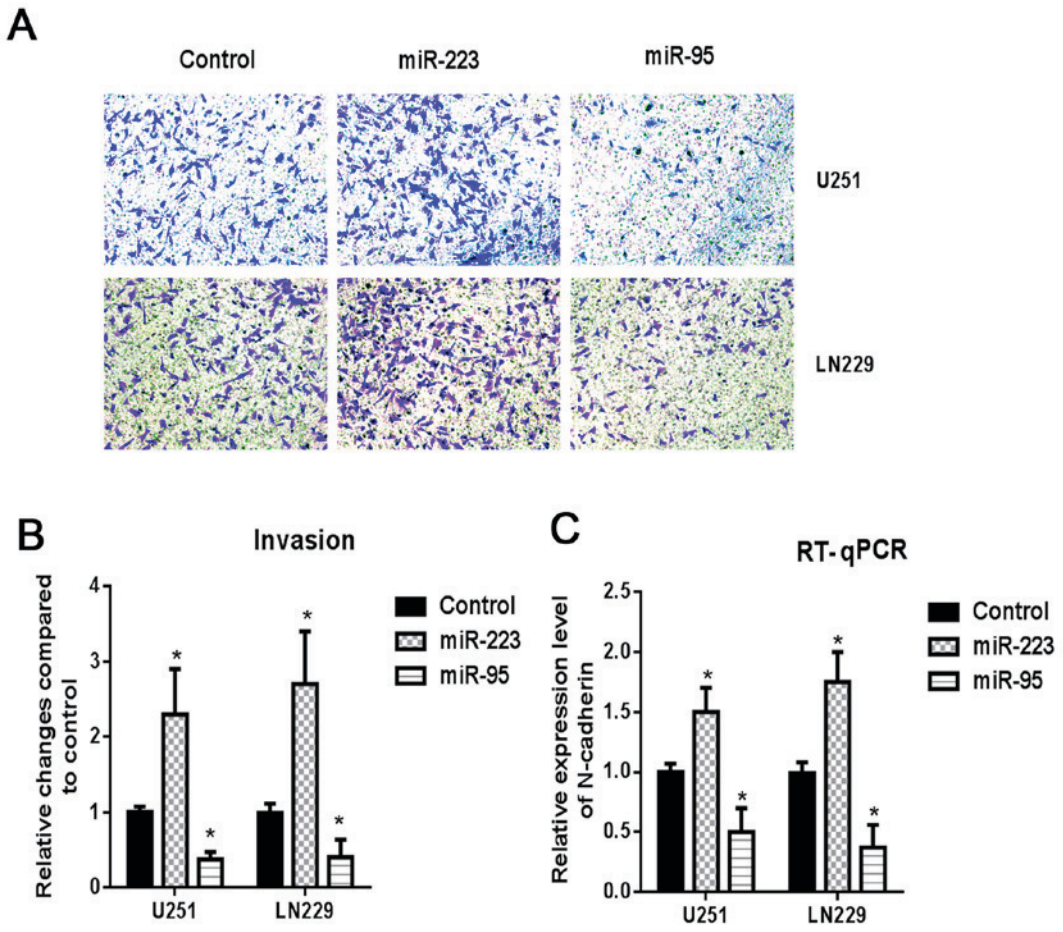

Figure 4. Influences of miR-95 and miR-223 on the expression and invasion of N-cadherin in glioma cells. (A) Representative cell images of the effect of miR-95 and miR-223 on the cellular invasion of U251 and LN229 glioma cells. (B) The quantitative data of the effect of miR-95 and miR-223 on the cellular invasion of U251 and LN229 glioma cells. All experiments were repeated three times. (C) Following the transfection of miR-95 and miR-223 into U251 and LN229 glioma cells, Reverse transcription-quantitative polymerase chain reaction demonstrated that the N-cadherin mRNA level was significantly regulated. The data are obtained from representative experiments repeated in triplicate. *P<0.05 vs. the control. miRNA/miR, microRNA.

The progression of the majority of carcinomas towards malignancy is associated with an increase in the mesenchymal phenotype and a loss of epithelial differentiation, accompanied by increased invasion and mobility (17). In the present study, the clinical significance of EMT was analyzed in three independent glioma datasets, GSE16011, Rembrandt and TCGA. The results demonstrated that EMT was closely associated with malignant progression in gliomas, as previously proposed (8,9). These results indicated that EMT was also involved in the malignant transformation of glioma.

miRNAs inhibit EMT-associated phenotypic changes in a number of different cancer types, and can provide anticancer therapies with a novel target (18). In the present study, the EMT-associated miRNA profile was identified through the integrated analysis of miRNAs and mRNA profiling in 491 TCGA GBM samples. Among the miRNAs profiled in the current study, a number of well-characterized tumorigenesis-associated miRNAs, including miR-21, miR-181 and miR-128 family members, and numerous miRNAs with presently unknown functions, including miR-95, miR-155 and miR-223, also exhibited similarly decreased or increased expression during EMT. A number of previous studies have recognized EMT as an early event of invasion/metastasis (8,9,13). The upregulation of miR-95, which is negatively correlated with EMT, was demonstrated to inhibit cellular invasion in U251 and LN229 glioma cells and reduce the levels of N-catenin expression, whereas miR-223, which was positively correlated with EMT, produced the opposite effects. However, only the mRNA expression level of N-cadherin, and not that of the protein, was examined in the present study, which is a limitation of the current study. The protein expression of N-cadherin should therefore be studied further in future. These results indicated that the EMT-specific miRNA expression profile may provide potential targets for GBM therapy.

In summary, the present study demonstrated that EMT was closely associated with malignant progression and clinical outcome in glioma. To the best of our knowledge, the current study provided the first evidence of an EMT-specific miRNA expression profile, which may provide potential targets for GBM therapy. These data can aid the identification of therapeutic and prognostic markers. Additional studies are required to characterize the roles of the miRNAs involved and to identify further miRNAs as molecular targets of therapy for patients with GBM.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL and YY concieved and designed the study. YZ, AZ, SL, XW and WY downloaded the gene expression data and performed the bioinformatics analysis. RL, AZ and SL performed the *in vitro* experiments. YZ, RL, XW and WY wrote the manuscript. All authors gave the final approval of the version to be published.

Ethics approval and consent to participate

All patients provided signed informed consent and the study was approved by the institutional Review Board of Nanjing Medical University.

Consent for publication

Written informed consent was obtained from all participants for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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