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Original Article

MicroRNA-940 inhibits epithelial-mesenchymal transition of glioma cells via targeting ZEB2

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Abstract: MicroRNAs have been found ectopically expressed in many cancers and play essential roles in tumor EMT progress. Recent studies identified decreased miR-940 expression in glioma cells and may serve as a tumor-suppressor. However, whether miR-940 involve in glioma EMT remain poorly understood. Here we confirmed that miR-940 was significantly reduced in glioma cells and tissues. Introduction of miR-940 dramatically suppressed invasion and migration of glioma cells. Gain-of-function experiments showed ZEB2 as a direct target of miR-940, knockdown of ZEB2 evidently repressed invasive capacity of glioma cells through EMT. Moreover, reintroduction of ZEB2 effectively reversed the tumor suppressive effect of miR-940 treatment. In vivo study showed reduced tumor cell motion in miR-940-injected groups. Spearman's correlation analysis indicated inversely correlated expression of ZEB2 and miR-940 in gliomas and NBTs. Altogether, miR-940-ZEB2 cascade may play important roles in glioma cells invasion and EMT progression, and might provide new therapeutic approaches for better outcomes of GBM patients.

Keywords: miR-940, ZEB2, invasion/migration, EMT, glioma

Introduction

Glioblastoma has been characterized as the most malignant and lethal human brain tumor for many years [1-3]. Despite advanced treatments such as surgery, radiation and chemotherapy, patients generally show poor prognosis with high recurrence and mortality rates [4, 5]. The median survival of patients with GBM is less than 15 months [6, 7]. Incomplete understanding of GBM's malignancy and mechanisms of tumorigenesis leads to the limited treatment for clinical GBM patients. Therefore, effective identification and novel therapeutic strategies are required to elucidate mechanisms underlying GBM progression for improving survival outcomes of GBM patients.

MicroRNAs are well-documented small RNAs that play important parts in tumor development by binding to the 3's untranslated regions (3'UTRs) of their target genes [8]. Recent studies uncovered special functions of miRNAs in regulating cell proliferation, migration, invasion and differentiation in many types of cancers [9]. Furthermore, miRNAs have been demonstrated to be associated with identifying and judging the severities of diseases as diagnostic and prognostic markers [10]. In gliomas, many miRNAs have been identified to be ectopically expressed compared to normal brain tissues and thus play essential roles in regulating tumorigenesis and development [11-15]. MiR-940, a newly multifunctional microRNA has recently been demonstrated tumor-suppressor functions in many cancers. Of which, it can suppress pancreatic ductal adenocarcinoma growth by targeting MyD88 [16], inhibit the progression of non-small cell lung cancer by targeting FAM83F [17], and suppress hepatocellular carcinoma tumor cell migration and invasion by regulation of CXCR2 [18]. MiR-940 has also been revealed to have anti-tumor functions in ovarian cancer and breast cancer [19-21]. However, in cervical cancer and gastric cancer, miR-940 embodied tumor-promoting functions [22, 23]. A recent study identified miR-940 impedes glioma progression by blocking mitochondrial folate metabolism through targeting of MTHFD2, but specific mechanisms of miR-

940 regulated gain of glioma invasive capacity with EMT progress has not been clearly clarified. Our work focused on the EMT pathway mediated miR-940 suppress glioma invasion and further evaluated its potentials in glioma.

Epithelial-mesenchymal transition (EMT) occurs in the initiation of organisms and facilitates the progression of epithelial tumors to invasive phenotypes and play essential parts during this process [24]. EMT includes disruption of the cytoskeleton, intercellular adhesions and transcriptional factors expression, and is pivotal in promoting glioma invasive capacity as well as chemoresistance [25-27]. Many well-established EMT-inducing transcription factors (EMT-TFs) are essential in driving EMT process including Snail1, Slug, Twist1 and Twist2 which have been uncoverd in embryogenesis, tumorigenesis and progression directly or indirectly [28-32]. Furthermore, ZEB family members, such as ZEB1 and ZEB2, both are zinc finger proteins with basic helix-loop-helix (bHLH) structure, are also key EMT-TFs in glioma progression [33-35]. However, mechanisms of these TFs involved in EMT steps and glioma development remain dismal. Our study highlight decreased miR-940 expression in glioma cell lines and tissues, and confirmed that ectopic miR-940 expression induced glioma cell migration and invasion. Moreover, we found ZEB2 is a direct downstream target of miR-940, knockdown of ZEB2 remarkably dampened invasive capacity of glioma cells through EMT. Moreover, reintroduction of ZEB2 effectively reversed the tumor suppressive effect of miR-940. Recent studies demonstrated ZEB2 is highly associated with EMT process during tumorigenesis, indicating the essential role of ZEB2 in promoting glioma progression [34]. Our work established miR-940-ZEB2 cascade that play key roles in GBM invasion through EMT pathway, and revealed a new mechanism underlying glioma progression, thus may provide potential therapeutic strategies for improving survival outcomes of GBM patients.

Materials and methods

Cell lines and culture conditions

LN229, T98G, U118 and H4 glioma cells were purchased from American Type Culture Collection ATCC. U87, A172 and U251 human GBM cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Before each experiment, GBM cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Normal human astrocytes (NHAs) were gained from Lonza (Basel, Switzerland) and cultured in provided growth media supplemented with GA-1000, rhEGF, 5% FBS, ascorbic acid, insulin and L-glutamine.

Atlases of glioma samples and gene set enrichment analysis

Whole glioma miRNA microarray data of 158 specimens were obtained from the Chinese Atlas (CGGA) data portal (http://www.cgga.org.cn/portal.php). The Gene Set Enrichment Analysis (GSEA) of the differentially expressed genes was performed using software downloaded from the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp). All Clinical glioma samples were provided by the First Affiliated Hospital of Nanjing Medical University. 8 non-cancerous brain tissues, 12 low grade glioma tissues and 19 GBM tissues were included in this study. The use and study of all the specimens were approved by the Research Ethics Committee of Nanjing Medical University, and written informed consents were obtained from all participants. The procedures were conducted according to the approved guidelines.

Plasmids construction, transfection, and stable cell establishment

MiR-940 mimic or miR-NC were purchased from Ribobio (Guangzhou, China). ZEB2 plasmid was chemically synthesized and provided by Genechem (Shanghai, China). ZEB2 cDNA was cloned to the pGL3 vector to construct pGL3-ZEB2 recombinant plasmid according to the manufacturer's protocol. All plasmids were transfected into U87 and LN229 glioma cells using Lipofectamine 3000 Transfection Reagent (Invitrogen) accordingly. Premade lentiviral ZEB2 short hairpin RNA (shRNA) constructs and a shCtrl construct were purchased from Open Biosystems. The lentiviral packaging kit was purchased from Genechem (Shanghai, China) and stable cell lines were established by infecting U87 and LN229 glioma cells with lentiviruses according to the manufacturer's instructions.

RNA extraction and qPCR analysis

Total RNA was extracted using TRIzol Reagent (Invitrogen). cDNAs were amplified by following the manufacturer's protocol. Quantitative real-time PCR was run using SYBR Premix Dimer Eraser from Takara (Dalian, China) on a 7900HT reaction system. Primers of miR-940 were purchased from Ribobio (Guangzhou, China) and U6 used as a loading control. 2-ΔΔCt method was used to calculate the fold changes.

Orthotopic glioma model

Animal experiments were approved by the Nanjing Medical University Institutional Committee for Animal Research and the Animal Management Rule of the Chinese Ministry of Health. U87 glioma cells transfected with lentivirus overexpressing miR-940 or the negative control sequences were intracranially injected into 6-week-old Nude mice. Bioluminescence imaging was used to detect the Intracranial tumor growth volumes. Within 60 days, all the nude mice were sacrificed.

IHC assay

IHC assay was used to determine the expression of ZEB2, N-cadherin, E-cadherin and Vimentin of the brain tissues and glioma samples from nude mice. Briefly, Fresh nude mice brain tissues were under cryopreservation and processed into frozen sections and then fixed with 4% paraformaldehyde, incubated with primary antibodies overnight. antibody reactions were detected by exposure to 3,3-diaminobenzidine and hydrogen peroxide chromogen substrate. Slides were imaged under a light microscope (Leica, German) at 200 or 400 × magnification.

Reporter assay

The mutated putative and wild-type (WT) miR-940 target on ZEB2 were cloned into pGL3 Luciferase reporter vector (Invitrogen) to generate the pGL3-WT-ZEB2-3'UTR and pGL3-MUT-ZEB2-3'UTR plasmids. Luciferase reporter assay was performed according to the manufacturer's protocol. The Luciferase Assay System was used to measure the dual luciferase activity.

Western blot assay and antibodies

The proteins of glioma tissues or cell lines were loading onto 10% SDS-PAGE followed by transferring to a PVDF membrane. After blocking in 5% milk for 2 h, the membranes were blotted with primary antibodies. Antibodies against ZEB1 (ab203829), N-cadherin (ab18-203), E-cadherin (ab1416), Vimentin (ab8978), Snail1 (ab53519), Twist1 (ab50581), Twist2 (ab66031), SLUG (ab27568), MMP2 (ab37150), Fibronectin (ab32419) and α-SMA (ab32575) were purchased from Abcam, USA. ZEB2 (NB-P1-82991) was purchased from Novus, USA.

Transwell assays

Cell invasion potential was determined on plates with transwell inserts (Corning, New York, USA) which were pre-coated with 20 μg/μl Matrigel (BD Biosciences, New jersey, USA). For this, 2×10^4 cells were seeded in the upper well of the invasion chamber in DMEM without serum. The lower chamber well contained DMEM supplemented with 10% FBS to stimulate invasion. After incubation for 24 h at 37°C, non-invading cells from the upper surface of the membrane were removed with a cotton swab and the cells at the bottom of the upper chamber were fixed with 100% methanol for 10 min, and stained with 0.1% crystal violet for 20 min. 6 independent fields of cells were captured and counted randomly at × 20 magnification in each well.

Three-dimensional spheroid assays

Established U87 and LN229 glioma cell lines were cultured to 80% confluence. Cells were seeded at a 0.3×10^5 cells/ml density in 96-well ultralow adherence plates (#7007, Costar) and cultured for 96 h at 37°C. After these cells formed into a multicellular spheroid with an estimated density of 3000 cells, matrigel was added into each well and cultured for 72 h, motion of cells was confirmed as fully formed under light microscopy.

Wound healing assays

The migratory capacity of glioma cells was measured by wound healing assays. Briefly, U87 and LN229 glioma cells were seeded in six-well dishes. Upon confluency, the dish surface was scratched with a P200 pipette tip and the photographs of scratched areas were taken. After 24 h, nine scratched areas were selected randomly in each well and were photographed. The cells protruding from the border of the scratches were counted.

Statistics

All experiments were performed in triplicate with means and standard error of the mean or standard deviation subjected to Student's t-test for pairwise comparison or ANOVA for multivariate analysis. Kaplan-Meier survival analysis was performed using Graph-Pad 6.0 software. A significance level set at $P<0.05$ was considered significant for all the tests.

Results

MiR-940 levels are decreased in glioblastoma tissues and correlates with EMT process

Since previous study uncovered downregulated miR-940 levels in glioma tissues and to further prove this, we analyzed miR-940 expression in 158 patients based on the Chinese Glioma Genome Atlas (CGGA) database. Results indicated that miR-940 expression was negatively correlated with glioma grade (**Figure 1A**). Then we compared the expression of miR-940 of 158 glioma patients in four glioma subtypes (Classical, Proneural, Mesenchymal and Neural) based on CGGA. Result showed significantly decreased miR-940 expression level in mesenchymal subtype compared with proneural subtype (**Figure 1B**). Next, we determined miR-940 expression in 19 HGGs, 12 LGGs and 8 non-cancerous brain tissues. HGGs were found to decrease compared with LGG and NBTs, whereas NBTs shows the highest expression of miR-940 (**Figure 1C**). Using the expression data from the CGGA dataset, we identified 184 downregulated genes and 140 upregulated genes in the low miR-940 expression group (**Figure 1D**). All these genes were named "miR-940 differentially expressed genes". We used GSEA to testify if there are differences in the mesenchymal transition-associated genes listed by Cheng [36] in the CGGA dataset. Result showed that gene set of EMT was enriched in samples with low expression of miR-940 (**Figure 1E**). Furthermore, we performed gene annotation analysis using Metascape (provided by Zhou et al. [37]) based on expression level of miR-940 of glioma patients in CGGA, and found gene set of extracellular matrix organization was highly enriched in miR-940 downregulated genes (**Figure 1F** and **1G**). These results suggesting that miR-940 is downregulated in gliomas patients in comparison with normal brain tissues. Bioinformatic analysis indicated gene sets of EMT and extracellular matrix organization were highly enriched in low miR-940 expression samples. Thus, it may play a pivotal role in tumor-invasion via EMT and may have potential value in diagnosis and prognosis of clinical glioma patients.

Induced miR-940 suppresses glioma cells invasion and migration

To further investigate the potential effect of miR-940 on tumorigenesis and malignancy of glioma cells, we performed in vitro gain-of-function experiment by transfecting miR-940 mimic in two glioma cell lines. To test the overexpression efficiency, qPCR analysis was performed and results verified that miR-940 levels were significantly increased in both U87 and LN229 glioma cell lines compared with miR-ctrl group, results were showed in our previous paper which has not been published yet but in proofreading process. First, we tested effect of miR-940 on EMT markers. In agreement with our hypothesis, overexpressed miR-940 dramatically decreased levels of some mesenchymal biomarkers, such as N-cadherin, Vimentin, Fibronectin, α-SMA as well as MMP2, whereas the epithelial biomarker E-cadherin was significantly increased compared with the miR-ctrl groups (**Figure 2A**). Next, we conducted transwell assays to evaluate the influence of miR-940 on invasion and migration ability in glioblastoma. Results showed that ectopically expressed miR-940 with mimics strongly reduced U87 and LN229 glioma cells invasive capacity (**Figure 2B** and **2C**). Moreover, we performed a wound healing assay using miR-940 mimic-transfected U87 and LN229 cells and control cells. Overexpressing miR-940 markedly inhibited the migration of U87 and LN229 glioma cells into the scratch-wounded area (**Figure 2D** and **2E**). To further confirm our conclusion, we conducted a 3D spheroid BME cell invasion assay and the same result was detected (**Figure 2F** and **2G**). Collectively, these results verified that miR-940 suppresses invasion and migration of glioma cells through EMT pathway.

ZEB2 silencing is involved in the miR-940 repressed invasive and migratory capacity

MiRNAs are well-documented noncoding RNAs composed of 18-23 nucleotides and exert their functions by binding to specific 3'UTR regions

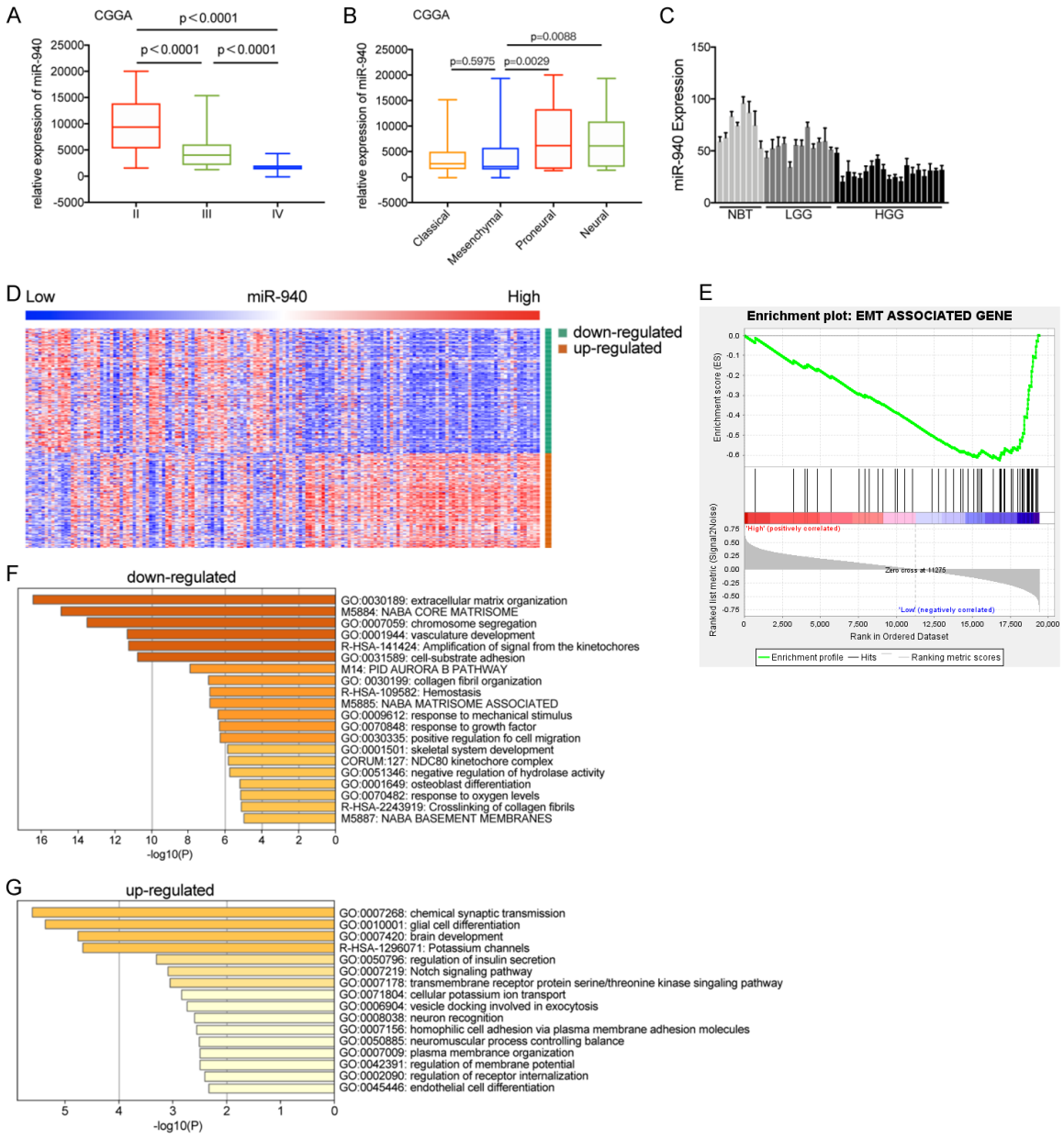

Figure 1. MiR-940 is decreased in GBM tissues and correlates with EMT. A. Comparison of miR-940 levels among grade II, grade III and grade IV gliomas in CGGA database. B. Expression levels of miR-940 in four glioma subtypes (Classical, Mesenchymal, Proneural and Neural) based on CGGA. C. miR-940 expression in 8 non-cancerous brain tissues, 12 low-grade glioma tissues and 19 high-grade glioma tissues was detected by qRT-PCR. D. In silico analysis of miR-940 expression and the correlation with its associated genes in glioma of CGGA datasets. A heatmap of miR-940 relative level differentially expressed genes in glioma tissues sorted by miR-940 expression level. E. GSEA analysis indicated enriched expression of EMT-associated genes in low miR-940 expression samples of glioma. F, G. Gene annotation analysis of genes sorted by relative miR-940 level in glioma of CGGA database using Metascape.

of downstream targets. As we uncovered that miR-940 can suppress glioma cells invasion and migration, we explored its potential mechanisms. Through bioinformatics, we used TargetScan to predict its potential downstream target, and we focused on ZEB2, an EMT transcription factor. To verify if miR-940 can directly binding to 3'UTR region of ZEB2, we performed dual luciferase reporter assays in U87 cells co-transfected with miR-940 mimic and vectors harboring wild-type or mutant ZEB2 3'UTRs (**Figure 3A**). As shown in **Figure 3B**, luciferase

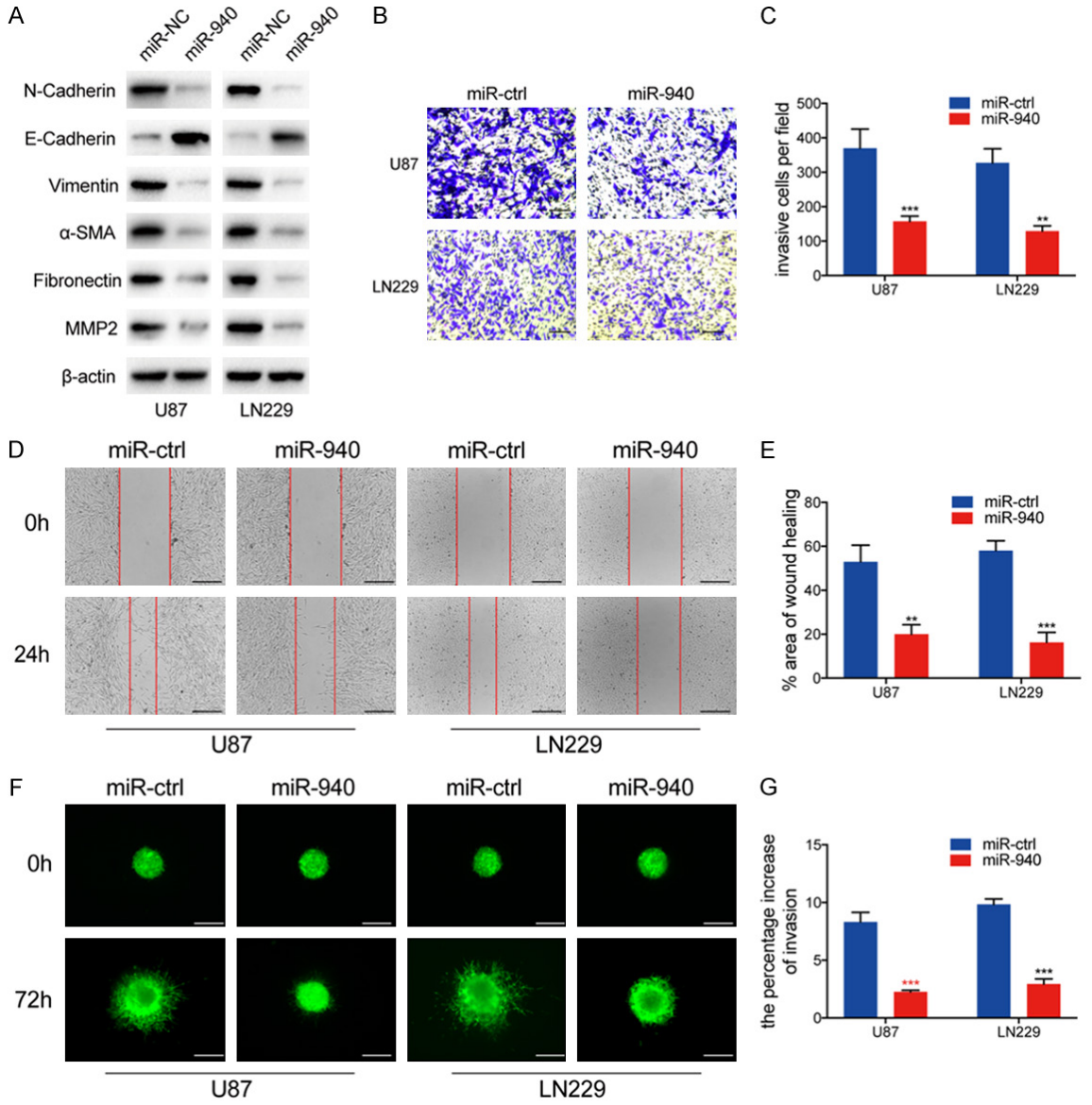

Figure 2. MiR-940 overexpression inhibits glioma invasion/migration in vitro. A. Western blot analysis of N-cadherin, E-cadherin, Vimentin, MMP2, Fibronectin and α-SMA expression in U87 and LN229 cells after overexpression of miR-940. B, C. Transwell invasion assay showing the invasive capacity of U87 and LN229 cells transfected with miR-940 mimic and miR-ctrl. Data are means of three independent experiments ± SD (**P<0.01, ***P<0.001). D, E. Wound healing assay showing the migration capacity of U87 and LN229 glioma cells transfected with miR-940 mimic and miR-ctrl. Data are means of three independent experiments ± SD (**P<0.01, ***P<0.001). F, G. Representative images of 3D spheroid BME cell invasion assays using LN229 and U87 cells with indicated plasmids. Data are means of three independent experiments ± SD (***P<0.001).

activity was strongly suppressed by miR-940 but not changed obviously with mutated 3'UTR and this result was verified in LN229 cells (**Figure 3B**). To evaluate the relationship between ZEB2 and miR-940 in gliomas, we conducted western blotting to examine the protein levels of ZEB2 in glioma tissues and non-cancerous brain tissues. We found ZEB2 was obviously increased in glioma tissues as compared with that of the non-cancerous brain samples. Low grade gliomas also showed decreased ZEB2 levels than High grade gliomas (**Figure 3C** and **3D**). To address the relationship between ZEB2 and miR-940, we performed Spearman's correlation analysis and compared ZEB2 and miR-940 levels in each group, result showed

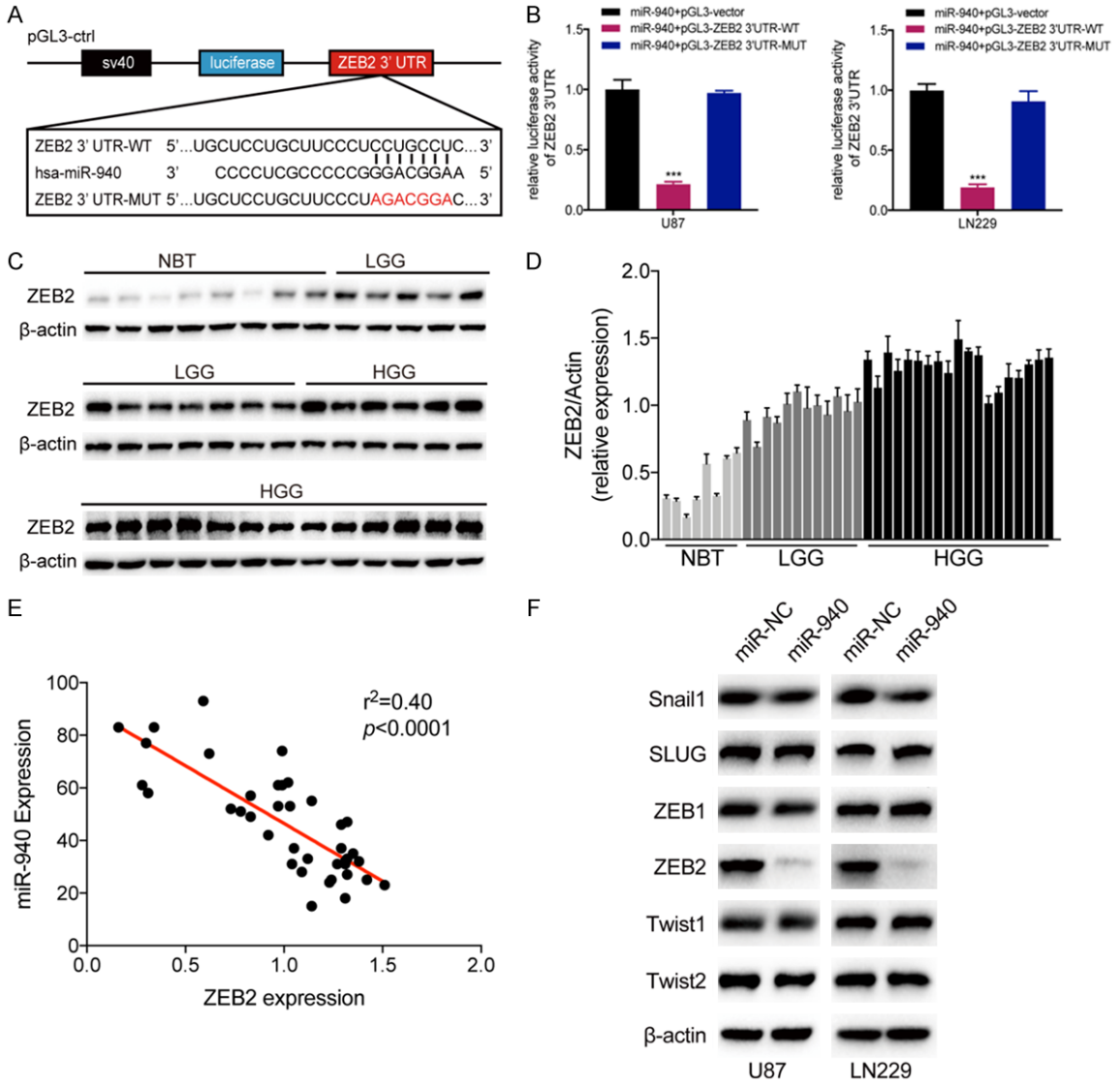

Figure 3. ZEB2 is a downstream target of miR-940. A. Predicted binding sites of wild-type (WT) and mutated sequences of miR-940 in the 3'-UTR of ZEB2 mRNA. B. Luciferase reporter assay was used in U87 and LN229 cells transfected with pGL3-Vector, pGL3-ZEB2-3'UTR-WT, or pGL3-ZEB2-3'UTR-MUT reporter with miR-940 mimic. Data are means of three independent experiments ± SD (***P<0.001). C, D. ZEB2 protein levels in NBTs and glioma tissues were tested by western blotting and β-actin served as the loading control. Eight non-neoplastic brain tissues were collected from brain trauma surgery. Twelve low-grade glioma samples were derived from grades I and II glioma tissues, and nineteen high-grade glioma samples represent grade III and IV glioma tissues. Data are means of three independent experiments ± SD (***P<0.001). E. The correlation between the relative miR-940 expression and ZEB2 expression was tested using Pearson's correlation analysis. F. Snail1, SLUG, ZEB1, ZEB2, Twist1 and Twist2 expression levels in indicated cells were determined by western blotting.

significantly negative correlated miR-940 and ZEB2 expression levels in samples (**Figure 3E**). To confirm ZEB2 as the specific target of miR-940 that inducing EMT in glioma, we performed western blot assay and tested other important EMT-inducing transcription factors (Snail1, Twist1, Twist2, ZEB1 and SLUG), in line with our hypothesis, only ZEB2 levels were dramatically downregulated (**Figure 3F**). Together, these results showed miR-940 can direct binding to the 3'UTR region of ZEB2 and thus suppresses glioma cells invasion and migration through ZEB2-dependent EMT pathway.

ZEB2 knockdown suppresses glioma cells invasion/migration through EMT

As we have demonstrated ZEB2 is a direct target of miR-940 in glioma cells, we next exam-

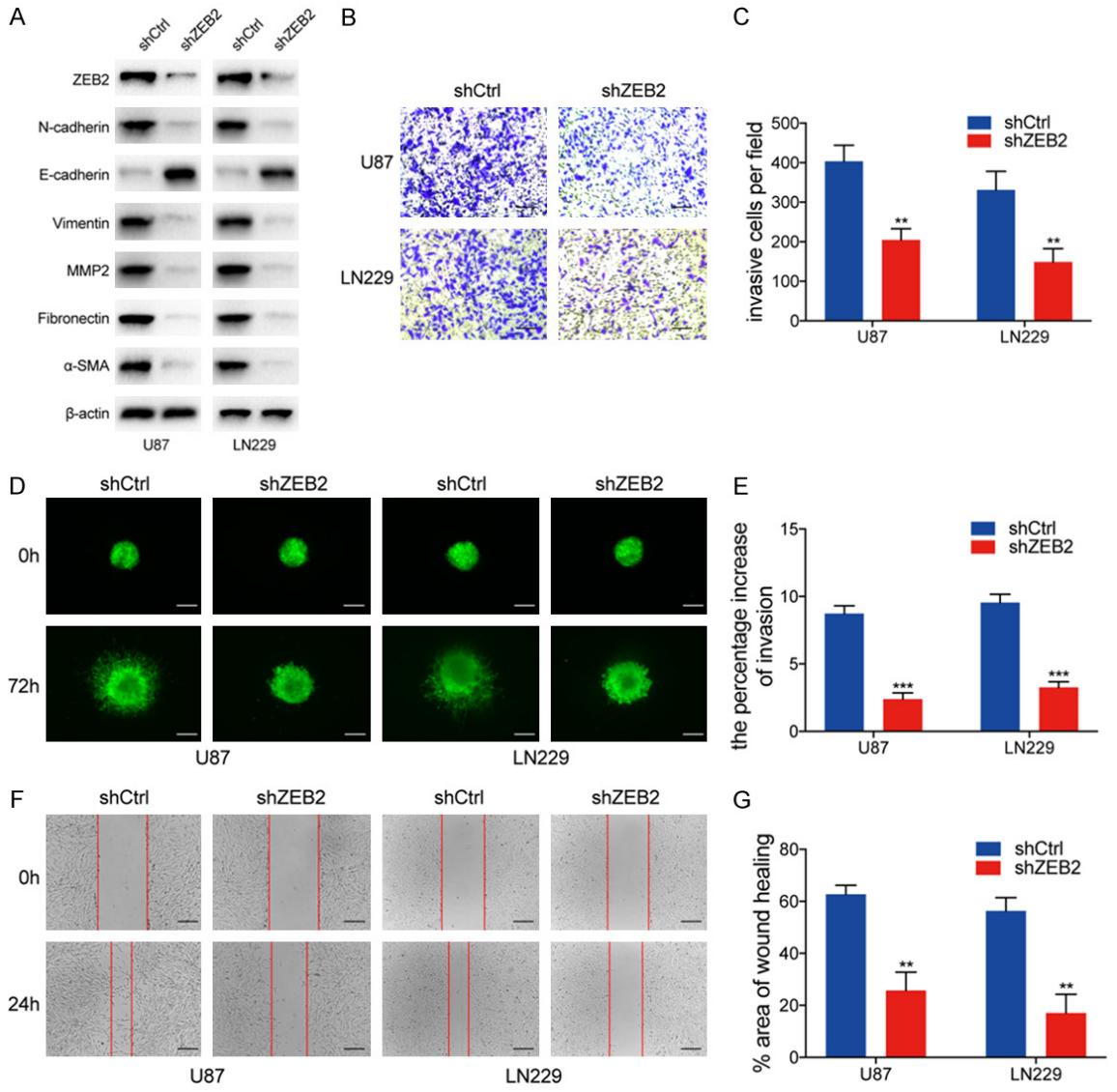

Figure 4. ZEB2 knockdown suppresses GBM cells invasion/migration through EMT. A. Western blot analysis of ZEB2, N-cadherin, E-cadherin, Vimentin, MMP2, Fibronectin and α-SMA expression in U87 and LN229 cells after knockdown of ZEB2. B, C. Transwell invasion assay was used to evaluate the invasive ability using U87 and LN229 glioma cells transfected with shCtrl or shZEB2 plasmids. Data are means of three independent experiments ± SD (**P<0.01). D, E. Representative images of 3D spheroid BME cell invasion assays using U87 and LN229 cells with the indicated plasmid. Data are means of three independent experiments ± SD (**P<0.01, ***P<0.001). F, G. Representative images of wound healing assay using U87 and LN229 cells with indicated plasmids. Data are means of three independent experiments ± SD (***P<0.001).

ined its function on EMT in gliomas. To prove this, we transfected shCtrl and shZEB2 plasmids into U87 and LN229 cells, western blotting indicated that the protein levels of ZEB2 were obviously decreased by shRNAs in both cell lines. Consistent with previous studies, EMT biomarkers such as N-cadherin, Vimentin, Fibronectin, α-SMA as well as MMP2 were significantly downregulated by ZEB2 knockdown, while E-cadherin was markedly upregulated (**Figure 4A**). The effect of ZEB2 knockdown on invasive and migratory capacity was tested in U87 and LN229 cells using transwell assays, wounding assays and 3D spheroid BME cell invasion assays. Similar results were noted in all these experiments that knockdown of ZEB2 dramatically dampened the invasion and migration ability of GBM cells (**Figure 4B-G**).

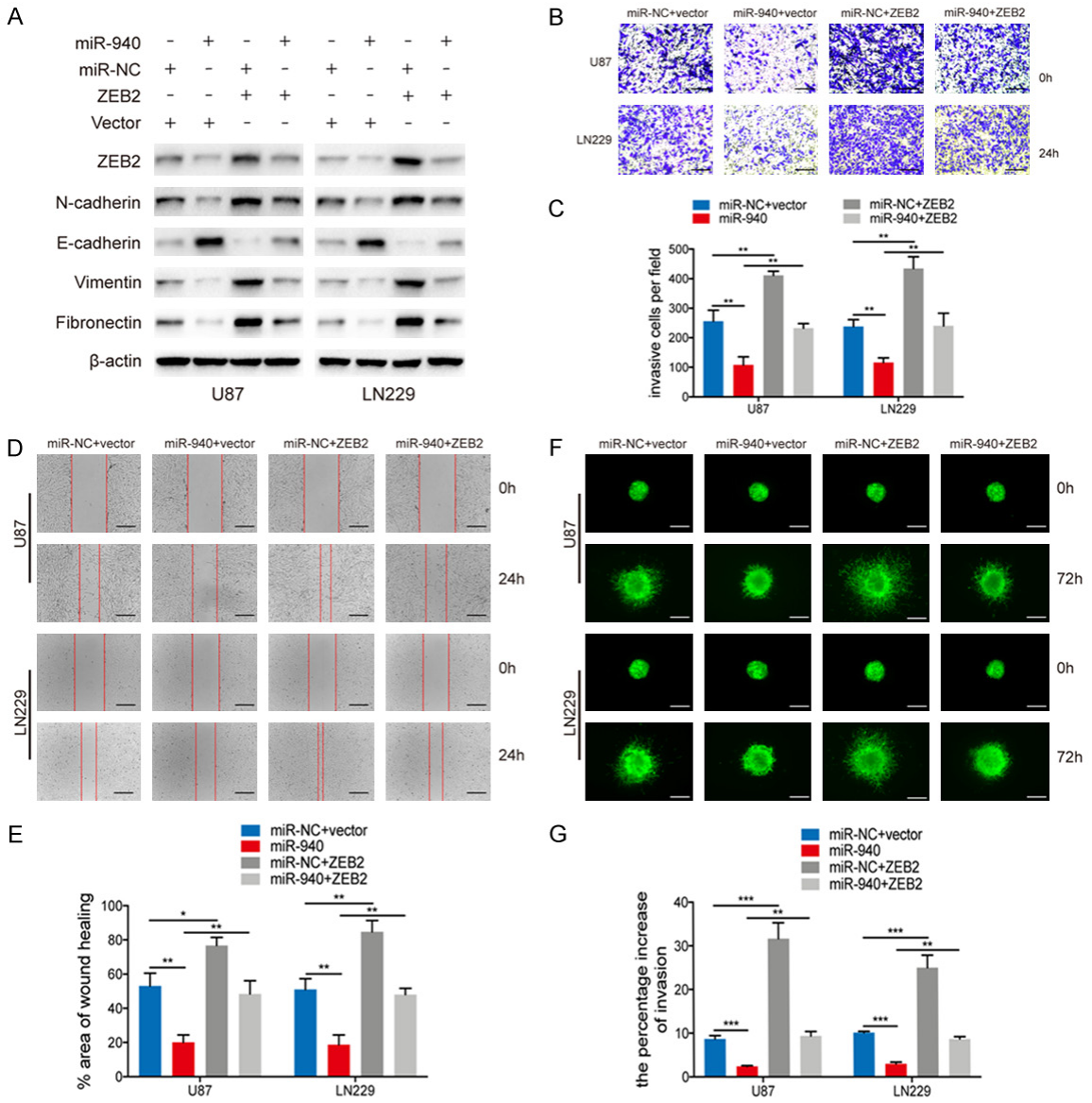

Figure 5. ZEB2 is involved in the function of miR-940. A. Western blot analysis of ZEB2, N-cadherin, E-cadherin, Fibronectin and Vimentin in U87 and LN229 glioma cells transfected with vector or ZEB2 in the presence of miR-NC or miR-940. β-actin used as the loading control. B, C. Cell invasion ability was detected by transwell assays using U87 and LN229 glioma cells with the indicated plasmids. Data are means of three independent experiments ± SD (**P<0.01). D, E. Cell migration ability was tested vie wound healing assays using u87 and LN229 glioma cells with the indicated plasmids. Data are means of three independent experiments ± SD (*P<0.05, **P<0.01). F, G. Representative images of 3D spheroid BME cell invasion assays using U87 and LN229 cells with the indicated plasmid. Data are means of three independent experiments ± SD (**P<0.01, ***P<0.001).

Reintroduction of ZEB2 restored invasion potential on glioma cells

As we have verified in our previous experiments that miR-940 suppressed the invasive and migratory capacity of glioma cells through directly binding to the 3'UTR region of ZEB2, we wondered if ZEB2 could reverse these phenotype changes caused by miR-940 overexpression. To evaluate our thought, we transduced ZEB2 plasmids which were stably expressing miR-940 or miR-NC and conducted western blot assays to test changes of some EMT biomarkers. In glioma cells in which miR-940 were stably highly expressed, the alterations in EMT biomarkers were reversed by ZEB2 overexpression (**Figure 5A**). We next performed transwell assays, wound healing assays to demonstrate the function of ZEB2 in miR-940 dependent invasion and migration. Notably, results indi-

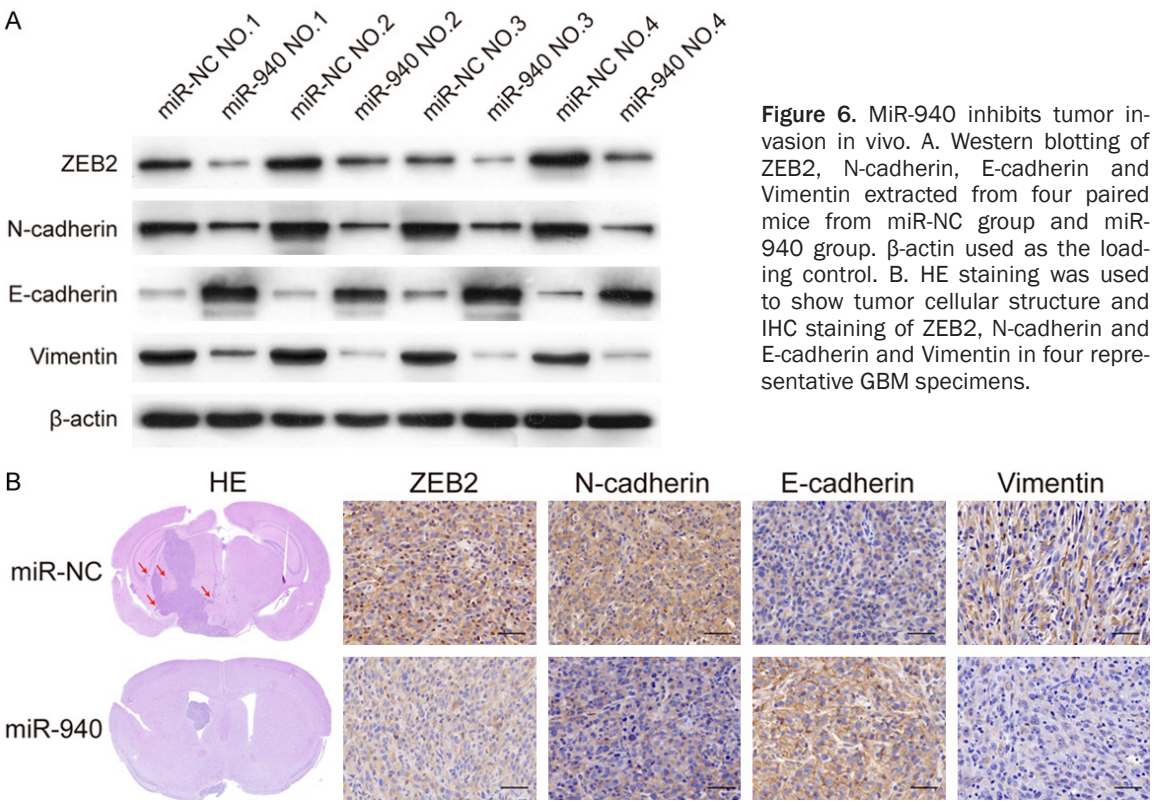

Figure 6. MiR-940 inhibits tumor invasion in vivo. A. Western blotting of ZEB2, N-cadherin, E-cadherin and Vimentin extracted from four paired mice from miR-NC group and miR-940 group. β-actin used as the loading control. B. HE staining was used to show tumor cellular structure and IHC staining of ZEB2, N-cadherin and E-cadherin and Vimentin in four representative GBM specimens.

cated that overexpressing ZEB2 completely restored the invasive capability of U87 and LN229 cells suppressed by induced miR-940 (**Figure 5B-E**). All results were consistent with the results of 3D spheroid BME cell invasion assays (**Figure 5F** and **5G**). Taken together, our findings further confirmed the result that ZEB2 is a direct target of miR-940 and involves in the miR-940 dependent invasive phenotype of glioma cells vie EMT.

Intracranial injection mouse model and IHC

Our previous work has shown that miR-940 decreases the expression of ZEB2 and blocks glioma cells invasion and migration by directly binding to the 3'UTR region of ZEB2, which was confirmed in U87 and LN229 glioma cell lines. Further studies should be performed in vivo. The U87 cell transfected with miR-940 or miR-NC were transplanted in orthotopic nude mice model. On days 30, mice were euthanatized and brains were taken out and subjected to further tests. To investigate the ZEB2 expression in the orthotopic nude mice model. we randomly selected four pairs of nude mice model from miR-940-injected groups and miR-NC-injected groups. Western blotting showed dramatically decreased ZEB2, N-cadherin, Vimentin expression and increased E-cadherin expression in miR-940 groups as compared to the mice in miR-NC groups (**Figure 6A**). Moreover, HE staining showed cell motion was weaken in miR-940-injected groups. IHC staining also showed strikingly reduced ZEB2, N-cadherin, Vimentin expression and increased E-cadherin expression in nude mice of miR-940-injected groups compared to miR-NC groups (**Figure 6B**). Taken together, in vivo studies also demonstrated the anti-EMT potential of miR-940 in gliomas.

Discussion

High invasive potential accounts for large parts of the leading cause of mortality in GBM patients, but molecular mechanisms underlying this carcinogenic phenotype remain incompletely clarified [38]. EMT, the transformation progress from epithelial to mesenchymal phenotype change, has been implicated in promoting invasive capacity of glioma cells, in which TFs are key factors during this process [30, 34]. MicroRNA-940, which has been characterized as a dual-functional RNA and involved in the proliferation, invasion, migration in many cancers [17-19, 39]. Recent studies revealed ectopically expressed miR-940 in glioma tissues and inhibits glioma progression through

blocking mitochondrial folate metabolism by targeting of MTHFD2 [40]. However, the invasive capacity repressed by miR-940 through EMT has not been verified completely.

Our study report for the first time that miR-940 functions as a tumor invasion inducer in glioma through EMT. Our data confirmed a decreased expression level of miR-940 in glioma, indicating its tumor suppressor potential in glioma. We tested clinical glioma tissues and found downregulated miR-940 levels in gliomas. In line with this finding, miR-940 expression levels were obviously low in glioma cell lines compared with NHA. Moreover, functional experiments showed miR-940 overexpression significantly repressed glioma cell invasive and migratory capacity. Additionally, we noticed this phenotype change correlates with EMT closely. To address this, we examined several EMT markers and found downregulated N-cadherin, Vimentin and upregulated E-cadherin protein levels in glioma cells treated with miR-940 overexpression. In vivo study further confirmed the anti-invasive capacity of miR-940 and its influence on EMT markers. Although more tests should be made to further explore the role of miR-940, the striking impairment of wound healing and 3D sphere formation in glioma cell lines induced by miR-940 strongly support our hypothesis that miR-940 suppresses invasion and migration of glioma cells.

Zinc finger E-box binding homeobox 2 (ZEB2), a member of the zinc finger family, is a transcription factor that play important roles in EMT process [41] and is well-documented in regulating cell invasion, migration, proliferation and apoptosis in many types of cancer [34]. Here, we report miR-940 silences ZEB2 expression by directly binding to the 3'UTR region of ZEB2, and found miR-940-suppressed invasive capacity via EMT is dependent on ZEB2 to alter EMT-relevant markers. All the data was consistent with our previous observation that miR-940 is crucial in regulating glioma cells invasive and migratory capacity through EMT.

In conclusion, our work revealed miR-940 play an essential part in inhibiting glioma cells invasion and migration through EMT by directly targeting ZEB2, indicating it as a potential molecular biomarker for glioblastoma patients. Despite the striking findings, more tests should be employed to further explore the specific mechanism of anti-tumor functions of miR-940 for improving survival outcomes of patients with glioma.

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Disclosure of conflict of interest

None.

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