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Original Article

miR-181d/MALT1 regulatory axis attenuates mesenchymal phenotype through NF-κB pathways in glioblastoma

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ABSTRACT

The mesenchymal (MES) subtype of glioblastoma (GBM) indicated a more malignant phenotype and worse prognosis compared with their proneural (PN) counterpart. The plasticity between PN and MES transcriptome signatures provided an approach for clinical intervention. However, few miRNAs have been identified to participate in the shift between subtypes. Here, we utilized transcriptomic data and experimental evidences to prove that miR-181d was a novel regulator of NFκB signaling pathway by directly repressing MALT1, leading to induced PN markers and reduced MES genes. Functionally, ectopic expression of miR-181d suppressed GBM cell proliferation, colony formation and anchor-independent growth, as well as migration, invasion and tube formation. Moreover, miR-181d overexpression increased radio- and chemo-sensitivity for GBM cells. Rescue of MALT1 could partially reverse the effects of miR-181d in GBM malignant behaviors. Clinically, miR-181d could serve as a prognostic indicator for GBM patients. Taken together, we concluded that loss of miR-181d contributes to aggressive biological processes associated with MES phenotype via NFκB signaling, which broaden our insights into the underlying mechanisms in subtype transition and miRNA-based tailored medicine for GBM management.

Introduction

Glioblastoma (GBM; World Health Organization grade IV), the most common and lethal primary brain tumor, carries an invariably poor clinical outcome in adults [1]. Despite treatment involving surgery, ionizing radiation, and chemotherapy with the alkylating agent temozolomide (TMZ), patients with GBM only have an average survival of slightly more than 1 year after the initial diagnosis [1,2]. The unfavorable prognosis is largely attributed to the highly proliferative, invasive and therapeutic resistant nature of glioma. Hence, an understanding of the biomolecular mechanisms involved in glioma malignant behaviors is urgently needed.

To comprehensively explore the molecular mechanism of GBM, many groups have turned to high-throughput profiling researches [3]. Although inconsistencies were observed across various classification schemes, the mesenchymal (MES) and proneural (PN) subtypes were robust and generally consistent [4–6]. GBM patients in the MES subtype exhibit radio- and chemo-resistant properties, increased invasiveness, reduced cell stiffness, and relatively worse prognosis than PN tumors [7]. Moreover, PN tumors were proved to give rise to MES recurrences, indicative of a MES transition during glioma progression [5]. Following studies further confirmed the occurrence of MES shift in GBM and identified several master transcription factors, including NFκB, STAT3, were responsible for this procedure [7,8]. Considering the malignant behaviors of MES GBMs, clarifying the mechanistic basis activating MES phenotype may aid in the development of tailored therapy for GBM patients.

Abbreviations: GBM, glioblastoma; MES, mesenchymal subtype; PN, proneural subtype; TMZ, temozolomide; NC, negative control; FACS, fluorescence-activated cell sorting; GO, Gene Ontology; GSEA, gene set enrichment analysis; GSVA, gene set variation analysis.

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MicroRNAs (miRNAs) are endogenous small noncoding RNAs, which could act as regulators of gene expression by post-transcriptionally reducing target mRNAs with partial complementarity in their 3′-untranslated regions (UTRs) [9]. A spectrum of miRNAs have been demonstrated to be altered in multiple cellular processes including proliferation, apoptosis, differentiation, migration and angiogenesis, affecting the hallmarks of tumorigenic processes [10,11]. Similarly, several miRNAs have been found to participate in the pathogenesis of GBM, including oncogenic miR-21 and tumor-suppressive miR-181 family [12–14]. Besides of the classification and prognostic significance, these miRNAs could also serve as biomarker for glioma diagnosis and therapeutics [15]. In the present study, we aimed to investigate the expression and function of miR-181d in both glioma patients and cell lines. By comprehensively analyzing the miRNA/mRNA profiles, we revealed that miR-181d was highly expressed in PN subtype and could serve as a prognostic indicator for glioma patients. *In vitro* and *in vivo* experiments both demonstrated that miR-181d restrained the malignancy of GBM cells via its direct target mucosa associated lymphoid tissue lymphoma translocation gene 1 (MALT1) and NFκB signaling pathway. Notably, NFκB pathway inhibition by miR-181d inhibited the expression of MES signature, which will increase the sensitization of GBM cells to chemo- and radio-therapy. Collectively, our results will help us comprehend the underlying mechanisms of miR-181d in glioma and provided an alternative miRNA-based approach for glioma precision oncology.

Materials and methods

Tissue specimens

The mRNA/miRNA profiles and relevant clinical parameters were obtained from the Chinese Glioma Genome Atlas (CGGA, www.cgga.org) and the Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov). Human glioma samples used in the present study were confirmed by pathologist according to WHO criteria [16]. This study was approved by the Ethics Committee of Beijing Tiantan Hospital, Capital Medical University, and written informed consents were obtained from all patients.

Cell culture and establishment of TMZ-resistant cell line

The human GBM cell lines LN229, U87MG, SNB19 and U251 were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Cells were grown in DMEM (Dulbecco's modified Eagle's medium, Gibco) containing 10% of fetal bovine serum (FBS, Gibco). Human umbilical vein endothelial cell (HUVEC) were obtained from Harbin Medical University and cultured in endothelial cell basal medium supplemented with 1% endothelial cell growth supplement and 5% FBS. To establish the TMZ-resistant cells, the U87MG parental cells were first maintained at a low dose of TMZ (5 μM, Sigma–Aldrich) and then successively exposed to incremental doses of TMZ (10 μM, 20 μM, 40 μM, 80 μM). Each time, the surviving cells were maintained until a normal rate of growth was obtained. TMZ-resistant U87MG cells with more than 20 passages were maintained at corresponding doses and used for subsequent experiments [17]. Cells were maintained at 37 °C under a humidified atmosphere containing 5% CO_2.

Lentivirus preparation

The lentiviral packaging kit was purchased from Open GeneCopoeia. Lentivirus carrying hsa-miR-181d or hsa-miR-negative control (NC) was packaged in 293T cells and concentrated from the supernatant, as instructed by the manufacturer's manual. Stable cell lines were established by infecting lentivirus into U87MG, LN229, SNB19 and U251 followed by puromycin (2 μg/ml) selection. These established stable cell lines were maintained in DMEM containing 10% FBS and puromycin (0.5 μg/ml) for further experiments.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from frozen tumor samples was extracted using the Trizol reagent (Ambion). RNA concentration and quality were measured using the ND-1000 spectrophotometer (NanoDrop Technologies). qRT-PCR was performed with 7500 fast (Applied Biosystems) and used to analyze the expression of miRNA and mRNA in human glioma samples and cell lines. Each cDNA sample was run as triplicates. Expression of U6 was used as an endogenous control for miR-181d, while GAPDH was used as an internal control for normalization and quantification of mRNA expression (MALT1, IL-6, Olig2, PDGFRa, CD44, Vimentin). All primer sequences are listed in Supplementary Table 1.

Protein extraction and western blot

Protein were extracted using RIPA buffer according to the manufacturer's protocol. Expression of protein was confirmed by MALT1 (rabbit, 1:1500, ABclonal), P65 (rabbit, 1:1500, Cell signaling technology), phoshorylated-P65 (p-P65; rabbit, 1:1500, Cell signaling technology), CD44 (mouse, 1:2000, Cell signaling technology), Vimentin (rabbit, 1:1000, Cell signaling technology), Olig2 (rabbit, 1:1500, Abcam), PDGFRa (rabbit, 1:1000, Abcam) and GAPDH (mouse, 1:5000, Proteintech), followed by incubation with appropriate correlated HRP-conjugated secondary antibody. Representative images from 2 or 3 independent experiments are shown.

Luciferase reporter assay

To determine whether miR-181d directly binds to the MALT1 3′-UTR, dual luciferase reporter assays were performed. The pmirGLO (Promega) vector was used to construct MALT1 3′-UTR containing reporter. In brief, 293T and U87MG cells seeded in 96-well plates (8×10^3 cells/well) were co-transfected with MALT1 3′-UTR reporters, miR-181d plasmid or control miRNA. Similarly, to determine the NFκB pathway transcriptional activity, a reporter containing NFκB response element was used (pGL4.32[luc2P/NFκB-RE/Hypro] Vector, Promega). The plasmids were transfected into cells treated with miR-181d plasmid or negative control, and an internal control vector expressing Renilla luciferase (Promega). At 48 h after transfection, cells were lysed and subjected to luciferase assays using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase values.

Cell proliferation assay

Cell proliferation was conducted using the Cell-Counting Kit 8 (CCK8; Dojindo Laboratories) according to the manufacturer's instructions. After planting cells in 96-well microtiter plates (Corning Costar) at 1.0×10^3/well, 10 μL of CCK8 was added to each well (medium 100 μL) at the time of harvest. A half-hour after adding CCK8, cellular viability was determined by measuring the absorbance of the converted dye at 450 nm.

Colony formation and soft agar assay

Previously established stable cell lines were planted in 6-well microtiter plates (Corning Costar) at 500/well. The assay should be stopped when the colonies are clearly visible even without looking under the microscope. Cell colony were fixed with 4% paraformaldehyde and observed by staining with 0.1% crystal violet. For soft agar assay, GBM cells (8×10^2 cells/well) were suspended in 1.5 mL complete medium supplemented with 0.7% agarose (Gibco). The cells were placed in 6-well plate containing 1.5 mL complete medium (double concentration) and agarose (1.2%) on the bottom layer. The plates were incubated at 37 °C with 5% CO_2 for 20 days. Each experiment was performed at least three times.

Apoptosis detection and transwell migration/invasion assays

The apoptosis assay was tested in U87MG and LN229 cells after transfection using the Annexin Alexa Fluor647/PI (4A Biotech) and analyzed by fluorescence-activated cell sorting (FACS). The cells were harvested by trypsinization and washed once with phosphate buffer saline (PBS). To measure cell migration, 8-mm pore size culture inserts (Transwell; Costar) were placed into the wells of 24-well culture plates, separating the upper and the lower chambers. In the lower chamber, 600 μL of DMEM/F12 containing 10% FBS was added. Then, serum-free medium containing 3×10^4 cells were added to the upper chamber for migration assays, whereas 1×10^5 cells were used for Matrigel invasion assays. Incubation at 37 °C with 5% CO_2, the number of cells that had migrated through the pores was quantified by counting 5 independent visual fields under the microscope (Zeiss). Cell morphology was fixed with 4% paraformaldehyde and observed by staining with 0.1% crystal violet. Each experiment was performed at least three times.

Tube formation assays

Tube formation assays were performed on growth-factor-reduced (GFR) Matrigel Matrix (BD Biosciences) and visualized using either phase contrast microscopy. HUVECs (2×10^5/mL) were cultured in 96-well plate with GBM cell supernatant that were collected from previously established stable cell lines, respectively. Then putted the 96-well plate into incubator (37 °C, 5% CO_2) and visualized by microscope after 8 h.

Determination of interleukin 6 (IL-6) in supernatant of cultured GBM cells

Human IL-6 quantikine ELISA kits (R&D systems) were used to determine the level of corresponding protein. Briefly, 100 μL of assay diluent and 100 μL of standard, control or sample were pipetted to cover with plate sealer and incubate at room temperature for 2 h. Then, aspirate each well and add 200 μL of Conjugate to incubate at room temperature for another 2 h on the shaker. Afterwards, 200 μL Substrate Solution were added and kept from the exposure of light for 30 min before the addition of Stop Solution. The plate was read at 450 nm within 30 min. A standard curve was drawn to calculate the protein concentrations for each sample.

Nude mouse tumor intracranial model

BALB/c female athymic mice were implanted in the brain with established stable cell U87MG-miR-181d and U87MG-negative control. Briefly, mice were anesthetized with 5% chloral hydrate and cells were implanted using cranial guide screws as previously described [15]. A TJ-4A Syringe Pump Controller and micro-infusion syringe pump (1 μL/min) were used to implant 1×10^6 cells into the brain of mice. After 40 days, mice brains' tumors were detected by MRI for animal. Then, mice were sacrificed simultaneously and brains were extracted and fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned into 5 μm slices for immunohistochemistry.

Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 6.0 and SPSS, version 16.0. Heat maps were constructed by Gene Cluster 3.0 and Gene Tree View software. Kaplan–Meier survival analysis was used to estimate the survival distributions. Then, the log-rank test was applied to assess the statistical significance between stratified survival groups. Differential genes between high- and low-expression groups were selected using significance analysis of microarray (SAM) algorithm. The miR-181d negatively associated genes were identified as $R < -0.4$ using Pearson correlation analyses. Gene Ontology (GO) analysis was performed using DAVID (http://david.abcc.ncifcrf.gov/) [18]. Gene set enrichment analysis (GSEA) was performed using GSEA software downloaded from the Broad Institute (www.broadinstitute.org/gsea) [19]. Gene set variation analysis (GSVA) was analyzed using the GSVA package [20] of R. miRWalk 2.0 [21] and miRanda [22] were utilized for miR-181d potential targets screening. All differences were considered statistically significant at the level of two-side $P < 0.05$.

Results

Aberrant expression and prognostic significance of miR-181d for patients with GBMs

To assess the expression pattern of miR-181d between PN and MES subclass, miRNA array data for 45 GBM patients (35 MES and 10 PN patients) have been obtained from CGGA dataset (Supplementary Table 2). The results demonstrated that GBM patients in PN subtype showed high-level of miR-181d expression (Fig. 1A–D). The consistent expression pattern was validated using TCGA miRNA array data (177 MES and 106 PN patients) (Fig. 1A–D). Afterwards, we divided GBM patients from TCGA cohort into two groups according to the expression of miR-181d. Similarly, GSEA between different groups demonstrated that patients with high-level of miR-181d were enriched in PN-associated gene set (Fig. 1E). Moreover, patients in the miR-181d high-expressed group had significantly longer overall survival than those in the miR-181d low-expressed group, which further highlight the suppressive character of miR-181d for glioblastoma patients (CGGA, $P = 0.0028$; TCGA, $P = 0.0095$) (Fig. 1F).

Identification of miR-181d related pathways and biological processes

To clarify the cellular functions of miR-181d, we first explored the miR-181d associated biological processes and KEGG pathways in TCGA and CGGA datasets. GO analyses of miR-181d negatively related genes demonstrated that KEGG pathways, including JAK-STAT signaling pathway, cell adhesion molecules and focal adhesion was significantly enriched, while the biological processes mainly contained immune response, cell adhesion, cell migration, regulation of apoptosis, cell proliferation, angiogenesis and NFκB cascade (Fig. 2A and B, Fig. S1). Consistently, GSEA results showed that JAK-STAT and NFκB related terms were significantly enriched (Fig. 2C and D). GSVA results further confirmed that NFκB and JAK-STAT signaling pathways associated gene sets were significantly associated with miR-181d expression (Fig. 2E).

Ectopic overexpression of miR-181d suppresses glioma cell malignant behaviors

To confirm the expression pattern of miR-181d in glioma, we detected the expression of miR-181d in a panel of adult glioma tissues using qRT-PCR. As expected, miR-181d was gradually down-regulated in glioma samples along with WHO grade (Fig. 3A). Then, the miR-181d stable expressed cells, which showed significantly higher level of miR-181d NC group, were subjected to further experimental analyses (Fig. 3B). The results demonstrated that cell proliferation, colony formation and anchor-independent cell

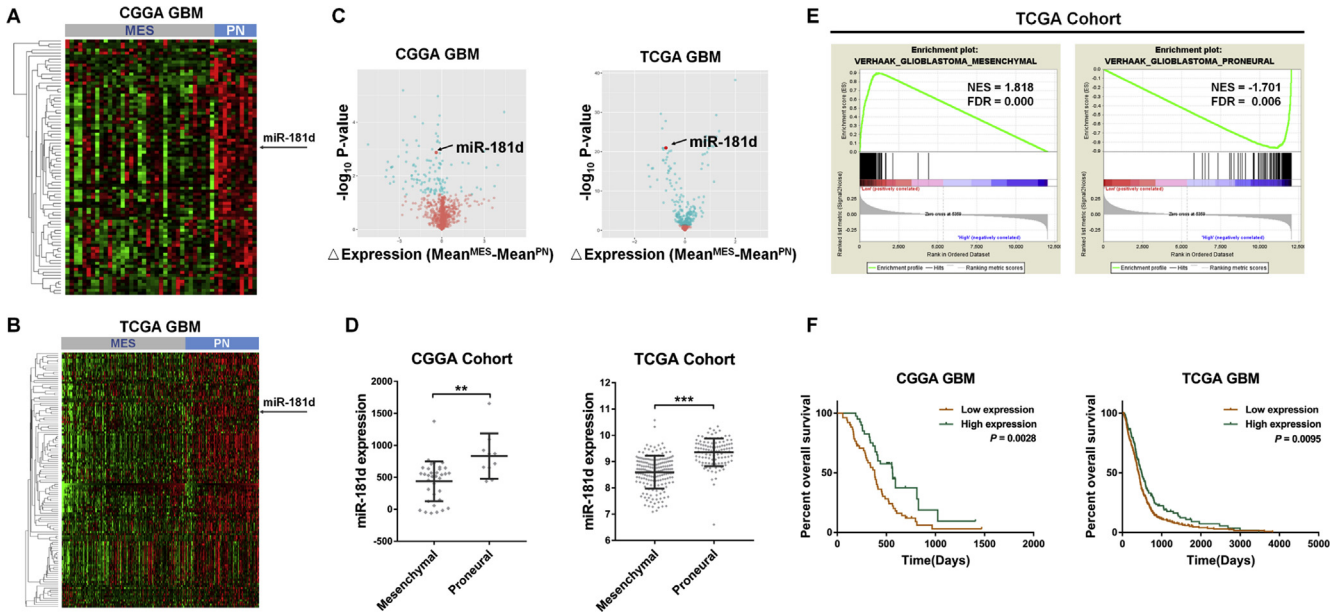

Fig. 1. miR-181d expression and prognostic significance in CGGA and TCGA datasets. **A and B**. Heatmaps were constructed using the differential miRNAs between proneural and mesenchymal subtypes identified by significance analysis of microarray algorithm. **C**. Volcano plot indicated that miR-181d were differential expressed between the two group. **D**. The expression pattern of miR-181d between proneural and mesenchymal subtypes were compared. **E**. Gene set enrichment analysis of proneural and mesenchymal gene sets was applied for patients with different miR-181d expression. **F**. Kaplan–Meier algorithm was performed for evaluating overall survival time between miR-181d high- and low-expression groups. **, $P < 0.01$, ***, $P < 0.001$.

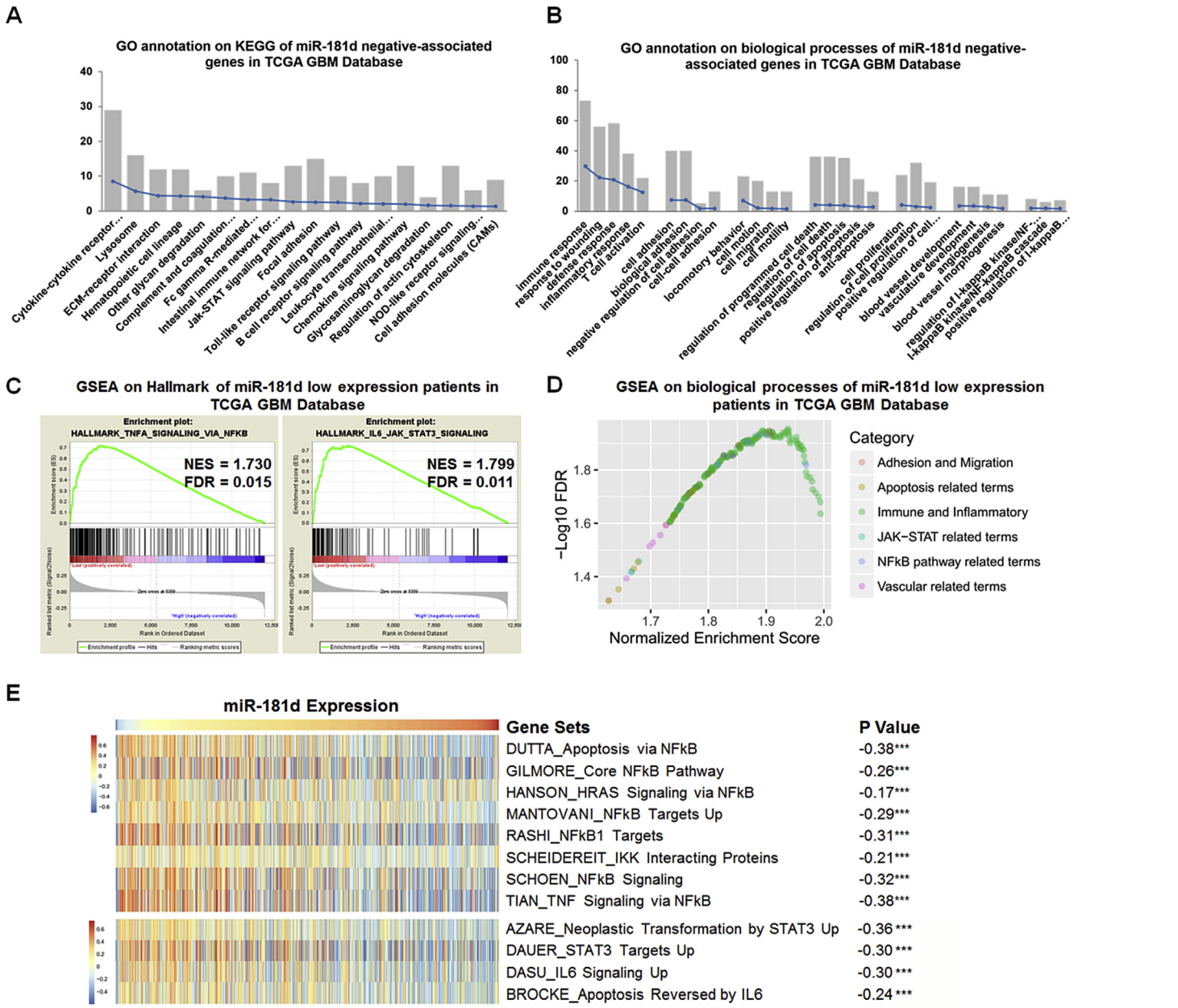

Fig. 2. miR-181d associated biological processes and KEGG pathways. **A and B**. Gene ontology analyses of miR-181d negatively associated genes revealed several malignant pathways in cancer and cellular functions. **C**. The hallmark gene sets were analyzed using Gene Set Enrichment Analysis (GSEA). **D**. The genesets regarding biological processes were subjected to GSEA and the significant processes were delineated. **E**. JAK-STAT3 and NFκB related genes sets were obtained for Gene Set Variation Q. Mao, analysis. NES, normalized enrichment score, FDR, false discovery rate, ***, $P < 0.001$.

growth were suppressed in the miR-181d overexpressed cells (Fig. 3C–E, Figs. S2A and S2B). Transwell assays revealed that both invasive and migrative capabilities decreased in miR-181d transfected cells compared with control groups (Fig. 3F and G, Fig. S2C). There was also a significantly suppressive effect of miR-181d on cell angiogenesis with shorter tube length (Fig. 3I). In addition, miR-181d overexpression notably enhanced glioma cells apoptosis (Fig. 3H). Thus, these results indicated that miR-181d overexpression suppressed glioma cell proliferation, invasion, migration, angiogenesis and facilitated apoptosis *in vitro*.

MALT1 is a direct target of miR-181d

To further investigate the biomolecular mechanism of miR-181d in GBM cells, we screened for potential targets of miR-181d using several methods. As shown in technology roadmap, the U87MG cells stably expressed miR-181d or NC were subjected to microarray and RNA sequencing, respectively (Fig. 4A). By overlapping the miR-181d negatively associated genes, potential target genes, and downregulated genes identified by microarray and RNA-sequencing detection, 122 genes were chosen as candidates (Fig. 4B). Wherein, MALT1 was significantly down-regulated and served as a crucial regulator for NFκB signaling pathway. In databases and glioma patients' tissues, MALT1 expression was gradually upregulated along with the WHO grade and negatively associated with miR-181d expression (Fig. 4C and D, Fig. S3). To confirm the direct binding of miR-181d in MALT1 3′UTR, we co-transfected U87MG and 293T cells with the MALT1 3′UTR luciferase reporter vector along with miR-181d or NC plasmid for 48 h and then evaluated the luciferase activities of those cells. miR-181d

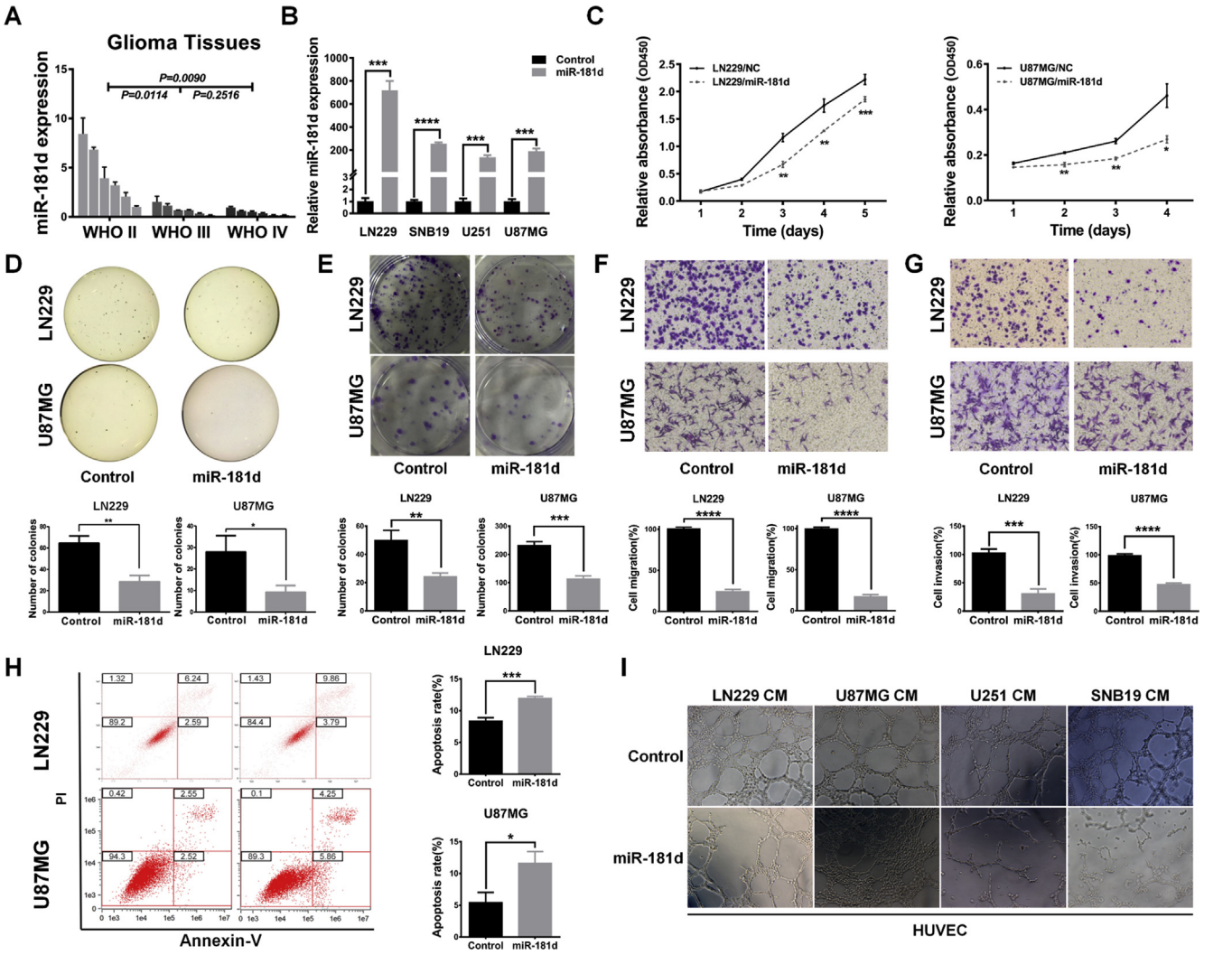

Fig. 3. miR-181d repressed the malignancy of GBM cells. **A**. The expression of miR-181d was analyzed in glioma samples. **B**. The expression of miR-181d was detected in miR-181d stably expressed cells and corresponding control cells. **C**–**G**. CCK-8 assay, Colony formation, soft agar assay, transwell assay with or without matrigel were performed to evaluate the proliferative and invasive abilities between control and miR-181d overexpressed cells. **H**. The apoptosis rates of miR-181d and control transfected cells were detected. **I**. Human umbilical vein endothelial cells were added to matrigel-coated 96 wells plate. Conditioned medium from GBM cells were collected and added to the wells to evaluate the effect of miR-181d on tube formation ability of endothelial cells. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

transfected cells observed a remarkable reduction of luciferase activities of the MALT1 reporter in both U87MG and 293T cells (Fig. 5E). Meanwhile, we found that the mRNA and protein expression of MALT1 was obviously downregulated in miR-181d overexpressed cells (Fig. 4G and H). In addition, the MALT1 associated biological processes mainly included immune response, cell cycle, cell adhesion, vascular development and several malignant functions for cancer (Fig. 4F). Collectively, these results support the notion that MALT1 serves as a direct target of miR-181d and participates the progress and malignancy of GBM.

miR-181d represses NFκB transcription activity

Previously reports suggested that MALT1 functions as a scaffold protein in NFκB pathway [23]. Hence, to explore the effect of miR-181d on NFκB pathway, phosphorylated p65 (p-P65) and NFκB luciferase reporter activity were detected. The results indicated that cellular level of p-P65 was significantly decreased and the NFκB transcriptional activity was suppressed under miR-181d overexpression (Fig. 5A and B). Moreover, the mRNA expression and protein secretion of IL-6, which was a direct target of NFκB pathway [24], was decreased in miR-181d overexpressed cells (Fig. 5C and D). Furthermore, rescue of MALT1 expression could partially reverse the miR-181d mediated proliferative inhibition of GBM cells (Fig. 5E).

miR-181d overexpression attenuates MES profile and increased sensitivity to therapies

Considering the importance of NFκB pathway in PN-MES shift, we thought to explore the functional implication of miR-181d during the this process. Our results showed that both mRNA and protein expression of PN markers (Olig2 and PDGFRa) were upregulated, while the MES markers (CD44 and Vimentin) were attenuated by ectopic overexpression of miR-181 (Fig. 6A and B). Then, we explored the impact of miR-181d on glioma cell therapeutic resistance, which are two properties caused by MES- transition [7,8]. miR-181d overexpressed or NC GBM cells were treated with 0gy, 2gy, 4gy and 8gy, respectively. Two days after irradiation, cell apoptosis and colony-formation capability were detected. The

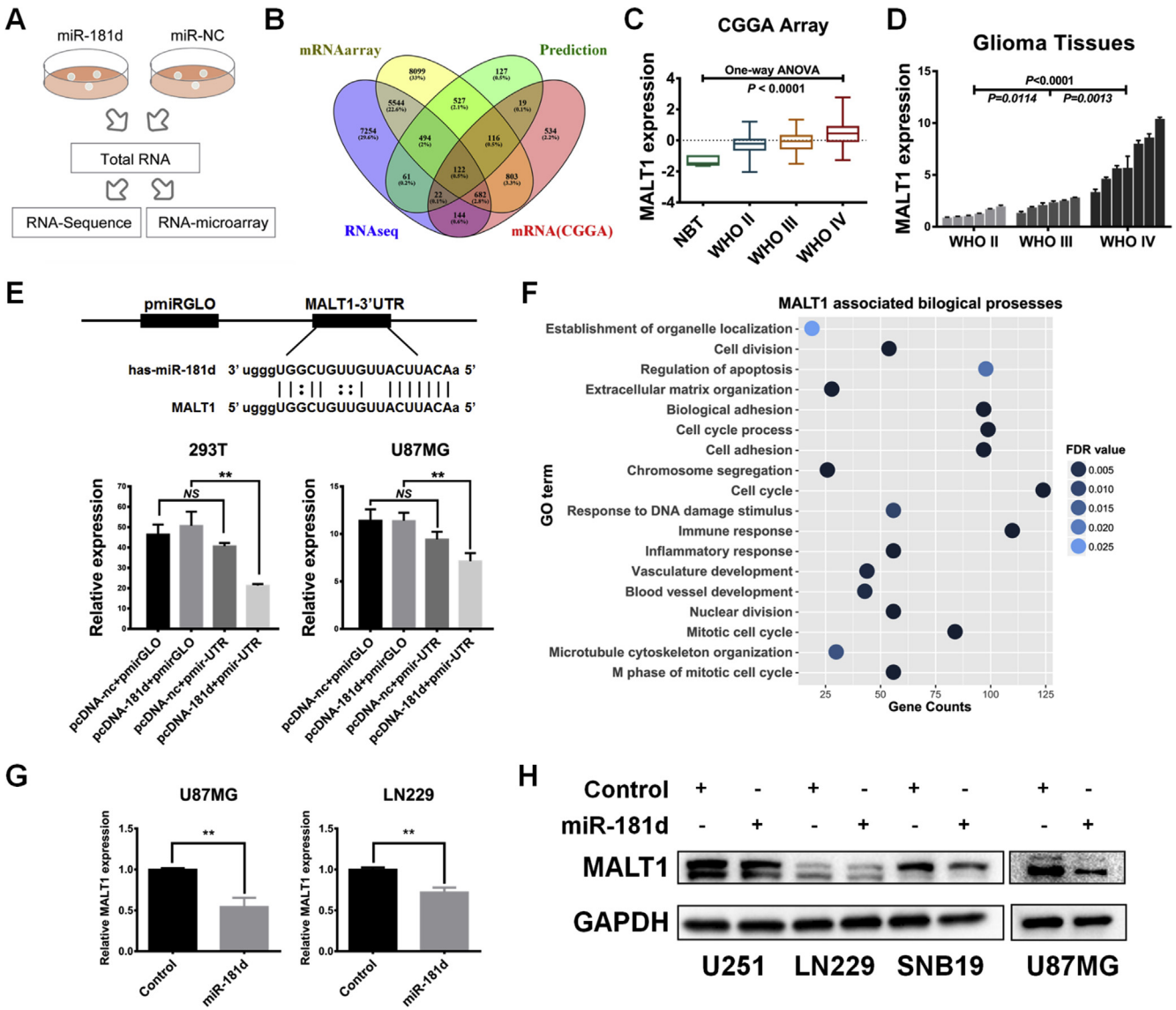

Fig. 4. MALT1 is a direct target of miR-181d. **A**. The roadmap of RNA microarray and sequencing analyses. **B**. Venn diagram were drawn to identify the overlapped genes. **C**. In CGGA dataset, the expression pattern of MALT1 for all grade glioma samples were compared. **D**. RNA from frozen samples was extracted and the mRNA expression of MALT1 were detected. **E**. MALT1 3′UTR-containing pmiRGLO plasmid were constructed and transfected to identify the directly binding of miR-181d. **F**. Gene ontology of MALT1 positively associated genes were analyzed. **G and H**. The mRNA and protein level of MALT1 were detected in miR-181d or control transfected GBM cells. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

combination treatment of miR-181d and radiotherapy showed more cell apoptosis and fewer cell clones when compared with that of NC plus radiotherapy, at 2gy. Then, when radiation measurement increased to 8gy, the two group were both shown a high percentage of cell apoptosis rates (Fig. 6C). It may be the cytotoxic effect of high-dose radiotherapy, rather than miR-181d induced increase in radiosensitization. Notably, the TMZ-resistant U87MG cells showed a reduced expression of miR-181d along with the increasing of TMZ dose or compared to the dimethylsulfoxide (DMSO) treated cells (Fig. 6D), while miR-181d overexpression significantly increased the sensitivity of U87MG and U251 cells to TMZ treatment (Fig. S4).

miR-181d inhibited tumorigenicity, and invasion in vivo

In light of the suppressive effects of miR-181d on GBM cells *in vitro*, we extended our investigation to examine if miR-181d could restrain tumor growth *in vivo*. BALB/c female athymic mice were implanted in the brain with established stable cell U87-miR-181d and U87-control. MRI and HE showed the inhibition of tumor growth in the miR-181d – overexpressing group compared with the control group (Fig. 7A and B). The overexpression of miR-181d could reduce the protein level of Ki-67 and MMP9, the molecular biomarkers for proliferation and invasion, which further confirmed the biological function of miR-181d.

Discussion

miRNAs act as posttranscriptional gene regulators in modulating various physiological and pathological events. Several studies have reported that miR-181d functions as a biomarker and has important roles in many types of cancers, inclusive of glioma [13,25–27]. However, there is no research to date that refers to the role of miR-181d in the process of MES transition. In our study, we report that miR-181d is down-regulated in GBMs and affects a

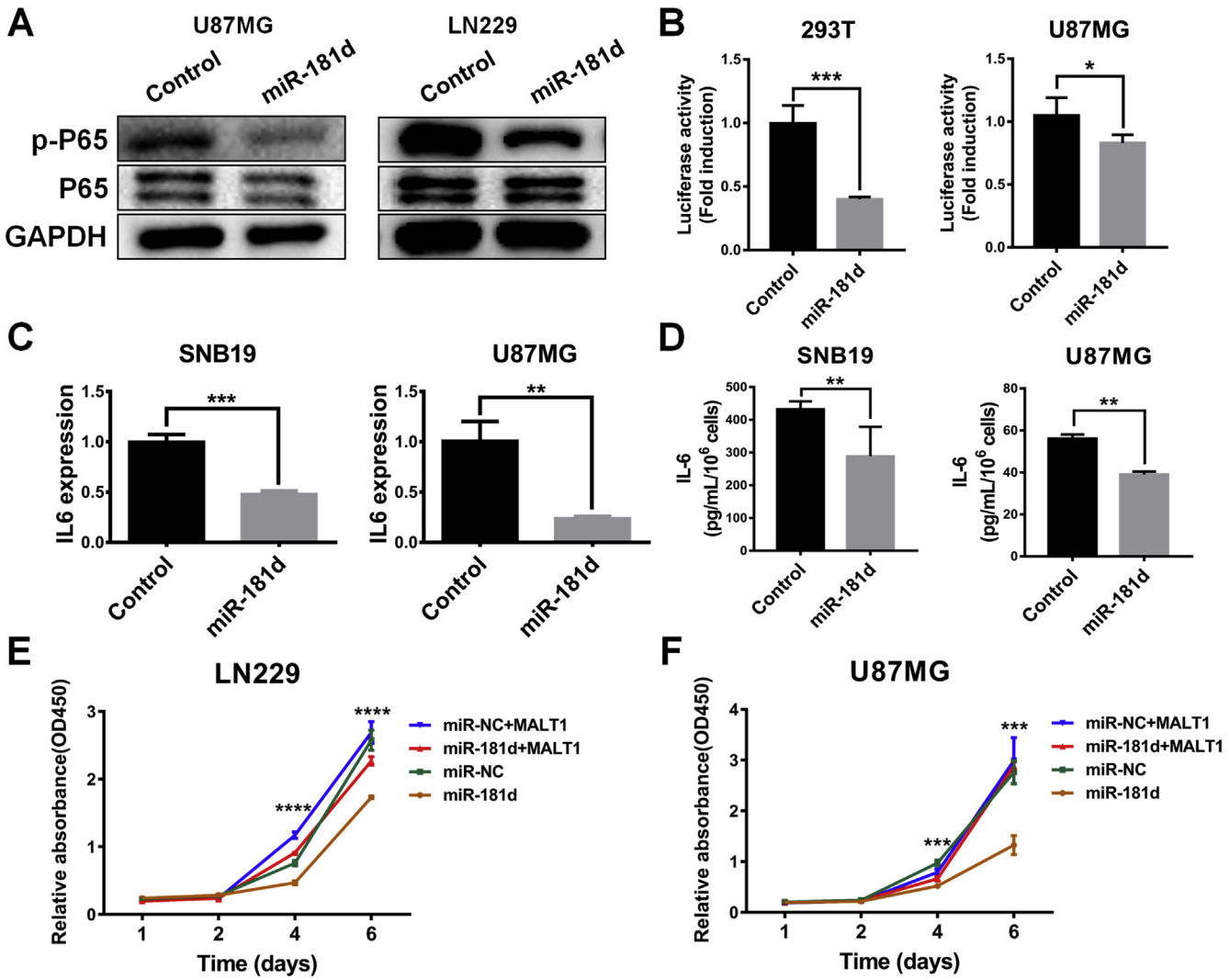

Fig. 5. miR-181d inhibits NFκB transcriptional activity and target genes. **A**. The protein level of transcription factor p65 and phosphotylated-p65 were detected. **B**. NFκB luciferase reporter plasmid and Renilla control were transfected to evaluate the influence of miR-181d on NFκB transcriptional activity. **C and D**. The IL-6 mRNA expression and protein secretion were analyzed between miR-181d overexpressed and control groups. **E and F**. The cell viability of miR-181d overexpressed, MALT1 rescued and relevant controls were detected. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

spectrum of cellular functions via directly inhibiting MALT1 and NFκB transcriptional activity. Notably, miR-181d increased the cell sensitivity to radio- and TMZ-treatment by attenuating MES subtype related gene signature, providing an alternative approach for glioma tailored medicine.

MALT1 is a well-documented effector protein in immune cells and mediates critical NFκB-driven adaptive immune responses [28]. Recent progress revealed that MALT1 targeted molecules suppress the growth of activated B-cell subtype of diffuse large B-cell lymphomas both *in vitro* and *in vivo* [29], suggesting that MALT1 may serve as a therapeutic target for the treatment of cancer. In addition to the tumor-promotive role in lymphoid system, investigations demonstrated that MALT1 is required for EGFR-induced NFκB activation and contributes to EGFR-driven lung cancer progression [29], which firstly identify the functional role of MALT1 in solid tumor. Mechanistically, MALT1 augments NFκB-dependent IL-6 production, of which triggers STAT3 activation [29]. Moreover, usage of miR-26 abolished IL-6 transcription by directly targeting MALT1, suggesting a possibility of miRNA-based treatment for MALT1-related disease [30]. In the present study, we showed that MALT1 was upregulated along with glioma malignancy. High expression of MALT1 was associated with immune response, in accordance with its intrinsic functions. Besides, The MALT1 could also promote glioma progression through several hallmarks of cancer, including cell adhesion, vascular development. Therefore, targeting MALT1 by miRNA-based therapy or small molecules may hold great promising in development of individual treatment for glioma patients.

MES differentiation induced therapeutic resistances are critical for glioma progression and recurrence. Studies indicated that activation of NFκB pathway using TNF-α or MLK4 contributes to this malignant process by induction of YKL40, STAT3, C/EBPβ, and TAZ [7,31]. Experimental evidences further revealed that STAT3 blockage abrogated the MES transition and extended survival [8]. Bioinformatic analyses suggested that miR-181d expression were tightly associated with NFκB and JAK-STAT3 signaling pathways. Combined with the observation that IL-6, the activator for JAK-STAT3 pathway, were significantly downregulated under miR-181d overexpression, it is reasonable to speculate that miR-181d may function on JAK-STAT3 pathway through IL-6 inhibition. Intriguingly, IL-6 was proved as a NFκB target inflammatory factors [32]. In turn, STAT3 could directly interact with the NFκB family

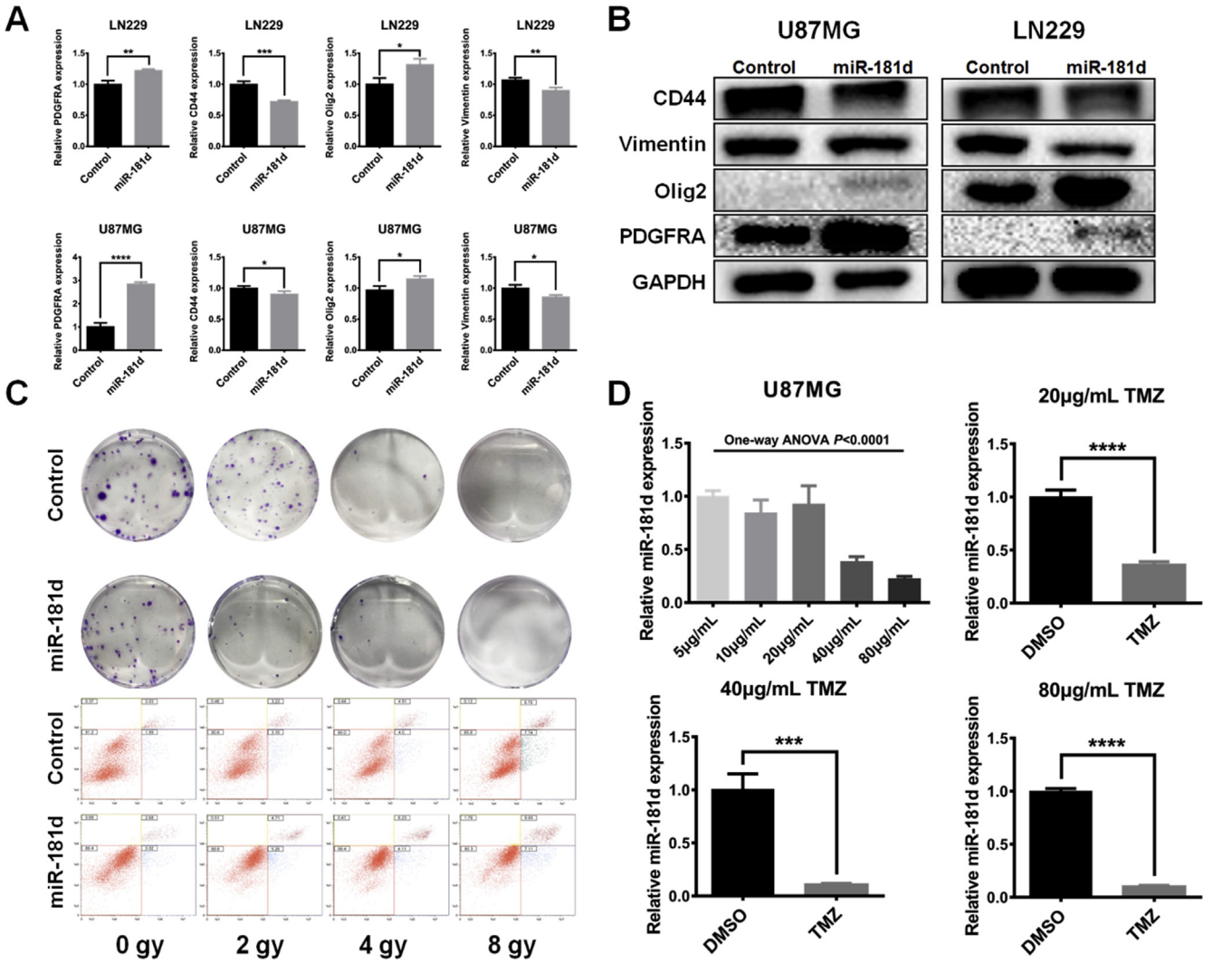

Fig. 6. miR-181d attenuates mesenchymal profiles and therapeutic resistance. **A and B**. The mRNA and protein expression of PDGFRa, Olig2, CD44 and Vimentin were detected. **C**. The colony formation ability and apoptosis of U87MG cells were detected after 0gy, 2gy, 4gy and 8gy radiotherapy. **D**. The expression of miR-181d was analyzed in TMZ-resistant U87MG cells compared to dimethylsulfoxide treat cells. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

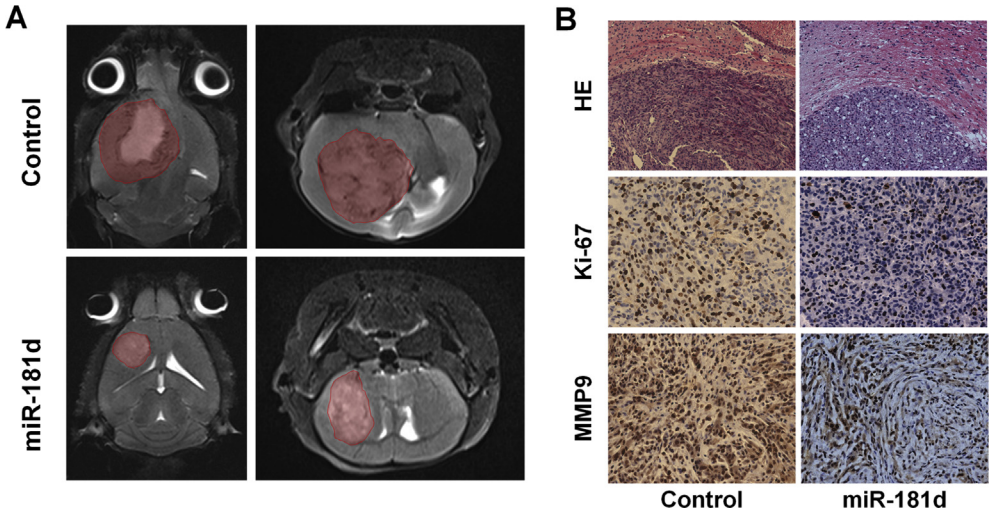

Fig. 7. miR-181d inhibits glioma growth *in vivo*. **A**. U87 cells stably expressing miR-181d and control were injected into the brain in two groups respectively. 7T MRI were performed to evaluate the volume of xenograft tumors after 40 days. **B**. HE staining and Ki-67, MMP9 immunochemistry assays were used to assess the inhibitory effects of miR-181d.

member RELA, thereby contributing to constitutive NFκB activation [33]. This reinforced crosstalk might boost downstream biological effects caused by NFκB and STAT3 pathways. Experimental evidence is need for clarifying the latent relationship between miR-181d and STAT3 pathways.

In summary, our results thoroughly show that miR-181d acts as a tumor suppressor by targeting MALT1 to restrain numerous tumor-related malignancy. We also demonstrated that miR-181d overexpression confers increased therapeutic sensitization to GBM cells. Our results highlight the regulatory mechanisms of miRNA implicated in MES differentiation and could offer more inspiration to miRNA-based glioma therapy.

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Conflict of interest statement

None declared.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2017.03.002.

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