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TUBB2B regulates epithelial-mesenchymal transition via interaction with Vimentin to promote glioma migration and invasion

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Abstract

Background Epithelial-mesenchymal transition (EMT) plays a crucial role in the migration and invasion capabilities of glioblastoma (GBM) cells. Several studies have established tubulin as a significant regulator of the EMT process. Tubulin beta 2B class IIb (TUBB2B), a critical component of microtubules, has been linked to the prognosis of various tumors. However, the specific biological function and mechanism of TUBB2B in GBM remain unclear.

Methods In vitro experiments demonstrated that TUBB2B knockdown inhibited the migration and invasion of GBM cells, while its overexpression enhanced these capabilities. Western blot, immunofluorescence (IF) and co-immunoprecipitation (Co-IP) assays revealed that TUBB2B interacts with Vimentin. Molecular docking and residue mutation scanning indicated that TUBB2B interacts with Vimentin at the R391/K392/A393/F394 sites. In vivo experiments using nude mice confirmed that TUBB2B knockdown inhibited GBM cell invasion and migration.

Results TUBB2B was upregulated in GBM tissue samples compared with normal tissues. The sites of TUBB2B(R391/K392/A393/F394) physically interacts with Vimentin to induce EMT, which promotes migration and invasion.

Conclusion TUBB2B may regulate EMT and promote the migration and invasion of GBM cells through its interaction with Vimentin, highlighting TUBB2B as a potential therapeutic target for GBM.

Keywords TUBB2B, Epithelial-mesenchymal transition, Glioblastoma, Vimentin

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Introduction

Glioblastoma (GBM) is the most invasive and common type of intracranial malignant brain tumor [1]. Although clinicians and specialists have used various treatments such as surgery, chemotherapy or radiotherapy for patients with GBM. However, the prognosis of GBM is still unsatisfactory [2]. The strong migration and invasion ability is the difficulty of GBM treatment, and it is also the key to the recurrence and low survival rate of patients [3]. Therefore, it is great significance to understand the mechanism of GBM invasion and migration ability.

Epithelial-mesenchymal transition (EMT) refers to the process in which epithelial cells acquire a mesenchymal phenotype through specific changes in gene expression [4]. The decrease of epithelial markers E-cadherin and the increased expression of mesenchymal proteins Vimentin and N-cadherin, as well as EMT transcription factors Snail and Slug can demonstrate EMT-induced epithelial plasticity changes [5–7]. During the EMT process, the cytoskeleton of epithelial cells undergoes reorganization, leading to the loss of the cell polarity and the connection between cells and basement membrane, resulting in enhanced cell metastasis ability [8]. Emerging evidence suggests that EMT is involved in GBM invasion and migration, and tubulin may be involved in this EMT process [9–13]. In addition, EMT plays an important role in the therapeutic response of GBM and is associated with the resistance to GBM chemotherapy [14]. Therefore, regulating EMT to inhibit tumor invasiveness and increase chemosensitivity may be a promising strategy for treating GBM.

Tubulin beta 2B class IIb (TUBB2B) encode a beta isoform of tubulin that are essential components of microtubules. Many studies have shown that upregulation of its expression suggests poor prognosis in patients with colorectal Cancer, lung cancer, prostate cancer, pancreatic ductal adenocarcinoma, adenocarcinoma and hepatocellular carcinoma [15–17]. In addition, TUBB2B can interact with antitumor drugs, such as paclitaxel and vinblastine, leading to the poor prognosis of prostate cancer, breast Cancer and endometrial cancer [17, 18]. TUBB2B is associated with neuronal migration and is involved in a wide range of neuronal migration disorders [19]. However, the potential mechanism, clinical significance and biologic functions of TUBB2B in GBM are still unclear.

The purpose of this study is to explore the role of TUBB2B in GBM. The results showed that compared to normal tissues, TUBB2B was upregulated in GBM tissue samples. TUBB2B promotes the invasion and migration of GBM by inducing EMT. Moreover, TUBB2B interacts with Vimentin to promote the migration and invasion of EMT in GBM cells. Our experiments verified the molecular function and mechanism of TUBB2B in GBM and provided a possible target for the treatment of GBM.

Materials and methods

Human samples

Glioma samples ($n=80$) and non-neoplastic brain tissue samples ($n=8$) were collected from postoperative glioma patients at the Affiliated Hospital of Southwest Medical University. All glioma samples were histologically confirmed. Patients did not undergo radiotherapy, chemotherapy, or any other treatments prior to surgery. All surgical procedures were conducted by a qualified neurosurgeon. This study received approval from the Ethics Committee of the Affiliated Hospital of Southwest Medical University (approval number: KY2019030) and adhered to all relevant ethical guidelines and regulations.

Cell culture and treatment

HA1800, U87, U251, T98, and LN229 cell lines were obtained from BeNa Culture Collection (BNCC, Henan, China). These cell lines cultured in DMEM (HyClone, USA) containing 1% penicillin-streptomycin (PS) and 10% foetal bovine serum (FBS, Gibco, USA) and maintained at 37 °C in a humidified atmosphere containing 5% CO2. Stable LN229 and T98 cells with silenced or overexpressed TUBB2B, along with their control cells, were selected using 4 mg/mL puromycin. Stable HEK293T cells expressing TUBB2B-Mut and their control cells were also selected using 4 mg/mL puromycin.

Animals and glioma model

Healthy female nude mice were purchased from Chengdu GemPharmatech Co. Ltd (Chengdu, China), housed in the SPF-grade animal room of the Southwest Medical University Laboratory Animal Center. They were kept at a room temperature of 20±2 °C, provided with adequate feed and water, and maintained on a 12-hour light/dark cycle. This study was approved by the Ethics Committee of Southwest Medical University (approval number: 20231008-001) and complied with the guidelines for the management and use of laboratory animals at Southwest Medical University. Before surgery, mice were anesthetized with 2,2, 2-tribromoethanol (also known as Avertin, 100 mg/kg). A total of 5×10^6 of the indicated cells were collected and resuspended in 10 μL PBS and then injected into the caudate nucleus of 4-week-old female nude mice. When all mice died due to tumor progression, the brains of all mice were collected, then fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). All animal experiments adhered to the principles outlined in the Guidelines for the Care and Use of Laboratory Animals of Southwest Medical University.

TUBB2B silencing, overexpression, and mutation

We used the lentiviral vector pLV-hU6-TUBB2BshRNA03(Human)-hef1a-mNeongreen-P2A-

Puro (SyngenTech, Beijing, China) to knockdown TUBB2B expression. The sequence of the nucleotide TUBB2B shRNA03 (Human) targeting TUBB2B was G CTGGAGAGAATCAATGTTTA and the negative control (NC) targeting TUBB2B was AAACGTGACACGT TCGGAGAA. TUBB2B was overexpressed using Ubi-MCS-3FLAG-CBh-gcGFP-IRES-puromycin (Genechem, Shanghai, China). Lentivirus-mediated RNA construction mutants including TUBB2B-NC, TUBB2B-Mut1 (N99P/N100W), TUBB2B-Mut2 (P173Y/K174P/V175W/S176Y/D177R/T178Y/V179D/V182Y), TUBB2B-Mut3 (T218G/T219S/P220H), and TUBB2B-Mut4 (R391D/K392W/A393H/F394E) were obtained from OBiO Technology Co. Ltd (Shanghai, China). Transfections were performed according to the manufacturer's instructions. Experiments were conducted 48 h post-transfection with lentivirus. LN229, T98, and HEK293T cells were transfected with TUBB2B short hairpin RNA (shRNA), overexpression RNA (OERNA), mutant RNA (MutRNA), and control RNA. These cells were then cultured in a puromycin-containing medium (2 μg/mL; Thermo Fisher Scientific) for one week, and the medium with the highest positive rate was selected for subsequent experiments, which were further validated by Western blotting. The sequences used are listed in Supplementary Table 1.

Immunohistochemistry (IHC)

Initially, paraffin-embedded tissue blocks were placed in a 60 °C incubator for 30–60 min to facilitate dewaxing and rehydration. Subsequently, the tissue sections were subjected to antigen retrieval by placing them in a repair box containing a citric acid antigen retrieval buffer (pH 6.0) and heating in a microwave. The sections were then incubated in a 3% hydrogen peroxide solution in the dark to quench endogenous peroxidase activity. Following this, 3% bovine serum albumin was added dropwise to the immunohistochemistry circle to ensure complete and uniform coverage of the tumor tissue, and the sections were sealed at room temperature for 30 min. After blocking, the sections were incubated with the primary antibody in phosphate-buffered saline (PBS) at 4 °C overnight. The next day, the sections were rinsed with PBS and incubated with the secondary antibody for 50 min at 37 °C. The sections were then treated with 3,3′-diaminobenzidine (DAB) for 3 min to visualize the antigen-antibody complexes. Following DAB incubation, the sections were rinsed and counterstained with hematoxylin. The expression of TUBB2B was observed and analyzed under a microscope.

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA of glioma cells was extracted with TRIzol reagent (Vazyme, Nanjing, China) and reverse transcribed into cDNA (Vazyme, Nanjing, China). After that, SYBR Green Master Mix (Vazyme, Nanjing, China) was used for quantitative reverse transcription polymerase chain reaction to analyze TUBB2B expression. The Primer sequences were as follows: TUBB2B, F: 5'-GCAC GATGGATTCGGTTAGGTC-3', R: 5'-TCGGCTCCCTC TGTGTAGTGG-3'; GAPDH, F: 5'-ATGGGGAAGGTG AAGGTCG-3', R: 5'-GGGGTCATTGATGGCAACAAT A-3'.

Western blotting

Cultured cells were washed three times with PBS at 4 °C and lysed using RIPA buffer (Beyotime, Shanghai, China) supplemented with 1% protease and phosphatase inhibitor mixture. Protein concentrations were determined post-centrifugation using the BCA assay. Extracted proteins were separated on SDS-PAGE gels of varying concentrations (8%, 10%, and 12%) and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated overnight at 4 °C with primary antibodies, including mouse anti-GAPDH (ab8245, 1:2000), mouse anti-TUBB2B (MBS200174, 1:1000), mouse anti-3xFlag (Cat No. 66008-3-Ig, 1:1000), rabbit anti-vimentin (Cat No. 10366-1-AP, 1:1000), rabbit anti-E-cadherin (Cat No. 20874-1-AP, 1:1000), rabbit anti-N-cadherin (Cat No. 22018-1-AP, 1:1000), rabbit anti-Snail (Cat No. 13099-1-AP, 1:1000), and rabbit anti-Slug (Cat No. 12129-1-AP, 1:1000). After washing three times with TBST, the membranes were incubated with secondary antibodies for 60 min at room temperature. Enhanced chemiluminescence was used to visualize immunoreactive bands.

Wound-healing assay

LN229 and T98 cells were cultured in 6-well plates until they reached 90% confluence. Three straight gaps were created using a sterile 1000 μL pipette tip. The cells were then gently rinsed three times with PBS and incubated in culture medium containing 1% FBS at 37 °C with 5% CO2. The wound areas were photographed under a microscope at 0, 24, 48, and 72 h in the marked area.

Cell invasion assay

Cells with good growth and normal morphology were used for the cell invasion assay using a 24-well Matrigel invasion chamber. Cells were washed with PBS, and 5×10^4 cells were added to the upper chamber, while 500 μL of 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation, cells that migrated through the porous membrane were stained. The cells in the upper chamber were wiped off with cotton swabs, and the lower chamber was fixed with methanol and stained with 0.3% crystal violet for 30 min. After

washing three times with PBS, images were taken under an inverted microscope.

Immunofluorescence (IF)

Cells were seeded at a density of 2×10^4 per well onto glass cover slips in a 12-well plate. Cells were fixed with paraformaldehyde, permeabilized with Triton X-100 for 30 min, and washed three times with PBS for 10 min. The sections were blocked with 10% goat serum and incubated with the primary antibodies (rabbit anti-β-Actin (CST8457,1:500), mouse anti-α-Tubulin (CST3873, 1:500), mouse anti-TUBB2B (MBS200174, 1:200), rabbit anti-vimentin (Cat No. 10366-1-AP, 1:200)) for two days at 4 °C. After washing with PBS, the sections were incubated overnight with secondary antibodies (Alexa Fluor 647 goat anti-rabbit IgG (ab150083, 1:500) and Alexa Fluor 488 goat anti-rabbit IgG (ab150077, 1:500)) at 4 °C. The sections were finally sealed with an anti-fluorescence quenching solution containing DAPI.

Co-immunoprecipitation (Co-IP)

Cells were lysed using lysis buffer (Beyotime, Shanghai, China) containing a protease inhibitor cocktail. The total lysates were centrifuged at 14,000 × g at 4 °C for 5 min, and the supernatant was collected. The lysate was incubated with primary antibody-conjugated magnetic beads at room temperature for 2 h. Following thorough washing, the potential interacting proteins were analyzed by Western blotting.

Molecular docking and residue mutation scanning

Protein-protein docking in ClusPro was used for molecular docking simulation of protein TUBB2B and Vimentin [20]. The crystal structure of TUBB2B (PDB code 6E7C) and the AlphaFold2 predicted model of Vimentin were used in the docking simulation [21]. For protein docking, Vimentin was set as receptor and TUBB2B as ligand. The most probable poses were further analyzed for intermolecular contacts, with molecular graphics generated using PyMOL. Virtual saturation mutational scanning of complex TUBB2B and Vimentin was carried out in the FoldX web server. We performed a virtual residue scan of the binding surface between TUBB2B and Vimentin [22].

Data and statistical analysis

All experiments were conducted at least three times independently. Data are presented as the mean±standard deviation (SD). Comparisons between two groups were made using a two-sided t-test or two-way analysis of variance (ANOVA). Data analysis was performed using GraphPad Prism 7 (GraphPad Software, USA). Statistical significance was set at $p<0.05$, *; $p<0.01$, **; $p<0.001$, ***; $p<0.0001$, ****.

Results

TUBB2B is upregulated in GBM and correlates with poor prognosis

To elucidate the role of TUBB2B in GBM development, we analyzed TUBB2B mRNA levels in GBM and normal brain tissues using the GEPIA dataset. The results indicated significantly higher TUBB2B mRNA levels in GBM tissues compared to normal brain tissues (Fig. 1A-B). Prognostic analysis using TCGA, CGGA, and Rembrandt datasets revealed that high TUBB2B expression correlated with poor patient prognosis (Fig. 1C-E). Immunohistochemical data from the Human Protein Atlas (HPA) further confirmed elevated TUBB2B protein levels in GBM tissues (Fig. 1F). Quantitative real-time PCR (qRT-PCR) analysis of 8 normal brain tissues and 80 glioma tissues showed higher TUBB2B mRNA expression in GBM tissues (Fig. 1G). Kaplan–Meier survival curves demonstrated a clear association between TUBB2B expression and poor prognosis in GBM patients (Fig. 1H). These findings suggest that TUBB2B may serve as a novel prognostic biomarker in gliomas.

TUBB2B promotes GBM cell migration and invasion in vitro

To investigate the functional role of TUBB2B in GBM cells, we measured TUBB2B mRNA levels in four GBM cell lines (U87, U251, T98, LN229) and an immortalized human astrocyte cell line (HA1800). Consistent with the findings in GBM tissues, all GBM cell lines exhibited higher TUBB2B protein levels than HA1800, with T98 and LN229 showing the highest levels (Fig. 2A). TUBB2B knockdown was established in T98 and LN229 cells via stable expression of shRNA targeting TUBB2B (Fig. 2B). Transwell and wound healing assays indicated that TUBB2B knockdown significantly reduced invasion and migration in T98 and LN229 cells (Fig. 2C-D). Conversely, TUBB2B overexpression was achieved in T98 and LN229 cells by stably expressing TUBB2B-targeting OERNA (Fig. 2E-F). Overexpression significantly enhanced migration and invasion capabilities in these cells, as demonstrated by Transwell and wound-healing assays (Fig. 2G-H). These results suggest that TUBB2B promotes GBM cell migration and invasion.

TUBB2B induces EMT in GBM cell

To explore the molecular mechanisms underlying TUBB2B function in GBM cells, we conducted RNA sequencing (RNA-seq) to identify TUBB2B-regulated pathways in LN229 and T98 cell lines. In LN229 cells, 31,117 genes were detected, with 1,138 differentially expressed genes (DEGs) identified—567 upregulated and 571 downregulated. According to the RNAseq results, TUBB2B was reduced by 73%. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated that DEGs were enriched in ECM-receptor

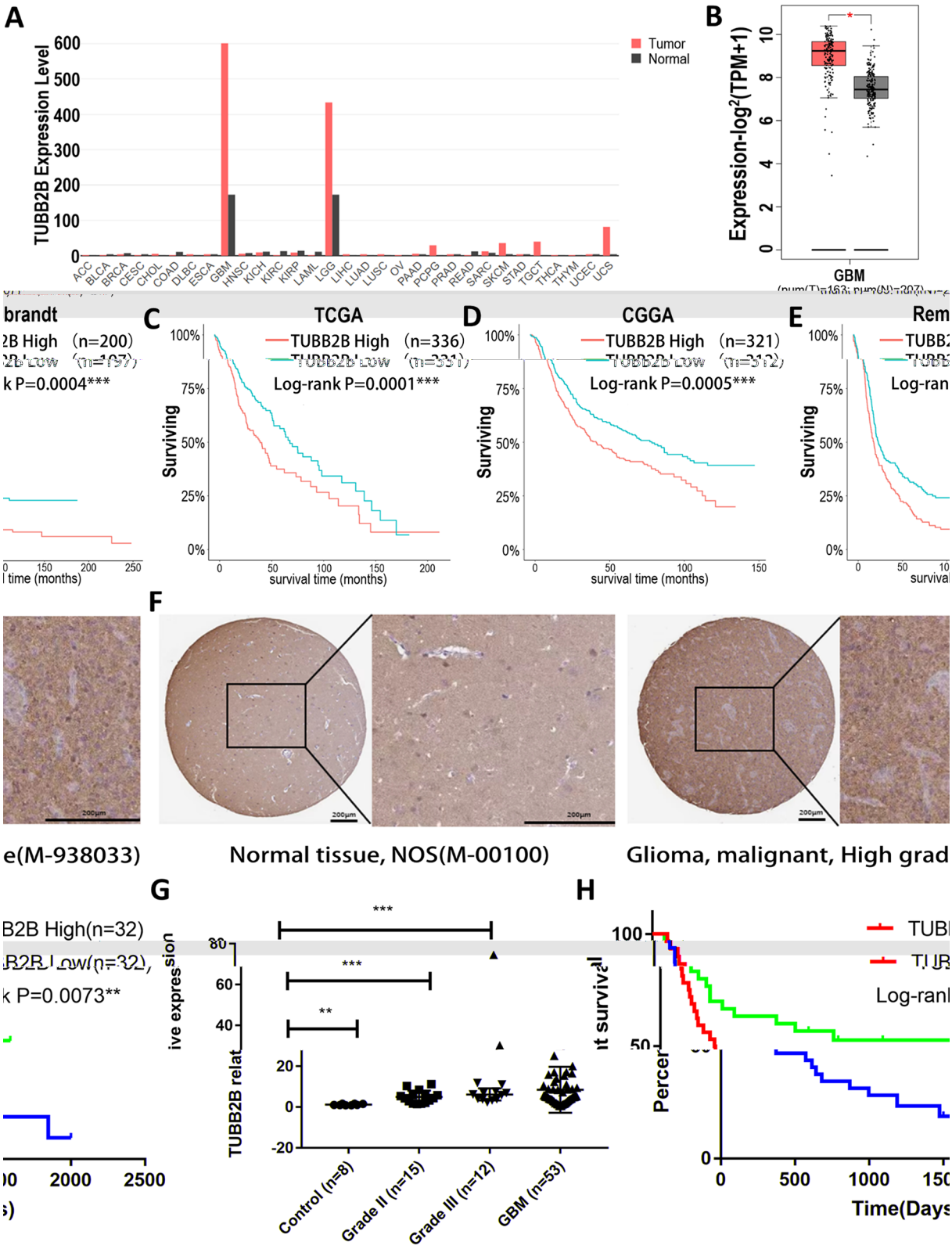

Fig. 1 TUBB2B is upregulated in GBM tissues. (**A**-**B**) Based on GEPIA database analysis, TUBB2B was significantly upregulated in LGG and GBM; $P < 0.05$. (**C**-**E**) Prognostic value of TUBB2B in TGGA, CGGA, Rembrandt datasets. (**F**) Immunohistochemical detection of TUBB2B protein levels in glioma tissues and normal brain tissues (Scale bar, 200 μm). (**G**) Expression of TUBB2B in 8 normal tissues and 80 GBM tissues. (**H**) Kaplan Meier analysis was used to evaluate the overall survival of GBM patients based on TUBB2B expression ($n = 64$, $^{**}P = 0.0073$). All results are displayed as the mean ± SD. $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***

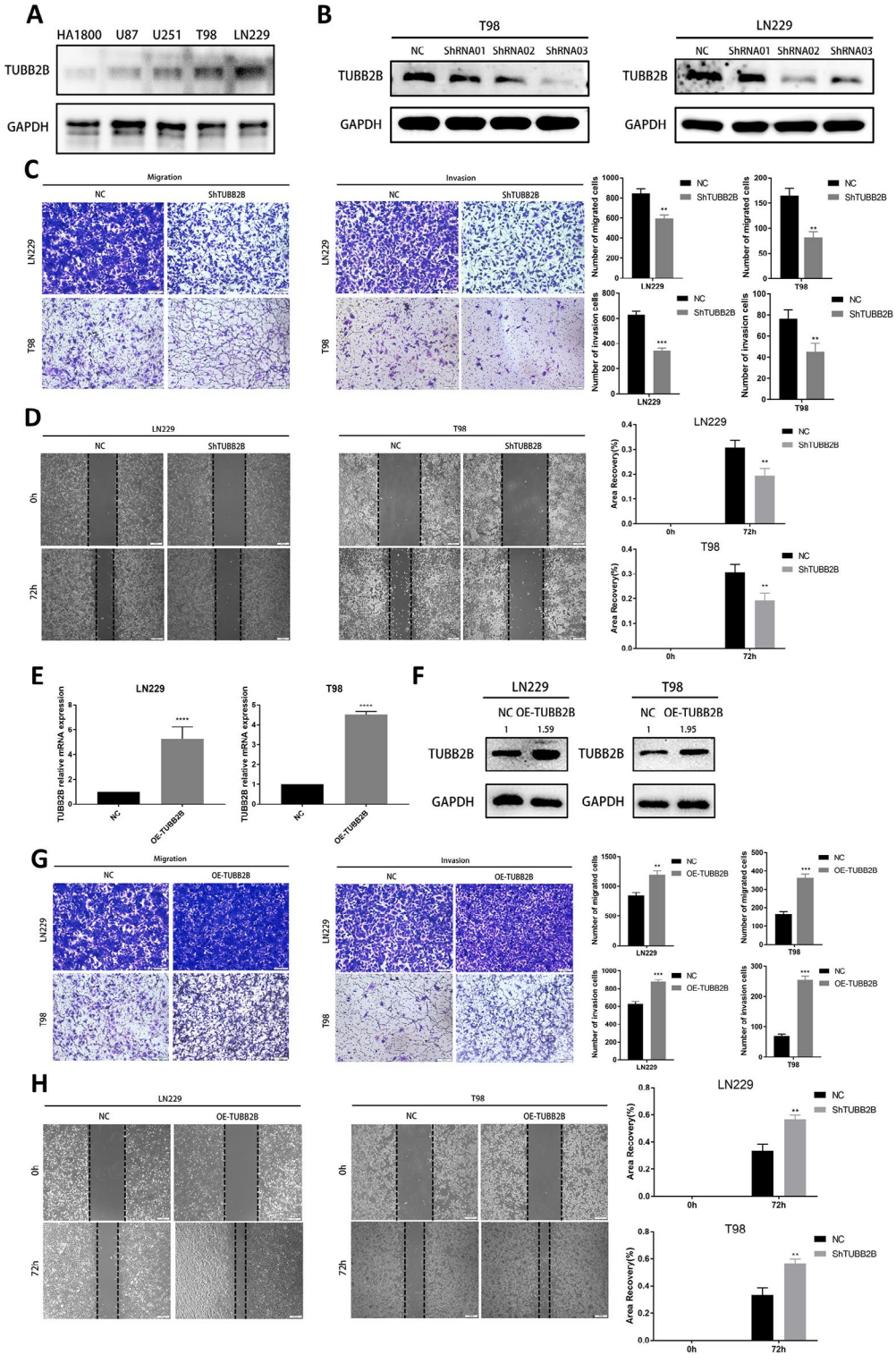

Fig. 2 Effect of TUBB2B knockdown and overexpression on the proliferation and migration of glioma cells. (**A**) Expression levels of TUBB2B in glioma cell lines and astrocyte cell lines. (**B**) Western blot analysis of TUBB2B expression. (**C**) Cell migration and invasion were assessed in GBM cells expressing TUBB2B shRNA using Transwell assay (Scale bar, 100 μm). (**D**) The cell migration ability of GBM cells expressing TUBB2B shRNA was detected by wound healing assay (Scale bar, 200 μm). (**E**) The efficiency of TUBB2B overexpression after transfection with TUBB2B OERNA were evaluated by qRT-PCR. (**F**) Western blot analysis of TUBB2B expression. (**G**) Cell migration and invasion were assessed in GBM cells expressing TUBB2B OERNA using Transwell assay (Scale bar, 100 μm). (**H**) The cell migration ability of GBM cells expressing TUBB2B OERNA was detected by wound healing assay (Scale bar, 200 μm). All results are displayed as the mean ± SD. $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****

interaction (hsa04512, padj=1.01×10^{-10}) and (hsa0451, padj=4.05×10^{-5}). Meanwhile, Gene Ontology (GO) analysis indicated that DEGs were enriched in extracellular matrix organization (GO:0030198, padj=7.23×10^{-20}) and cell adhesion molecule binding (GO:0050839, padj=1.00×10^{-8}) (Fig. 3A-B). Downregulated DEGs were associated with extracellular matrix (ECM), such as collagen family (COL4A1, COL8A1, COL11A1, COL11A2, COL12A1) and matrix metalloproteinases family (MMP2, MMP15). Genes related to cell migration were also downregulated, such as integrin alpha family (ITGA4, ITGA6). Members of the laminin family involved in cell adhesion (LAMA4, LAMB1, LAMB2, LAMC1) were also downregulated. We focused on genes related to cell migration, ECM, and cell adhesion among the top 400 significant DEGs. A protein interaction network was constructed using Cytoscape's string plugin, revealing key interactions (Fig. 3C-D). Literature review suggested that ECM properties are critical in regulating EMT, which in turn influences ECM structure and composition, forming a feedback loop crucial in healthy tissues. Given that GBM invasion and metastasis depend on EMT, we hypothesized that TUBB2B might induce EMT in GBM, leading to poor patient prognosis.

To further investigate the relationship between TUBB2B and EMT, we assessed EMT markers in GBM cells. TUBB2B knockdown cells exhibited significantly elevated epithelial marker E-cadherin levels, while

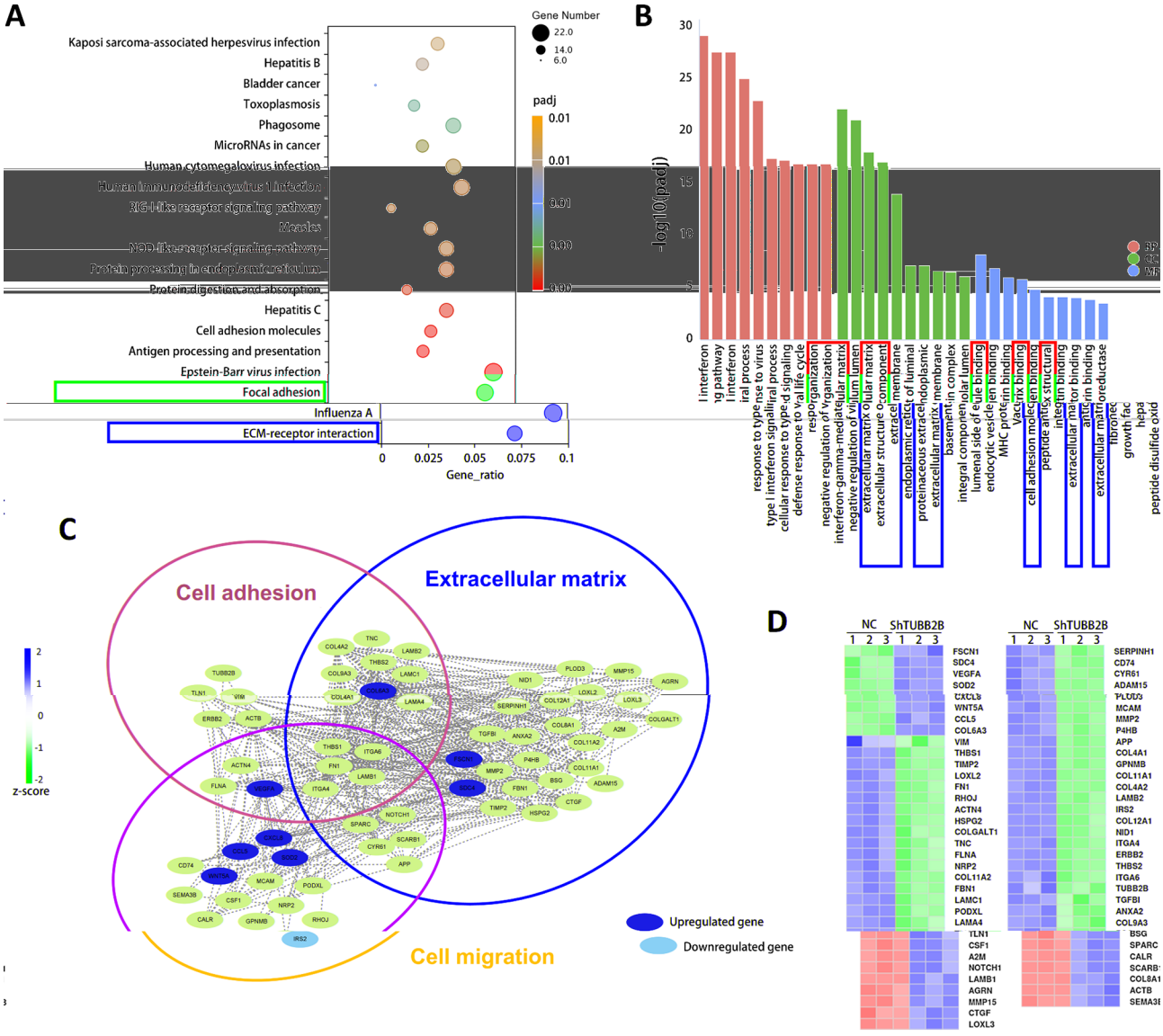

Fig. 3 Transcriptome analysis of LN229 cell line. (**A**) KEGG enrichment analysis of the RNA-seq results. (**B**) GO enrichment analysis of the RNA-seq results. (**C**) The PPI network of TUBB2B and Vimentin (scorevalue ≥ 0.3). (**D**) RNA-seq expression of heatmaps of genes involved in extracellular matrix, focal adhesion, and cell migration

mesenchymal markers N-cadherin, vimentin, snail, and slug were decreased (Fig. 4A). Conversely, TUBB2B overexpression led to opposite changes. Immunofluorescence staining for actin and tubulin revealed that TUBB2B knockdown cells displayed rounded morphology, reduced cell matrix, cytoskeletal recombination, and non-polarized tubulin rearrangement, characteristic of EMT (Fig. 4B-C). These findings suggest that TUBB2B induces EMT in GBM cells.

TUBB2B interacts with Vimentin to induce EMT and promote invasion and migration of GBM cells

Based on our RNAseq findings, we conducted protein-protein interaction analysis using the STRING database, revealing a potential interaction between TUBB2B and vimentin (Fig. 5A). Previous studies have shown that vimentin expression in mesenchymal cells can enhance cell invasion and migration by inducing EMT. Building on this knowledge, we hypothesized that TUBB2B might interact with vimentin to facilitate GBM cell migration and invasion. Reciprocal coimmunoprecipitation experiments confirmed the interaction between TUBB2B and vimentin in GBM (Fig. 5B). Additionally,

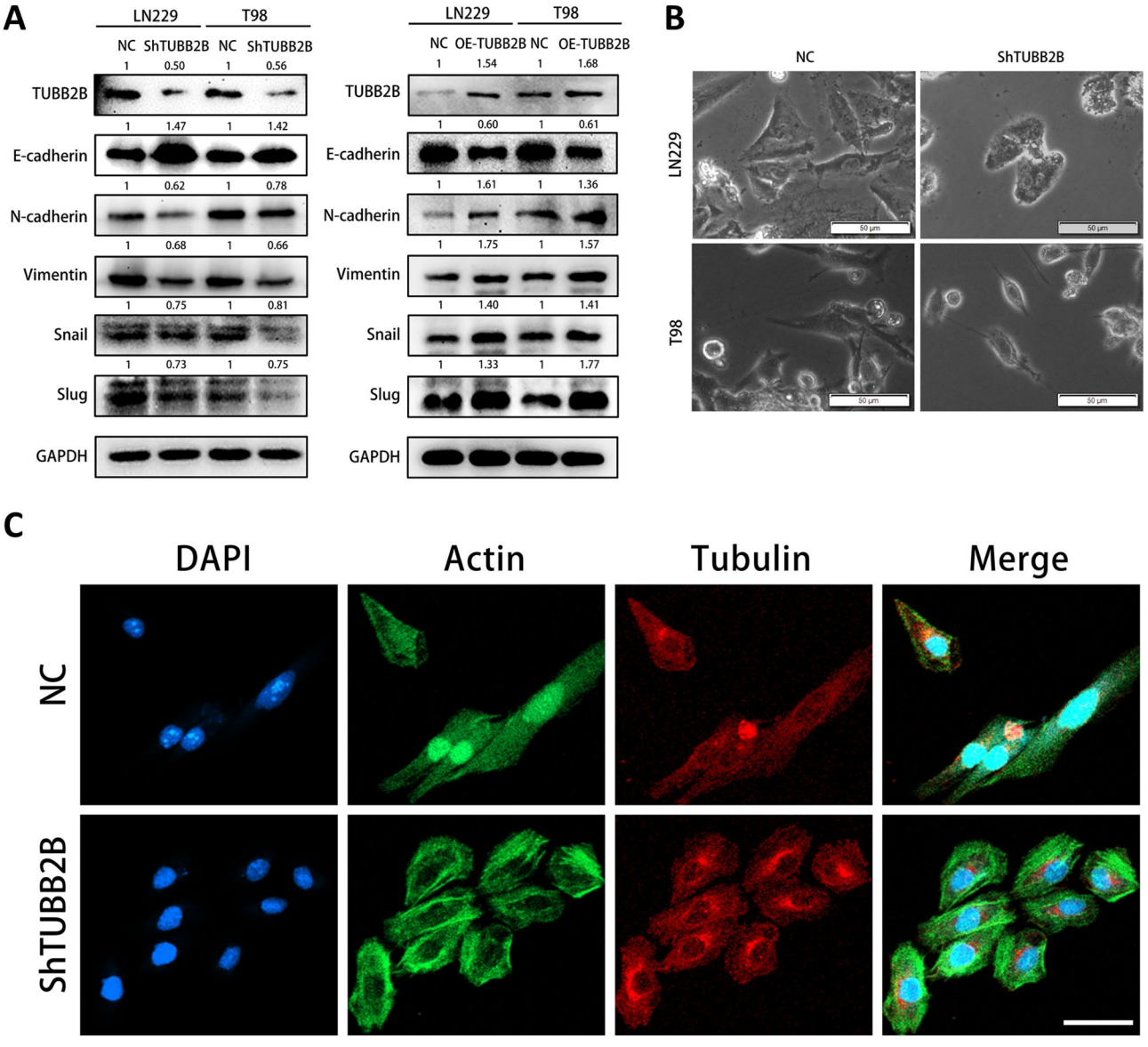

Fig. 4 TUBB2B promotes EMT in GBM cells (**A**) The levels of EMT markers and TUBB2B in the LN229 and T98 cells. (**B**) Representative morphology of LN229 and T98 cells silenced for TUBB2B expression (Scale bar, 50 μm). (**C**) Representative images (20 × magnification) of immunofluorescence staining patterns for nucleus (DAPI, blue), Actin(green), and Tubulin (red) evaluated in control (NC) and shRNA cells (Scale bar, 20 μm)

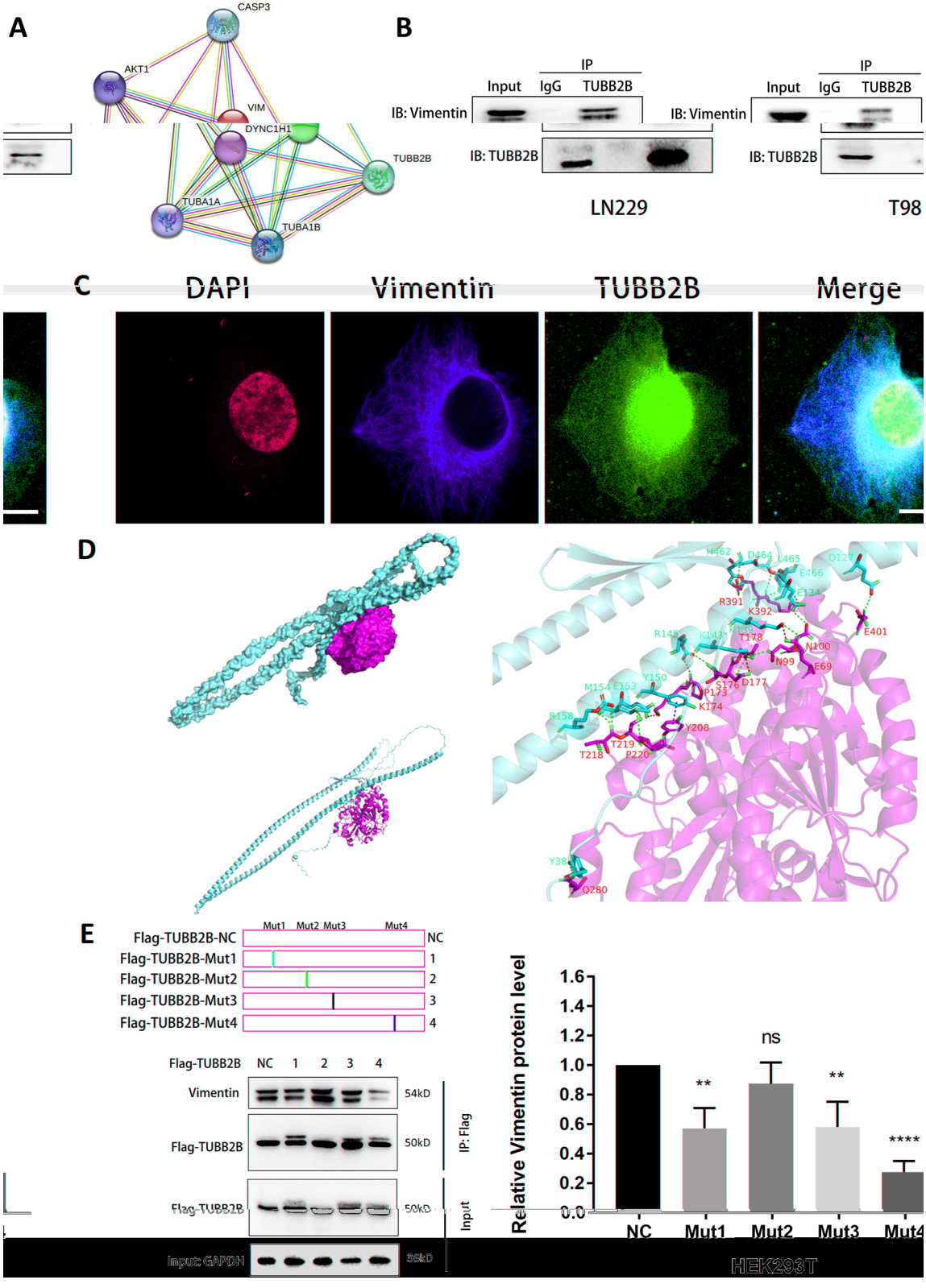

Fig. 5 TUBB2B interacts with Vimentin in GBM cells (**A**) STRING database protein interaction network of TUBB2B. (**B**) The interaction between TUBB2B and Vimentin inLN229 and T98 cells was determined using Co-IP. (**C**) Double staining of GBM cells with TUBB2B and Vimentin was assessed using confocal microscopy (Scale bar, 10 μm. Pearson's R value = 0.60). (**D**) The predicted binding mode of TUBB2B and Vimentin, as well as the detail interactions between two proteins. (**E**) Schematic representation of Flag-TUBB2B-NC and the Flag-TUBB2B mutants. All results are displayed as the mean ± SD. $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****

confocal fluorescence microscopy revealed colocalization of TUBB2B and vimentin in the cytoplasm of GBM cells (Fig. 5C). Subsequent protein-protein docking experiments between TUBB2B and vimentin predicted their binding mode and detailed interactions (Fig. 5D and Supplementary Table 2).

In detail, vimentin formed sixteen hydrogen bonds, five salt bridges, and one pi-pi stacking interaction with TUBB2B. The docking results indicated that two regions of vimentin (amino acids 127–158, 462–466) bound to TUBB2B, while both N-terminal (amino acids 69–220) and C-terminal (amino acids 391–392, 401) regions of TUBB2B interacted with vimentin.

Furthermore, to explore this interaction further, we conducted mutational scanning of TUBB2B. Four TUBB2B mutants were constructed: TUBB2B-Mut1(N99P/N100W), TUBB2B-Mut2(P173Y/K174P/V175W/S176Y/D177R/T178Y/V179D/V182Y), TUBB2B-Mut3(T218G/T219S/P220H), and TUBB2B-Mut4(R391D/K392W/A393H/F394E). Flag-tagged versions of these mutants were transfected into HEK293T cells, followed by reciprocal coimmunoprecipitation experiments. Interaction between TUBB2B-Mut1 and vimentin remained unchanged compared to TUBB2B-NC, while interaction with TUBB2B-Mut3 and TUBB2B-Mut4 was diminished (Fig. 5E).

To assess the functional significance of these mutants in GBM, Flag-tagged TUBB2B-Mut4 and TUBB2B-NC were transfected into LN229 and T98 cell lines. Transwell and wound healing assays revealed that TUBB2B-Mut4 attenuated invasion and migration ability compared to TUBB2B-NC (Fig. 6A-B). These findings highlight the critical role of TUBB2B-Mut4 (R391D/K392W/A393H/F394E) in the interaction between TUBB2B and vimentin, underscoring its importance in GBM progression.

Knockdown of TUBB2B reduces GBM cell migration and invasion in vivo

To delve deeper into the impact of TUBB2B on GBM invasion and migration in vivo, we utilized lentivirus-mediated shRNA targeting TUBB2B (TUBB2B-shRNA) and non-targeting shRNA (NC) cells, which were injected into the caudate nucleus of female nude mice. Notably, tumors with depleted TUBB2B exhibited distinct margins, with significantly reduced invasive fingers compared to control tumors (Fig. 7A). Moreover, the decrease in TUBB2B expression prolonged the survival of nude mice (Fig. 7B).

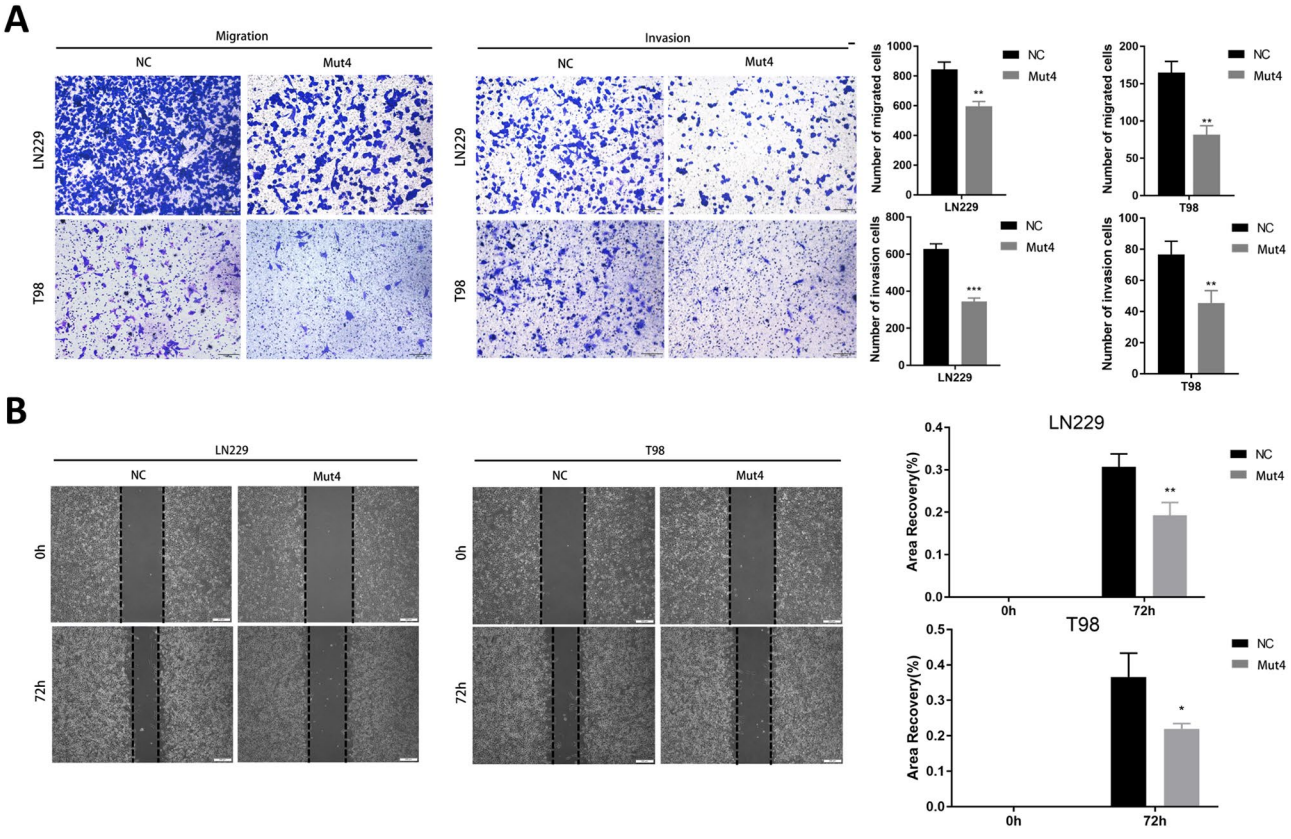

Fig. 6 TUBB2B-Mut4 inhibits GBM cell migration and invasion in vivo. (**A**) Migration and invasion ability of TUBB2B-Mut4 in GBM cells (Scale bar, 10 μm). (**B**) The cell migration ability of GBM cells expressing TUBB2B-Mut4 was detected by wound healing assay (Scale bar, 10 μm). All results are displayed as the mean ± SD. $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***

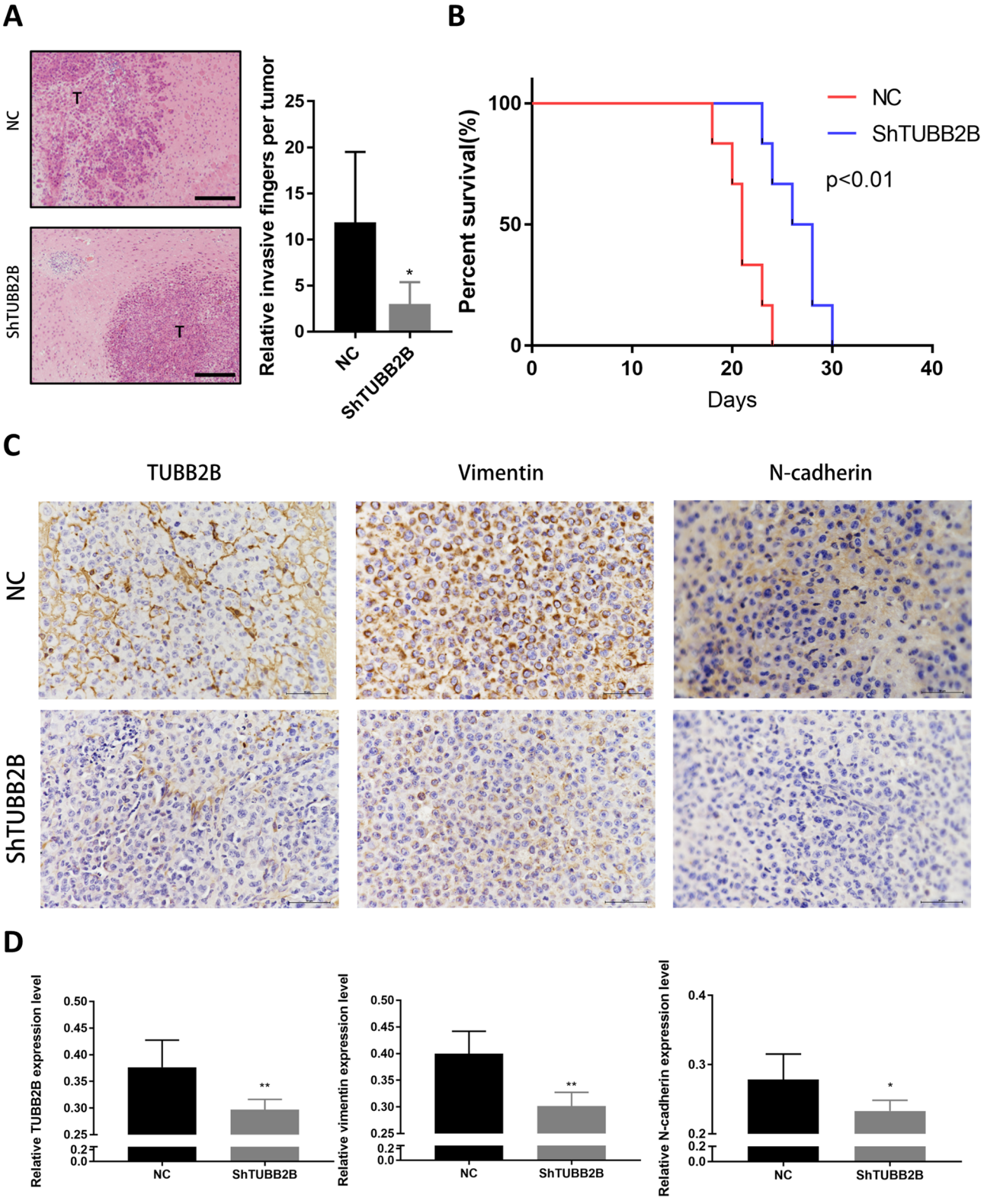

Fig. 7 Effect of tubb2b knockdown on GBM cell migration and invasion in vivo. (**A**) In vivo invasion assay for NC and ShTUBB2B LN229 cells (Scale bar = 200 μm). (**B**) Overall survival of mice. (**C**) IHC analysis of TUBB2B, Vimentin and N-cadherin levels in mice brain tumors (Scale bar, 50 μm). (**D**)Relative expression levels of TUBB2B, Vimentin and N-cadherin in IHC. All results are displayed as the mean ± SD. $p < 0.05$, *; $p < 0.01$, **

Furthermore, immunohistochemical staining of brain tissue sections from mice revealed that the reduction of TUBB2B led to decreased expression of vimentin and N-cadherin (Fig. 7C-D). And we demonstrated through immunofluorescence experiments that the TUBB2B-Vimentin complex can exist in mouse tumor tissues, as indicated by the white arrow in Supplementary Figure S1. These findings collectively suggest that knockdown of TUBB2B diminishes the migratory and invasive capacities of GBM cells in vivo.

Discussion

GBM is well-known the most invasive type of intracranial malignant brain tumor [1]. Strong migration and invasion ability is the key to the recurrence and poor survival of GBM patients. EMT is a complex process consisting of a complex network of interactions between multiple proteins and effector factors. Inducers and signaling pathways of EMT include activator of transcription 3 (STAT3), notch, signal transducer and Wnt/β-Catenin, transforming growth factor β (TGF-β), extracellular matrix (ECM)-mediated and hypoxia signaling pathways [23–27]. These EMT inducers activate the expression and function of EMT core regulators, including Snail, ZEB, and TWIST. During the EMT process, cancer cells acquire a mesenchymal phenotype. This shape transition is mainly due to the loss of cell junctions and the reorganization of the cytoskeleton, which enable tumor cells to have a stronger ability to invade and migrate. EMT may be a key breakthrough point in the treatment of GBM, but there is still no effective method to cure GBM and much work remains to be done to understand the mechanism.

Microtubules are known to participate in cytoskeleton formation together with microfilaments and intermediate filaments [28]. Tubbs coding β-Tubulin is associated with α-Tubulin forms dimers and serves as a structural component of microtubules [29]. Currently, evidence suggested that they play a vital role in various types of tumors. The cytoskeleton plays a critical role in the migration of normal cells and microtubules are an integral component of the cytoskeleton. Previous studies have shown that TUBA1C and TUBA1A can promote glioma cell proliferation and lead to poor prognosis by regulating the cell cycle and β3-tubulin is integrated with PRL-3 interactions promote glioma cell invasion [30–32]. TUBB2B is a member of the TUBBs family. It has been documented that TUBB2B expression is increased in endometrial cancer and neuroblastoma, and is associated with adverse OS [18, 33]. Our study indicates that in TCGA, CGGA and Rembrandt, we found that TUBB2B is highly expressed in GBM tissue, and the expression was associated with GBM prognosis. These results were confirmed in tissue samples from 80 patients with glioma. Next, we found that the invasion and migration of GBM cells were significantly inhibited when we silenced TUBB2B, while TUBB2B overexpression had the opposite effect. Moreover, in nude mice model of tumorigenesis, TUBB2B knockdown significantly reduced the invasive fingers of tumor. These results suggested that TUBB2B promoted GBM migration and invasion. Many previous studies have reported that TUBB2B is involved in the migration and invasion of tumor cells [15–18]. Our research results show that TUBB2B is involved in the migration and invasion of GBM cells, suggesting that TUBB2B is a therapeutic target for GBM.

Vimentin is an important component of the cytoskeleton. It can interact with other cytoskeletal proteins to reorganize the cytoskeleton, change cell morphology, and regulate cell migration and adhesion. Vimentin is considered to be an important reprogramming marker protein for EMT, and can directly enhance cell migration [34, 35]. Vimentin is regulated by many genes: CMTM6 and FOXK1 can interact with Vimentin to stabilize Vimentin and promote EMT [36]. The EMT process is related to the polymerization ability of microtubules. The reason for the difference between different subtypes of Tubbs may be related to the microtubule polymerization ability, and the increase of TUBB2B and TUBB3 levels can lead to the increase of microtubule polymerization, thus affecting the difference of cell invasion and migration ability [37]. Many literatures have reported that Vimentin expression increases in many cancers, such as colorectal, breast, gastric and liver cancers. The cytoskeleton is essential for cell migration, and Vimentin can maintain the integrity of the cytoskeleton [38, 39]. The interaction between Vimentin and microtubules is critical for mechanical cell integrity. Vimentin can function as an adaptor between microtubules and actin. Vimentin has also been implicated as a modulator of cell polarity and motility by maintaining the stability of the cytoskeletal structure and mechanical forces [40]. In our study, morphologically, GBM cells silenced for TUBB2B presented a rounded morphology and reduced cell matrix, exhibiting a reorganization of the cytoskeleton and nonpolarized tubulin rearrangement, whereas control cells maintained a spindle-shaped mesenchymal cell morphology. And immunofluorescence and immunoprecipitation were used to confirm the co-localization and interaction relationship between TUBB2B and Vimentin. In the interaction mechanism, we performed Protein-protein docking experiment and found the mutant TUBB2B-Mut4(R391D/K392W/A393H/F394E) that could significantly inhibit the migration and invasion of GBM. Thus, we propose that TUBB2B-Vimentin interaction is critical for migration and invasive in GBM cells. Based on this reasoning, we found that the MUT4 (R391D/K392W/

A393H/F394E) region of TUBB2B plays an important role in the control of Vimentin through direct interaction.

Conclusions

In summary, compared with normal tissues, TUBB2B expression was up-regulated in GBM tissue samples. Functionally, TUBB2B contributed to GBM cell invasion, migration, and EMT. In terms of mechanism, our results revealed the regulatory mechanism between TUBB2B and Vimentin. The MUT4 (R391D/K392W/A393H/F394E) region of TUBB2B plays an important role in the control of Vimentin through direct interaction. Although we have investigated the possible sites of interaction between TUBB2B and Vimentin, the mechanism of interaction between TUBB2B and Vimentin is not completely clear, which may be related to the stability of Vimentin and the polymerization and depolymerization mechanism of microtubules. In addition, TUBB2B is also widely distributed in the extracellular matrix, which may also be related to the effect of tumor microenvironment on cell migration and invasion. There is still much work to be done to understand its mechanism. Our results show that TUBB2B may be a novel prognostic biomarker and a promising therapeutic target for GBM.

Abbreviations

EMT	Epithelial-mesenchymal transition
GBM	Glioblastoma
TUBB2B	Tubulin beta 2B class IIb
IF	Immunofluorescence
Co-IP	Co-immunoprecipitation
shRNA	Short hairpin RNA
OERNA	Overexpression RNA
MutRNA	Mutant RNA
IHC	Immunohistochemistry
PBS	Phosphate-buffered saline
DAB	Diaminobenzidine
qRT-PCR	Real-time quantitative RT-PCR
PVDF	Polyvinylidene difluoride
SEM	Standard error of the mean
SD	Standard deviation
ANOVA	Analysis of variance
HPA	Human Protein Atlas
RNA-Seq	RNA sequencing
GO	Gene Ontology
ECM	Extracellular matrix
DEGs	Differentially expressed genes
NC	Non-targeting shRNA cells

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

JXL, ZJZ, JRZ and MW designed research; JXL, ZJZ, JRZ, MKZ and GJ performed research; JXL, XZL and GYW analyzed data; JXL, ZJZ and JRZ wrote the manuscript; SJL and WX made an important contribution to the article revision. JZ and LGC provided feedback on the report.

Data availability

Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information with the the project number PRJNA1181537.

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee and Institutional Review Board of the Affiliated Hospital, Southwest Medical University.

Competing interests

The authors declare no competing interests.

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