NRBP1 Promotes the Malignant Phenotypes of Glioblastoma by Regulating PI3K/Akt Activation

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Research Article

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Abstract

Glioblastomas (GBMs) are intracranial gliomas with the highest aggressiveness. Despite maximal treatment intervention, GBM patients’ median survival duration remains at approximately 14–16 months. Nuclear receptor-binding protein 1 (NRBP1) could stimulate the growth of cells. In this study, we investigated whether NRBP1 promotes malignant glioblastoma phenotypes and its potential mechanisms. High NRBP1 expression correlated with higher-grade glioma and shorter duration of overall and disease-free survival. NRBP1 knockdown via short hairpin RNAs caused suppression of cell proliferation, invasion, migration and triggered apoptotic cell death in vitro, whereas its overexpression, through plasmid transfection, showed the opposite effect. GO enrichment and KEGG analysis revealed that NRBP1 regulated differentially expressed gene clusters involved in the PI3K/Akt signaling pathway. Additionally, NRBP1 regulated epithelial–mesenchymal transition mediated by this pathway. Moreover, MK-2206 and SC79, which are respectively an inhibitor and an activator of PI3K/Akt signaling, reversed the effect of NRBP1 knockdown and overexpression on GBM, respectively. Thus, NRBP1 promotes malignant phenotypes in GBM by activating the PI3K/Akt pathway, thereby serving as a prognostic indicator and new target for GBM treatment.

Introduction

Glioblastoma (GBM), which accounts for 45–50% of central nervous system (CNS) malignant tumors, is among the most frequently diagnosed adult CNS cancers [1]. In the past decades, despite having access to optimal multimodal treatments, such as surgery removal, chemotherapy and radiotherapy, GBM outcomes have remained dismal [2]. Malignant phenotypes, such as strongly invasive, anti-apoptotic, and proliferative abilities of GBM, are associated with failed therapeutic regimens and recurrent lesions [3]. However, the precise mechanisms behind the malignant phenotype of GBM remain poorly investigated. Hence, the mechanisms responsible for tumor cell proliferation, invasion, and apoptosis, as well as reliable therapeutic biomarkers of GBM, warrant further exploration.

Although they share structural similarities with kinases, pseudokinases lack kinase activity. In contrast to the previously held belief that pseudokinases merely serve as redundant kinases, several pseudokinases have important physiological activities and/or demonstrate kinase activity, despite not having a conserved sequence of the kinase domain [4]. Additionally, pseudokinases are crucial players in cancer development and spread. The nuclear receptor-binding protein 1 (NRBP1) gene located on 2p23 of human genome encodes a highly conserved adaptor protein that exhibits ubiquitous expression [5]. This protein is known as a pseudokinase as the kinase core lacks residues essential for substrate phosphorylation. However, NRBP1 is structurally similar to a kinase in eukaryotic cells and may be implicated in other cellular activities [6].

NRBP1 has varied roles in the development of various cancers. For instance, Nrbp1 exhibited anti-tumor functions in intestinal cells as Nrbp1-null mice were more prone to intestinal malignancies [7]. In addition, the protein has been shown to promote prostate and bladder cancer progression [8, 9] while inhibiting the
development of breast and colorectal cancers [10, 11]. NRBP2 is another pseudokinase that is highly homologous to NRBP1. Both proteins lack the complete serine/threonine kinase domain with a sequence homology of 55.4% [12]. NRBP2 plays a key role in neural progenitor cell survival and has been proved to mutate in gliomas [13]. Nevertheless, its function and expression in gliomas remain unclear.

Here, we report aberrantly high NRBP1 mRNA levels in GBM tissues relative to low-grade glioma and normal tissues, indicating worse prognosis. We silenced or overexpressed NRBP1 in GBM cell lines and found that NRBP1 promotes malignant phenotypes and epithelial–mesenchymal transition (EMT) in GBM by modulating PI3K/Akt activation in vitro. Hence, we hypothesize that NRBP1 functions as an oncogene in GBM.

Materials And Methods

Bioinformatical analyses

Data for bioinformatics analysis were obtained from Chinese Glioma Genome Atlas (CGGA; http://www.cgga.org.cn/) and The Cancer Genome Atlas (TCGA; https://cancergenome.nih.gov/). Using GEPIA (http://gepia.cancer-pku.cn), we examined NRBP1 mRNA expression levels in gliomas with CNS WHO grade, 1p/19q co-deletion status, IDH1 mutation status, CDKN2A/2B deletion, overall survival (OS), and disease-free survival (DFS). The R package "cluster Profiler" was used to conduct GO and KEGG enrichment analysis.

Clinical tissue sample collection

From January 2021 to June 2022, 10 normal tissue samples and 20 low- and 20 high-grade glioma samples were harvested from the First Affiliated Hospital of USTC. The freshly collected samples were immediately frozen at −80°C. In accordance with the WHO Classification of Central Nervous System Tumours (5th Edition), two pathologists classified 305 surgically-removed paraffin-embedded intracranial glioma tissues collected between October 2019 and July 2022. These samples included 30 pilocytic astrocytomas (CNS WHO grade 1), 34 IDH-mutant astrocytomas (CNS WHO grade 2), 30 IDH-mutant astrocytomas (CNS WHO grade 3), 47 oligodendrogliomas (CNS WHO grade 2), 32 oligodendrogliomas (CNS WHO grade 3), and 132 IDH wild-type glioblastomas (CNS WHO grade 4). Patients' clinical data were collected and summarized. The hospital's ethics committee granted approval for the experimental procedures of this research (approval number: 2022-RE-298).

Quantitative real-time PCR (qRT-PCR)

Total RNA extraction from human glioma tissue samples was carried out with the TRIzol reagent (Thermo Fisher Scientific, USA). A reverse transcription kit from TaKaRa, Japan was employed to generate cDNA, which was subsequently subjected to qRT-PCR assays for quantification of the relative mRNA levels of NRBP1 based on the $2^{-\Delta\Delta Ct}$ algorithm. The reaction systems for these qRT-PCR assays were established with the SYBR Green Real-Time PCR Master Mix (Roche, Switzerland), and all the reactions were
performed with the 7500 Fast Real-Time PCR System. The following oligonucleotide primers were used in these assays: \( \beta \)-actin, sense primer: 5'-CATGTACGTTGCTATCCAGGC-3' and antisense primer: 5'-CTCCTTAATGTCACGCACGAT-3' and NRBP1, sense primer: 5'-GGACTCATCAAGATTGGCTTG-3' and antisense primer: 5'-TCTTCTGCTCTTCTCGACAAGT-3'. \( \beta \)-actin served as the internal reference gene.

**Immunohistochemistry and staining**

Paraffin-embedded intracranial glioma specimens were sectioned at 4-µm thickness. The sections were removed from xylene and immersed in graded alcohol for rehydration. Citric acid buffer (pH 6.0) was used for antigen fixing. The activity of endogenous peroxidase was stopped by washing the slides with phosphate-buffered saline (PBS). Then, the sections were subjected to overnight incubation in an NRBP1 primary antibody (GeneTex, Beijing, China) solution at 1:250 dilution at 4°C, then in a horseradish peroxidase-labeled goat anti-mouse IgG antibody (Beyotime, Beijing, China) for 30 min at ambient temperature. As per the proportion of stain-positive tumor cells, staining extent was calculated as 0% (0), 1–5% (1), 6–25% (2), 26–75% (3), and 76–100% (4). For staining intensity, scores of 0–3 were respectively assigned to negatively, weakly, mediumly, and strongly stained samples. The scores from 0 to 12 were determined with staining extent score×staining intensity score. For the statistical analysis of cytoplasmic expression, low expression was indicated by a score of 0–4 indicated, and high expression by a score of 5–12.

**Cell line culturing**

We purchased human GBM cell lines A172, SHG44, U87, and U251, and the normal astrocyte cell line HA1800 from the Institute of Biophysics, Chinese Academy of Sciences (Beijing, China). All cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, China) added with fetal calf serum (10%) and antibiotics (streptomycin and penicillin at 1%, Amimed, Switzerland) in a moisturized incubator filled with 5% CO\(_2\).

**Western blotting**

Cell lysis was induced by incubating the samples for 30 min in ice-cold radioimmunoprecipitation assay lysis buffer. The bicinchoninic acid assay was conducted for measuring total protein concentrations (Beyotime, Beijing, China). All protein samples underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis before their electrotransfer to a polyvinylidene difluoride (PVDF) membrane. The blots were immersed for 60 min in 5% BSA in Tris-buffered saline with Tween-20 (TBST, pH 7.0) and then subjected to overnight incubation (at 4°C) with primary antibodies. The primary antibodies included those raised against NRBP1 (1:1000) from GeneTex, China. Bcl-2, Bax, PI3K, p-PI3K, Akt, and p-Akt (1:1000) from Thermo Fisher Scientific, USA, and N-cadherin, E-cadherin, MMP-7(1:1000) and \( \beta \)-actin (1:5000) from Cell Signaling Technology, USA. The blots were visualized by HRP-labeled goat anti-mouse or goat anti-rabbit IgG (1:5000) from CoWin Biosciences, China, with enhanced chemiluminescence kits from Millipore, USA. \( \beta \)-actin bands served as the loading control.

**Lentiviral infection**
We purchased the lentivirus plasmid from Beyotime Biotechnology (Shanghai, China). For lentiviral infection, $1 \times 10^6$ U87 cells were grown in the presence of 4 µL lentivirus with NRBP1 for 12 h, and lentivirus without NRBP1 was the negative control. After culturing for 48 h at 37°C, puromycin (4 µg/mL) was utilized to sift the cells that were successfully transferred into the plasmid. We constructed three short hairpin RNAs (shRNAs) targeting NRBP1 mRNA, with the following sequences: shNRBP1-1, GACCTTGAACAAGTTCAATTT; shNRBP1-2, GCAATGGAGAGTCCTCATATG; and shNRBP1-3, CCAACACATGATCCCAGAGAA. Western blotting was carried out to determine the transfection rate. For subsequent experiments, the two groups of cells with superior transfection efficiency were chosen. NRBP1-overexpressing (pcDNA-NRBP1) and empty plasmids were bought from GenePharma (Shanghai, China) and transfected into cells that maintained in Opti-MEM by utilizing Lipofectamine 2000. 48-h after transfection, the cells were harvested for transfection efficiency determination.

**Cell proliferation assay and colony formation**

Cell counting kit-8 (CCK-8) assays were implemented to appraise cell proliferation ability. Briefly, logarithmically growing cells were inoculated in 96-well plates at a density of 3000 cells per well and grown for 0, 24, 48, 72 or 96 h. In each well, the CCK-8 reagent (Dojindo Laboratories, Kyushu Island, Japan) was supplemented. After incubation for 4 h. Optical density at 450 nm was recorded with a Synergy H1 microplate reader (BioTek, USA).

Five hundred cells were maintained in one well of a 6-well plate, with the medium refreshed once on the fourth day of culture for two weeks. Cloned cells were fixed in 4% paraformaldehyde for 15 min, dried, and subjected to crystal violet staining. Six randomly picked fields were observed under an Olympus optical microscope for colony number counting (colonies with $\geq 50$ cells were considered a single clone).

**Wound healing assay**

$2 \times 10^6$ cells were allowed to proliferate for 12 h in each well of a 6well cell culture plate to achieve 90–100% confluence. Afterwards, scratch wounds were generated with a sterilized pipette tip. After two PBS washes, the cells were maintained in a culture medium without serum for evaluating wound closure. A phasecontrast microscope ($\times100$) was utilized to visualize the cultured cells immediately and 24 after the wounds were created to assess the percentage of wound healing that was determined as $[(A_i - A_t)/A_i] \times 100$, in which $A_i$ is the wound area determined immediately while $A_t$ denotes the wound area after cell culture.

**Analysis of migratory and invasive abilities of glioma cells**

The migratory and invasive abilities of glioma cells were appraised through Transwell assays, for which the Matrigel was applied in the upper chamber only in the cell invasion analysis. Cells that had undergone starvation treatment were inoculated into an even distribution in the upper chamber containing culture medium without serum. Then the lower chamber was added with cell culture medium plus 10% FBS, which served as a chemoattractant. The Transwell chambers were then incubated for 24 h at 37°C, after
which cells in the lower chamber were subjected to fixation and crystal violet (1%) staining. Five fields of view were used for cell counting.

**Cell apoptosis assay**

A TUNEL kit from Beyotime, China was used to assess DNA fragmentation, which is a hallmark of apoptosis, in accordance with the provider’s protocol. 2×10^6 cells were placed onto a glass slide in each well of a 6-well plate and grown for 4 days. The cells were then mounted in a solution that prevents quenching of fluorescence and observed with an Olympus fluorescence microscope. We randomly picked five high-power fields (×200 magnification) to determine the numbers of TUNEL- and Hoechst-positive cells, the proportion of which reflects the amount of apoptotic cells.

**AKT phosphorylation experiment**

NRBP1-overexpressing U251 cells (pcDNA-NRBP1) were incubated with 10 µM MK-2206 (Selleck, Houston, USA), and U87 cells with NRBP1 knockdown (sh-NRBP1) were incubated with 10 µg/mL SC79 (Calbio, La Jolla, CA). U251 and U251 (pcDNA-NRBP1) cells without MK-2206 incubation served as controls, whereas U87 and U87 (sh-NRBP1) cells that were not incubated with SC79 were used as controls. Next, CCK-8, Transwell assay, and western blotting were conducted to determine cell proliferation, invasion, and EMT in GBM.

**Statistical analysis**

We employed GraphPad Prism 8.0 (GraphPad Software, USA) for analysis of statistical data. The data from three assays performed in triplicate are displayed as mean ± SEM. The Kaplan–Meier method was employed for plotting DFS and OS curves, and intergroup differences were analyzed by the log-rank test. One-way ANOVA or two-tailed Student’s t-test was carried out to decide whether differences were statistically significant. Values with ∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001 indicate statistical significance.

**Results**

**NRBP1 expression in glioma**

To explore the NRBP1 function in gliomas, we compared NRBP1 mRNA expression in gliomas and normal tissues using the TCGA/GEPIA and CGGA datasets. Significantly upregulated mRNA expression of NRBP1 was noted in GBM tissues than in normal or low-grade glioma tissues (Fig. 1A); this expression was positively correlated with the glioma grade (Fig. 1B). Additionally, the mRNA level of NRBP1 was negatively correlated with the IDH mutant status (Fig. 1C) and positively correlated with CDKN2A/2B deletion. Immunohistochemistry identified significantly higher positive staining intensity of NRBP1 in GBM than in normal or low-grade glioma tissues (Fig. 1D). Higher mRNA expression of NRBP1 was found in high-grade glioma tissues relative to normal or low-grade glioma tissues in freshly collected samples (Fig. 1E). Clinicopathological features of the 305 patients with gliomas are displayed in Table 1.
Univariate analysis demonstrated significant correlations between NRBP1 expression levels and age, WHO grade, IDH mutant status, and 1p/19q co-deletion (Table 1). The prognostic significance of NRBP1 was assessed using Kaplan–Meier survival analysis in 132 patients with GBM, and 115 patients had follow-up data. High NRBP1 expression had positive correlations with reduced OS and DFS. The results are consistent with those in the TCGA database (Fig. 1F). Therefore, our data suggest that high NRBP1 expression predicts poor survival in GBM.

### Table 1

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NRBP1 silencing suppressed cell proliferation, invasion, and migration and induced apoptosis

The biological roles of NRBP1 in GBM were analyzed by measuring NRBP1 expression in GBM cell lines U251, SHG44, U87, and A172 and a noncancerous astrocyte cell line HA1800. Glioma cells showed significantly higher NRBP1 expression than HA1800 cells (Fig. 2A). U87 cells with the highest expression and U251 cells with the lowest expression were selected for NRBP1 knockdown and overexpression assays, respectively. Next, we transfected lentivirus particles packaged with three different shRNAs
targeting NBRP1, which resulted in significantly reduced NRBP1 protein expression in U87 cells (Fig. 2B). We selected shNRBP1-2 and shNRBP1-3 for cell function experiments because of their high knockdown efficiency. Growth curves for shNRBP1-1, shNRBP1-2, and control cells were generated using CCK8 assay results. Cell growth was markedly suppressed in both shNRBP1s-transfected cell lines (Fig. 2C). Decreased proliferation upon NRBP1 knockdown was further confirmed by colony formation (Fig. 2D). Transwell and wound healing assays demonstrated significantly decreased cell invasion and migration ability of the sh-NRBP1 groups relative to control (Fig. 2E and 2F). TUNEL staining and western blotting revealed that the sh-NRBP1 groups exhibited significantly increased cell apoptosis than control (Fig. 2G), along with lower Bcl-2 expression and higher Bax expression (Fig. 2H).

**NRBP1 overexpression induced cell proliferation, invasion, and migration and suppressed apoptosis**

To validate the function of NRBP1 in GBM cells, we conducted overexpression experiments on U251 cells. Colony formation and CCK-8 assays identified an obvious effect of NRBP1 overexpression on U251 cell proliferation (Fig. 3A and 3B). Wound healing assay results revealed significantly increased wound closure area at 24 h in pcDNA-NRBP1 cells relative to the negative control cells (Fig. 3C); Transwell experiments exhibited consistent results. In the pcDNA-NRBP1 group, significantly increased cell invasion ability and migration ability were noted, compared with the vector group (Fig. 3D). TUNEL staining and western blotting revealed significantly decreased cell apoptosis in the pcDNA-NRBP1 group (Fig. 3E), accompanied by decreased Bax expression and increased Bcl-2 expression (Fig. 3F), relative to the vector group. These observations indicate that NRBP1 promotes the malignant phenotype of GBM in vitro.

**NRBP1 regulated EMT marker expression and induced phosphorylation of PI3K/Akt**

The relationship between NRBP1 and EMT was evaluated by determining the expression of MMP-7, N-cadherin, and E-cadherin, which are markers for the EMT process, before and after NRBP1 knockdown and overexpression. NRBP1 knockdown augmented E-cadherin expression while reduced that of N-cadherin and MMP-7, whereas NRBP1 overexpression resulted in opposite effects (Fig. 4A). These data verified that NRBP1 induces EMT in GBM cells. Functional annotation analyses of GO and KEGG reflected that NRBP1 regulated differentially expressed gene clusters implicated with the PI3K/Akt signaling cascade (Fig. 4B). To test the impact of NRBP1 on PI3K/Akt activation, we evaluated the protein levels of total and phosphorylated PI3K and AKT before and after NRBP1 knockdown and overexpression, which revealed that the phosphorylation levels of the two proteins were markedly higher than in the shNRBP1 group and lower than in the pcDNA-NRBP1 group, while total PI3K and AKT expression remained unchanged (Fig. 4C).

**PI3K/Akt inhibitor or activator affected cell proliferation, invasion, and EMT via NRBP1**
To investigate whether the impact of NRBP1 silencing or overexpression on GBM occurred via the PI3K/Akt signaling cascade, U251 cells overexpressing NRBP1 (pcDNA-NRBP1) were treated with 10 µM AKT inhibitor MK-2206, and U87 cells with NRBP1 knockdown (sh-NRBP1) were incubated with 10 µg/mL AKT activator SC79. Notably, MK-2206, an inhibitor against AKT, suppressed the proliferation and invasion of the pcDNA-NRBP1 group, whereas the AKT activator SC79 promoted this in the sh-NRBP1 group (Fig. 5A–5D). Moreover, MK-2206 significantly inhibited N-cadherin and MMP-7 expression but upregulated E-cadherin expression in the pcDNA-NRBP1 group. This effect was reversed with SC79 in the sh-NRBP1 group (Fig. 5E). Our results reveal the possible involvement of NRBP1 in the progression of GBM via inducing EMT, which is triggered by phosphorylation of AKT.

Discussion

In GBM, treatment failure and recurrence are caused by the highly malignant phenotype of GBM cells invading the surrounding brain parenchyma [14]. Therefore, there is a need to elucidate the complicated pathology of GBM so as to obtain novel insights into accurate prognostic prediction that is crucial for designing more effective therapeutic strategies.

Recombinant proteins from bacterial and mammalian sources have been used to assess NRBP1 kinase activity, which identified NRBP1 as an adaptor protein [15]. Although NRBP1 does not phosphorylate substrates, it is crucial for homeostasis of cells and cancer pathophysiology [16]. The conserved structure of NRBP1, as well as its binding interaction with several important transcription factors, such as activated Rac3, the MLF1 oncoprotein, and JAB1, makes it an essential component of cellular activity [17]. Although NRBP1 reportedly plays a role in cancer progression, its precise function remains unclear.

Herein, we provide preliminary findings for NRBP1 expression in clinical samples of glioma tissues and cultured GBM cell lines. Our analysis of the TCGA and CGGA databases revealed poor outcomes for patients with a high NRBP1 expression. Our findings from clinical cases, along with bioinformatics analysis, indicated a relationship between high NRBP1 expression and a higher grade of glioma and poor OS and DFS. We noted that NRBP1 expression levels were significantly correlated with age, WHO grade, IDH status, and 1p/19q co-deletion in the univariate analysis, consistent with previous findings showing upregulated NRBP1 expression in diverse cancers, including prostate cancer and bladder cancer [8, 9]. These data suggest the role of NRBP1 in glioma tumorigenesis.

To confirm whether NRBP1 advances GBM progression, GBM cell lines with NRBP1 overexpression and knockdown were used, and cell proliferation, migration, invasion, and apoptosis were examined. We selected U87 cells with the highest expression for NRBP1 knockdown assay and U251 cells with the lowest expression for NRBP1 overexpression assay. NRBP1-transfected U87 cells showed significant inhibition of proliferation, invasion, and migration, and induced apoptosis. A significant increase in cell proliferation, invasion, and migration was noted when NRBP1 was overexpressed in U251 cells. These data indicate that NRBP1 promotes the malignant phenotype of glioblastomas.
EMT plays a crucial role in embryonic development [18]. EMT is a significant molecular event in tumor invasion, including that of GBM [19]. We observed that NRBP1 knockdown enhanced E-cadherin expression and reduced that of MMP-7 and N-cadherin; the reverse effect was noted with NRBP1 overexpression. Thus, NRBP1 may regulate EMT in GBM cells. EMT is under the regulation of a number of signaling cascades [20] and is characterized by AKT protein kinase activation, which is oncogenic [21]. The PI3K/Akt signaling affects cell proliferation, invasion, and migration in astrocytomas, melanomas, endometrial cancer, breast cancer, renal cancer, ovarian cancer, pulmonary cancer, and lymphoid cancer [22]. GO enrichment and KEGG analyses revealed that NRBP1 regulates differentially expressed gene clusters implicated with the PI3K/Akt signaling cascade. We also verified whether NRBP1 expression is associated with Akt phosphorylation at the molecular level and identified the inhibition of AKT and PI3K phosphorylation after NRBP1 downregulation in U87 cells, which increased after NRBP1 overexpression. In addition, treatment with the specific inhibitor MK-2206 or the activator SC79 reversed the effect of NRBP1 silencing or overexpression on GBM. Overall, our results strongly indicate the involvement of NRBP1 in the progression of GBM, possibly through EMT induction, which is activated by AKT phosphorylation.

Conclusion

In this study, NRBP1 overexpression was noted in GBM tissues. Our data suggest that NRBP1 overexpression was correlated with progression and dismal clinical outcomes of GBM. Thus, NRBP1 was identified as a crucial factor in the malignant progression of GBM and has great potential as a therapeutic target for GBM. Moreover, NRBP1 may participate in the progression of GBM via inducing EMT, which is activated through AKT phosphorylation.

Declarations

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Author Contributions A.Z., S.P., S.S and S.Y. carried out the experiments; A.Z. and S.P. performed data analysis; A.Z. and Y.Z. were responsible for manuscript drafting. Y.Z. and Q.W. were responsible for study conceiving and experimental design. All authors read and approved the fnal manuscript.

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Conflict of interest The authors declare that there are no competing interests associated with the manuscript.
**Ethical approval** Our current study was approved by the Ethics Committee of The First Affiliated Hospital of USTC with the date 04/09/2022, and No: 2022-RE-298.

**Consent for publication** Not applicable.

**References**


**Figures**
Figure 1

NRBP1 expression in glioma. (A) NRBP1 mRNA levels of glioma and normal tissues obtained from TCGA and CGGA (B). (C) Correlations between NRBP1 and IDH status in gliomas determined using CGGA. (D) Representative IHC staining images for NRBP1 in gliomas and normal tissues. (E) qRT-PCR conducted to measure relative expression levels of NRBP1 in different grade glioma and normal tissue samples. (F)
Kaplan–Meier analysis showing the prognostic significance of NRBP1 using data from the TCGA dataset.

Figure 2

NRBP1 knockdown inhibited cell proliferation, invasion, and migration while inducing apoptosis. (A) Immunoblotting conducted to evaluate NRBP1 expression in GBM cell lines (U251, SHG44, A172, and...
U87MG) and normal astrocyte cell line (HA1800). (B) Western blotting results of NRBP1 levels after sh-NRBP1-1, sh-NRBP1-2, and sh-NRBP1-3 transfection in cells. (C, D) CCK-8 and colony formation assay for estimating cell proliferation in sh-NRBP1-transfected cells. (E) Transwell assay for comparing cell migration and invasion ability of sh-NRBP1-transfected cells. (F) Wound healing assay to compare cell migration ability of sh-NRBP1-transfected cells. (G, H) TUNEL staining and western blotting for determining cell apoptosis. β-Actin was used as the internal control.
Cell proliferation, invasion, and migration were promoted and apoptosis suppressed by NRBP1 overexpression. (A, B) CCK-8 and colony formation assay conducted to estimate cell proliferation in pcDNA-NRBP1-transfected cells. (C) Wound healing assay to compare migration ability of pcDNA-NRBP1-transfected cells. (D) Transwell assay for comparing invasion and migration abilities of pcDNA-NRBP1-transfected cells. (E, F) TUNEL staining and western blotting conducted to assess cell apoptosis. β-Actin was the loading control.
Figure 4

EMT marker expression was regulated and PI3K/Akt phosphorylation induced by NRBP1. (A) Western blotting with corresponding antibodies for determining EMT marker protein levels in shNRBP1- and pcDNA-NRBP1-treated U87 and U251 cells. (B) Enriched GO term of NRBP1 molecular function indicated by dot plot. Dot size reflects the number of genes in NRBP1 that are associated with the GO term, and the

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Figure 4

EMT marker expression was regulated and PI3K/Akt phosphorylation induced by NRBP1. (A) Western blotting with corresponding antibodies for determining EMT marker protein levels in shNRBP1- and pcDNA-NRBP1-treated U87 and U251 cells. (B) Enriched GO term of NRBP1 molecular function indicated by dot plot. Dot size reflects the number of genes in NRBP1 that are associated with the GO term, and the
Figure 5

PI3K/Akt inhibitor or activator affected cell proliferation, invasion, and EMT via NRBP1. (A, B) CCK-8 analysis for estimating cell proliferation in pcDNA-NRBP1- or shNRBP1-transfected cells with AKT.
inhibitor MK-2206 or AKT activator SC79. (C, D) Transwell assay comparing the invasion ability of pcDNA-NRBP1- or shNRBP1-transfected cells added with AKT inhibitor MK-2206 or AKT activator SC79. (E) Western blotting with corresponding antibodies showing the expression levels of EMT markers in shNRBP1- or pcDNA-NRBP1-treated U87 and U251 cells added with AKT inhibitor MK-2206 or AKT activator SC79. β-Actin was the loading control.

**Supplementary Files**

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- WesternblotOriginalImages.pdf