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ORIGINAL ARTICLE

# DDX46 accelerates the proliferation of glioblastoma by activating the MAPK-p38 signaling

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## Summary

***Purpose:*** *To analyze the influence of DDX46 on the proliferative and migratory potentials of glioblastoma (GBM).*

***Methods:*** *Differential levels of DDX46 in GBM cases and controls were examined by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. By intervening DDX46 in U87 and U251 cells, proliferative and migratory changes were determined by colony formation assay, 5-Ethynyl-2'- deoxyuridine (EdU) assay and Transwell assay, respectively. Protein levels of p-p38, p38, cyclin D1 and MMP7 in GBM cells intervened by DDX46 or the inhibitor of p38 MAPK were detected.*

***Results:*** *DDX46 was upregulated in GBM cases. Knockdown of DDX46 attenuated the proliferative capacity of GBM cells, and its overexpression enhanced the proliferative rate. The migratory capacity of GBM was not affected by DDX46. Overexpression of DDX46 upregulated p-p38 and cyclin D1 in GBM cells. The regulatory effect of DDX46 on GBM proliferation could be partially reversed by the treatment of doramapimod.*

***Conclusions:*** *DDX46 is upregulated in GBM, which strengthens the proliferative capacity of GBM by activating the MAPK-p38 signaling.*

***Key words:*** *DDX46, MAPK-p38, GBM, proliferation*

## Introduction

Gliomas, especially glioblastomas (GBMs), are highly invasive primary malignant tumors in the brain, which are the third leading cause of global cancer deaths in middle-aged people. Surgical resection combined with postoperative temozolomide (TMZ)-based therapy is currently the standard treatment for glioma [1,2]. However, the prognosis of GBM is extremely poor because of the high rates of invasion and recurrence. The median overall survival of high-grade glioma is only about 14.6 months [3]. Targeted therapy and individualized therapy based on gene targets of GBM have been well concerned in medical research of glioma treatment.

In recent years, the importance of RNA in life activities has been highlighted. RNA helicases are necessary for RNA-involved biological processes. RNA unwinding and ribonucleoprotein structural reorganization require energies produced by RNA helicases through ATP hydrolysis, which are involved almost in all aspects of RNA metabolism [4,5]. Besides, RNA helicases participate in tumor development by mediating expressions and mutations of tumor-associated genes or initiating tumorigenesis [6].

DEAD-box helicases (DDX) family members are important mediators in tumor development. It is reported that DDX3 and DDX5 are abnormally

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upregulated in lung cancer cases [7,8]. DDX3 and DDX39 have a close relation to liver cancer development [9,10]. DDX43 is involved in breast cancer development [11]. An abnormal expression of DDX27 is identified in gastric cancer patients [12,13].

DDX46, also known as PRPF5 or hPrp5, is related to multiple types of tumors and their prognosis [14-16]. The biological function of DDX46 in GBM is largely unclear. This study intended to demonstrate the role of DDX46 in influencing malignant phenotypes of GBM and the underlying mechanism.

## Methods

*Clinical samples*

GBM tissues (n=26) were collected during surgery. None of the recruited patients received preoperative chemotherapy. During the same period, non-tumor brain tissues (n=26) were collected from the decompression of the cranium in brain trauma patients. This study was approved by the Ethics Committee of our hospital. Signed written informed consents were obtained from all participants before the study entry.

*Cell culture*

Glioma cell lines (U87, U251 and A172) and the astrocyte cell line (NHA) were provided by Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1000 U/mL penicillin and 100 mg/mL streptomycin in an incubator containing 5% $CO_2$ at 37°C. Cell passage was conducted at 48 h.

*Cell transfection*

DDX46 shRNA (5'-AGAAATCACCAGGCTCATA-3') and pcDNA3.0 were synthesized by GeneChem (Shanghai, China). Cell preparations (3-5×$10^4$ /mL) were implanted in a 6-well plates, and transfected with DDX46 shRNA or pcDNA3.0 (DOI=10). Twelve h later, fresh medium was replaced. GFP-labeled cells were observed under a fluorescence microscope, and 70% of GFP-positive cells was qualified for the following experiments.

*Quantitative real-time polymerase chain reaction (qRT-PCR)*

Total RNAs were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and the concentrations were measured using NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Using the PrimeScript™RT Master Mix, reversely transcribed complementary DNAs (cDNAs) were further subjected to qPCR. Relative level was calculated using ABI Step One (Applied Biosystems, Foster City, CA, USA). Primer sequences were: DDX46, forward, 5'-AAAATGGCGAGAAGAGCAACG-3' and reverse, 5'-CATCATCGTCCTCTAAACTCCAC-3'; glyceraldheyde 3-phosphate dehydrogenase (GAPDH), forward, 5'-TGACTTCAA-CAGCGACACCCA-3' and reverse, 5'-CACCCTGTTGCTG-TAGCCAAA-3'.

*Western blot*

Cells or tissues were lysed on ice for 30 min using RIPA and the mixture was centrifuged at 4°C, 12,000 rpm for 15 min. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking non-specific antigens on membranes, they were induced with primary and secondary antibodies under indicated conditions. Protein signals were detected using Luminol substrate solution.

*Transwell assay*

300 μL of serum-free cell suspension was seeded in an insert (1×$10^6$ cells/L) placed in each well of 24-well plates, where 500 μL medium with 10% FBS per well was contained. After 24-h cell culture, penetrating cells were fixed in 4% paraformaldehyde. Twenty min later, cells were dyed in 0.4% trypan blue for 20 min and observed under a microscope (40×).

*Colony formation assay*

Cells were seeded in 12-well plates with 200 cells/well. After cell culture for 12-14 days, colonies (>50 cells) were fixed in paraformaldehyde, dyed in Giemsa and captured for counting.

*5-Ethynyl-2'- deoxyuridine (EdU)*

After 24-h cell culture, cells were induced in 100 μL of EdU solution (Beyotime, Shanghai, China) for 48 h, paraformaldehyde for 15 min and 0.2% glycine for 10 min. Cells were induced in 0.5% Triton X-100, dyed in Apollo in the dark for 30 min, and Hoechst in the dark for 10 min. PBS washing twice was conducted after each procedure. Lastly, cells were washed in 0.5% Triton X-100 for three times, and captured using a fluorescence microscope.

*Statistics*

SPSS 21.0 (IBM, Armonk, NY, USA) and Graph Pad 8.0 (La Jolla, CA, USA) were used for statistical analyses and figure formatting, respectively. Data were expressed as mean±SD. Differences between groups were compared by the t-test and $p<0.05$ was considered as statistically significant.

## Results

*Upregulation of DDX46 in GBM*

Compared with non-tumor tissues, DDX46 was highly expressed in GBM tissues at both mRNA and protein levels (Figure 1A,1B). Similarly, it was upregulated in glioma cell lines (Figure 1C, 1D). U87 and U251 cell lines were used because of their relatively high abundance of DDX46.

*Knockdown of DDX46 suppressed the proliferative potential of GBM*

To investigate the biological function of DDX46 in GBM, its expression level was intervened by

transfection of DDX46 shRNA (Figure 2A). The results of colony formation and EdU assay demonstrated that knockdown of DDX46 suppressed the proliferative potential of U87 and U251 cells (Figure 2B, 2C). Nevertheless, the migratory rate of GBM cells was not affected by DDX46 (Figure 2D).

*Overexpression of DDX46 accelerated proliferative potential of GBM*

Furthermore, overexpression of DDX46 was achieved by transfection of DDX46 pcDNA3.0 (Figure 3A). As expected, overexpression of DDX46 elevated the colony number and EdU-positive rate,

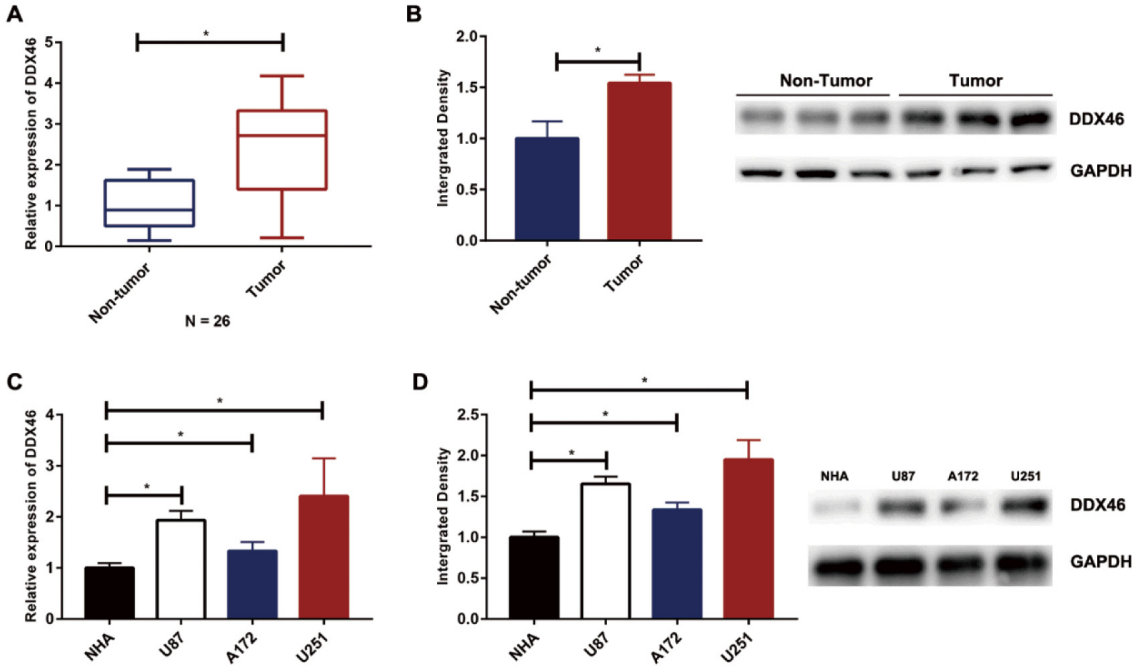


**Figure 1.** Upregulation of DDX46 in GBM. **A:** Relative level of DDX46 in GBM tissues (n=26) and non-tumor tissues (n=26) detected by qRT-PCR. **B:** Protein level of DDX46 in GBM tissues (n=3) and non-tumor tissues (n=3) detected by Western blot. **C:** Relative level of DDX46 in glioma cell lines and the astrocyte cell line detected by qRT-PCR. **D:** Protein level of DDX46 in glioma cell lines and the astrocyte cell line detected by Western blot (*p<0.05).

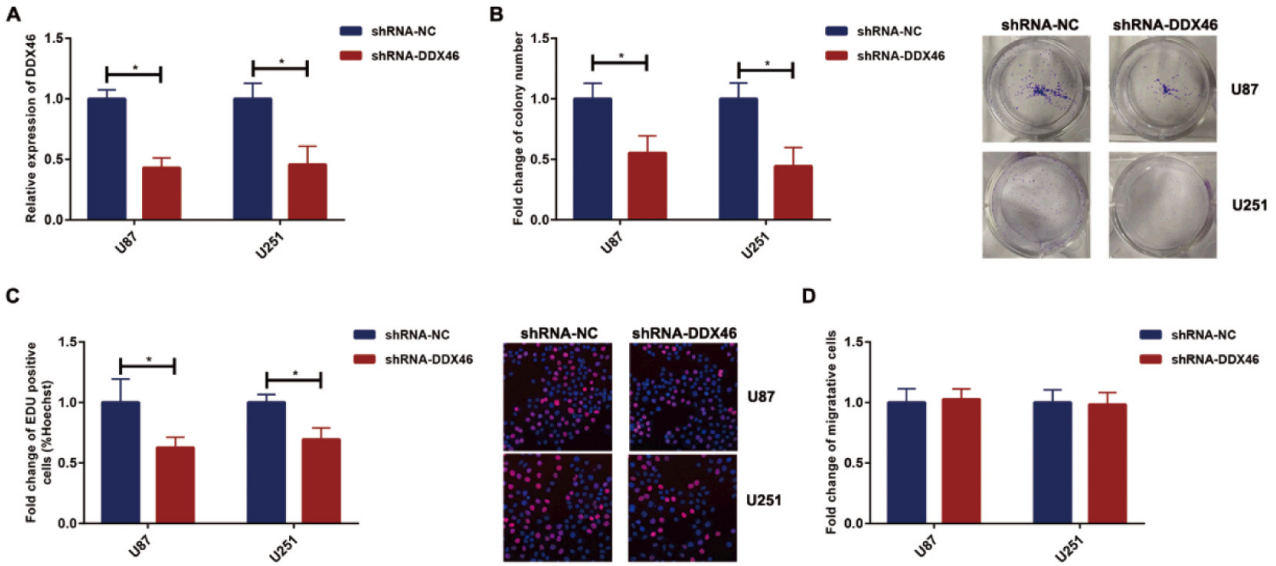


**Figure 2.** Knockdown of DDX46 suppressed proliferative potential of GBM. **A:** Transfection efficacy of DDX46 shRNA in U87 and U251 cells. **B:** Colony number in U87 and U251 cells with DDX46 knockdown (40×). **C:** EdU-positive rate in U87 and U251 cells with DDX46 knockdown. **D:** Migratory cell rate in U87 and U251 cells with DDX46 knockdown (40×) (*p<0.05).

indicating the accelerated proliferative potential of GBM (Figure 3B, 3C). The number of migratory cells was similar between cells transfected with DDX46 pcDNA3.0 and those of controls (Figure 3D). Therefore, the migratory potential of GBM was not influenced by DDX46.

*DDX46 activated the MAPK-p38 signaling*

The relative levels of key genes in the MAPK-p38 signaling were detected by Western blot. Protein levels of p-p38 and its downstream protein cyclin D1 were upregulated by overexpression of DDX46. However, the protein level of MMP7 was not obviously regulated by DDX46, which was consistent with the previous conclusion that the migratory potential of GBM was not affected by DDX46. To verify the involvement of the MAPK-p38 signaling in GBM development, cells were induced with doramapimod, the inhibitor of p38 MAPK, which showed that doramapimod treatment partially reversed the regulatory effects of DDX46 on protein levels of p-p38 and cyclin D1, as well as the proliferative potential of GBM (Figure 4A, 4B).

## Discussion

GBM is a highly aggressive tumor of the brain, and is categorized into classical, neural, mesenchymal and proneural subtype based on differential expressions of the gene transcriptome [17]. However, the prognosis of GBM is extremely poor even after a comprehensive treatment, which is mainly caused by the polyclonal origin, high heterogeneity and resistance to radiotherapy and chemotherapy. Conventional pathologic examination of GBM is unable to satisfy the demand for individualized treatment. Seeking for gene targets of GBM is essential to develop targeted therapy, thus improving the clinical outcomes of GBM patients.

DDX proteins are a type of highly conserved RNA helicases that are distributed in most of prokaryotes and nearly all eukaryotes. In 1982, Grifo et al [18] first identified eIF4A in rabbit red blood cells, which is the first member of the DDX family associated with the initiation of translation. In 1988 Lane et al [19] proposed that some proteins with similar structures and functions of eIF4A can be classified to the same protein family. Later, Linder et al [20] nominated the DDX protein family, where all family members contain the same conserved amino acid fragments (Asp-Glu-Ala-Asp, D-E-A-D). DDX46 is a multifunctional RNA helicase with a typical, highly conversed helicase domain. It exerts a key role in RNA-RNA or RNA-protein interaction and alteration of the secondary structure of RNA [21,22]. *In vitro* evidence has proven that DDX46 is a core mediator in mRNA precursor splicing and ribosome assembly [23,24]. In a zebrafish model, DDX46 is necessary for the directed differentiation of hematopoietic stem cells [25], digestive system organs and brain development [26]. DDX46 locates on chromosome

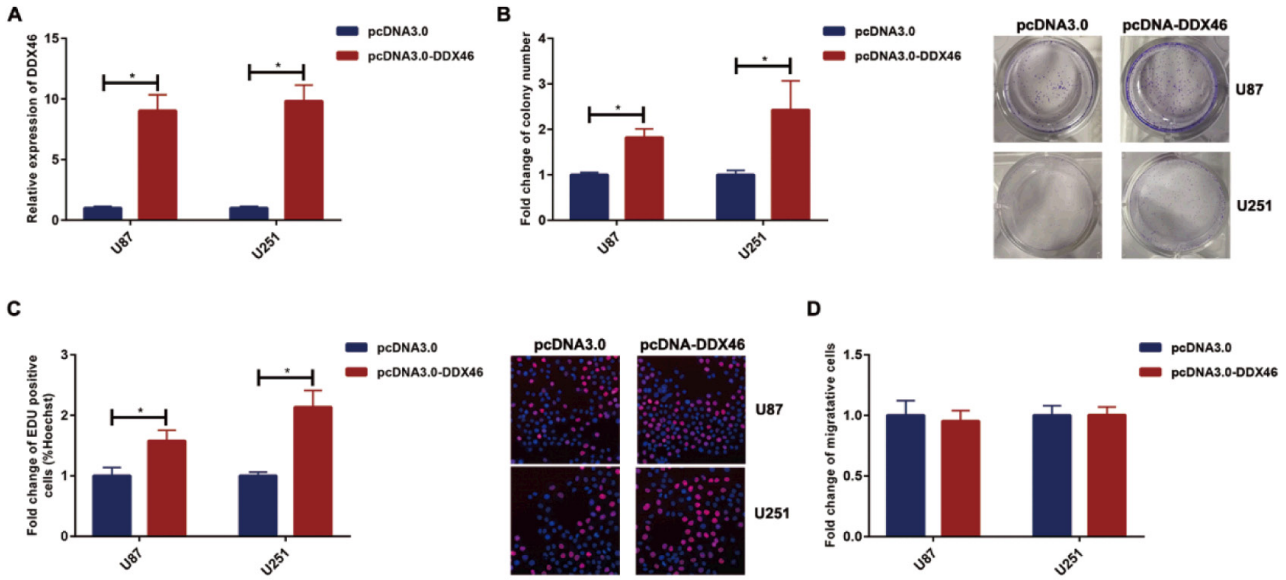


**Figure 3.** Overexpression of DDX46 suppressed proliferative potential of GBM. **A:** Transfection efficacy of DDX46 pcDNA3.0 in U87 and U251 cells. **B:** Colony number in U87 and U251 cells overexpressing DDX46 (40×). **C:** EdU-positive rate in U87 and U251 cells overexpressing DDX46. **D:** Migratory cell rate in U87 and U251 cells overexpressing DDX46 (40×) (*p<0.05).

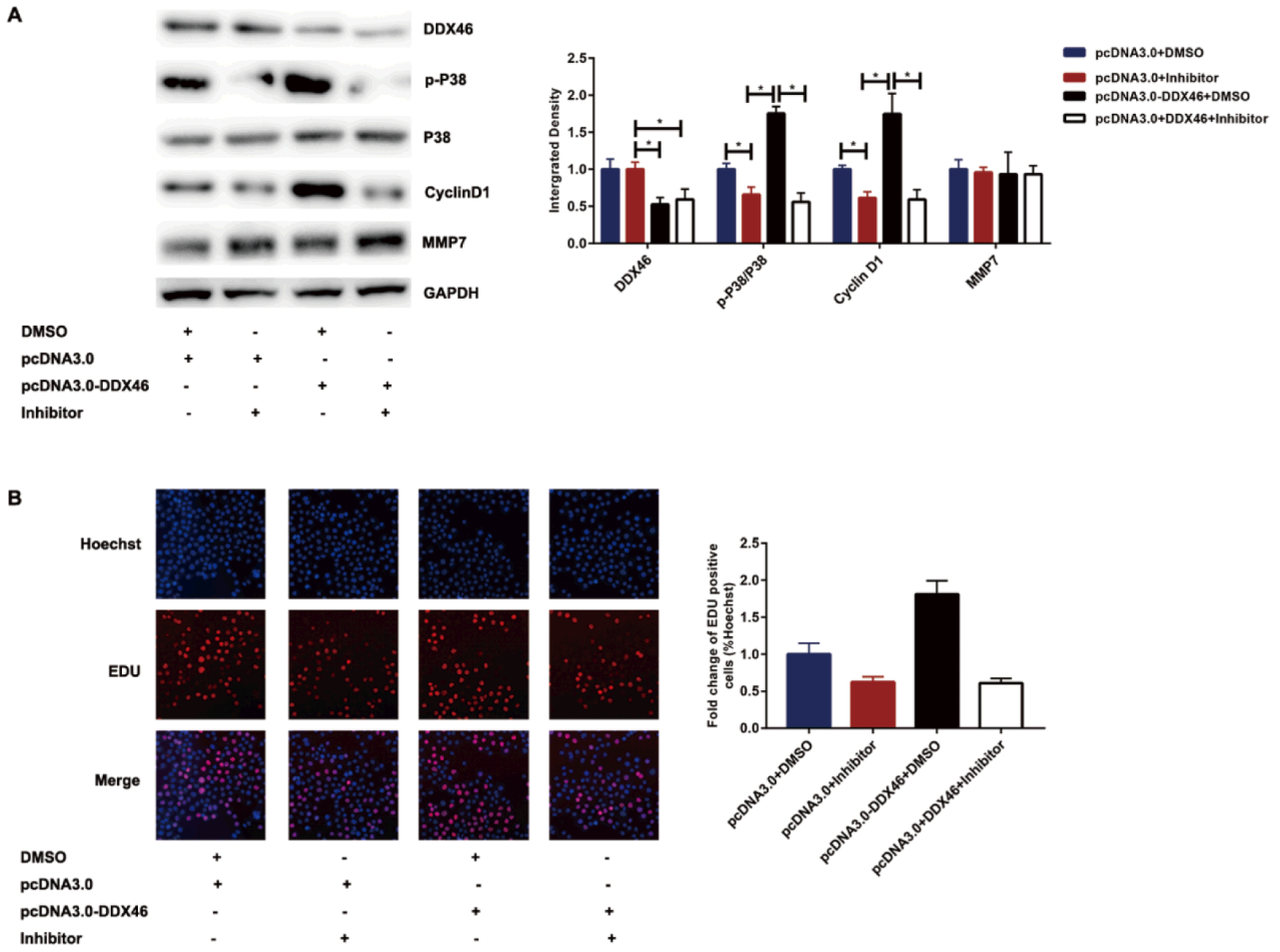


**Figure 4.** DDX46 activated the MAPK-p38 signaling. U87 cells were intervened by DDX46 overexpression or doramapimod (the inhibitor of p38 MAPK). **A:** Protein levels of DDX46, p-p38, p38, cyclin D1, and MMP7 in U87 cells. **B:** EdU-positive rate in U87 cells (40×) (*p<0.05).

5q31.1, which contains 26 exons and encodes a 131 kDa protein [27]. Here, DDX46 was upregulated in GBM cells. Knockdown of DDX46 in U87 and U251 cells markedly suppressed the proliferative capacity. However, the migratory potential of GBM was not affected by DDX46.

The MAPK-p38 signaling is the mostly analyzed one that is involved in diverse pathological and physiological activities [28]. There are four isomers of MAPK-p38, including p38α, p38β, p38δ and p38γ. The famous MAPK-p38 signaling is activated by various intracellular and extracellular stimuli like hypertonic environment, radiation exposure, heat shock, etc [29]. Through activating a cascade of cellular signals, the MAPK-p38 is responsible for inducing inflammatory response, apoptosis, differentiation and other activities in the tumor microenvironment [30]. The MAPK-p38 signaling is capable of promoting the osteogenic differentiation of human periodontal ligament stem cells [31]. Abundant clinical evidence has proven the close relationship between the MAPK-p38 signaling and cancer cell behaviors [32]. Our findings consistently found that DDX46 could regulate the MAPK-p38 signaling in GBM, which was essential for the positive regulation of DDX46 in GBM cell proliferation.

Two limitations in this paper should be further improved. First, *in vivo* function of DDX46 requires to be investigated by establishing a xenograft model. Second, the potential correlation between DDX46 and prognosis of GBM patients needs to be clarified by collecting more clinical samples.

## Conclusions

DDX46 is upregulated in GBM, which strengthens the proliferative capacity of GBM by activating the MAPK-p38 signaling.

## Conflict of interests

The authors declare no conflict of interests.

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