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Aberrantly high *DEPDC1B* expression leads to poor prognosis in patients with lower-grade gliomas

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Abstract. – OBJECTIVE: ***DEPDC1B*****, which encodes DEP domain-containing protein 1B, exerts pathogenic effects in diverse cancers, but no such effect has been reported in the case of lower-grade glioma (LGG). Therefore, we sought to investigate the relationship between *DEPDC1B* expression and the prognosis of patients with LGG and reveal the underlying molecular mechanism.**

MATERIALS AND METHODS: First, RT-qPCR and immunohistochemical staining were used to examine *DEPDC1B* mRNA and protein expression in LGG. Second, transcriptomic data were collected from The Cancer Genome Atlas and Chinese Glioma Genome Atlas databases to investigate the impact of *DEPDC1B* expression on LGG patients by using the Kaplan-Meier survival analysis, receiver operating characteristic analysis and Cox models. Third, the effects of *DEPDC1B* on LGG cell proliferation and migration were revealed using wound-healing and Cell Counting Kit-8 assays and Ki67 immunofluorescence staining. Fourth, the Tumor Immune Estimation Resource database was used to examine how *DEPDC1B* affects the LGG immune microenvironment, and gene set enrichment analysis was used to uncover the signaling pathways in which *DEPDC1B* is involved in LGG.

RESULTS: *DEPDC1B* was significantly upregulated in both LGG cells and tissues, and high expression of *DEPDC1B* contributed to poor prognosis of LGG patients and represented an independent risk factor for LGG. Moreover, *DEPDC1B* knockdown reduced the proliferation and migration abilities of LGG cells. Lastly, *DEPDC1B* was found to be positively associated with multiple immune infiltrates and immune-checkpoint markers.

CONCLUSIONS: Our findings indicate for the first time that *DEPDC1B* is a pathogenic gene in LGG. More importantly, we provide a new biomarker and immunotherapeutic target for improving the diagnosis and treatment of LGG patients.

Key Words:
DEPDC1B, Lower-grade glioma, Pathogenic gene, Biomarker.

Abbreviations

CCK-8: Cell Counting Kit-8; CGGA: Chinese Glioma Genome Atlas; 95%-CI: 95% confidence interval; DEPDC1B: DEP domain-containing protein 1B; GBM: glioblastomas; GEO: Gene Expression Omnibus; GSEA: gene set enrichment analysis; HPA: Human Protein Atlas; HR: hazard ratio; IHC staining: immunohistochemical staining; KEGG: Kyoto Encyclopedia of Genes and Genomes; LGG: lower-grade glioma; OS: overall survival; ROC: receiver operating characteristic; TCGA: The Cancer Genome Atlas; TIMER: Tumor Immune Estimation Resource; WHO: World Health Organization.

Introduction

Gliomas, which are derived from neural precursor cells, are the most common intracranial primary malignant tumors and include low-

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er-grade gliomas (LGGs, WHO Grades I-III) and glioblastomas (GBMs, WHO Grade IV), according to the World Health Organization (WHO) classification system[1,2]. LGG is an indolent tumor that exhibits low heterogeneity and aggressiveness, but even after comprehensive treatment, such as neurosurgical resection combined with adjuvant radiotherapy and chemotherapy, high recurrence and disability rates remain high in LGG[3]. Furthermore, recurrent LGG progresses, in most of the cases, to the most malignant GBM, producing a heavy economic burden on families and society[4]. Therefore, the identification of a highly specific and sensitive biomarker for LGG holds major implications for improving the quality of life of patients with gliomas.

The fundamental characteristics of malignant tumors are unlimited proliferation, angiogenesis, activation, invasion, and metastasis; these characteristics represent a major obstacle to cancer treatment, and among these, the most basic feature of cancer cells is their continuous and long-term proliferation ability. Therefore, DEP domain-containing protein 1B (encoded by *DEPDC1B*) as a key protein that participates in cell cycle progression and regulates mitotic dynamics to accelerate cell growth[5], has been extensively investigated for its tumor-promoting character. For example, *DEPDC1B* is reported to be highly expressed in pancreatic cancer and to promote the migration and invasion abilities of pancreatic cancer cells, which lead to a significant shortening of the disease-free survival time of patients[6]. Moreover, *DEPDC1B* expression can enhance epithelial-to-mesenchymal transition and thereby accelerate the distant metastasis of prostate cancer cells, which significantly reduces the post-treatment survival rate of patients[7]. Similar results were also obtained in a study[8] on malignant melanoma, where *DEPDC1B* knockdown was accompanied with increased expression of proapoptotic proteins and reduced expression of antiapoptotic proteins in malignant melanoma cells. Most importantly, *DEPDC1B* has been found to play a pathogenic role in GBM[9]. These findings raise the question of what role *DEPDC1B* plays in LGGs exhibiting lower malignancy and a more stable genome than GBMs. However, to our knowledge, few studies have investigated the function of *DEPDC1B* in LGG.

In this study, we discovered, using big-data analyses, that *DEPDC1B* expression was correlated with the clinical characteristics and prognosis of LGG patients. More importantly, we found that *DEPDC1B* expression was markably associated with immune-cell infiltration and immune-checkpoint markers in LGG. Therefore, our study provides not only a new direction for elucidating the complex malignant progression of LGG, but also a novel biomarker for predicting LGG prognosis – a biomarker that could serve as a potential target for the development of targeted therapy and immune therapy for LGG patients.

Materials and Methods

Data Collection

The Cancer Genome Atlas (TCGA, available at: https://portal.gdc.cancer.gov/), a public database established by the National Cancer Institute and the National Human Genome Research Institute for tumor research, contains transcriptome data, DNA methylation data, and genomics data[10]. After excluding samples for which incomplete clinical information was available, we collected the data on mRNA expression in brain tissue and the corresponding clinical information of 503 patients with LGG from the TCGA RNA-seq database to investigate the expression and prognostic value of *DEPDC1B* in LGG; the detailed clinical information is displayed in **Supplementary Table I**.

Chinese Glioma Genome Atlas (CGGA, available at: https://www.cgga.org.cn/), a public database containing 2,000 samples of distinct types of gliomas, is also a key tool for glioma research[11]. After excluding GBM samples and samples recorded with incomplete clinical information, we collected CGGA RNA-seq data on 403 LGG cases and CGGA mRNA-array data on 142 LGG cases to verify the results obtained using the TCGA database; the detailed clinical information is shown in **Supplementary Tables II** and **III**.

To compare *DEPDC1B* protein expression levels in normal brain tissues and LGG brain tissues, we collected immunohistochemical (IHC) data recorded in the Human Protein Atlas (HPA, available at: https://www.proteinatlas.org/) database. Lastly, to perform a meta-analysis by using data from the Gene Expression Omnibus (GEO, available at: https://www.ncbi.nlm.nih.gov/) database[12], we selected these two datasets: GSE43378 (18 LGG patients) and GSE50025 (34 LGG patients).

Six LGG brain tissues and five para-tumor tissues of LGG were obtained from the neurosurgery department of Henan Provincial People's

Hospital. All brain tissues were obtained from the operating room and placed in liquid nitrogen. The study was approved by the Ethics Committee of Henan Provincial People's Hospital (2020107), and all selected patients signed informed consent.

Cell Culture and Transfection

The human LGG cell line SHG44 was purchased from Qingqi Cell Bank (Shanghai, China) and tested for mycoplasma contamination. SHG44 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Procell, Wuhan, Hubei Province, China) containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin mixture (Procell, Wuhan, Hubei Province, China) in an incubator with 5% CO_2 at 37°C. After seeding SHG44 cells into 6-well plates, we transiently transfected the cells with a DEPDC1B-targeting siRNA (siDEPDC1B) or control siRNA (siNC) and used the cells in the subsequent experiments. First, we added 3.75 µL of Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) and 7.5 µL of 20 µmol/L siRNA (GenePharma, Shanghai, China) into two 1.5 mL tubes containing 125 µL of Opti-MEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). After mixing the solution in the two tubes and allowing the mixture to stand for 15 min, we added the Lipofectamine 3000 plus siRNA mixture to 2 mL of serum-free DMEM and used this to culture SHG44 cells. Lastly, after 6 h of culture, the transfection medium was replaced with complete medium. The siRNA sequences were the following: siNC: sense, 5′-UUCUCCGAACGUGUCACGUTT-3′, and antisense, 5′-ACGUGACACGUUCGGAGAATT-3′; siDEPDC1B: sense, 5′-GCCUCUACUUACAUUUCAUTT-3′, and antisense, 5′-AUGAAAUGUAGAGGCTT-3′.

Extraction of Total RNA and RT-qRCR

Total RNA from each brain tissue sample was obtained by using a Total RNA Kit I (R6834-02; Omega Bio-tek Inc., Norcross, GA, USA), according to the reagent manufacturer's instructions. After measuring the RNA concentration by using a NanoDrop One instrument (Thermo Fisher Scientific, Waltham, MA, USA), cDNA was synthesized using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions for NovoScript Plus All-in-one 1st Strand cDNA Synthesis SuperMix (Novoprotein, Shanghai, China). RT-qPCR was performed on the obtained cDNA by using NovoStart® SYBR qPCR SuperMix Plus (Novoprotein, Shanghai, China), as per the manufacturer's instructions, on a StepOne Plus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). After *DEPDC1B* expression was standardized against that of the internal-reference gene *18S*, the -ΔCT value was used to compare the mRNA expression of *DEPDC1B* in LGG brain tissue and para-tumor tissues of LGG. The following specific primer sequences were used: *18S* – forward, 5′-GTAACCCGTTGAACCCCATT-3′, and reverse, 5′-CCATCCAATCGGTAGTAGCG-3′; *DEPDC1B* – forward, 5′-AGCTACCAGGCTGTGGAATG-3′, and reverse, 5′-AGCTCTTGAAACGACAGCGA-3′.

IHC Staining

IHC staining was performed according to standard procedures. First, the collected brain tissues were sectioned, and the sections were placed in xylene for dewaxing and then in gradient absolute ethanol for hydration. Subsequently, after boiling at high temperature with an EDTA antigen-repair solution (ZSGB-BIO, Beijing, China) and blocking endogenous peroxidase, nonspecific antigens were blocked using a 10% BSA solution. Next, a primary antibody against *DEPDC1B* (1:500; Bioss, Woburn, MA, USA) was added to the sections and incubated at 4°C overnight, and, on the next day, *DEPDC1B* protein was stained brown after reaction enhancement and incubation with an appropriate secondary antibody, according to the manufacturer's instructions for Mouse/Rabbit enhanced polymer detection system (ZSGB-BIO, Beijing, China). Lastly, after the nuclei were dyed blue with hematoxylin, the sections were dried, dehydrated, and sealed to complete the IHC staining, and then the protein expression of *DEPDC1B* in LGG and normal brain tissue was examined and photographed under the 200× lens of a microscope. The IHC staining results were analyzed using Image-Pro Plus software (version 6.0, Meyer Instruments Inc., Houston, TX, USA).

Wound-Healing Assay

SHG44 cells transfected with the siRNAs (siDEPDC1B and siNC) were used as the experimental and control groups, respectively; when the two groups of cells were full confluent in 6-well plate, a 200 µL sterile pipette tip was used to scratch the cell monolayer in each well of the plates. After removing necrotic cells by washing with 1× phosphate-buffered saline, the cells in the 6-well plates were incubated with serum-free

medium for 0, 24, and 48 h at 37°C and photographed in parallel under the 100× lens of a microscope. Lastly, the relative migration distance of the siDEPDC1B group and siNC group was calculated to compare the effect of *DEPDC1B* knockdown on the migration ability of the LGG cell line SHG44.

Cell Counting Kit-8 (CCK-8) Assay

The transfected SHG44 cells were seeded into five 96-well plates at 1,000 cells/well in 100 μL of complete medium. After the cells adhered to the wells, the supernatant was removed, and a mixture of 100 μL of complete medium and 10 μL of CCK-8 reagent (Dojindo Laboratories, Mashiki, Kumamoto, Japan) was added to each well and the plates were placed at 37°C; subsequently, the optical density at 450 nm was measured after 4 h for one of the plates, and the remaining four 96-well plates were incubated for 24, 48, 72, and 96 h, and the respective optical density values at 450 nm were measured in parallel. The effect of *DEPDC1B* knockdown on the proliferation of SHG44 cells was analyzed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA).

Ki67 Immunofluorescence Staining

Ki67 immunofluorescence staining, a crucial index used to evaluate the proliferation ability of cells, was performed on the siDEPDC1B and siNC groups of SHG44 cells. After removing the supernatant from the culture dishes of cells in the logarithmic growth stage, 4% paraformaldehyde was used to fix the cells and 0.5% Triton X-100 was used to permeabilize the cell membrane. Subsequently, nonspecific antigens were blocked using a 5% BSA solution and the cells in the culture dishes were incubated overnight at 4°C with 1:200 Ki67 primary antibody (Abcam, Shanghai, China). On the next day, after incubating the cells with a fluorescein-conjugated secondary antibody for 1 h and staining nuclei with DAPI for 10 min at room temperature, Ki67 protein expression in the siDEPDC1B and siNC groups was examined and photographed under the 200× lens of a fluorescence microscope. The results of Ki67 immunofluorescence staining were analyzed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA).

Meta-Analysis

In this study, we performed a meta-analysis to increase the credibility of *DEPDC1B* as a risk factor for the overall survival (OS) of LGG patients. First, we found few published studies on the relationship between *DEPDC1B* and OS of LGG patients by searching PubMed, Web of Science, Cochrane Library databases, and Embase. Therefore, for further research, we performed the meta-analysis, using R software (version 4.0.3, R Foundation, Vienna, Austria), on 1,100 samples collected from the TCGA, CGGA, and GEO databases (503 LGG patients with TCGA RNA-seq data, 403 LGG patients with CGGA RNA-seq data, 142 LGG patients with CGGA mRNA-array data, 18 LGG patients with GSE43378 data, and 34 LGG patients with GSE50025 data). Subsequently, we tested for the heterogeneity of the five databases by using the Q test and I-squared (I^2) statistics. In these tests, $I^2 > 50\%$ and $p < 0.05$ indicated that the data were heterogeneous, and the random-effect model was selected; conversely, when $I^2 < 50\%$, the fixed-effect model was selected. Lastly, the joint hazard ratio (HR) and 95% confidence interval (95% CI) were calculated using R software (version 4.0.3; R Foundation, Vienna, Austria) to evaluate the correlation between *DEPDC1B* expression and the prognosis of LGG patients.

Immune-Infiltration Analysis

The Tumor Immune Estimation Resource (TIMER, available at: https://cistrome.shinyapps.io/timer/) database is an international public data analysis platform for detecting immune-cell infiltration in tumor tissue through TCGA RNA-seq expression profiling[13]. Therefore, we used three modules of the TIMER database to perform a correlation analysis between *DEPDC1B* expression and immune-cell infiltration in LGG tissue. First, we used the "Gene" module to visualize the correlation between *DEPDC1B* expression and six types of immune cells (B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells) in the LGG immune microenvironment. Second, we used the "Survival" module to predict the effects of the six immune-cell infiltration levels and *DEPDC1B* expression on the OS of LGG patients. Third, we used the "correlation" module to draw the relationship between *DEPDC1B* and well-known immune-checkpoint markers such as *CTLA4*, *PDCD1*, *CD274* and *PDCD1LG2* in LGG samples.

Gene Set Enrichment Analysis (GSEA)

GSEA includes various datasets to indirectly predict the function of target genes[14]. The Kyoto Encyclopedia of Genes and Genomes (KEGG),

the most commonly used gene set, is used to reveal the cell signaling pathways in which genes might participate in[15]. We divided the *DEPDC1B* mRNA expression levels of LGG samples from the TCGA RNA-seq database into low-expression and high-expression groups according to the median value, and then we performed KEGG enrichment analysis in GSEA software (version 4.0.3; Broad Institute, Inc., Cambridge, MA, USA) to predict the cell signaling pathways that *DEPDC1B* might regulate in the pathological process of LGG. After 1,000 gene permutations, a nominal (NOM) p-value < 0.05 and a false-discovery rate (FDR) Q-value < 0.25 were used the criteria for the inclusion of cell signaling pathways.

Statistical Analysis

All experiments were repeated at least thrice and compared using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA), where unpaired *t*-tests and multiple unpaired *t*-tests were used for analyzing the results. In the study, the data from the TCGA and CGGA databases were statistically analyzed using R software (version 4.0.3, R Foundation, Vienna, Austria). First, the Chi-squared test was used to investigate the correlation between *DEPDC1B* mRNA expression and the clinical characteristics of patients with LGG. Second, the Kaplan-Meier curve was used to examine the relationship between *DEPDC1B* expression and the OS of LGG patients. Moreover, univariate and multivariate analyses were used to evaluate whether *DEPDC1B* was an independent risk factor for LGG. Third, the receiver operating characteristic (ROC) curve was used to verify the diagnostic value of *DEPDC1B* expression for LGG. Lastly, the Pearson's correlation coefficient was used to obtain genes positively and negatively correlated with *DEPDC1B* in order to reveal the gene function through this relationship. All results were expressed as means ± SD, and $p < 0.05$ was considered statistically significant.

Results

Highly Expressed DEPDC1B in LGG Patients

A literature review revealed that *DEPDC1B* expression is markedly increased in the transcriptome disorders of various tumor pathological processes[7,16]; however, as neurosurgeons, we found few reports on the disruption of *DEPDC1B* mRNA expression in LGG. Therefore, to ascertain whether *DEPDC1B* expression is disordered in LGG patients, we performed RT-qPCR and IHC staining to reveal the expression at, respectively, the mRNA and protein levels: *DEPDC1B* mRNA expression in brain tissues from LGG patients was significantly higher than that in normal brain tissues (Figure 1A), and *DEPDC1B* protein was highly expressed in LGG patients' tissues but expressed at low levels in normal tissues (Figure 1B-C). Moreover, the IHC results collected from the HPA database agreed with the IHC results that we obtained (**Supplementary Figure**

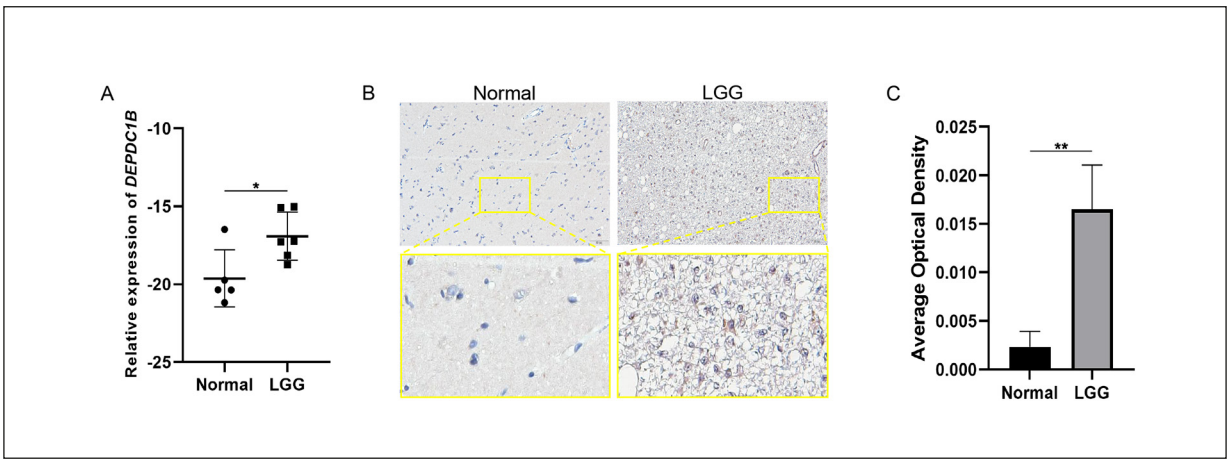


Figure 1. In LGG, *DEPDC1B* was highly expressed at mRNA and protein levels. **A**, The result of RT-qPCR of *DEPDC1B* in 5 normal brain tissues and 6 LGG tissues. **B**, Immunohistochemical staining photographs of DEPDC1B in normal brain tissue and LGG tissue under 200× microscope. **C**, Statistical diagram of immunohistochemical results in 3 normal brain tissues and 3 LGG tissues. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001. p<0.05 was considered statistically significant.

S1A-B). Thus, we found that *DEPDC1B* expression was aberrantly elevated in the complex malignant progression of LGG, and we sought to further study the role of DEPDC1B in LGG.

Correlation Between DEPDC1B Expression and Clinical Characteristics

The high correlation between LGG pathogenic genes and clinical characteristics led us to investigate the relationship between *DEPDC1B* expression and the clinical features of LGG patients by using the TCGA and CGGA databases. First, *DEPDC1B* expression was higher in the older patients than in younger patients, based on the median age of the LGG patients in the TCGA RNA-seq database (Figure 2A). Second, *DEPDC1B* expression in LGG of WHO grade III was significantly higher than that in LGG of WHO grade II, as per the results of our analyses of TCGA RNA-seq, CGGA RNA-seq, and CGGA mRNA-array databases (Figure 2B and **Supplementary Figure S2A-B**). Third, according to the WHO histological classification of gliomas, *DEPDC1B* expression was the highest in anaplastic astrocytomas (AA) and the lowest in astrocytomas (A) in the TCGA RNA-seq database (Figure 2C). Fourth, *DEPDC1B* expression was significantly higher in LGG patients in the recurrence group, radiotherapy group, and chemotherapy group than in LGG patients in the primary group, non-radiotherapy group, and no-chemotherapy group, based on the data in the TCGA and CGGA databases (Figure 2D-F and **Supplementary Figure S2C-E**). Fifth, *DEPDC1B* expression was higher in LGG patients with no 1p19q codeletion than in patients with 1p19q codeletion, according to the CGGA RNA-seq database (**Supplementary Figure S2F**). Thus, high expression of *DEPDC1B* was found to be positively correlated with the risk factors for LGG, and we therefore hypothesized that high *DEPDC1B* expression might affect the prognosis of LGG patients.

High Expression of DEPDC1B Leads to Poor Prognosis of LGG Patients

We first used Kaplan-Meier analysis to verify a potential relationship between *DEPDC1B* expression and the OS (and thus the prognosis) of LGG patients by using the TCGA RNA-seq, CGGA RNA-seq, and CGGA mRNA-array databases. In all the three these databases, the OS of LGG patients with high *DEPDC1B* expression was shorter than the OS of patients with low *DEPDC1B* expression, which revealed that high

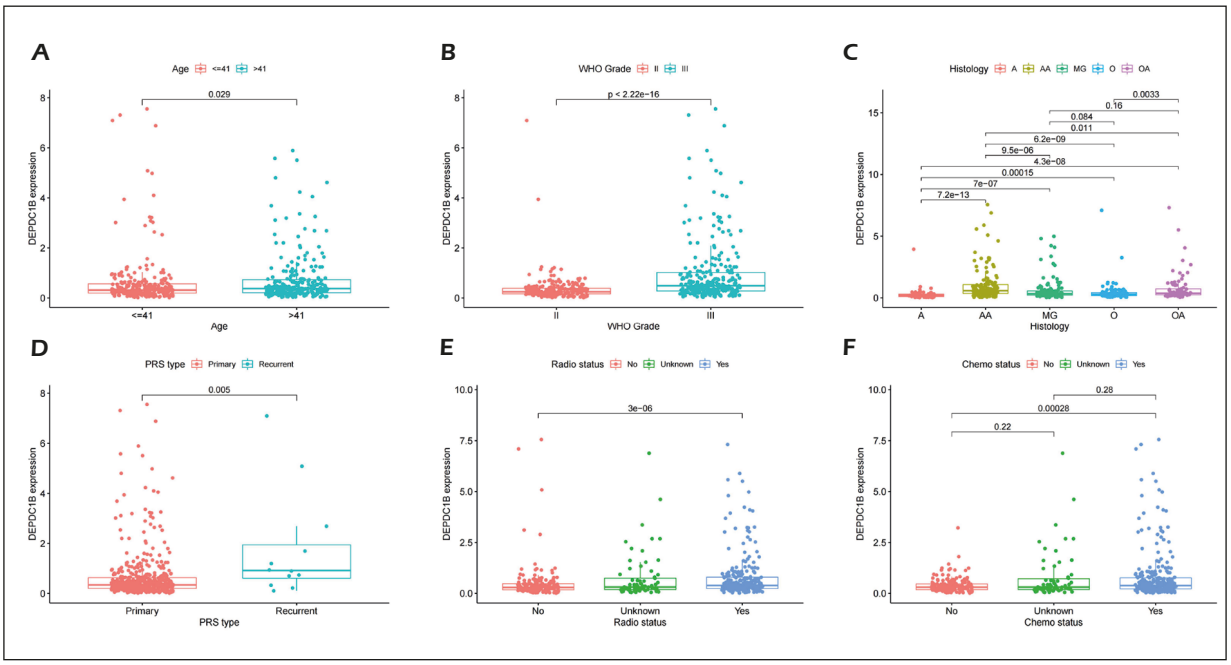


Figure 2. Correlation between *DEPDC1B* and clinical characteristics of LGG patients based on TCGA RNA-seq database: age (**A**), WHO grade (**B**), histological type (**C**), PRS type (**D**), radio status (**E**), chemo status (**F**). $p<0.05$ was considered statistically significant.

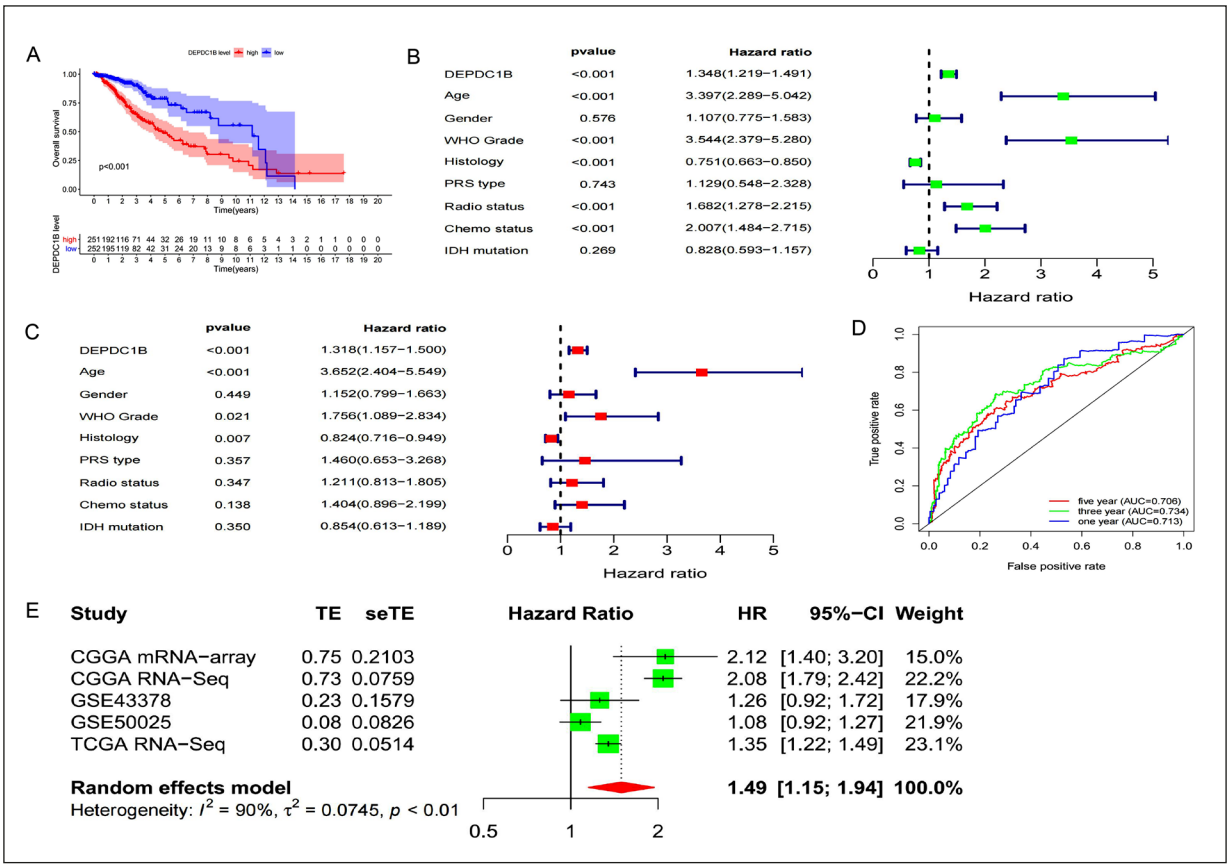


Figure 3. Correlation between *DEPDC1B* and prognosis of LGG patients based on TCGA RNA-seq database. **A**, Kaplan-Meier survival curve showed that LGG patients with high *DEPDC1B* expression had a short overall survival time. **B**, Univariate analysis showed that *DEPDC1B* was a risk factor for LGG. **C**, Multivariate analysis showed that *DEPDC1B* was an independent risk factor for LGG. **D**, ROC curves showed that *DEPDC1B* had good diagnostic value in 1, 3, 5 years. **E**, Meta-analysis between *DEPDC1B* expression of LGG and overall survival time. $p<0.05$ was considered statistically significant.

expression of *DEPDC1B* leads to poor prognosis in LGG (Figure 3A and **Supplementary Figure S3A-B**). Next, the results of univariate and multivariate analyses performed using the three aforementioned databases yielded an HR value >1 ($p < 0.05$) for *DEPDC1B* expression in LGG, which suggested that *DEPDC1B* expression is an independent risk factor for LGG (Figure 3B-C and **Supplementary Figure S3C-F**). Moreover, we generated a ROC curve to investigate the diagnostic value of *DEPDC1B* expression in LGG survival rate. The area under the curve value was higher than 0.7 in the 1-year, 3-year, and 5-year survival rates of LGG patients, based on the data in all three databases (Figure 3D and **Supplementary Figure S3G-H**); this result showed that *DEPDC1B* expression is a reliable diagnostic index for LGG. Lastly, the joint HR obtained in our meta-analysis was 1.49 (95% CI: 1.15-1.94), which also showed that *DEPDC1B* expression is a risk factor for LGG (Figure 3E). These results indicated that *DEPDC1B* is a pathogenic gene in patients with LGG, and we thus concluded that it is critical to examine how *DEPDC1B* plays a pathogenic role in LGG.

High DEPDC1B Expression Increases the Malignant Behavior of LGG Cells

LGG cells constitute the main tumor component affecting the pathological process of LGG, and the migration and proliferation abilities of LGG cells are closely related to the prognosis of LGG patients. Therefore, we used standard experiments to reveal the effect of *DEPDC1B* on the malignant biological behavior of LGG cells. First, the wound-healing assay revealed that relative to control SHG44 cells, *DEPDC1B*-knockdown cells moved a shorter distance by 24 and 48 h (Figure 4A); this showed that high *DEPDC1B* expression can increase the

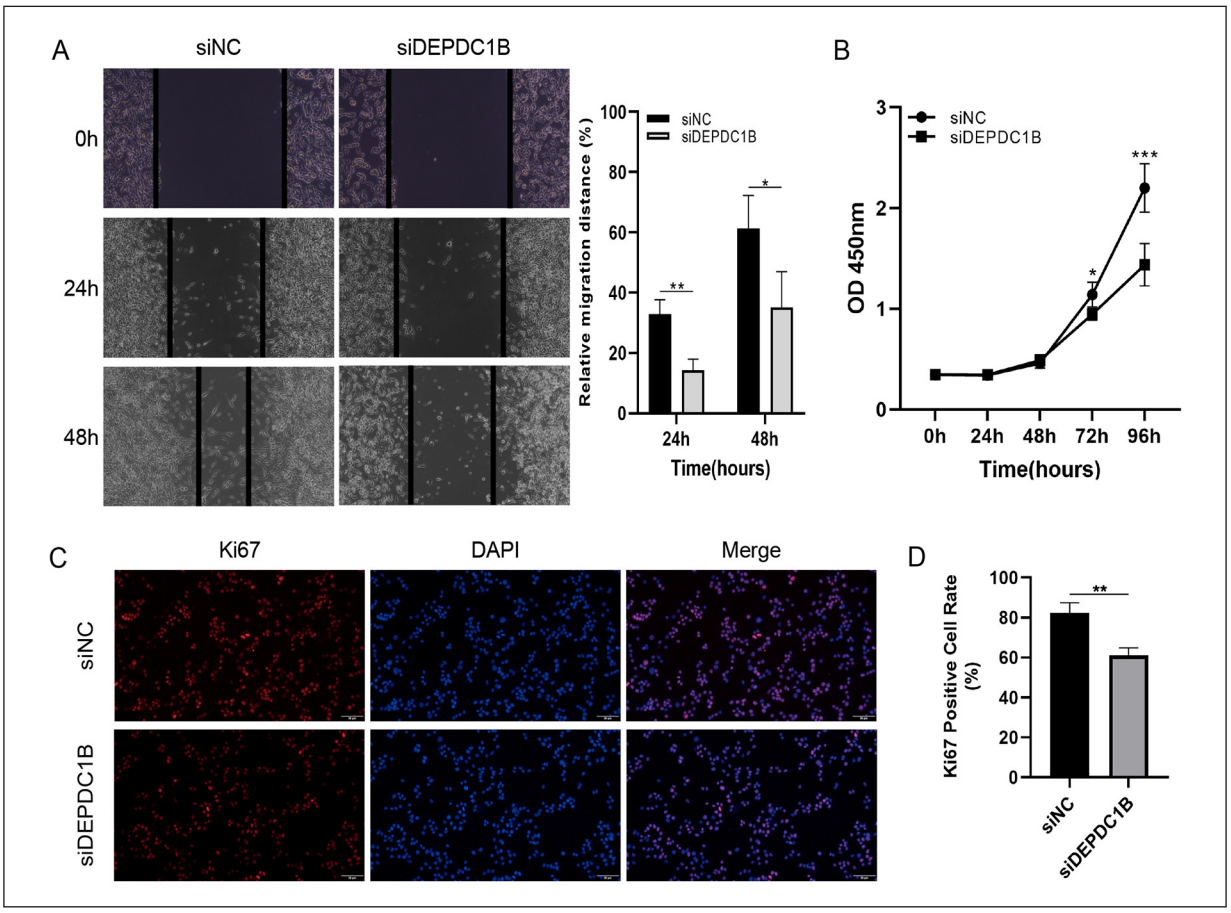


Figure 4. *DEPDC1B* affected the malignant biological behavior of LGG cells. **A**, Wound healing assay showed that knockdown of DEPDC1B reduced the migration ability of LGG cells, and these photographs were taken under 100× microscope. **B**, CCK8 assay showed that knockdown of *DEPDC1B* decreased the proliferation ability of LGG cells. **C**, Photograph of Ki67 immunofluorescence staining under 200× microscope. **D**, The result of Ki67 immunofluorescence staining. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. $p<0.05$ was considered statistically significant.

migration ability of LGG cells. Next, in the CCK-8 assay, high *DEPDC1B* expression was found to significantly enhance the proliferation ability of LGG cells (Figure 4B). Lastly, the results of the Ki67 immunofluorescence assay on SHG44 cells showed that high expression of *DEPDC1B* also significantly increased the proliferation ability of LGG cells (Figure 4C-D). Collectively, these results showed for the first time that high *DEPDC1B* expression enhances the proliferation and migration abilities of LGG cells and thereby leads to the poor prognosis of LGG patients.

Relationship Between DEPDC1B Expression and Immune Infiltration in LGG

Tumor-infiltrating immune cells are widely recognized to critically affect cancer-treatment outcome and patient prognosis[17]. Therefore, we examined the correlation between *DEPDC1B* expression and six types of immune cells (B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells) in the LGG tumor microenvironment by using the TIMER database. First, Spearman's correlation analysis revealed that *DEPDC1B* expression increased together with the infiltration level of the six types of immune cells in LGG tissues ($p < 0.05$) (Figure 5A). Second, Kaplan-Meier analysis revealed a shortened OS time in the case of LGG patients with a high level of immune-cell infiltration and high *DEPDC1B* expression (Figure 5B). Lastly, the results of multivariate analysis showed that macrophages (HR = 1,681.809; 95% CI: 25.156-112,435.624; $p < 0.05$) and *DEPDC1B* (HR = 1.683; 95% CI: 1.368-2.070; $p < 0.05$) were independent prognostic indicators for LGG pa-

tients (**Supplementary Table IV**). Because immune checkpoints are currently a major focus of antitumor therapy, we further performed a correlation analysis between *DEPDC1B* expression and four widely recognized immune-checkpoint markers (CTLA4, PD-1, PD-L1, and PD-L2), which revealed a positive correlation between *DEPDC1B* and all four markers (Figure 5C-F). These results led us to hypothesize that *DEPDC1B* can affect the prognosis of LGG patients by regulating immune-cell infiltration and can be used as a potential immunotherapeutic target.

DEPDC1B-Related Cell Signaling Pathways and Co-Expressed Genes in LGG Pathological Process

Our study thus far revealed that *DEPDC1B* affects the prognosis of LGG patients. This raised the question of how *DEPDC1B*, as a pathogenic gene, participates in regulating and affecting the pathological process of LGG. Therefore, we performed GSEA to reveal the cell signaling pathways in which *DEPDC1B* might participate. By using the RNA-seq database of TCGA, we screened out four signaling pathways – those associated with the cell cycle, DNA replication, neurotrophin, and Notch – as the pathways that high *DEPDC1B* expression might activate in LGG (Figure 6A-D and **Supplementary Table V**). These results suggested that high *DEPDC1B* expression leads to the poor prognosis of LGG patients by activating the aforementioned cell signaling pathways in the malignant progression of LGG. The results further suggested that the activation of these four pathways might represent the molecular mechanism through which high expression of *DEPDC1B* enhances the proliferation and migration abilities of LGG cells.

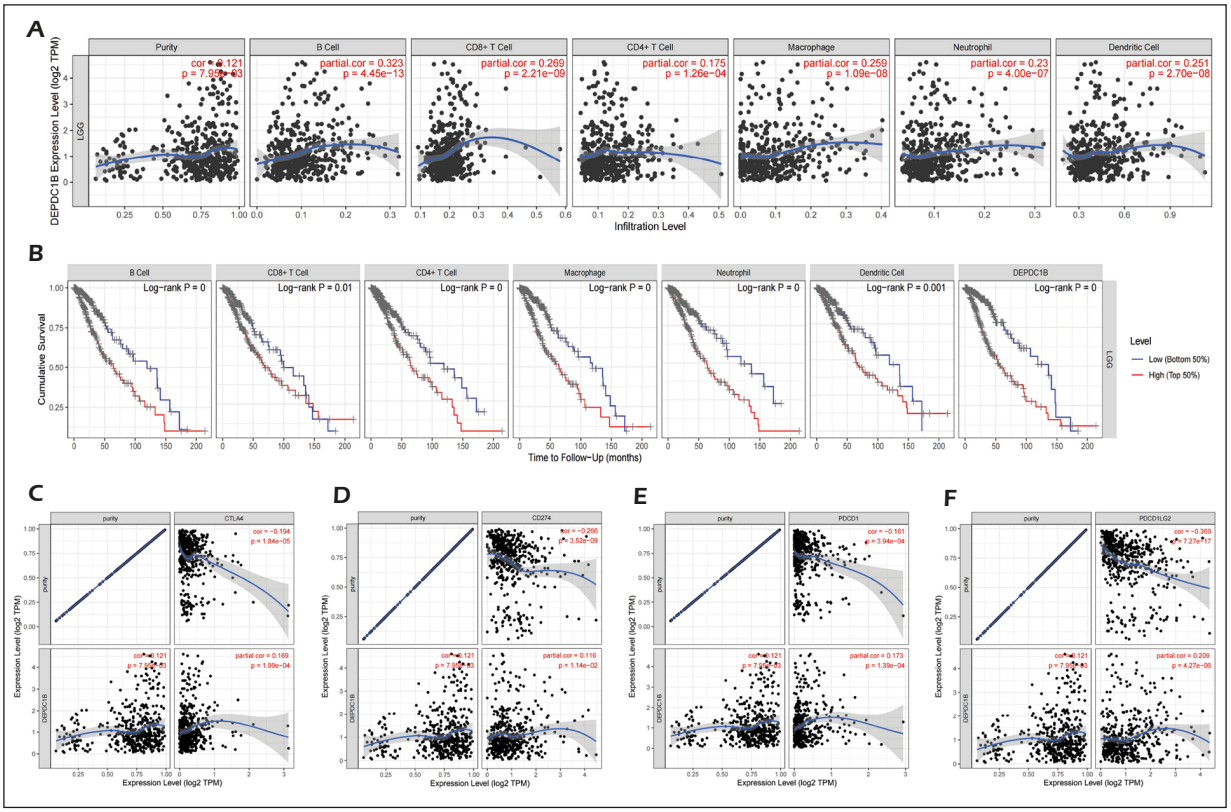


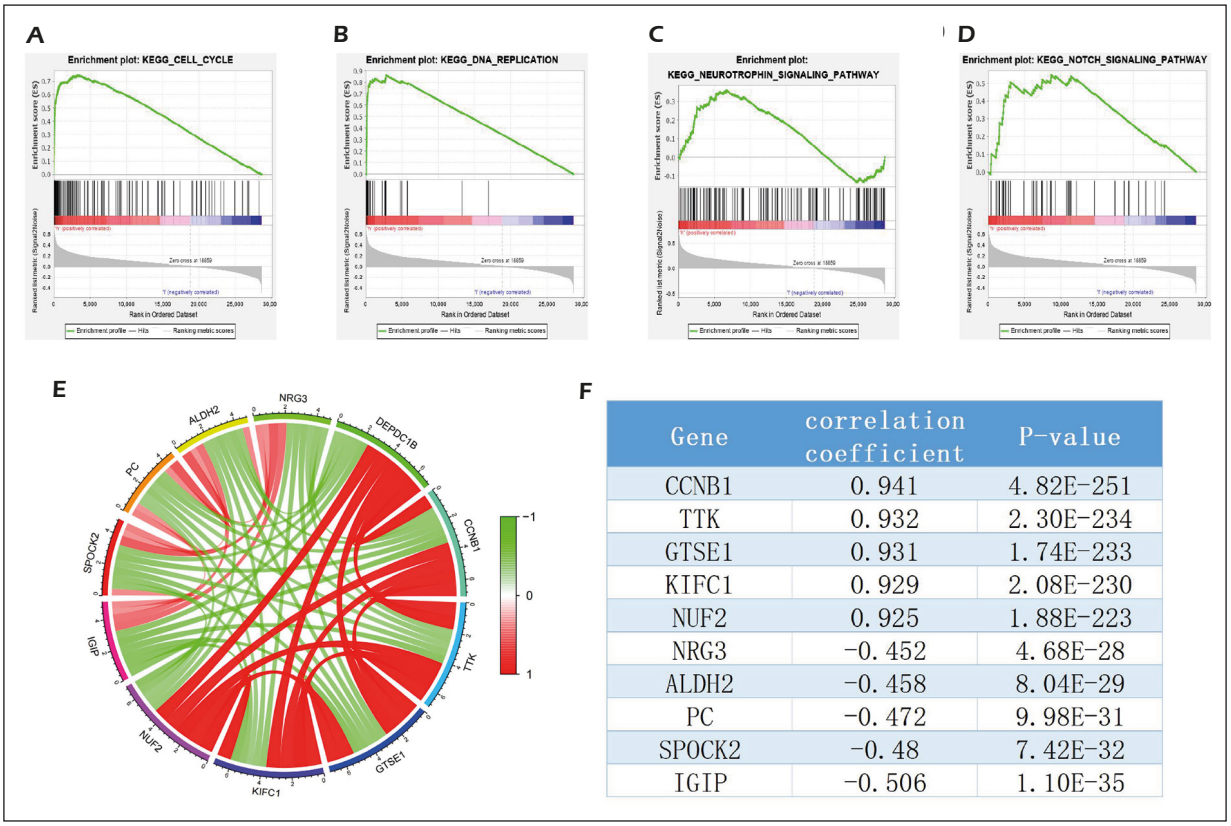
Figure 5. Correlation between *DEPDC1B* of LGG and immune cell infiltration based on TIMER database. **A**, *DEPDC1B* expression was positively correlated with the immune infiltration level of 6 immune cells (B cells, CD8+T cells, CD4+T cells, macrophages, neutrophils, and dendritic cells). **B**, High immune infiltration level or *DEPDC1B* led to poor prognosis of LGG patients. **C**, *DEPDC1B* and CTLA4 were positively correlated. **D**, *DEPDC1B* and CD274 (PD-L1) was positively correlated. **E**, *DEPDC1B* and PDCD1 (PD-1) were positively correlated. **F**, *DEPDC1B* and PDCD1LG2 (PD-L2) were positively correlated. $p<0.05$ was considered statistically significant.

Genes that are co-expressed in LGG formation are expected to both play crucial synergistic or antagonistic roles[18]. Therefore, we used Pearson's correlation analysis to screen for genes related to *DEPDC1B* in LGG in the TCGA database, which yielded thousands of genes with correlation coefficients of more than 0.4 or less than -0.4. Ultimately, we selected the five most positive genes (*CCNB1*, *TTK*, *GTSE1*, *KIFC1*, and *NUF2*) and five most negative genes (*NRG3*, *ALDH2*, *PC*, *SPOCK2*, and *IGIP*) and used these to generate a Circos diagram (Figure 6E-F).

Discussion

Recently, a role of *DEPDC1B* in malignant tumors has been increasingly reported, with *DEPDC1B* being shown to act as a pathogenic gene when expressed at aberrantly high levels in various cancers such as bladder cancer and pancreatic ductal adenocarcinoma[16,18,19]. Moreover, *DEPDC1B* expression was found to be increased in non-small cell lung cancer, and this elevated expression was positively correlated with the migration ability of lung cancer cells and negatively correlated with patient survival time[20]. Similar results were also obtained in the case of prostate cancer, where the expression of *DEPDC1B*, as an oncogene, was negatively correlated with recurrence-free survival of patients[21]. However, the relationship between *DEPDC1B* and the pathogenic process of LGG has remained unclear. To the best of our knowledge, this is the first study to describe the role of *DEPDC1B* in LGG.

In this study, we demonstrated for the first time that *DEPDC1B* expression was higher in LGG tissue than in normal brain tissue, and further that elevated *DEPDC1B* expression was positively correlated with the malignant characteristics of LGG cells and led to poor prognosis of patients with LGG. First, RT-qPCR and IHC staining results showed that *DEPDC1B* expression was



F

Gene	correlation coefficient	P-value
CCNB1	0.941	4.82E-251
TTK	0.932	2.30E-234
GTSE1	0.931	1.74E-233
KIFC1	0.929	2.08E-230
NUF2	0.925	1.88E-223
NRG3	-0.452	4.68E-28
ALDH2	-0.458	8.04E-29
PC	-0.472	9.98E-31
SPOCK2	-0.48	7.42E-32
IGIP	-0.506	1.10E-35

Figure 6. GSEA enrichment analysis and co-expression analysis in TCGA RNA-seq database. The results of GSEA enrichment analysis of *DEPDC1B*: Cell cycle (**A**), DNA replication (**B**), Neurotrophin signaling pathway (**C**), Notch signaling pathway (**D**), Diagram of co-expression analysis (**E**), Correlation coefficients and *p*-value of the 5 most positively and negatively correlated genes with DEPDC1B in LGG (**F**).

increased in both the transcriptome and the proteome of LGG tissue. Next, the results of univariate and multivariate logistic regression analyses confirmed that *DEPDC1B* expression represents an independent risk factor for LGG. Furthermore, to ensure scientific preciseness, we performed a meta-analysis and determined that *DEPDC1B* expression serves as a risk factor in relation to the prognosis of LGG patients. *DEPDC1B* has also been widely reported[22-24] previously to function as an oncogene in several cancers, such as hepatocellular carcinoma, sarcoma, and oral cancer. Lastly, we found that *DEPDC1B* knockdown significantly reduced the migration and proliferation abilities of LGG cells. Considering these findings, we propose that high expression of *DEPDC1B* leads to the poor prognosis of LGG patients by increasing the malignant biological behavior of LGG cells, and that *DEPDC1B* is a highly promising candidate biomarker for use as a therapeutic target in LGG.

Immune therapy has emerged as a clinically effective cancer treatment based on the understanding of the immunosuppressive microenvironment of gliomas[25]. One aspect of this field is the infiltration of immune cells, which has been demonstrated[26] to serve as a predictor of the OS and therapy response in patients with glioma. Thus, we examined the relationship between *DEPDC1B* expression and the level of immune-cell infiltration in LGG: *DEPDC1B* expression in LGG showed a markedly positive association with the infiltration level of six widely recognized types of immune cells, while also concurrently being negatively correlated with the OS time of LGG patients. These findings clearly indicated that high expression of *DEPDC1B* could promote the formation of a suppressive immune microenvironment in LGG and then promote the malignant progression of LGG.

Immune-checkpoint markers are widely reported[27] to play a critical role in glioma development and lead to immune escape. Unexpectedly, we found a highly positive correlation between *DEPDC1B* expression and four well-known immune-checkpoint markers (CTLA4, PD-1, PD-L1, and PD-L2) in LGG, which suggests that *DEPDC1B* could represent a sensitive immunotherapeutic target. Thus, our study not only indirectly suggests that *DEPDC1B* could regulate the tumor immune microenvironment and thereby play a pathogenic role, but also identifies a potential immunotherapeutic target for improving the prognosis of LGG patients.

To further investigate the potential mechanism of action of *DEPDC1B* in LGG, we performed GSEA, which revealed that the main enrichment was in cell cycle, DNA replication, neurotrophin, and Notch cell signaling pathways; this bolsters the role of *DEPDC1B* in protumor progression. The cell cycle signaling pathway exerts a critical effect on cancer formation, with abnormal activation of the pathway potently increasing the proliferation characteristics of cancers[28]. Therefore, molecular drugs targeting the cell cycle could be used in the clinical treatment of LGG. For example, *miR-188* and moxidectin can regulate glioma proliferation by acting through the cell cycle pathway[29,30]. DNA replication is also a crucial element of the malignant characteristics in LGG; genomic instability caused by DNA-replication stress in gliomas is a critical factor in the development of extreme chemoradiotherapy resistance and high vascularization[31]. Moreover, neurotrophin and Notch cell signaling pathways play a key regulatory role in the pathological process of LGG. For example, neurotrophins and their receptors can act on the $p75^{NTR}$ receptor system to strengthen the proliferation and invasion abilities of glioma cells and the interaction between immune cells in the tumor microenvironment, leading to the promotion of glioma development[32]; moreover, activation of a NOTCH1-SOX2 positive-feedback loop in glioma stem cells can function as a critical regulatory mechanism for controlling the distant invasion of gliomas along the white matter trace[33]. Together, these findings suggest that high *DEPDC1B* expression can promote the tumor evolution of LGG by acting through the aforementioned key signaling pathways, and this helps considerably enhance current understanding of the function of *DEPDC1B*.

Gene interactions and networks constitute another crucial mechanism involved in influencing the malignant behavior of tumors. Transcriptionally synergistic genes typically play similar roles in the malignant progression of cancers or participate in the same regulatory processes[18]. Therefore, we examined here the genes that were co-expressed with *DEPDC1B* in order to indirectly reveal the function of *DEPDC1B* in LGG. The most positively related gene, *CCNB1*, functions as a critical regulator in the cell cycle to control mitosis and thereby promote cell growth[34]. Furthermore, *CCNB1* has also been reported[35-37] to play a carcinogenic role in various cancers, such as hepatocellular carcinoma, pancreatic cancer, and breast cancer. As a protu-

mor factor, *CCNB1* is positively associated with tumor resistance and inhibits apoptosis through the p53 signaling pathway[36,38]. Moreover, the co-expressed gene *TTK* is another recognized oncogene that is highly expressed in breast cancer and non-small cell lung cancer and leads to poor prognosis of patients[39,40]. *TTK* not only regulates epithelial-to-mesenchymal transition, but also enhances homologous recombination in breast cancer and leads to radiotherapy resistance[41,42]. In conclusion, we have indirectly revealed that *DEPDC1B* might produce function synergistically with co-expressed genes and participate in the pathogenic process of LGG.

Conclusions

In this study, we demonstrated for the first time that high expression of *DEPDC1B* leads to poor prognosis of LGG patients. Notably, we reduced the occurrence of false-positive data by analyzing multiple databases and performing mutual verification by using standard experiments and biological information analysis; therefore, the results of this study are highly reliable. Most importantly, our findings not only enhance current understanding of the pathological mechanism of LGG, but also provide a potential biomarker and immunotherapy target, and this holds substantial practical significance for improving the prognosis of LGG patients and reducing the medical burden on society.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

ZL, WL and XC contributed equally to this work. ZL and YG designed the research; XC and RQ performed the research; WL and XC wrote the paper. All authors read and approved the final manuscript.

Ethics Approval

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Henan Provincial People's Hospital (2020, Ethical Review No. 107).

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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Availability of Data and Material

The data set analyzed during the current study is available in the TCGA database (The Cancer Genome Atlas Program, available at: https://cancergenome.nih.gov/) and the CGGA database (CGGA, available at: http://www.cgga.org.cn/). Besides, the datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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