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ZNF480 influences the prognosis, pathogenesis, and immune microenvironment in patients with lower-grade glioma

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ABSTRACT

ZNF480 has not yet attracted attention in the study of malignant tumors. Therefore, this study attempts to explain the significance of *ZNF480* in the pathological process of lower-grade gliomas (LGG) based on large-scale samples from public database sources and *in vitro* experiments. Reverse transcription quantitative real-time polymerase chain reaction and immunohistochemistry confirmed that *ZNF480* was highly expressed at both the mRNA and protein levels in LGG. Prognostic correlation analysis confirmed that the high expression of *ZNF480*, as an independent pathogenic gene, significantly correlates with poor survival in patients. Furthermore, the expression level of *ZNF480* was significantly inhibited in SHG-44 cells treated with ademetionine disulfate tosylate. Gene set enrichment analysis showed that *ZNF480* exists in multiple tumor-related signaling pathways, including the Notch signaling pathway. Immunological correlation analysis showed that *ZNF480* can promote the LGG microenvironment to a high immune state and significantly enhance the infiltration of various immune cells, such as M2 macrophages. Finally, Spearman analysis showed a positive correlation of *ZNF480* with many immune checkpoints, such as PD-L1. Overall, this study reveals for the first time the adverse effects of *ZNF480* on the prognosis of tumor patients, which expands our understanding of the molecular mechanisms behind the regulation of *ZNF480*. We believe that the high expression of *ZNF480* in LGG may be valuable for molecular targeted therapy or combined immunotherapy.

1. Introduction

Glioma is a malignant tumor in the central nervous epithelial system, with high morbidity and mortality rates. As one of the main

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types of gliomas, lower-grade gliomas (LGG) are defined as World Health Organization (WHO) Grade I–III neoplasms [1]. Despite the adoption of new diagnostic methods and multidisciplinary comprehensive treatment of LGG [1], the 5-year survival rate of LGG patients has not shown major improvement [2]. With advances in transcriptomics, multiple studies have shown disordered transcriptome as an important reason for the malignant progression of tumors [3]. However, the specific regulatory mechanism of LGG is still unclear, which hinders the development of effective targeted drugs to correct transcription disorders and improve the prognosis of LGG. Therefore, molecular exploration of LGG pathogenesis and identification of new potential therapeutic targets are of great significance in promoting precision medicine-targeted therapy and in improving long-term survival outcomes for patients.

Zinc finger proteins (ZNFs), the largest family of transcription factors and epigenetic inhibitors in mammals, are involved in gene expression regulation in a wide range of life events, including embryonic development, epigenetic modification, immune regulation, and carcinogenesis [4,5]. About one-third of ZNFs have the Krüppel-associated box (KRAB) domain, collectively known as the Krüppel-associated box zinc finger proteins (KRAB-ZNFs), making them the largest subfamily of ZNFs [6]. In recent years, there has been increasing evidence that KRAB-ZNFs promote tumor cell proliferation, invasion, and migration and help tumors evade the killing effects of host immune surveillance [7,8]. For example, multiple ZNF genes play key roles in the regulation of the tumor immune microenvironment (TME) in breast carcinoma, shortening the overall survival (OS) of patients [9]. Moreover, overexpression of *ZNF655* promotes the proliferation and migration of glioma cells and inhibits apoptosis of cells by activating *AURKA* [10]. However, while an increasing number of KRAB-ZNFs are being identified, their complex regulatory mechanisms and diverse biological roles require further exploration.

ZNF480 is a member of the KRAB-ZNF family with one KRAB-A box and 12 C2H2 motifs, which are widely distributed in the brain and are involved in the proliferation and differentiation of stem cells *in vivo* [11]. More importantly, *ZNF480* plays a key regulatory role in brain and nerve development and cognitive function improvement, as abnormal mutations in *ZNF480* are associated with the development of schizophrenia and intellectual disability [12,13]. However, to date, there has been no report on the prognosis of tumor patients with mutations in *ZNF480*, which has aroused our research interest. Based on the important role of *ZNF480* in the embryo and the close relationship between tumorigenesis and embryonic development, it may become a potential target for tumor treatment.

In this study, the diagnostic and prognostic value of *ZNF480* in LGG was explored, for the first time, by combining bioreliability analysis and *in vitro* experiments. In addition, this is the first record of the potential molecular mechanism underlying the role of *ZNF480* in LGG, determined by combining upstream methylation regulation, gene coexpression, and signal pathway enrichment analysis. Furthermore, by exploring the relationship between *ZNF480* and tumor-infiltrating immune cells (TILs), we elucidated the role of the TME in promoting LGG for the first time. This comprehensive analysis, from a multi-level and multidimensional perspective, reveals that *ZNF480* promotes the malignant progression of LGG. This study not only contributes to the improvement of the understanding of *ZNF480*, but also brings hope for providing new potential diagnostic biomarkers and therapeutic targets.

2. Materials and methods

2.1. Data collection

Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/), an online public platform developed by the team of Professor Zhang Zemin [14], was used to explore the changes in the expression level of *ZNF480* mRNA in LGG. The sample sizes of the LGG and control groups were 518 and 207, respectively. Subsequently, transcriptome sequencing data and clinical information of 503 LGG patients (Table S1) were collected from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) to study the correlation of *ZNF480* expression with clinical characteristics, survival, and TME in LGG patients. We collected DNA methylation sequencing data for 511 LGG patients from TCGA to explore the effect of DNA methylation status on the expression of *ZNF480*. To verify the accuracy of TCGA data, we analyzed the microarray data of 142 cases (see detailed patient information in Table S2) and the RNA sequencing data of 403 cases (see detailed patient information in Table S3) from the Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/) database [15]. GSE4 3378 dataset [16], with a sample size of 18 LGG patients from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database was mainly used for meta-analysis to verify the effect of *ZNF480* on LGG prognosis.

2.2. Patients and tissue specimens

Human tissue samples, collected from Henan Provincial People's Hospital and cryopreserved in liquid nitrogen after surgical resection, were used for the experiments. Control brain (10 cases) and tumor tissues (31 cases) were collected from patients with epilepsy and LGG, respectively and used for quantitation of *ZNF480* expression by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). Brain tissues from three epilepsy patients and tumor tissues from three LGG patients were used for detecting the expression of ZNF480, CD163, and CD174 proteins using immunohistochemistry (IHC). All patients participating in this study signed written informed consent before participating, and this project was approved by the Ethics Committee of Henan Provincial People's Hospital (ethics number: 2020107).

2.3. Cell culture and treatment

We used WHO Grade III glioma primary cells, a low-grade glioma cell line SHG44 (Cat BFN608006389, Bluefbio, China), and a normal human astrocytic (HA) cell line (Cat HTX2627, Otwo Biotech, China) for *in vitro* experiments to study the expression of *ZNF480*

in LGG cells. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Cat PM150210, Procell, China) containing 10% fetal bovine serum (Cat 10099141, Gibco, USA) and 1% penicillin and streptomycin (Cat P1400, Solarbio, China), and then incubated at 37 °C with 5% carbon dioxide. To examine whether the mRNA expression of *ZNF480* is regulated by DNA methylation, SHG-44 cells were treated with 100 μM ademetionine disulfate tosylate (SAM, Cat 97540-22-2, Topscience, China) for 10 h. Total RNA was extracted from SAM-treated and blank control groups, and the expression level of *ZNF480* mRNA was measured.

2.4. RNA isolation and RT-qPCR

Total RNA was extracted from cells using RNA Kit I (Cat R6834-02, Omega, USA), following the manufacturer's instructions, and quantitated using NanoDrop (Cat ND-ONEC-W, Thermo, USA). The cDNA was synthesized using NovoScript Plus All-in-one 1st Strand cDNA Synthesis SuperMix (Cat E047-01B, Novoprotein, China) and RT-qPCR was performed using NovoStart® SYBR qPCR SuperMix Plus (Cat E096-01A, Novoprotein, China), as per the manufacturer's instructions. The expression of *ZNF480* mRNA was quantitatively analyzed using the StepOne Plus real-time PCR system (Thermo, USA). The experiment was repeated three times, and the expression of *ZNF480* in tissues or cells was calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of specific primers for the housekeeping gene (18S) and the target gene (*ZNF480*) were as follows: 18S forward: 5′-GTAACCCGTTGAACCCCATT-3′, 18S reverse: 5′-CCATCCAATCGG-TAGTAGCG-3′; *ZNF480* forward: 5′-TCCTCAGGGACACTTAACATTC-3′, *ZNF480* reverse: 5′-GGGAGACCAGGTTCCTGTAGTT-3′.

2.5. IHC staining

The levels of ZNF480, CD163, and PD-L1 proteins were determined using IHC staining. First, the tissue sections were deparaffinized and dehydrated using xylene and gradient ethanol solution (100% anhydrous ethanol twice, 95% anhydrous ethanol once, and 85% anhydrous ethanol once). Subsequently, to fully expose the antigen sites, the sections were immersed in EDTA antigen repair solution, microwaved for 20 min, and then sealed with 10% serum solution for 30 min. The sections were then incubated overnight with the primary antibody (*ZNF480*, 1:50 dilution, Proteintech, China; CD163, 1:250 dilution, Invitrogen, USA; PD-L1, 1:250 dilution, Proteintech, China) at 4 °C and subsequently with the secondary antibody at 30 °C for 1 h. Finally, the sections were developed using a chromogenic solution, and the stained sections were visualized under a 200 × microscope and photographed. The IHC results were analyzed using ImagePro-Plus software (version 6.0), and the experiment was repeated three times.

2.6. Meta-analysis

A meta-analysis was used to increase the confidence of considering *ZNF480* as a risk factor in LGG patients. We searched literature related to *ZNF480* and LGG through MeSH terms on multiple literature retrieval platforms. Unfortunately, no relevant literature was available. Therefore, we collected four datasets for meta-analysis, including TCGA RNA seq: 503 cases, CGGA microarray: 142 cases, CGGA RNA seq: 403 cases, and GSE43378: 18 cases. First, the Cox regression method was used to analyze these four independent datasets, after which the R software (version number: R x6444.0.3) was used to calculate the HR for each dataset separately. The Q-test was used to detect heterogeneity between data. According to $I^2 > 50\%$ and $p < 0.05$, a random-effects model was selected for data integration. Finally, the combined HR and 95% CI were calculated.

2.7. GSEA analysis

Gene set enrichment analysis (GSEA) is a gene enrichment method developed by the Broad Institute [17]. First, the data from TCGA were divided into upregulated and downregulated groups according to the median value of *ZNF480* expression in LGG. Thereafter, GSEA 4.0.2 jar software was used to explore the relevant signaling pathways for *ZNF480* in LGG. For each analysis, 1000 gene sets were sequenced. The results were considered significant at nominal (NOM) $p < 0.05$ and false positive rate (FDR) < 0.25.

2.8. Immune infiltration analysis using the TIMER database and ESTIMATE software

Tumor Immune Estimation Resource (TIMER, https://cistrome.shinyapps.io/timer/) is a free online service platform that uses the RNA-seq data information from TCGA to detect the infiltration of immune cells through a variety of algorithms [18]. We explored the relationship between the expression of *ZNF480* in LGG, the infiltration level of six immune cells, and the prognosis of patients through the GENE and SURVIVAL modules of TIMER. The somatic copy number alterations (SCNA) module was used to investigate immune infiltration after a change in gene copy number.

Estimation of STromal and Immune cells in MAlignant Tumour tissues using Expression data (ESTIMATE), developed at the MD Anderson Cancer Center [19], estimates the proportion of tumor cells, immune cells, and stromal cells. We used the ESTIMATE algorithm to evaluate the effect of differential expression of *ZNF480* on cell composition in the tumor tissue in LGG.

2.9. Statistical analysis

We used R (version 4.0.3) and GraphPad Prism 9 software for the statistical analysis and visualization. Non-paired *t*-test and one-way analysis of variance (one-way ANOVA) were used to estimate the differences between two and more than two groups. The Chi-squared test was used to detect the correlation of *ZNF480* mRNA expression with clinical features. Kaplan–Meier analysis was used to

compare the differences in survival between patients with high and low *ZNF480* expression. Multivariate Cox analysis was used to assess the prognostic value of risk scores. Finally, the receiver operating characteristic (ROC) curve was used to evaluate the prognostic diagnostic value of *ZNF480* in patients. The above statistical results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Differential expression of ZNF480 in LGG

The GEPIA database showed that *ZNF480* had abnormally higher expression in LGG than in control brain tissue (Fig. 1A and B). This was verified using RT-qPCR, which showed high expression of *ZNF480* mRNA in primary glioma cells and LGG tissue compared with normal human astrocytes and control brain tissue from epilepsy patients (Fig. 1C and D). The IHC results confirmed the differences in *ZNF480* expression at the protein level (Fig. 1E). These results indicate the high probability of *ZNF480* overexpression being closely related to the occurrence and development of LGG.

3.2. Upregulation of ZNF480 is associated with malignant clinical characteristics and poor prognosis in LGG patients

By analyzing data from two RNA-seq databases, TCGA and CGGA, the overexpression of *ZNF480* was found to be closely related to the adverse clinical features of LGG (Fig. 2A–F; Fig. S1A-D). In WHO Grade III and higher malignant histological types such as anaplastic astrocytoma (AA), the expression level of *ZNF480* was significantly higher than that in WHO Grade II and other histological types, such as astrocytoma (A), oligodendroglioma (O), anaplastic oligodendro (AO), recurrence of strocytoma (rA), recurrence of oligodendroglioma (rO), and recurrence of anaplastic oligodendroglioma (rAO) (Fig. 2A, B, D, E). Moreover, *ZNF480* was significantly overexpressed in the radiotherapy, chemotherapy, and relapse groups compared to the non-radiotherapy, non-chemotherapy, and primary groups. (Fig. S1A, B, D). The isocitrate dehydrogenase (IDH) mutation type and the 1p19q co-deletion type were significantly negatively correlated with *ZNF480* expression (Fig. 2C, F; Fig. S1C), which further demonstrated that the high expression of *ZNF480* is disadvantageous in LGG patients.

To further explore the effect of *ZNF480* expression on the prognosis of patients, we obtained three datasets from TCGA and CGGA databases to compare the OS in the *ZNF480* high-expression and low-expression groups. OS was significantly reduced in the group with high *ZNF480* expression compared to the group with low expression ($p < 0.001$). The results of the Kaplan–Meier survival analysis for the three datasets were highly consistent (Fig. 2G, H, I), suggesting that *ZNF480* overexpression is associated with poor prognosis in

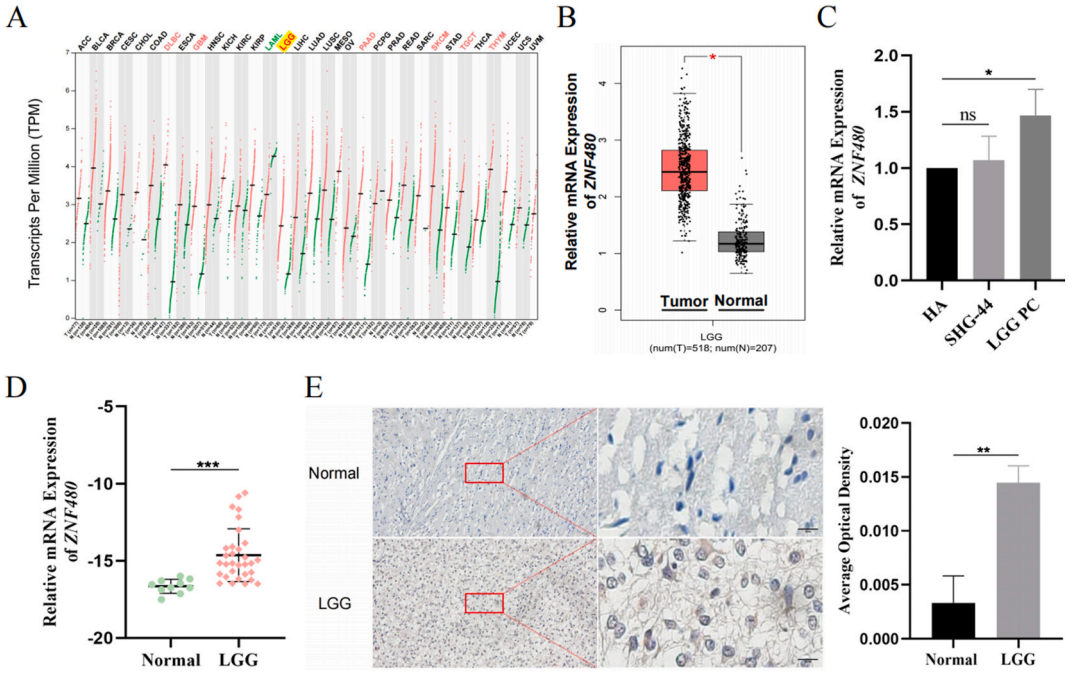

Fig. 1. Comparison of the expression of *ZNF480* in tumor and normal tissues. (A–B) The expression level of *ZNF480* in LGG based on analysis of the GEPIA database. Red represents high expression, green represents low expression, and LGG is highlighted in yellow. (C) RT-qPCR was used to detect *ZNF480* expression in normal human astrocytic (HA), SHG44 glioma, and primary cells. (D) RT-qPCR was used to detect the expression of *ZNF480* in the LGG tissues. (E) Representative images of IHC staining with *ZNF480* protein expression level in LGG and non-tumor tissue samples; brown represents positive staining. The experiments were repeated three times. Scale bar = 6 mm *: $p < 0.05$ and **: $p < 0.01$; $p < 0.05$ is considered statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

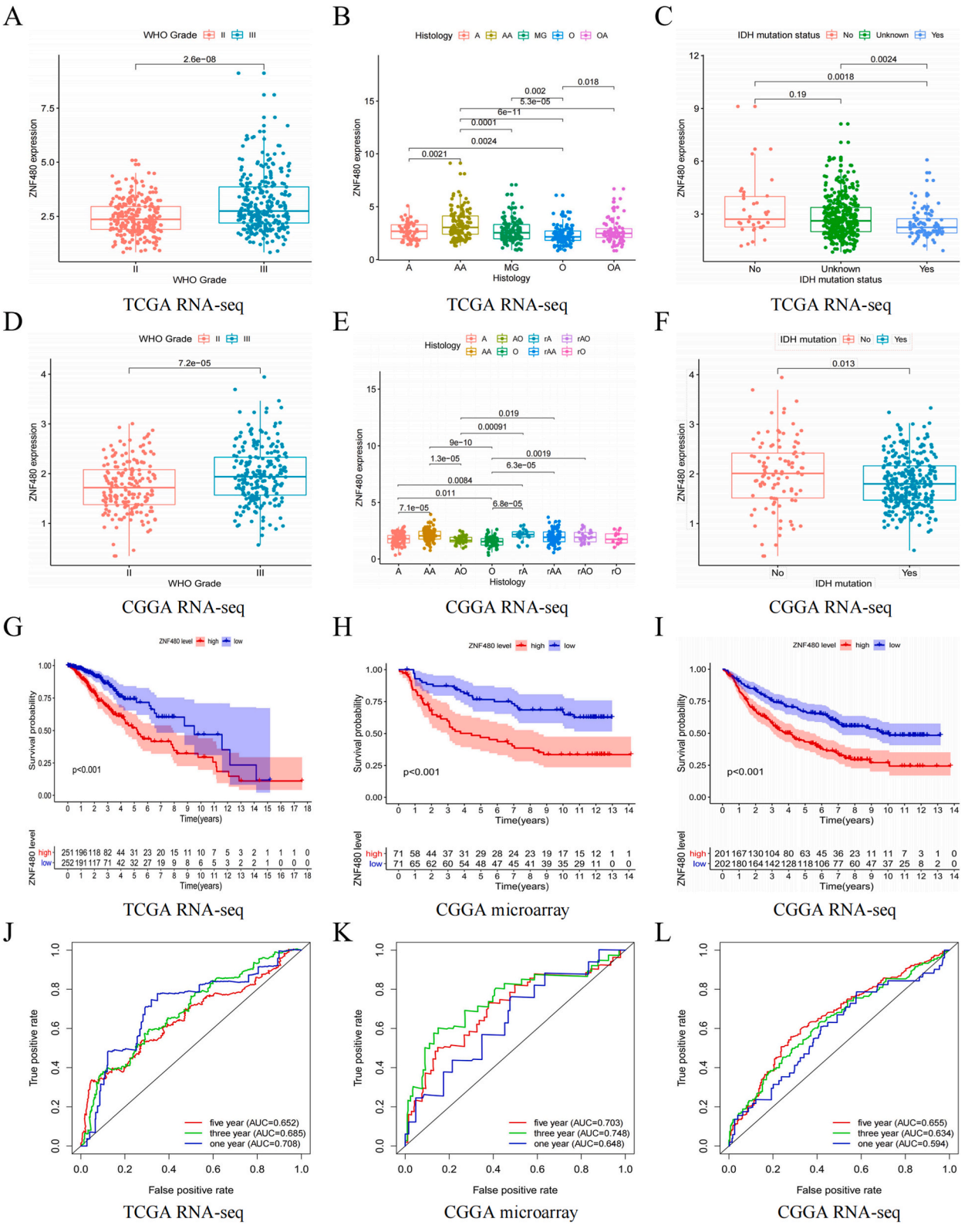

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Fig. 2. Correlation of *ZNF480* expression in TCGA RNA-seq database with clinical features: (A) WHO Grade, (B) histotype, and (C) IDH mutation. Correlation of *ZNF480* expression in the CGGA RNA-seq database with clinical features: (D) WHO Grade, (E) histology, and (F) IDH mutation. (G–I) Kaplan–Meier survival analysis based on TCGA RNA-seq, CGGA microarray, and CGGA RNA-seq datasets, respectively. (J–L) ROC based on TCGA RNA-seq, CGGA microarray, and CGGA RNA-seq datasets, respectively. $p < 0.05$ is considered statistically significant.

LGG patients. In addition, the results of the ROC curve analysis of the three datasets showed that the expression of *ZNF480* had moderate diagnostic significance for the patients (Fig. 2J, K, L). Thus, the high expression of *ZNF480* predicts the poor prognosis of LGG patients, and has diagnostic value for the prognosis of patients.

3.3. High expression of ZNF480 is an independent risk factor in LGG patients

Considering the existence of confounding factors, we further explored whether the high expression of *ZNF480* is an independent risk factor for the prognosis of LGG patients. Based on TCGA RNA-seq dataset, the results of univariate analysis showed that *ZNF480* (*p*

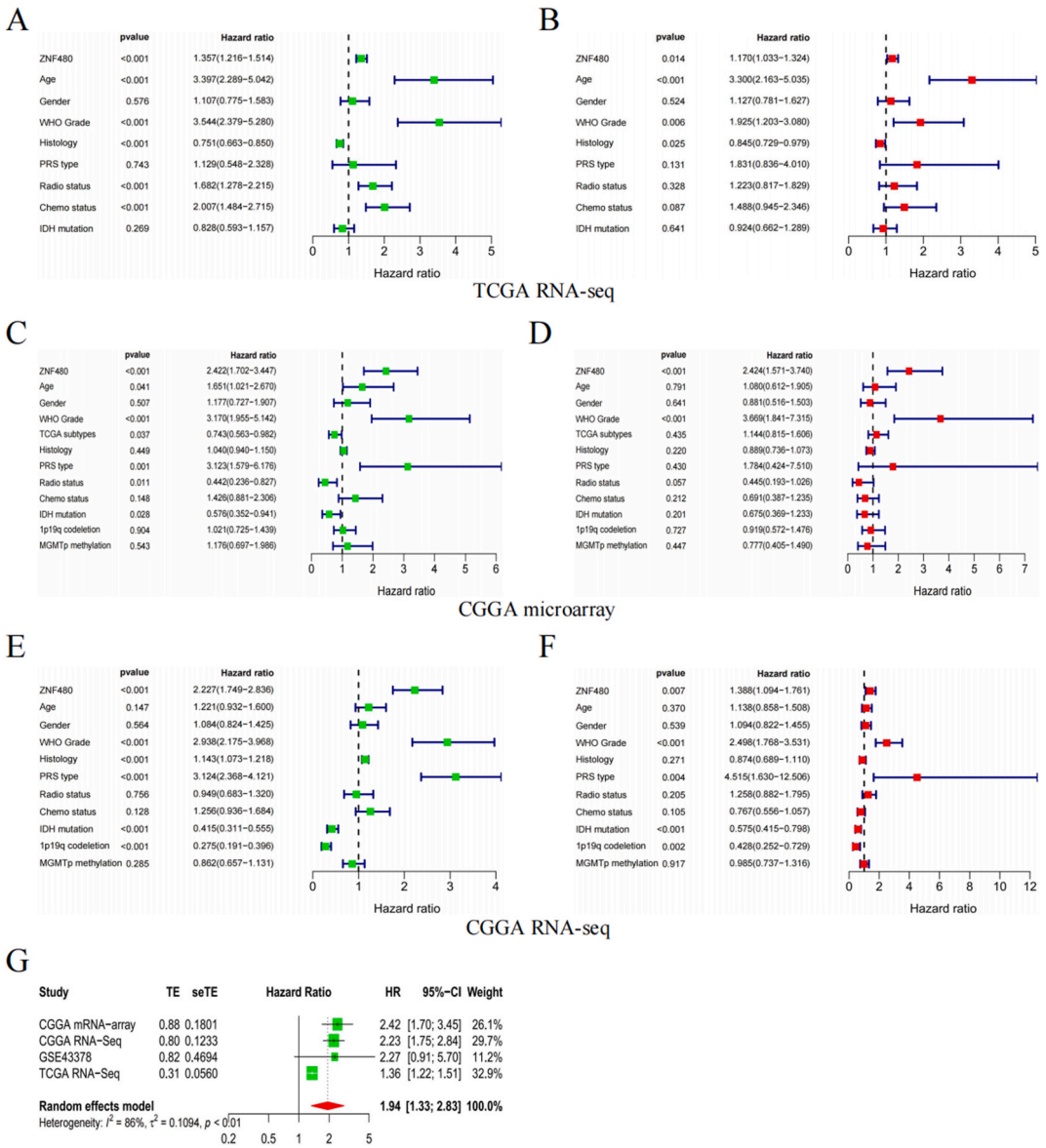


Fig. 3. *ZNF480* as an independent risk factor in LGG patients. (A, C, E) Results of univariate regression analysis based on TCGA RNA-seq, CGGA microarray, and CGGA RNA-seq datasets, respectively. (B, D, F) Results of multivariate regression analysis based on TCGA RNA-seq, CGGA microarray, and CGGA RNA-seq datasets, respectively. (G) Forest plot of high *ZNF480* expression and poor prognosis in LGG patients based on four datasets (TCGA RNA seq: 503 cases; CGGA microarray: 142 cases; CGGA RNA seq: 403 cases; GSE43378: 18 cases).

< 0.001; hazard ratio [HR], 1.357; 95% confidence interval [CI], 1.216–1.514) and WHO Grade ($p < 0.001$; HR, 3.544; 95% CI, 2.379–5.280) were prognostic risk factors (Fig. 3A). Univariate analyses of both CGGA RNA-seq and CGGA microarray datasets showed statistically significant results for *ZNF480* and WHO Grade as a risk factor ($p < 0.001$) (Fig. 3C, E). To confirm whether *ZNF480* is an independent risk factor, the TCGA RNA-seq dataset were subjected to multivariate analysis, and it was confirmed that *ZNF480* ($p = 0.014$; HR, 1.170; 95% CI, 1.033–1.324) and WHO Grade ($p = 0.006$; HR, 1.925; 95% CI, 1.203–3.080) were independent risk factors (Fig. 3B). As expected, *ZNF480* and WHO Grade were also validated as independent risk factors in the other two datasets (Fig. 3D, F), and the reliability of the results was further confirmed by meta-analysis (Fig. 3G). Thus, we revealed that *ZNF480* expression is an independent risk factor for the prognosis of LGG patients, suggesting that it may hold promise as a potential biomarker for the prognosis of LGG patients.

3.4. Co-expression analysis, methylation regulation, and signaling pathway enrichment of ZNF480 in LGG

To explore the mechanism behind *ZNF480* regulation, this study first used co-expression analysis to explore the synergistic or antagonistic expression relationships between *ZNF480* and other genes based on TCGA RNA-seq data. The results showed that hundreds of genes were co-expressed with *ZNF480* during the pathological process of LGG. Subsequently, we presented the top five genes with the highest positive (*ZNF45*, *ZNF701*, *ZNF616*, *ZNF227*, *ZNF845*) and negative (*MAPK3*, *FAIM2*, *LYRM9*, *APBB1*, *AVPI1*) correlations in the manuscript (Fig. 4A and B).

Abnormal DNA methylation is crucial to the malignant transformation of normal cells and in resistance to chemotherapy [20]. Therefore, this study explored the effects of DNA methylation on the expression of *ZNF480*. We obtained DNA methylation data from the TCGA database for LGG patients, and subsequently extracted 10 DNA methylation sites that may have regulatory effects on the expression of *ZNF480* (Fig. 4C). In addition, Kaplan–Meier survival analysis showed that LGG patients with hypermethylated DNA at methylation sites cg05638056 and cg222877 had significantly higher OS than those with hypomethylated DNA at these sites (Fig. 4D and E), while alterations at the remaining methylation sites did not have a significant effect on OS. Therefore, the low methylation state of *ZNF480* could be related to the poor prognosis of LGG patients. To further clarify how *ZNF480* methylation affects survival and prognosis, we used SHG-44 cells that were divided into control and methylation treatment groups. The expression of *ZNF480* mRNA

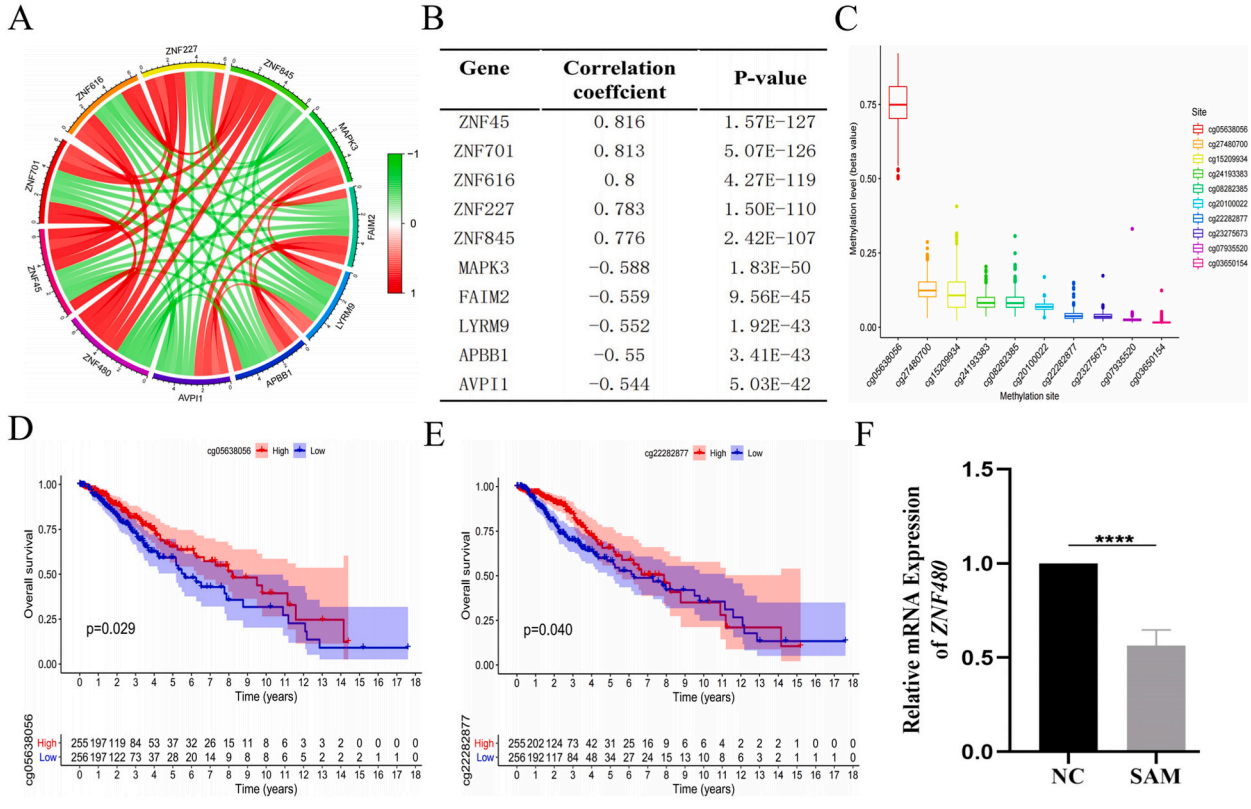
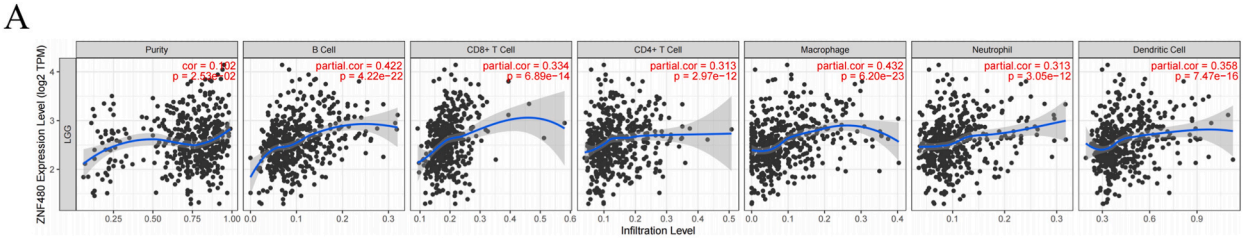
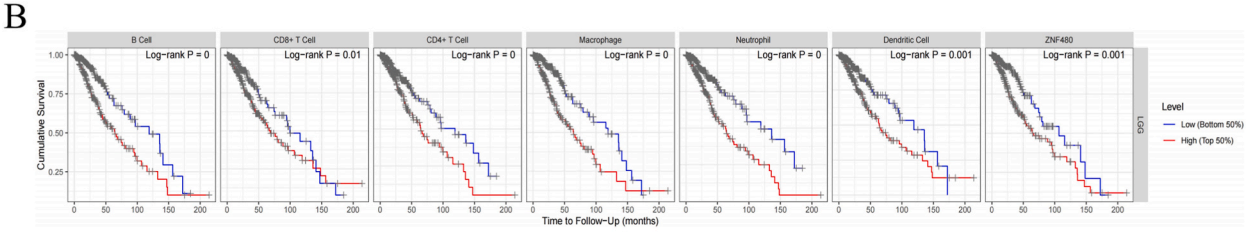
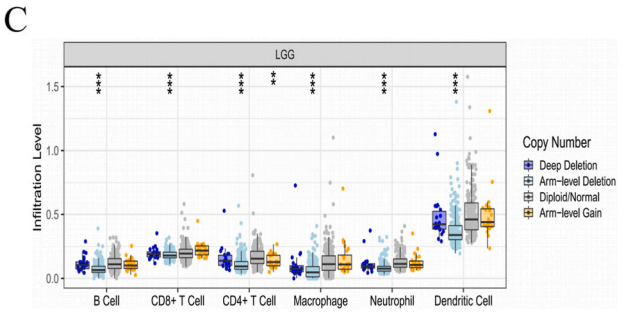
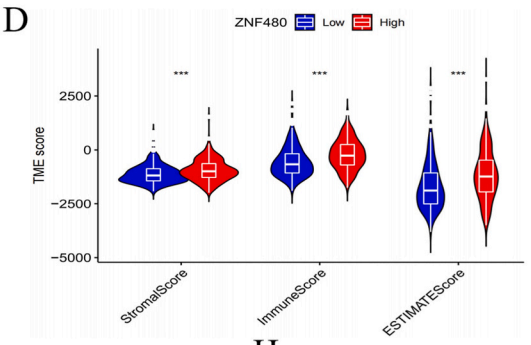


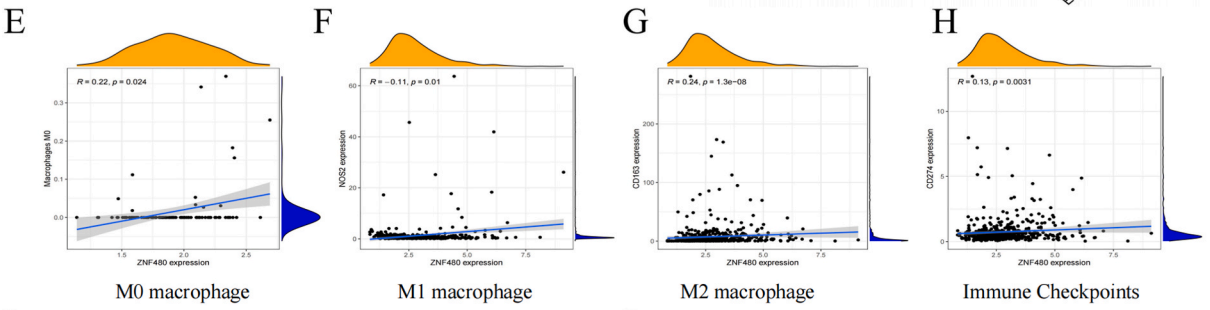
Fig. 4. Co-expression analysis and regulation of *ZNF480* by DNA methylation. (A) Visualization of the expression relationships of the top five genes with synergistic or antagonistic expression with *ZNF480*. (B) Summary table of the top five genes with synergistic or antagonistic expression with *ZNF480*. (C) Ten methylation sites in *ZNF480* based on TCGA data. (D–E) Kaplan–Meier survival analysis of ZNF480CpG sites (cg05638056 and cg22282877). (F) RT-qPCR detection of decreased expression of *ZNF480* in LGG cell lines treated with a methylating drug. *: $p < 0.05$, **: $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; $p < 0.05$ is considered statistically significant.











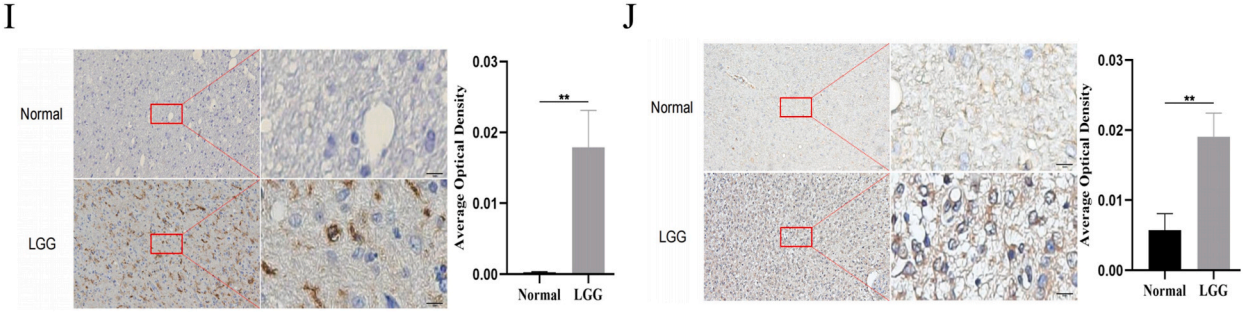


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Fig. 5. The regulatory role of *ZNF480* in the immune microenvironment. (A) The expression level of *ZNF480* was positively correlated with the infiltration level of six types of immune cells (B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells). (B) High infiltration levels of the six types of immune cells were negatively correlated with the survival time of LGG patients. (C) Relationship between the copy number variation of *ZNF480* and the infiltration level of the six types of immune cells. (D) The results of the ESTIMATE algorithm analysis, showing increased StromaScore, ImmuneScore, and ESTIMATE Score with high *ZNF480* expression. (E) Spearman analysis showing a positive correlation between high *ZNF480* expression and increased M0 macrophage infiltration levels. (F–G) Spearman analysis showed that *ZNF480* expression level was positively correlated with the expression of *NOS2* (an M1 macrophage surface marker) and *CD163* (an M2 macrophage surface marker). (H) Spearman analysis showed that *ZNF480* expression levels were positively correlated with the expression of the immune checkpoint CD274. (I) Representative IHC images of CD163 (an M2 macrophage surface marker) expression level in LGG and non-LGG tissue samples (brown indicates positive staining). (J) Representative IHC images of PD-L1 expression levels in LGG and non-LGG tissue samples (brown indicates positive staining). Scale bars = 6 mm **: $p < 0.01$; $p < 0.05$ is considered statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

decreased significantly after methylation treatment (Fig. 4F), indicating that *ZNF480* methylation may be a potential new diagnostic and therapeutic target for LGG.

GSEA of the TCGA RNA-seq data showed that high expression of *ZNF480* was significantly correlated with the Notch signaling pathway, TGF-β signaling pathway, cell cycle, and DNA replication, and other signaling pathways (Fig. S2A, B, C, D; Table S4). A review of the relevant literature revealed that these four signaling pathways promote the acquisition of malignant features, such as infinite proliferation, distant metastasis, and immune escape. This finding inspired us to explore the relationship among the TME, glioma cells, and *ZNF480* expression.

3.5. Components of the tumor immune microenvironment are regulated by ZNF480

We found that *ZNF480* expression was significantly and positively correlated with six immune cell types (B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells), especially with macrophages (Fig. 5A). Thus, the high expression of *ZNF480* may be associated with increased infiltration levels of immune cells. Subsequently, we divided LGG patients into high-expression and low-expression groups according to the median expression level of these six immune cell types, and Kaplan–Meier survival analysis was used to explore their impact on the OS time of LGG patients. The results showed that the OS time in the high-expression group was significantly reduced compared to the low-expression group (Fig. 5B). With regard to the effect of alterations in the somatic copy number of *ZNF480* in LGG on the level of immune cell infiltration, we found that among the six immune cell types, significantly lower infiltration occurred after arm-level deletion of *ZNF480* (Fig. 5C).

To explore the purity of tumor cells, we analyzed the proportion of stromal cells, immune cells, and tumor cells in the tumor tissue using the ESTIMATE algorithm. The results showed that StromalScore, ImmuneScore, and ESTIMATE Score in the *ZNF480* high-expression group were significantly higher than those in the *ZNF480* low-expression group (Fig. 5D), indicating that the tumor purity decreased with the high expression of *ZNF480*. This confirmed that *ZNF480* overexpression promoted the increase in stromal and immune cells, and played a positive role in the malignant progression of LGG.

The above data reveal that macrophages have a non-negligible effect on LGG. Previous studies have also shown that M0 macrophages mainly polarize to M2 macrophages in the TME and promote the malignant progression of tumors [21]. Using Spearman correlation analysis, we verified that *ZNF480* expression had a significant positive correlation with M0 macrophages and $CD4^+$ T cells (Fig. 5E, Table S5). By detecting the relationship between *ZNF480* expression level and immune cell markers, we confirmed that *ZNF480* was positively correlated with B cell, $CD4^+$ T cell, M2 macrophage, neutrophil, and dendritic cell surface markers (Fig. 5G; Table S5), and it was negatively correlated with M1 macrophage surface markers (Fig. 5F; Table S5). In IHC staining, CD163 (an M2 macrophage marker) was significantly overexpressed in LGG tissues compared with normal tissues (Fig. 5I). Therefore, *ZNF480* overexpression promotes immune cell infiltration and affects the components of the TME.

3.6. Relationship between ZNF480 and immune checkpoints

With the increasing importance of immune checkpoints in the field of cancer, immune checkpoint inhibitors have become a trend in cancer treatment [22]. Based on the influence of *ZNF480* on the expression level of immune cells, the question of whether *ZNF480* also has an expression relationship with well-known immune checkpoints arises. The results of the co-expression analysis based on TCGA RNA-seq data showed that the expression of *ZNF480* was positively correlated with that of several immune checkpoints (Table S6), including PD-L1 (*CD274*) (Fig. 5H). Because PD-L1 is a well-known target for antitumor immunotherapy, we collected samples from LGG patients and used IHC to detect PD-L1 in LGG tissue. The results showed that the expression level of PD-L1 in the LGG tissue was significantly increased compared to the control group (Fig. 5J). Thus, *ZNF480* may be a potential target for immunotherapy.

4. Discussion

Abnormally expressed genes can be used as targets for anti-tumor therapy [23]. The level of gene expression is strictly regulated by transcription factors, which are closely related to the occurrence of cancer [24]. As a transcription factor, *ZNF480* plays a crucial role in embryos, but there is currently no research on its role in the pathological progression of cancer [25]. Thus, this study is the first to reveal the regulatory effects of *ZNF480* on the prognosis, mechanism, and immune microenvironment of patients with LGG, providing

a potential target for the clinical treatment of LGG.

4.1. Abnormal high expression of ZNF480 predicts poor prognosis in LGG patients

Previous studies suggest that abnormally high gene expression can impact the prognosis of tumor patients [26], and this study found abnormally high expression of *ZNF480* in LGG tissue; thus, *ZNF480* may have an impact on the prognosis of LGG. To confirm this speculation, we performed prognostic correlation analysis, which revealed that the high expression of ZNF480 significantly correlates with poor survival in patients thereby establishing the pathogenic role of *ZNF480* in the prognosis of LGG. Although previous research on *ZNF480* in cancer is lacking to substantiate our findings, members of gene families often exhibit similar biological effects [27]. However, we did find data indicating the roles of other members of the *ZNF* family in the prognosis of various types of cancer. For example, high expression of *ZNF71* promoted tumor progression and significantly shortened OS in lung cancer [28]. Therefore, the view proposed by this study that *ZNF480* is a risk factor for the prognosis of LGG patients is indirectly supported.

4.2. The potential regulatory mechanism of ZNF480 in LGG

DNA methylation has a significant regulatory effect on the expression of downstream genes [29]. Therefore, we investigated the effect of DNA methylation on *ZNF480* and found that the expression level of *ZNF480* in SHG44 cells was significantly inhibited with ademetionine disulfate tosylate (SAM), which is a methylating drug. This suggests that DNA methylation is indeed a negative regulator of *ZNF480*. In fact, studies have shown that hypermethylation in the promoter region is negatively correlated with the expression of corresponding genes and is closely associated with prognosis [30]. For example, glioma patients with *MGMT* promoter region DNA hypermethylation have significant increases in OS after radiotherapy and chemotherapy, and the hypermethylation of *MGMT* has been identified as a potential biomarker of glioma [31]. This result not only reveals the regulatory mechanism of *ZNF480* overexpression in LGG, but also suggests that methylation sites may be used as a target for therapy.

Our GSEA results revealed that cell signaling pathways activated by high *ZNF480* expression may contribute to the malignant progression of cancer cells. Almost all tumors have abnormal changes in cell cycle signaling pathways, which promote infinite proliferation of cancer cells [32]. Moreover, the TGF-β and Notch signaling pathways can not only promote the angiogenesis and invasive ability of tumor cells, but also regulate the immune status of tumors and participate in the immune escape mechanism of tumor cells [33–35]. Taken together, these results further confirm that *ZNF480* may promote the malignant progression of LGG through a series of oncogenic signaling pathways and may be involved in the regulation of the tumor immune microenvironment.

4.3. The regulatory effect of abnormally high expression of ZNF480 on the immune microenvironment in LGG

Considering the clinical success of antitumor immunotherapy [36], we further studied the effect of *ZNF480* on the TME in LGG. We found that high *ZNF480* expression was associated with high scores for StromalScore and ImmuneScore, which are highly correlated with tumor cells escaping immune cell surveillance and poor patient prognosis in various cancers [37–39]. Based on this, we further showed that *ZNF480* has a positive correlation with six common immune cells (especially macrophages), and increased infiltration levels of these immune cells significantly reduced the OS time of LGG patients. Tumor-associated macrophages, the main component of the tumor suppressive immune microenvironment, play an important role in the occurrence and development of cancers [21]. This demonstrates that the high expression of *ZNF480* not only promoted the infiltration of M0 macrophages but was also positively correlated with the M2-type polarization of macrophages. Research has shown that M1 macrophages mainly play an anti-tumor role in the pathological process of tumors [21], while the M2 type plays a role in promoting tumor cell immune escape and exacerbating tumor malignant progression [40]. Our IHC experiments also confirmed the increased expression of *ZNF480* and CD163, which indicates that an increase in *ZNF480* expression may promote tumor-associated macrophage infiltration and polarization to the M2 type. Therefore, we speculate that *ZNF480* has important regulatory effects on the immune microenvironment and is involved in exacerbating the malignant progression of tumors.

The latest theory of colloidal lymphatic vessels and meningeal lymphatic vessels in the central nervous system may provide a foundation for anti-immunotherapy of central nervous system malignant tumors [41]. Detecting immune checkpoints is an important indicator of the effectiveness of tumor immunotherapy, and this study used co-expression analysis to show a significant positive association between *ZNF480* and various immune checkpoints, such as PD-L1. PD-1/PD-L1 suppresses T cell activity in the TME at advanced stages and promotes the formation of an inhibitory immune environment in tumors [42]. Significant progress in the treatment of various tumors has been achieved with anti-PD-1/anti-PD-L1 therapy, which reverses T cell responses to achieve anti-tumor effects [42]. More importantly, PD-L1 expression levels affect the clinical response in patients receiving PD-1/PD-L1 immunoblockers [43]. Therefore, in this study, PD-L1 was selected for IHC, and we found that the increased PD-L1 expression level correlated with that of *ZNF480*. Based on the above findings, a combination of anti-*ZNF480* and anti-PD-L1 antibody therapy may be a promising approach for prolonging the OS of LGG patients. Thus, our study may provide new immunotherapeutic targets for LGG patients, which could lead to an improved quality of life for these patients.

4.4. Limitations of the study

Although this study conducted a comprehensive analysis of the effects of *ZNF480* on the pathological process of LGG, there are still unavoidable limitations. First, retrospective analysis has inherent limitations and is incapable of compensating for the integrity of the

sample collection process and clinical data. Therefore, we tried our best to compensate for this shortcoming by using partial experiments to verify the key results of our analyses. However, due to the limitations of objective conditions in our laboratory, we could not comprehensively verify all our results. Second, the complex composition of the tumor immune microenvironment makes it difficult for a single study to comprehensively reveal the complex regulatory network in such an environment. Therefore, our study only discusses the relationship between *ZNF480* and immune cell infiltration and immune checkpoint sites in the LGG immune microenvironment, which requires further exploration in the future.

5. Conclusions

To the best of our knowledge, this is the first study of *ZNF480* in tumors. We systematically elucidated the impact of *ZNF480* on the prognosis, mechanism, and immune microenvironment of LGG patients. We propose that *ZNF480* plays a role as a pathogenic gene in the process of LGG. Moreover, our findings indicate a relationship between *ZNF480* and the effectiveness of antitumor immunotherapy, broadening the molecular understanding of *ZNF480* and providing a potential diagnostic biomarker and therapeutic target for LGG.

Declarations

Author contribution statement

Qingyun Zhu: Zhendong Liu: Xingbo Cheng: Wenjia Liang: Hongbo Wang: Pengxu Li: Jiangfen Zhang: Yusheng Chen: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yanzheng Gao: Rongjun Qian: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ethics statement

All patients participating in this study signed written informed consent before participating, and this project was approved by the Ethics Committee of Henan Provincial People's Hospital (ethics number: 2020107).

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Data availability statement

The datasets involved in this study can be found in the TCGA database (https://cancergenome.nih.gov/) and the CGGA database (http://www.cgga.org.cn/) and the GEO database (https://www.ncbi.nlm.nih.gov/geo/). At the same time, the data generated in this study can be provided by the corresponding authors.

Declaration of competing interest

There are no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e18185.

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