

Original Research

# MAPK9 is Correlated with a Poor Prognosis and Tumor Progression in Glioma

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## Abstract

**Background:** Glioma has a high incidence in young and middle-aged adults and a poor prognosis. Because of late diagnosis and uncontrollable recurrence of the primary tumor after failure of existing treatments, glioma patients tend to have a poor prognosis. Recent advances in research have revealed that gliomas exhibit unique genetic features. Mitogen-activated protein kinase 9 (MAPK9) is significantly upregulated in mesenchymal glioma spheres and may be a new target for glioma diagnosis. This study aimed to investigate the potential diagnostic significance and predictive value of MAPK9 in glioma. **Methods:** Paraffin-embedded tumor tissues and paraneoplastic tissues were collected from 150 glioma patients seen at the General Hospital of Northern Theater Command. Immunohistochemistry and western blot assays were used to detect the expression levels of MAPK9. Prognosis and survival analyses were performed using SPSS 26 software for univariate/multivariate analysis and log-rank analysis. Cellular models were used to assess the effect of MAPK9 overexpression and knockdown *in vitro*. **Results:** MAPK9 expression was higher in glioma tissues than in paraneoplastic tissues. Prognostic and survival analyses revealed that the MAPK9 expression level is an independent prognostic factor in glioma patients. In addition, overexpression of MAPK9 significantly promoted the proliferation and migration of primary glioma cells, possibly via the Wnt/ $\beta$ -catenin-regulated EMT pathway. **Conclusions:** MAPK9 is an independent prognostic factor in glioma and is involved in tumor progression.

**Keywords:** MAPK9; glioma; clinicopathological variables; prognosis

## 1. Introduction

Glioma has been studied extensively in recent years. However, despite many significant research advances [1,2], insufficient progress has been made in addressing the disease, and patient outcomes remain poor. The global prevalence of glioma is approximately 6 in 100,000. The number of glioma cases among men is 1.6 times that among women [3]. In accordance with the most recent guidelines in the 2021 WHO Classification of Tumors of the Central Nervous System, the glioma classification system has been significantly updated. Common subtypes include astrocytoma with the isocitrate dehydrogenase (IDH) mutation (which is usually WHO grade 2, 3, or 4), oligodendroglioma with IDH mutation and 1p/19q codeletion (usually WHO grade 2 or 3), and glioblastoma with wild-type IDH (typically in WHO grade 4) [4,5]. Molecular information on glioma, such as IDH mutation status and 1p/19q codeletion status, has become increasingly important in diagnosis and treatment, as these features provide information on the molecular mechanism of glioma development [6,7].

In histological classification, gliomas are commonly classified into high-grade gliomas (HGGs), whose most common type is glioblastoma, and low-grade gliomas (LGGs). Although studies in recent years have improved the treatment of glioma, the overall survival rate of glioma remains low. Glioblastoma (GBM) has the lowest overall survival among brain tumors, with a 5-year survival rate of only 5.8% after diagnosis [8]. Gliomas with an oligodendroglial component have a higher survival rate than gliomas with an astrocytic component. LGG has 5-year survival rates ranging from 55% to 86%, and the median survival time of LGG patients ranges from 7.5 to 10 years. The prognosis of gliomas commonly depends on multiple factors, such as cytological type, central nervous system (CNS) WHO grade, age, and Karnofsky performance score [9–11]. Current clinical diagnostic methods include imaging technology, intraoperative and postoperative histopathology, genetic testing [12], and molecular testing [13]. The current gold standard for diagnosing brain tumors remains brain MRI, including T2-weighted sequences, T2-weighted fluid-attenuated inver-



sion recovery sequences, and 3D T1-weighted sequences before and after gadolinium contrast [3,14]. However, imaging modalities for glioma classification, prognosis and judgment do not currently meet all needs, so more sensitive molecular-assisted diagnostic methods are still needed.

Mitogen-activated protein kinase 9 (MAPK9) belongs to the MAPK family and is also known as c-Jun N-terminal kinase (JNK2); the JNK signaling pathway at the core related pathway and can be activated in a variety of ways, such as by growth factors and multiple stressors [15]. MAPK9 participates in a variety of cellular regulatory mechanisms, such as stress responses, apoptosis, proliferation and differentiation, and has been found to promote tumor formation in mammalian studies [15]. Using tumor specimens from 18 proneural and 12 mesenchymal high-grade glioma patients, Kim *et al.* [16] compared the expression of 349 kinase-encoding genes in primary cultures of glioma stem cells in a genome-wide expression study and found that the *MAPK9* gene was significantly upregulated in mesenchymal tumor specimens. Recent studies have found that the MAPK9 inhibitor RGB-286638 causes loss of activity in a variety of basement membrane cell culture models and that RGB-286638 may retard tumor growth [17].

However, there is still a lack of more in-depth studies on the relationship between MAPK9 expression and the clinical features and prognosis of glioma and the possible mechanisms involved. Therefore, in our study, we examined glioma and paraneoplastic specimens from 150 clinical patients. Immunohistochemical staining revealed that MAPK9 protein expression was significantly enhanced in glioma tissue, could be used for diagnosis, and was closely related to a poor prognosis. We also established a primary glioma cell model of MAPK9 overexpression or knock-down and investigated the role of MAPK9 in glioma cell proliferation and migration as well as its possible mechanisms. The results of clinical analysis combined with cell studies suggest that MAPK9 may be a good prognostic predictor for glioma and is involved in tumor progression.

## 2. Materials and Methods

### 2.1 Patients and Tissue Samples

We collected paraffin-embedded tumor tissues from 150 glioma cases originally diagnosed and treated in the General Hospital of Northern Theater Command. Among them, 99 cases were LGG (WHO Grade 2–3), and 51 cases were GBM (WHO Grade 4). Tumors and paired paraneoplastic tissues from six glioma patients (4 LGG cases, 2 GBM cases) who underwent surgical treatment at the General Hospital of Northern Theater Command from March to June 2022 were also collected. These tissues were frozen in liquid nitrogen and analyzed by western blotting. We reviewed medical records to collect case information and to define overall survival from the date of diagnosis to the event endpoint or study completion date. In these

cases, we used immunohistochemistry to detect brain tumor molecular expression and WHO grade, next-generation sequencing to test IDH mutation status, fluorescence in situ hybridization to determine 1p/19q codeletion status, and methylation-specific PCR to detect MGMT methylation status. Ethical approval was obtained from the Ethics Committee of the General Hospital of Northern Theater Command, according to the principles of the Declaration of Helsinki. In addition, we included 147 cases from the CGGA database (<http://www.cgga.org.cn/index.jsp>) in the validation set.

### 2.2 Western Blot Assay

Six pairs of glioma tissues frozen in liquid nitrogen were first removed with paraneoplastic tissues, and MAPK9 expression was detected. Brain tissues were homogenized in RIPA lysis buffer (Cat. No.: P0013B, Beyotime Biotechnology, Shanghai, China) to lyse for 30 minutes. Next, the samples were centrifuged at 12,000 rpm for 10 minutes at 4 °C, and the supernatant was collected for measurement. The protein samples were initially separated using electrophoresis after using a BCA kit (Cat. No.: P0012, Beyotime Biotechnology) and transferred to Polyvinylidene Fluoride (PVDF) membranes. After Bovine Serum Albumin (BSA) blocking, the PVDF membranes (Cat. No.: ISE00010, Merck Millipore Ltd., Darmstadt, Germany) were incubated with MAPK9 antibody (Cat. No.: sc-7345, 1:200, Santa Cruz Biotechnology, Dallas, TX, USA) and secondary antibody of the corresponding species (Cat. No.: A0216, 1:1000, Beyotime Biotechnology) and exposed using BeyoECL luminescent buffer. Grayscale values were analyzed using ImageJ V1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

### 2.3 Immunohistochemistry

Immunohistochemistry was performed to assess MAPK9 expression in surgically resected specimens and normal brain tissues from 150 glioma patients. After the specimens were isolated, they were fixed in 10% formalin for 48 h. Then, we embedded the samples using paraffin and sectioned them at 4 μm. The tissue specimens were dewaxed and rehydrated, and sections were immersed in 0.01 M citrate buffer for thermal repair and blocked in 5% BSA (Cat. No.: ST023, Beyotime Biotechnology). MAPK9 antibody (Cat. No.: sc-7345, 1:50, Santa Cruz Biotechnology) was added dropwise and incubated overnight at 4 °C. Afterward, 3,3N-Diaminobenzidine (DAB) color development was performed, and the sections were observed under a microscope. Then, color development was terminated by rinsing with running water. The samples were stained with hematoxylin, dehydrated with an alcohol gradient, cleared with xylene, and sealed with neutral resin. Then, five fields of view per section were observed under the microscope, and at least 100 cells per sample were observed at high magnification. Specimen

assessment was performed by two senior pathologists.

#### 2.4 Immunohistochemical Evaluation

The expression score for MAPK9 was obtained by multiplying the percentage of tumor-positive cells by the staining intensity ranging from 0 to 12. We scored the samples as 0 when there was no staining under the microscope, as 1 when there was yellowish staining, as 2 when there was yellowish-brown staining, and as 3 when there was brown staining. The percentage of positive staining was scored as 1 for  $\leq 10\%$ , 2 for 11–50%, 3 for 51–80%, and 4 for  $\geq 81\%$ . The mean of the total score was used to distinguish between high- and low-expressing samples. Two pathologists blindly assessed and scored all IHC sections. When the expression scores for MAPK9 were  $< 7$ , we defined MAPK9 expression as negative or low, and when the scores were  $\geq 7$ , we defined MAPK9 expression as high.

#### 2.5 RT-PCR

We extracted total RNA with Beyozol reagent (Beyotime Biotechnology, Shanghai, China) and detected reverse transcription polymerase chain reaction (RT-PCR) products using SYBR Green fluorescent probes. Finally, the expression level of the *GAPDH* gene was used as the standard to compare the expression level of MAPK9 mRNA.

#### 2.6 Cell Culture and Lentivirus Infection

Recently we have isolated and reported two primary cell lines from GBM patients named G62 and G91 [18]. We used Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal bovine serum (FBS) to culture G62 and G91 cells routinely at 37 °C and 5% CO<sub>2</sub>. *MAPK9* knockdown (shMAPK9), overexpression (hMAPK9) and normal control cell models were generated by lentiviruses (GeneChem, Shanghai, China). At each passaging, we used 2.5  $\mu\text{g}/\text{mL}$  puromycin to select the stable cell lines, and the GFP fluorescence intensity of cells was observed each day using fluorescence microscopy until there was no significant change in fluorescence intensity and no dead floating cells. The transfection efficiency was confirmed by western blotting.

#### 2.7 Cell Counting Kit-8 (CCK-8) Assay

At 37 °C and 5% CO<sub>2</sub>, approximately 100  $\mu\text{L}$  of cell suspension was incubated with complete culture medium to approximately  $5 \times 10^3$  cells/well in a 96-well plate. After treatment with 10  $\mu\text{L}$  of CCK-8, the culture was continued under the same conditions, and the absorbance values of each well at 450 nm were measured at 0 h, 24 h and 48 h with an enzyme marker.

#### 2.8 Cell Transwell Assay

Stably transfected glioma cells were inoculated at  $1 \times 10^5$  cells/well on a transwell polycarbonate membrane coated with matrix gel, and 200  $\mu\text{L}$  of serum-free medium

was added; the lower chamber was filled with normal medium with 10% fetal bovine serum, and the cells were placed back in the incubator for an additional 48 h. At room temperature, we fixed cells in 3.7% formaldehyde for 15 minutes and stained them with a solution containing 0.1% crystalline violet 10% ethanol for 30 min. The excess staining solution was removed, and the cells were observed by light microscopy. At the same time, the number of invading cells was counted under a 400 $\times$  field of view.

#### 2.9 Statistical Analysis

We used SPSS 26 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA, USA) for statistical analysis. The relationship between MAPK9 expression and clinicopathological variables was assessed by the  $\chi^2$  test, survival rates were plotted, survival curves were calculated by the Kaplan–Meier method, and whether the differences were statistically significant was evaluated by the log-rank test. One-way analysis was used to perform correlation analysis of survival time with each clinicopathological variable.  $p < 0.05$  was considered to indicate statistical significance.

### 3. Results

#### 3.1 MAPK9 Expression is Correlated with Clinicopathological Variables of Glioma Patients

We divided 150 cases into two groups (high MAPK9 expression and low MAPK9 expression). Table 1 shows the statistical analysis and summary of the correlation between MAPK9 expression and glioma clinicopathological parameters. Statistical analysis showed that there were significant associations between the expression of MAPK9 and WHO grade ( $p < 0.001$ ), IDH mutation status and 1p/19q codeletion status ( $p < 0.001$ ), MGMT methylation status ( $p < 0.001$ ), 3-year survival ( $p < 0.001$ ), and 5-year survival ( $p < 0.001$ ). The expression of MAPK9 was not significantly related to age ( $p = 0.055$ ), sex ( $p = 0.625$ ), or tumor site ( $p = 0.343$ ) (Table 1).

#### 3.2 Expression of MAPK9 in Glioma Tissues of Different Grades

To verify whether MAPK9 expression was increased in glioma tissues, we used western blotting to detect MAPK9 expression in tumors and paired paraneoplastic tissues of six glioma patients. As shown in Fig. 1A,B, MAPK9 protein expression was significantly higher in glioma tissues than in paraneoplastic tissues. We further verified the results of MAPK9 in different grades of gliomas in 150 samples using immunohistochemical methods and revealed that MAPK9 expression was significantly higher in glioma tissues than in paraneoplastic tissues, and MAPK9 protein expression in GBM tumor tissue was significantly higher than that in LGG (Fig. 1C). Immunohistochemical evaluation more directly demonstrated the expression of MAPK9 in different grades of gliomas in 150 sam-

**Table 1. Correlation of MAPK9 expression with clinicopathological variables in glioma patients.**

Clinicopathologic variables	No. of case	MAPK9 expression		p-value
		Low	High	
Age				0.055
<60	129	89	40	
≥60	21	10	11	
Sex				0.625
Male	105	68	37	
Female	45	31	14	
WHO grades				<0.001
LGG	96	88	8	
GBM	54	11	43	
Site				0.343
Parietal and occipital lobe	27	13	14	
Frontal and temporal lobe	96	59	27	
Ventricle	12	7	5	
Cerebellar lobe	10	6	4	
Basis cranii	5	4	1	
IDH mutation status				<0.001
Mutant	103	93	10	
Wildtype	47	6	41	
IDH mutation status and 1p/19q codeletion status				<0.001
IDH-wildtype	47	6	41	
IDH-mutant and noncodel	9	4	5	
IDH-mutant and codel	94	89	5	
1p/19q codeletion status				<0.001
Codel	94	89	5	
Non-codel	56	10	46	
MGMT methylation status				<0.001
Methylated	93	71	22	
Un-methylated	57	28	29	
3-year survival rate				<0.001
<36 month	31	1	30	
≥36 month	119	98	21	
5-year survival rate				<0.001
<60 month	43	4	39	
≥60 month	107	95	12	

ples (Fig. 1D). In addition, the PCR results showed that the mRNA expression of MAPK9 in the IDH wild-type group was higher than that in the IDH mutant group (Fig. 1E,  $p < 0.05$ ), and the 1p/19q non-codel group had lower MAPK9 expression than the 1p/19q codel group (Fig. 1E,  $p < 0.05$ ). The association of MAPK9 expression with IDH mutation and 1p/19q codel were also supported by the CGGA database (Fig. 1F,  $p < 0.05$ ).

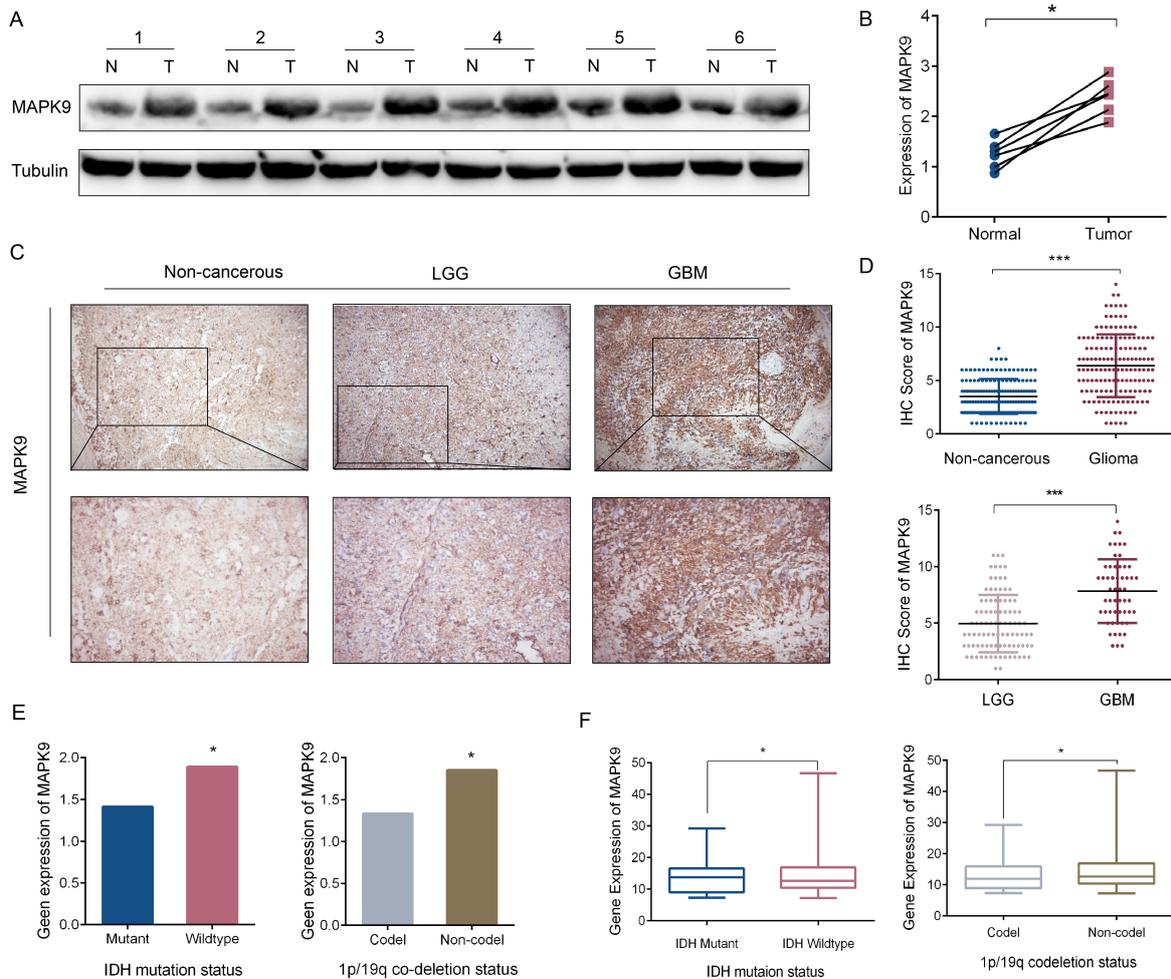
### 3.3 MAPK9 Expression is Correlated with Prognosis and Survival in Glioma Patients

To study the effect of MAPK9 on the prognosis of glioma patients, we used Kaplan–Meier analysis to analyze the association between MAPK9 expression level and patient survival. The results suggested that the patients with high MAPK9 expression had a worse prognosis than the

patients with low MAPK9 expression (Fig. 2A). In addition, MAPK9 remained a meaningful prognostic predictor in LGG and GBM patients (Fig. 2B,C). MAPK9 also predicted a worse prognosis in the IDH-wildtype and IDH-mutant/codel groups (Fig. 2D,F). Although not statistically significant, high MAPK9 expression showed a trend toward worse survival in IDH-mutant/non-codel group because of the small sample size ( $n = 9$ ) (Fig. 2E).

### 3.4 Overexpression of MAPK9 Promotes Glioma Cell Proliferation and Migration in Vitro

To explore the possible mechanism of MAPK9 in glioma progression, we used lentivirus to knock down and overexpress MAPK9 in primary glioma cell lines G62 and G91. First, the effectiveness of cell transfection was verified by western blotting. Compared with that in the control



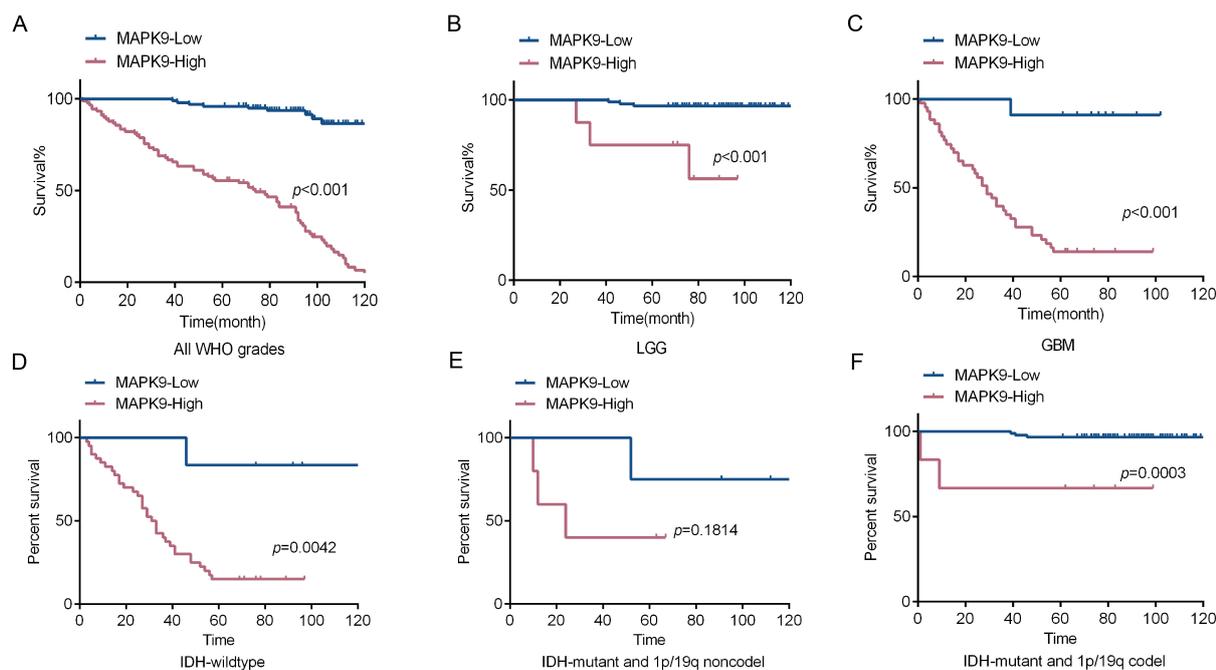
**Fig. 1. Expression of MAPK9 in different grades of glioma.** (A) Western blotting images of MAPK9 expression in 6 glioma cases and paired paracancerous tissues. (B) MAPK9 expression in glioma and paired paracancerous tissues,  $*p < 0.05$ . (C) Representative IHC staining images of MAPK9 (MAPK9 is low in noncancerous tissues and high in glioma tissues). (D) IHC scoring of glioma tissues and noncancerous tissues,  $***p < 0.001$ ; comparison of IHC scores of MAPK9 between WHO LGG and GBM tissues,  $***p < 0.001$ . (E) Comparison of MAPK9 expression between IDH mutant and IDH wild type, 1p/19q codel and 1p/19q non-codel in 150 samples,  $*p < 0.05$ . (F) Comparison of MAPK9 expression between IDH mutant and IDH wild-type, 1p/19q codel and 1p/19q non-codel samples from the CGGA database,  $*p < 0.05$ .

group, the expression level of MAPK9 in the shMAPK9 group was obviously decreased (Fig. 3A). Compared to those in control cells, MAPK9 expression levels were significantly increased in the MAPK9 overexpressing cells (Fig. 3A). We then used the CCK-8 assay to assess the proliferative capacity of glioma cells. The results suggested that the cell growth of the shMAPK9 group was significantly lower than that of the control group, while that of the MAPK9 overexpressing group was higher than that of the control group (Fig. 3B). In addition, the migration ability of glioma cells was assessed using a transwell assay. As shown in Fig. 3C, MAPK9 knockdown significantly reduced migration compared with that in the control group, and MAPK9 overexpression increased glioma cell migration. These results suggest that MAPK9 may be a potential promoter of glioma cell progression.

Furthermore, Western blot assays demonstrated that G62 cells with MAPK9 knockdown exhibited decreased N-cadherin and vimentin expression and increased E-cadherin expression, and MAPK9 overexpressing G91 cells exhibited the opposite trends (Fig. 3D). In general, these findings suggest that MAPK9 promotes glioma cell migration by altering EMT-related protein expression.

### 3.5 MAPK9 Promotes Glioma Cell Progression by Activating the $\beta$ -Catenin Signaling Pathway

To further investigate the mechanism of the cancer-promoting effect of MAPK9 on glioma cells, we assessed  $\beta$ -catenin expression and the expression of its downstream molecules CD44, MYC, and TCF1 by western blotting. Compared to that in the control group,  $\beta$ -catenin expression was slightly reduced in the MAPK9 knockdown group



**Fig. 2. The expression of MAPK9 affects the prognosis of glioma patients.** (A) Kaplan–Meier assay to assess MAPK9 expression in 150 glioma specimens. (B) Kaplan–Meier assay to detect MAPK9 expression in 96 LGG specimens. (C) Kaplan–Meier assay to detect MAPK9 expression in 54 GBM specimens. (D) Kaplan–Meier assay to assess MAPK9 expression in 47 IDH-wildtype specimens. (E) Kaplan–Meier assay to detect MAPK9 expression in 9 IDH-mutant/non-codel specimens. (F) Kaplan–Meier assay to detect MAPK9 expression in 94 IDH-mutant/codel specimens.

(Fig. 4A), while  $\beta$ -catenin expression in the nucleus of *MAPK9* knockdown cells was significantly reduced but slightly increased in the cytoplasm (Fig. 4B). *MAPK9* overexpression had the opposite effect (Fig. 4B). This suggests that  $\beta$ -catenin transcriptional activity is increased following overexpression of *MAPK9*.

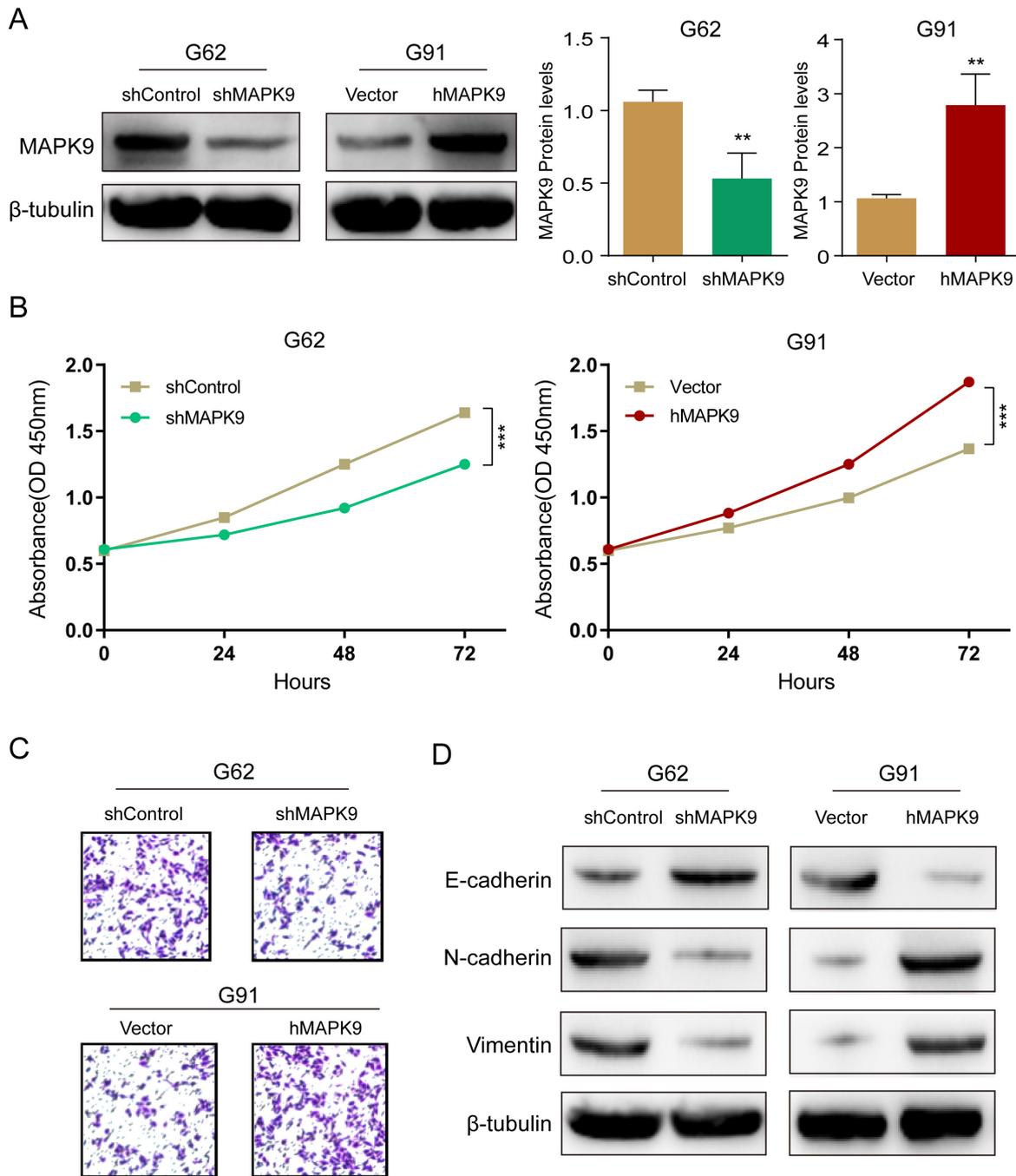
There was a significant decrease in the expression of the  $\beta$ -catenin signaling downstream molecules, CD44, MYC and TCF1 in the *MAPK9* knockdown group and a significant increase in the *MAPK9* overexpressing group (Fig. 4C), indicating that *MAPK9* promotes the progression of glioma cells through  $\beta$ -catenin signaling pathway and its downstream molecules CD44, MYC and TCF1.

#### 4. Discussion

Our study revealed that (1) the expression of *MAPK9* is high in glioma tissues and low in paracancerous tissues; (2) *MAPK9* overexpression is closely associated with WHO grade, IDH mutation and 1p/19q codel status, 3-year survival rate, and 5-year survival rate in glioma, providing new insights into the prediction of glioma prognosis; and (3) *MAPK9* overexpression increases the cell viability as well as the migration and invasion ability of glioma cells. Brain MRI, including T2-weighted sequences, T2-weighted FLAIR sequences, and 3D T1-weighted sequences, is the gold standard for diagnosing brain tumors. Electroencephalography can help monitor tumor-related epilepsy and

determine the cause of altered consciousness [3]. Genetic testing and molecular testing are helpful for the diagnosis and prognosis evaluation of glioma and, to a certain extent, can determine the follow-up treatment. However, genetic testing and molecular testing are not as popular as imaging diagnosis. Most importantly, the number of targets that can be used for clinical testing is too small, and they are not sensitive enough. Therefore, we need to identify target genes and molecules that can be applied in the clinic. Recently, Kim *et al.* [16] collected 18 proneural and 12 mesenchymal HGG tumor specimens. They found six significantly upregulated genes (*MAPK9*, *MLK4*, *LYN*, *MST4*, *VRK2* and *PRKCH*) from 349 protein kinase-encoding genes based on a genome-wide expression dataset for mesenchymal glioma spheres [16], and there is growing evidence that stem cells in these cancers significantly promote cancer initiation and treatment resistance [19]. Therefore, the expression of *MAPK9* in glioma warrants further investigation.

C-Jun amino-terminal kinases (JNKs) belong to the typical mitogen-activated protein kinase (MAPK) family. JNKs regulate multiple functions of cells. Stress, growth factors, and cytokines are common activators of JNK2 (*MAPK9*) expression in humans [20]. *MAPK9* has been shown to be overexpressed in various tumors and to promote tumor progression. Wang *Zet al.* [21] showed that CircRNF20 stimulates the proliferation of non-small cell lung carcinoma (NSCLC) through activation of *MAPK9*.

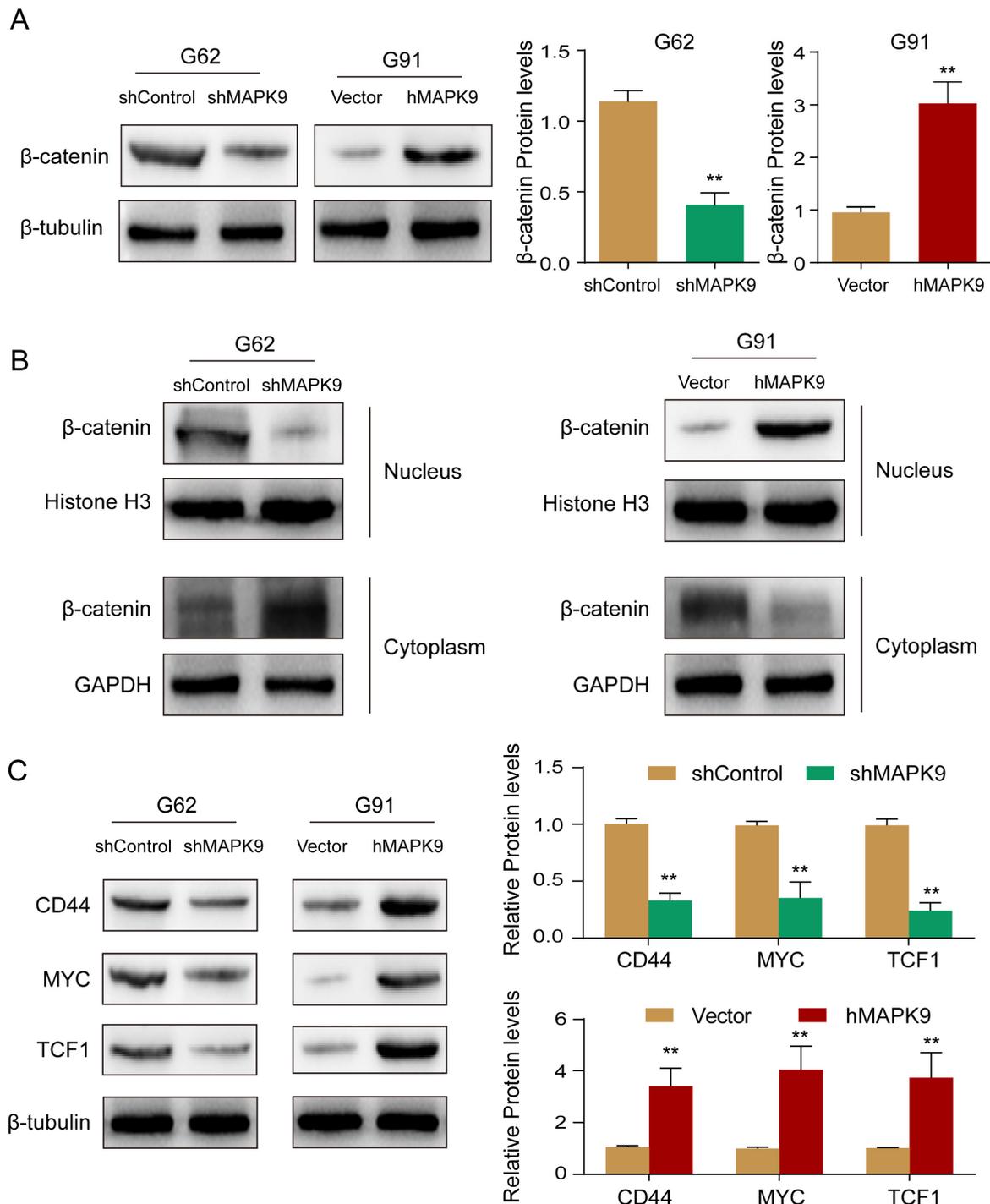


**Fig. 3. Overexpression of MAPK9 increases glioma cell proliferation and migration.** (A) Western blot validation of *MAPK9* knock-down and overexpression in G62 and G91 cell lines,  $**p < 0.01$  vs control group. (B) CCK-8 assay of the growth of *MAPK9* knockdown and overexpression glioma cells,  $***p < 0.001$  vs control group. (C) Transwell assay of indicated glioma cells for migration. (D) The protein expression of E-catenin, vimentin and N-cadherin of indicated groups.

MAPK9 expression is upregulated in NSCLC patients and correlates with T staging, and MAPK9 overexpression significantly promotes the proliferative capacity of NSCLC cells [21]. In human squamous cell carcinoma (SCC), JNK2 is activated in over 70% of cases, and genetic or pharmacological inhibition of JNK2 prevents tumorigenesis in human SCC cells. It is important to note that JNK2 binds to oncogenic Ras and transforms primary human epidermal

cells into SCC malignant tumors [22]. A study by Lili Du *et al.* [23] demonstrated that JNK2 activity promotes the proliferation and invasiveness of melanoma cells.

In our study, MAPK9 expression was first analyzed in relation to the clinical characteristics of 150 glioma patients, and we found that MAPK9 expression was correlated with IDH mutation and the 1p/19q codeletion, MGMT promoter methylation and glioma survival. IDH mutant and 1p/19q codeletion



**Fig. 4. MAPK9 increases glioma cell progression via  $\beta$ -catenin signaling and its downstream molecules.** (A) Typical western blot images showing  $\beta$ -catenin expression of indicated groups,  $**p < 0.01$  vs control group. (B) Typical western blot images showing  $\beta$ -catenin expression in the nucleus and cytoplasm. (C) Typical western blot images showing CD44, MYC and TCF1 expression,  $**p < 0.01$  vs control group.

are molecular phenotypes that have been included in the WHO classification of gliomas in recent years. IDH mutation, 1p/19q codeletion and MGMTp methylation are associated with better survival rate in glioma patients [4]. Our data suggest that MAPK9 is mostly expressed at low levels in IDH mutant and 1p/19q codeletion cases, whereas it is highly

expressed ( $p < 0.001$ ), which indicates that MAPK9 may have a certain diagnostic significance in glioma classification and prognosis. MAPK9 can predict prognosis independent of IDH mutation status, 1p/19q codeletion status and MGMTp methylation status. The results of western blotting and immunohistochemistry showed that MAPK9 ex-

pression in glioma tissue was obviously higher than that in normal tissue. This finding is in agreement with the observations of Cyrillo G *et al.* [17] using the MAPK9 inhibitor RGB-286638; these studies demonstrated decreased viability in multiple cell culture models of GBM, and there was some evidence that RGB-286638 can retard tumor growth.

Although there has been extensive research, the mechanism by which MAPK9 promotes tumor progression has not been elucidated and may have different pathways of action in different cancers. In our *in vitro* experiments, we demonstrated that *MAPK9* knockdown suppressed the tumor phenotype of primary glioma cells. Epithelial-mesenchymal transition (EMT) and  $\beta$ -catenin signaling pathway have been shown to be important in tumor progression [24,25]. EMT is an important process by which epithelial cells lose their cellular polarity and adhesion and thus transform into mesenchymal cells, which have a more aggressive or metastatic phenotype. The high invasive and migratory capacity resulting from EMT often limits overall surgical resection [26].  $\beta$ -Catenin was originally described as a membrane adhesion protein of the calmodulin-mediated cell-cell adhesion system. MAPK9 mediates  $\beta$ -catenin phosphorylation at Ser191 and Ser605, and nuclear localization of  $\beta$ -catenin promotes Wnt signaling, which is important for the regulation of cell proliferation [27–29]. In the current study, we found that upon knockdown of *MAPK9*,  $\beta$ -catenin transcriptional activity was inhibited, and  $\beta$ -catenin transcript levels were decreased.

Wnt/ $\beta$ -catenin is the classical pathway of glioma progression [30]. In this pathway, Wnt combines with the receptor proteins frizzled, LRP5/6 and casein kinase-1 to form a ternary cell surface complex that drives glycogen synthase kinase 3 $\beta$  phosphorylation and inactivation, preventing the phosphorylation and degradation of stable  $\beta$ -catenin. In the cytoplasm, stable  $\beta$ -catenin penetrates farther into the nucleus and combines with the transcription factor T-cell factor/lymphatic enhancer factor [31,32], leading to the transcription of molecules such as Wnt target genes MYC, CD44, and TCF1 [33,34]. Our findings show that MAPK9 increases the expression of downstream molecules of  $\beta$ -catenin (CD44, MYC, and TCF1), which facilitate glioma progression. It is possible that the Wnt/ $\beta$ -catenin pathway plays a significant role in this process.

In summary, MAPK9 may be a meaningful prognostic predictor in glioma and a molecular diagnostic target for glioma.

## 5. Conclusion

Our results showed that MAPK9 expression was significantly increased in glioma tissues compared to paraneoplastic tissues. By knocking down and overexpressing the MAPK9 gene in G62 and G91 cell lines, it was demonstrated that MAPK9 may have a facilitative effect on glioma development by activating the Wnt/ $\beta$ -catenin-regulated EMT signaling pathway. The detection of

MAPK9 could be used as a strategy to diagnose glioma and predict glioma prognosis.

## Availability of Data and Materials

All data generated or analyzed during this study are included in this published article. Some of the datasets generated and/or analyzed during the current study are available in the CGGA repository, [<http://www.cgga.org.cn/index.jsp>].

## Author Contributions

LC and DF conceived and designed the experiments; XY, XL, QJ, WW and ZZ performed the experiments; JS, XY, QJ and YH analyzed the data; XY and ML wrote the manuscript. LC gave the final approval for the manuscript to be published. The manuscript has been read and approved by all the authors, that the requirements for authorship have been met, and that each author believes that the manuscript represents honest work.

## Ethics Approval and Consent to Participate

The glioma tissue collection was approved by the Ethics Committee of the General Hospital of Northern Command (NO. (2022)028). Each patient has written an informed consent to use part of the tissue for scientific research. All experimental schemes are in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

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## Conflict of Interest

The authors declare no conflict of interest.

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