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ORIGINAL ARTICLE

High expression of *NLRP12* predicts poor prognosis in patients with intracranial glioma

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Abstract

Background: Intracranial gliomas are the most common primary central nervous system tumors in humans, and glioblastoma multiforme is the most malignant intracranial glioma. The nucleotide-binding domain leucine-rich repeat (NLR)-containing family are crucial regulators of inflammatory and innate immune responses. *NLRP12* codes for the monarch-1 protein, which regulates immune responses in humans. Data from a next-generation sequencing database indicated that *NLRP12* expression is increased in glioma cells. However, the relationship between NLRP12 levels and gliomas is unclear.

Methods: To explore the role of *NLRP12*-related translation factors and proteins in glioma, we evaluated the clinical data and paraffin sections from glioma patients. The expression of NLRP12 was evaluated using immunohistochemical analysis, and clinical parameters were analyzed using chi-square and Kaplan–Meier survival tests.

Results: The degree of malignancy and prognosis highly correlated with NLRP12 levels. In addition, the siRNA-mediated downregulation of *NLRP12* in glioma cell lines decreased proliferation, invasion, and migration. The levels of VEGF, N-cadherin, and cyclin D1 were downregulated after knockdown of NRLP12 in glioma cell lines, as observed using western blotting *in vitro*. Knockdown of NLRP12 attenuated the tumor progression *in vivo*.

Conclusion: The expression of *NLRP12* may be an independent prognostic factor and a potential target for the treatment of intracranial glioma.

Keywords: Biomarker; Immunohistochemistry; Intracranial glioma; *NLRP12*; Prognostic factor; Western blot

1. INTRODUCTION

Intracranial gliomas are the most common primary central nervous system tumors in humans[1] and are divided into four grades according to their pathological characteristics.[2] Grade I and grade II are low-grade gliomas (LGGs), while grade III and grade IV are high-grade gliomas. Grade IV gliomas, also known as glioblastoma multiforme (GBM), are the most aggressive and lethal type of glioma in humans.[3–5] The recurrence rate of GBM is high (>90%),[6,7] even after maximal surgical resection. Concurrent chemoradiotherapy (CCRT, Temodal, and radiation) significantly increases the survival rate and is included in the standard treatment guidelines.[8] In addition, post-operation conjugative therapy, such as CCRT and immunotherapy, is beneficial. However, the overall survival rate is still dismal; the average survival following diagnosis is 1.5 years.[9,10] Therefore, identifying prognostic biomarkers for glioma and elucidating the underlying pathogenic mechanism are crucial for the development of effective personalized treatments.[11]

The nucleotide-binding domain leucine-rich repeat (NLR)-containing family are crucial regulators of inflammatory and innate immune responses.[12,13] *NLRP12*, which is located on chromosome 19, encodes the monarch-1 protein. NLRP12 regulates immune reactions mainly in the cytoplasm of white blood cells, including NF-kB signaling, inflammasome activation, dendritic cell migration, and the transcription of MHC class I genes.[12,14,15] NLRP12 inhibits NF-κB signaling and alleviates inflammation in an *in vivo* model of colorectal cancer. The absence of the *NLRP12* inflammasome supports tumor neogenesis in colorectal cancer. Thus, *NLRP12* is considered a tumor suppressor gene.[14] Patients with glioma exhibit immune deficiency, and the tumor microenvironment tends to be immunosuppressive.[16] However, to our

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knowledge, few studies have examined the relationship between *NLRP12* expression and glioma development. Therefore, this study aimed to determine the correlation between NLRP12 protein expression and the clinicopathological features of gliomas.

2. METHODS

2.1. Extraction and analysis of The Cancer Genome Atlas datasets

The relationships between *NLRP12* mRNA expression and the clinicopathological characteristics (patient's race, gender, age, tumor grade, sample type, histological subtype, and survival time) were determined at the University of Alabama at Birmingham Cancer data analysis Portal (UALCAN) (http://ualcan.path.uab.edu). Transcripts per million (TPM) were compared between each group according to race, gender, age, tumor grade, histological subtype, and sample types. NLRP12 expression was divided into high expression (TPM values above the upper quartile) and low/median expression (TPM values below the upper quartile) groups.

2.2. Specimen collection and pathologic classification

One hundred patients, who underwent surgical excision for intracranial glioma at Kaohsiung Medical University Hospital between 2005 and 2015, were enrolled in the study. However, five patients were only biopsies or loss of follow-up data. Finally, 95 patients were involved in this study. Clinical information for all patients was collected from the medical records. Specimens were obtained and analyzed under a standard pathological processing protocol. All slides of glioma specimens were stained using hematoxylin and eosin to confirm the diagnosis and pathological grade. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUHIRB-E(I)-20200351).

2.3. Immunohistochemistry staining

Each tissue block was fixed in formalin, embedded in paraffin, and cut into 3-μm thick sections. The sections were deparaffinized, rehydrated, and autoclaved at 121°C for 10 min in Target Retrieval solution (pH 6.0) (DAKO; S2369). After 20 minutes at room temperature, endogenous peroxidase was blocked by treating it with 3% hydrogen peroxide for 5 minutes. The sections were washed twice with Tris buffer and incubated with anti-NLRP12 antibody (1:200; ABclonal, MA) for 1 hour at room temperature. The sections were washed twice with Tris buffer and incubated with horseradish peroxidase-conjugated secondary antibody for 30 minutes at room temperature. Finally, the slides were incubated in 3,3-diaminobenzidine (Dako; K5007) for 5 minutes, counterstained with Mayer's hematoxylin for 90 seconds, and mounted with Malinol. Tumor cells were scored based on the percentage and intensity of NLRP12 immunohistochemistry (IHC) staining, as follows: grade 0 (none), 1 (1%-9%), 2 (10%-50%), and 3 (>50%), based on positive staining; score 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining), based on staining intensity. The staining index was calculated by multiplying the intensity and percentage of positive tumor cells (score range 0-9). High expression of NLRP12 was defined as a score ≥4, and low expression was defined as a score ≤3.

2.4. Cell culture

All cell lines were obtained from the American Type Culture Collection Cell Line Bank and cultured in 5% CO_2 at 37°C. The GBM8401, GBM8901, and DBTRG-05MG cells were cultured in RPMI medium supplemented with 10% FBS. The U87-MG and SVGp12 cells were cultured in minimum essential medium supplemented with 10% FBS. The G5T/VGH, Hs683, and A172 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The M059K cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% FBS. The GBM8401, GBM8901, U87-MG, G5T/VGH, DBTRG-05MG, M059K, Hs683, and A172 cells were established from tissues excised from patients with GBM, and the SVG cell line was established from normal brain tissue, as a normal control.

2.5. Transfection

Glioma cells were transfected with NLRP12 siRNA using DharmaFECT transfection reagents (Dharmacon). The following siRNA sequences were used: human NLRP12 siRNA #1 (Sigma): 5′-GCUUGUCAGGAGAUGGCUUdTdT-3-′; human NLRP12 siRNA #2 (Sigma): 5′-CACCAACCCACAUCUGGUUdTdT-3′; and human negative control siRNA (Sigma): 5′-GAUCAUACGUGCGAUCAGAdTdT-3′. Cells were transfected with 5 μM siRNA and cultured for 2 days after transfection. NLRP12 expression in the transfected cells was evaluated using western blot analysis.

2.6. Western blotting

The samples were lysed in 200 μL lysis buffer; 50 μg of samples were loaded into the wells of an SDS-PAGE system and electrophoresed at 50 V for 4 h. The proteins were transferred to PVDF membranes. After blocking membranes for 1 h, the membranes were incubated with the respective primary antibody. The antibodies used for western blotting included anti-NLRP12 (1:200; ABclonal, A6671), anti-N-cadherin (1:1000; Proteintech; 22018-1-AP), anti-VEGF (1:500; ABGENT; AP6290b), anti-E-cadherin (1:1000; Proteintech; 22874-1-AP), anti-β-actin (1:20000; SIGMA; A5441), and anti-cyclin D1 (1:1000; Thermo; RM-9104). Membranes were incubated with the primary antibody for 2 h at room temperature, followed by incubation with the corresponding secondary antibody (goat anti-rabbit, 1:5000, Millipore, AP132P or goat anti-mouse, 1:5000, Millipore, AP124P) for 90 min. An enhanced chemiluminescence solution (Western Lightning; 205-14621) was used for detecting specific bands in a MINICHEMI (Thermo) system. Each western blot analysis was repeated thrice.

2.7. Cell viability

The GBM8401 and U87-MG cells were seeded into 24-well plates at 3×10^4 cells and 0.5 ml per well of RPMI medium without serum and cultured at 5% CO_2, saturated humidity, and 37°C for 24 h. The cells were co-cultured with 5 μM NLRP12 siRNA for 24 h, 48 h, and 72 h, and then counted using an MTT assay.

2.8. In vitro invasion assay

The in vitro cell invasion assay was performed using a Transwell invasion assay kit (CORNING; COR3452). Cells were seeded at 5×10^3 cells per insert, and the lower chamber of the Transwell was filled with 0.2 ml medium with nonsense siRNA (negative control) or NLRP12 siRNA. After 24 h of incubation, cells remaining on the upper surface of the Transwell membrane were removed with a cotton swab. Cells that passed through the Transwell to the bottom of the insert were fixed, stained, photographed, and quantified by counting the cells in six randomly selected high-power fields. The counting cells in every groups were normalization with control group.

2.9. In vitro migration assay

Cell invasion was analyzed using a wound healing assay (ibidi; 80209) in 6-well plates coated with culture inserts (Indio: LOT150831) and cultured at 37°C for 12 h. The cells were seeded at 3.5×10^5 cells per insert, and siRNA was added after 24 h. Cells were cultured and photographed at 6, 12, and 24 h. The area with cell migration was calculated following these pictures.

2.10. Animal model

GBM8401 cells including fluorescent (1×10^5 cells in 5 μl) were implanted intracranially in the striatum of immunodeficient mice from LASCO Laboratory Animal Center (Taipei, Taiwan). The animal protocol was approved by the Committee of Institutional Animal Research at Kaohsiung Medical University (IACUC 109082). All rats were housed at a constant temperature (24°C) and regular light/dark cycles (12 h/12 h), with free access to a standard diet. The control group was injected with GBM8401 cells (n = 12) and the knockdown NLRP12 group was injected with knockdown NLRP12 GBM8401 cells (NLRP12 shRNA: 5′-CACTCGGCTTCTCCTAGTAA-3′) (n = 12). Mice were anesthetized with isoflurane and detected the fluorescent using the Xenogen IVISR Spectrum Noninvasive Quantitative Molecular Imaging System (J&H; IVIS Lumina LT 2D) at 7, 14, and 21 days after injecting with GBM cells.

2.11. Data analysis

SPSS 24.0 (IBM, NY) software was used for statistical analysis. The chi-square test was used to determine the correlation between the expression of NLRP12 and specific clinicopathologic characteristics. The survival rate was analyzed using the Kaplan–Meier method with the log-rank test. Multivariate Cox regression analysis was used to verify the independent effects of each variable. A one-way analysis of variance was used to compare the results of the proliferation, migration, invasion, and western blot assays. For all analyses, a p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. NLRP12 was identified as the target using a bioinformatics approach

Patient data from the Cancer Genomic Atlas (TCGA) were analyzed using the UALCAN web resource and *NLRP12* gene that significantly influenced the overall survival in both LGG and glioblastomas (GBM) were identified.

In the TCGA database, *NLRP12* expression in LGG was the most variable in African Americans and the most consistent in Asians and Caucasians (Fig. 1A). No significant differences in *NLRP12* expression were detected between men and women (Fig. 1B). *NLRP12* expression in LGG tended to increase with age (Fig. 1C). *NLRP12* expression in grade 3 gliomas tended to be higher than the expression in grade 2 gliomas, but the difference was not statistically significant (Fig. 1D). No significant differences in *NLRP12* expression were detected between astrocytoma and oligoastrocytoma, but the expression in oligodendroglioma was lower (Fig. 1E). Patients with LGG in the high *NLRP12* expression group had longer survival than patients in the low/medium *NLRP12* expression group (Fig. 1F). In patients with GBM, the expression of *NLRP12* was significantly higher in primary tumors compared with expression in normal tissues (Fig. 1G). The average *NLRP12* expression in GBMs from African Americans was much higher than the expression in other ethnic groups (Fig. 1H). *NLRP12* expression in GBM had a higher degree of variation in females compared with the expression variation in males (Fig. 1I). *NLRP12* expression tended to increase with age in GBM (Fig. 1J). Higher *NLRP12* expression was significantly associated with poor prognosis in GBM (Fig. 1K).

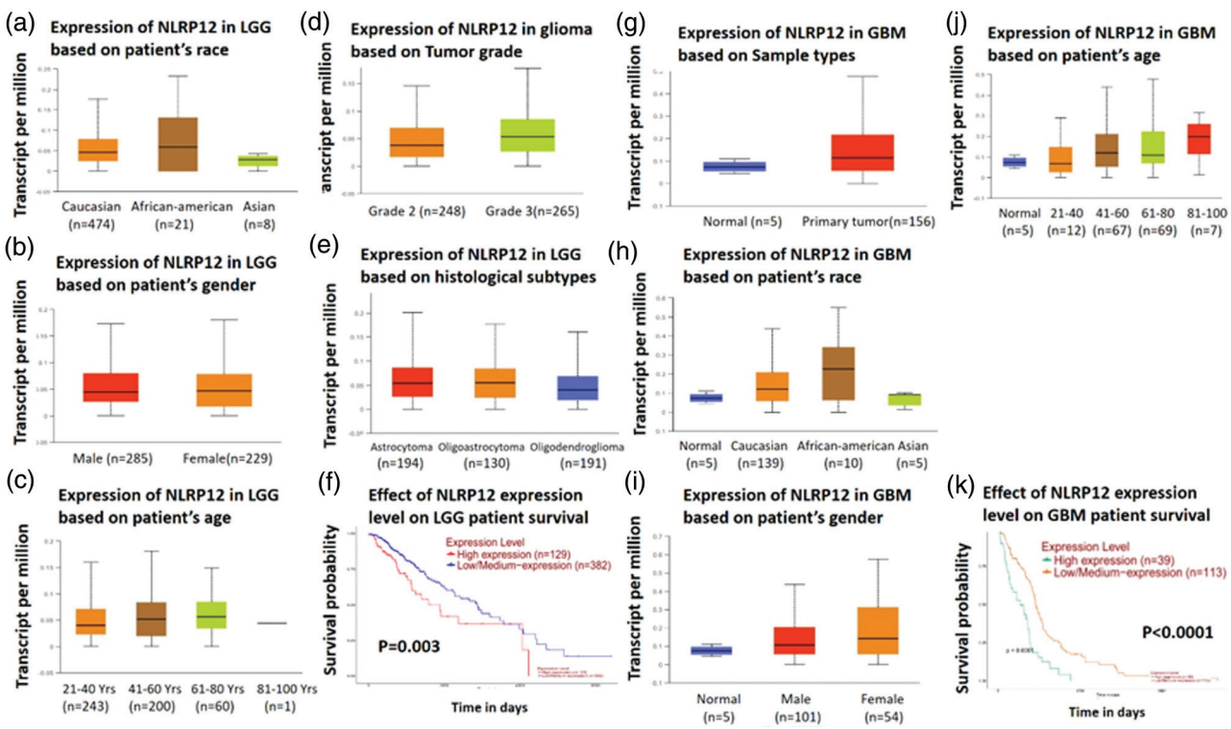

Fig. 1 The expression of NLRP12 in low-grade glioma (LGG) and glioblastoma multiforme (GBM) in TCGA samples and the survival analysis of glioma patients. A, NLRP12 expression of LGG in Caucasians, African Americans, and Asians. B, NRLP12 expression of LGG in male and female. C, NLRP12 expression of LGG in different ages. D, NLRP12 expression in grade 3 gliomas and grade 2 gliomas. E, The expression of NLRP12 in LGG between astrocytoma, oligoastrocytoma, and oligodendroglioma. F, The prognosis of LGG between patients with high and low/medium NLRP12 expression. G, NLRP12 expression in primary tumors (GBM) and normal tissues. H, NLRP12 expression of GBM in Caucasians, African Americans, and Asians. I, NRLP12 expression of GBM in male and female. J, NLRP12 expression of GBM in different ages. K, The prognosis of GBM between patients with high and low/medium NLRP12 expression. GBM = glioblastoma multiforme; TCGA = the Cancer Genomic Atlas.

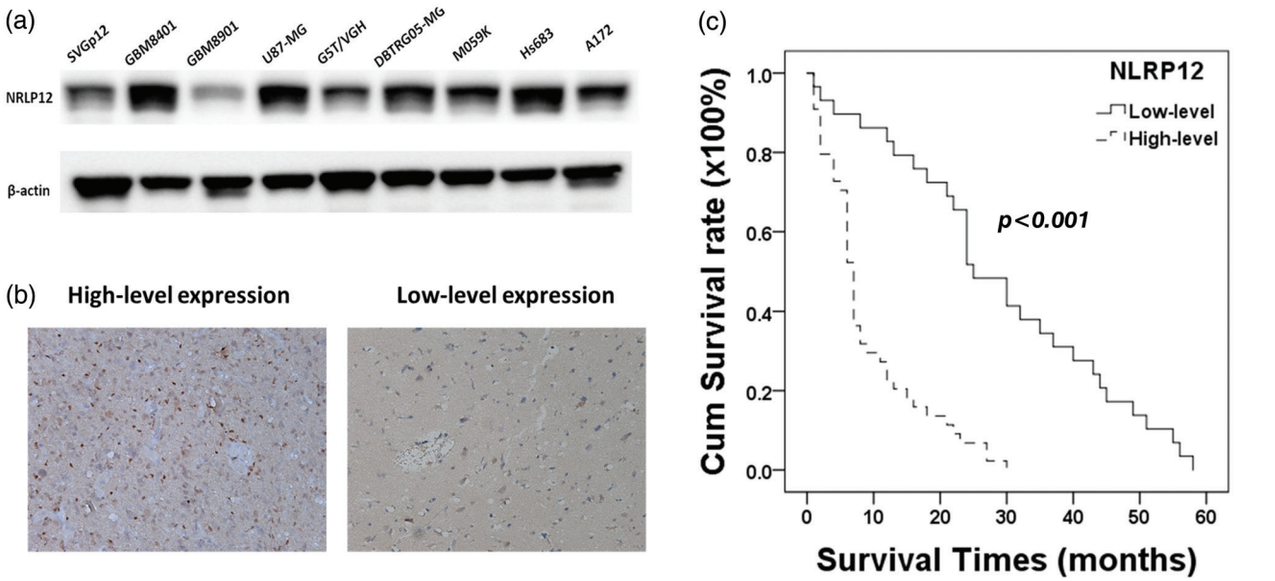

Fig. 2 NLRP12 protein levels in all GBM cell lines and glioma patients. A, Western blot of NLRP12 in all GBM cells and glial cells. SVGp12 was glial cell. GBM8401, GBM8901, U87-MG, G5T/VGH, DBTRG05-MG, M059K, Hs683, and A172 were GBM cells. B, IHC staining for high- and low-level expression in glioma patients. C, Kaplan–Meier analysis for overall survival following the IHC staining score of NLRP12 in glioma patients. GBM = glioblastoma multiforme; IHC = immunohistochemistry.

3.2. High NLRP12 expression in GBM cells

NLRP12 expression was evaluated in different glioma cell lines (GBM8401, GBM8901, U87-MG, G5T/VHG, DBTRG-05MG, Hs683, M059K, and A172) and normal primary human astrocytes (SVGp12). NLRP12 expression in GBM8401, U87-MG, G5T/VHG, DBTRG-05MG, Hs683, M059K, and A172 cells was significantly higher than expression in SVGp12 cells (Fig. 2A). Therefore, NLRP12 might play an important role in the development of gliomas. In addition, NLRP12 protein expression was higher in GBM8401 and U87-MG cells compared with expression in the other GBM cell lines. Therefore, GBM8401 and U87-MG were used to detect the tumor progression with NLRP12 siRNA. In GBM8401 cells, western blotting revealed that NLRP12 knockdown (48 h) decreased NLRP12 protein expression using both si-NLRP12#1 (p = 0.045) and si-NLRP12#2 (p = 0.026) compared with expression after transfection with the nonsense siRNA (negative group) (Fig. 3A). In U87MG cells, NLRP12 knockdown also decreased NLRP12 protein expression with both si-NLRP12#1 (p < 0.001) and si-NLRP12#2 (p < 0.001) (Fig. 3B).

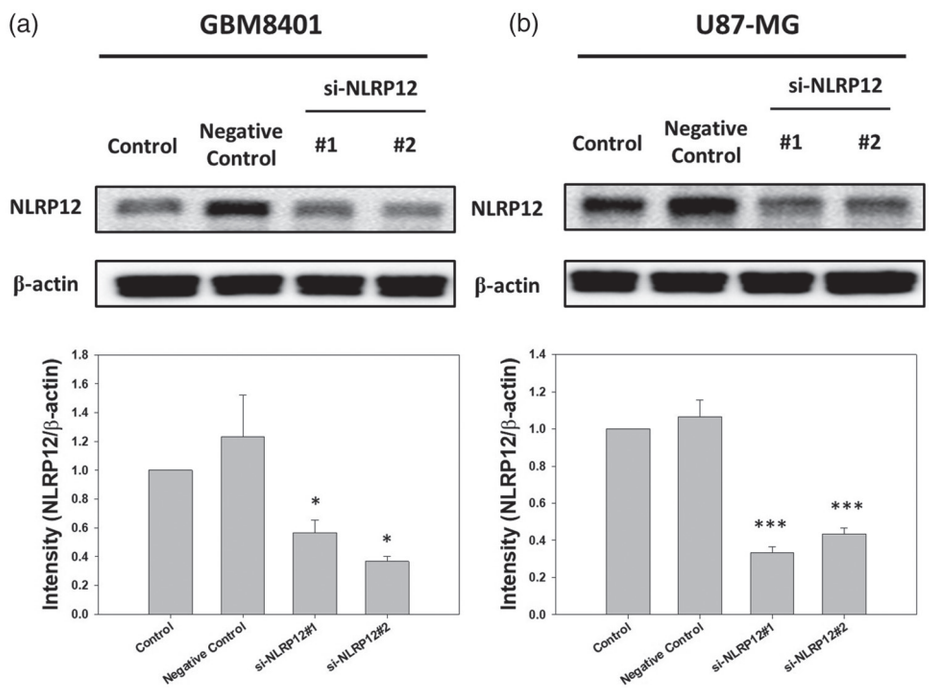

Fig. 3 Protein expression of NLRP12 following transfection with NLRP12 siRNA in GBM cells. NLRP12 expression in GBM8401 cells (A) and U87MG cells (B) in the control, negative control, si-NLRP12 #1, and si-NLRP12 #2 groups. *p < 0.05 and ***p < 0.001 compared with the control group. GBM = glioblastoma multiforme.

3.3. Survival analysis in glioma patients

Ninety-five patients from Kaohsiung Medical University Hospital who were diagnosed with intracranial gliomas were enrolled in the study (Table 1). Age, gender, World Health Organization (WHO) pathological classification, tumor size, whether they received chemotherapy and radiotherapy, and Karnofsky performance score quality of life index were evaluated. The tumors of most patients were classified as high-grade malignant tumors and grade III and grade IV gliomas (75.8%). The proportions of patients receiving radiotherapy and chemotherapy were 43.2% and 39.7%, respectively. Patients were divided into low and high NLRP 12 expression groups, based on IHC staining (Fig. 2B).

The clinicopathological characteristics in the low and high NLRP12 expression groups are summarized in Table 1. LGGs were associated with low NLRP12 expression (18.9% of the 95 glioma patients), while the high-grade malignancies were associated with high NLRP12 expression (61.1%) ($p < 0.001$). No significant differences in the other clinicopathological characteristics were detected. Kaplan–Meier analysis and the subsequent log-rank analysis confirmed the correlation between NRLP12 expression and survival in patients with gliomas (Fig. 2C). Specifically, the overall survival rate was significantly lower in patients with high NRLP12 expression compared with survival in patients with low NRLP12 expression ($p < 0.001$). Univariate analysis identified both the WHO grade ($p = 0.001$) and NLRP12 expression level ($p = 0.001$) as significant prognostic factors. Multivariate Cox regression analysis implicated the WHO grade (HR, 0.541; 95% CI, 0.292-1.101; $p = 0.051$) and NLRP12 expression level (HR, 0.234; 95% CI, 0.124-0.440; $p < 0.001$) as independent factors in the prognosis of patients with glioma (Table 2).

3.4. Knockdown of NLRP12 attenuated GBM cell proliferation

We evaluated the viability of GBM8401 and U87-MG cells and compared the differences in viability between cells transfected with si-NLRP12 and nonsense siRNAs (Fig. 4). After 24-, 48-,

Table 1

Correlation of NLRP12 expression with clinicopathologic parameters in patients with glioma

		NLRP12 expression, n (%)		
	No. of patients	Low	High	*p*
Age (y)				0.628
>60	24	7 (7.4%)	17 (17.9%)	
≤60	71	25 (26.3%)	46 (48.4%)	
Sex				0.831
Male	52	17 (17.9%)	35 (36.8%)	
Female	43	15 (15.8%)	28 (29.5%)	
WHO grade				<0.0001
II	23	18 (18.9%)	5 (5.3%)	
III/IV	72	14 (14.7%)	58 (61.1%)	
Tumor size				1
≤3 cm	59	20 (21.1%)	39 (41.1%)	
>3 cm	36	12 (12.6%)	24 (25.3%)	
Radiotherapy				0.664
No	54	17 (17.9%)	37 (38.9%)	
Yes	41	15 (15.8%)	26 (27.4%)	
Chemotherapy				0.379
No	59	22 (23.2%)	37 (38.9%)	
Yes	36	10 (10.5%)	26 (27.4%)	
KPS				0.482
≤70	67	21 (22.1%)	46 (48.4%)	
>70	28	11 (11.6%)	17 (17.9%)	

KPS = Karnofsky performance score; WHO = World Health Organization.

Table 2

Univariate and multivariate Cox regression analyses of prognostic parameters in patients with glioma

	Univariate analysis			Multivariate analysis		
	Relative risk	95% CI	*p*	Relative risk	95% CI	*p*
Age	0.672	0.386-1.169	0.160			
Sex	0.737	0.459-1.182	0.250			
WHO grade	2.680	1.515-4.741	0.001	0.541	0.292-1.001	0.051
Tumor size	1.313	0.797-2.164	0.284			
Radiotherapy	1.225	0.767-1.957	0.396			
Chemotherapy	1.604	0.626-1.610	0.987			
KPS	1.610	0.956-2.710	0.073			
NLRP12 expression	0.196	0.107-0.360	<0.001	0.234	0.124-0.440	<0.001

CI = confidence interval; KPS = Karnofsky performance score; WHO = World Health Organization.

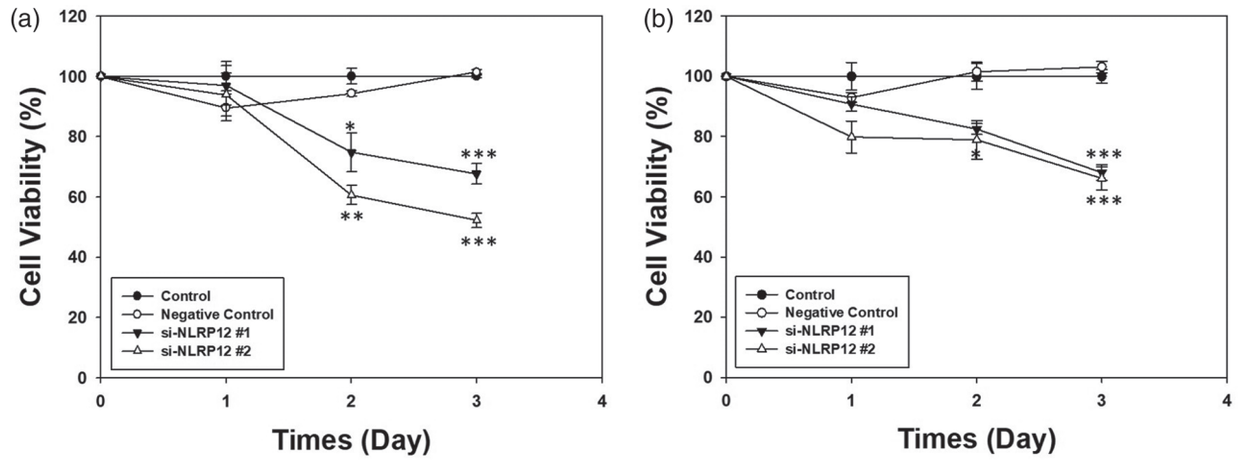

Fig. 4 Cell viability following transfection with NLRP12 siRNA in GBM cells. A, Cell viability in control, negative control, si-NLRP12 #1, and si-NLRP12#2 groups 24, 48, and 72 h after transfection in GBM8401 cells. B, Cell viability in control, negative control, si-NLRP12 #1, and si-NLRP12 #2 groups 24, 48, and 72 h after transfection in U87-MG cells. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with the control group. GBM = glioblastoma multiforme.

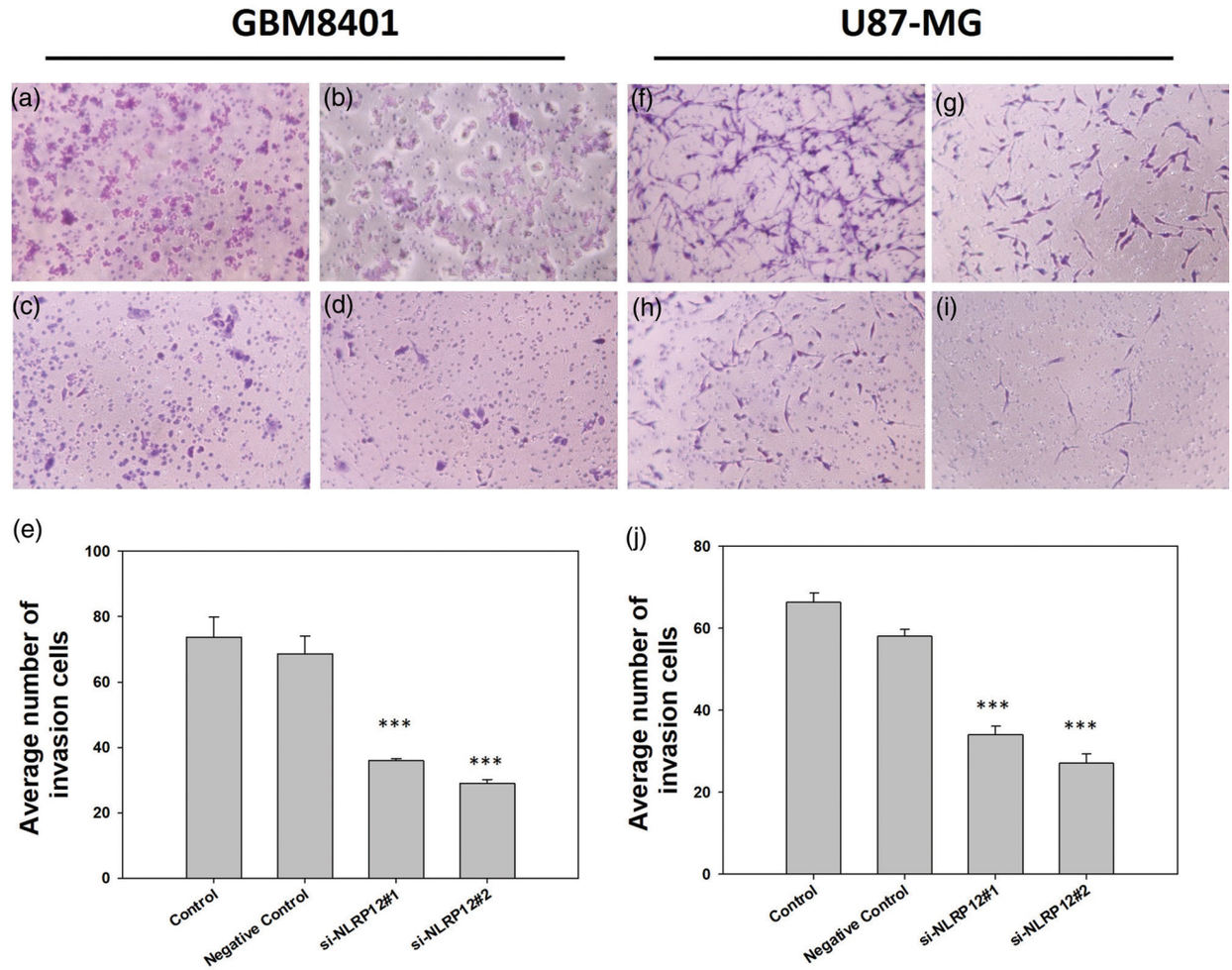

Fig. 5 Single-day Transwell invasion analysis following transfection with NLRP12 siRNA in GBM cells. Representative GBM8401 cells images from the (A) Control group, (B) Negative Control group, (C) si-NLRP12 #1 group, and (D) si-NLRP12 #2 group. E, The number of invading GBM8401 cells. Representative U87MG cells images from the (F) Control group, (G) Negative Control group, (H) si-NLRP12 #1 group, and (I) si-NLRP12 #2 group. J, The number of invading U87-MG cells. ***$p < 0.001$ compared with the control group. GBM = glioblastoma multiforme.

and 72-hour incubations with siRNA, cell viability was assessed using the MTT assay. Viability was significantly decreased in GBM8401 cells after transfection with si-NLRP12 #1 and si-NLRP12 #2 compared with viability in the control group at 48 h (NLRP12 #1: $p = 0.045$; NLRP12 #2: $p = 002$) and 72 hours (NLRP12 #1: $p < 0.001$; NLRP12 #2: $p < 0.001$) (Fig. 4A). In U87-MG cells, the viability decreased significantly after transfection with si-NLRP12 #1 and si-NLRP12 #2 compared with viability in the control group at 72 h (NLRP12 #1: $p < 0.001$; NLRP12 #2: $p < 0.001$) (Fig. 4B); however, 48 h after transfection, cell viability was significantly decreased only in the si-NLRP12 #2 group ($p = 0.035$), not the si-NLRP12 #1 group ($p = 0.077$) (Fig. 4B).

3.5. Knockdown of NLRP12 attenuated GBM cell invasion

Differences in cell invasion between the control and si-NLRP12 transfection groups were compared using a Matrigel invasion assay (Fig. 5). In both GBM8401 and U87-MG cells, siRNA-induced NLRP12 knockdown with both si-NLRP12 #1 and si-NLRP12 #2 markedly reduced cell invasiveness (GBM8401: si-NLRP12 #1, $p < 0.001$ and si-NLRP12 #2, $p < 0.001$; U87-MG: si-NLRP12 #1, $p < 0.001$ and si-NLRP12 #2, $p < 0.001$). These results indicate that NRLP12 knockdown inhibited the invasion of GBM cells.

3.6. Knockdown of NLRP12 inhibited GBM cell migration

Differences in cell migration between the control and si-NLRP12 groups were compared using a wound healing assay (Fig. 6). In GBM8401 cells, siRNA-induced NLRP12 knockdown with both si-NLRP12 #1 and si-NLRP12 #2 markedly reduced cell migratory ability 24 h after transfection (NLRP12 #1, $p < 0.001$; NLRP12 #2, $p < 0.001$). Migratory ability was significantly decreased after transfection with si-NLRP12 ($p = 0.031$), but not si-NLRP12 #1 ($p = 0.069$), 12 h after transfection in GBM8401 cells. In U87-MG cells, the migratory ability was significantly decreased 24 h after transfection with both si-NLRP12 #1 and si-NLRP12 #2 (NLRP12 #1, $P = 0.006$; NLRP12 #2, $p = 0.001$). The migratory ability was significantly decreased 12 h after transfection with si-NLRP12 #2 ($p = 0.025$), but not si-NLRP12 #1 ($p = 0.079$).

3.7. Knockdown of NLRP12 downregulated the expression of biomarkers associated with malignant changes in GBM

The effects of NLRP12 on VEGF, N-cadherin, and cyclin D1, which are associated with carcinogenesis, were evaluated using western blotting. In GBM8401 cells, the expression levels of VEGF (si-NLRP12 #1, $p < 0.001$; si-NLRP12 #2, $p < 0.001$), N-cadherin (si-NLRP12 #1, $p < 0.001$; si-NLRP12 #2, $p < 0.001$), and cyclin D1 (si-NLRP12 #1, $p < 0.001$; si-NLRP12 #2, $p < 0.001$) were significantly downregulated and the expression of E-cadherin (si-NLRP12 #1, $p < 0.001$; si-NLRP12 #2, $p < 0.001$) was significantly upregulated after transfection compared with expression levels in controls (Fig. 7A). In U87-MG, the expression levels of VEGF (si-NLRP12 #1, $p < 0.001$; si-NLRP12 #2, $p < 0.001$), N-cadherin (si-NLRP12 #1, $p < 0.001$; si-NLRP12 #2, $p < 0.001$), and cyclin D1 (si-NLRP12 #1, $p < 0.001$; si-NLRP12 #2, $p < 0.001$) were also significantly downregulated and the expression of E-cadherin (si-NLRP12 #1, $p = 0.001$; si-NLRP12 #2, $p = 0.005$) was significantly upregulated after transfection compared with expression levels in controls (Fig. 7B). These results suggest that the downregulation of NLRP12 inhibits carcinogenesis in gliomas.

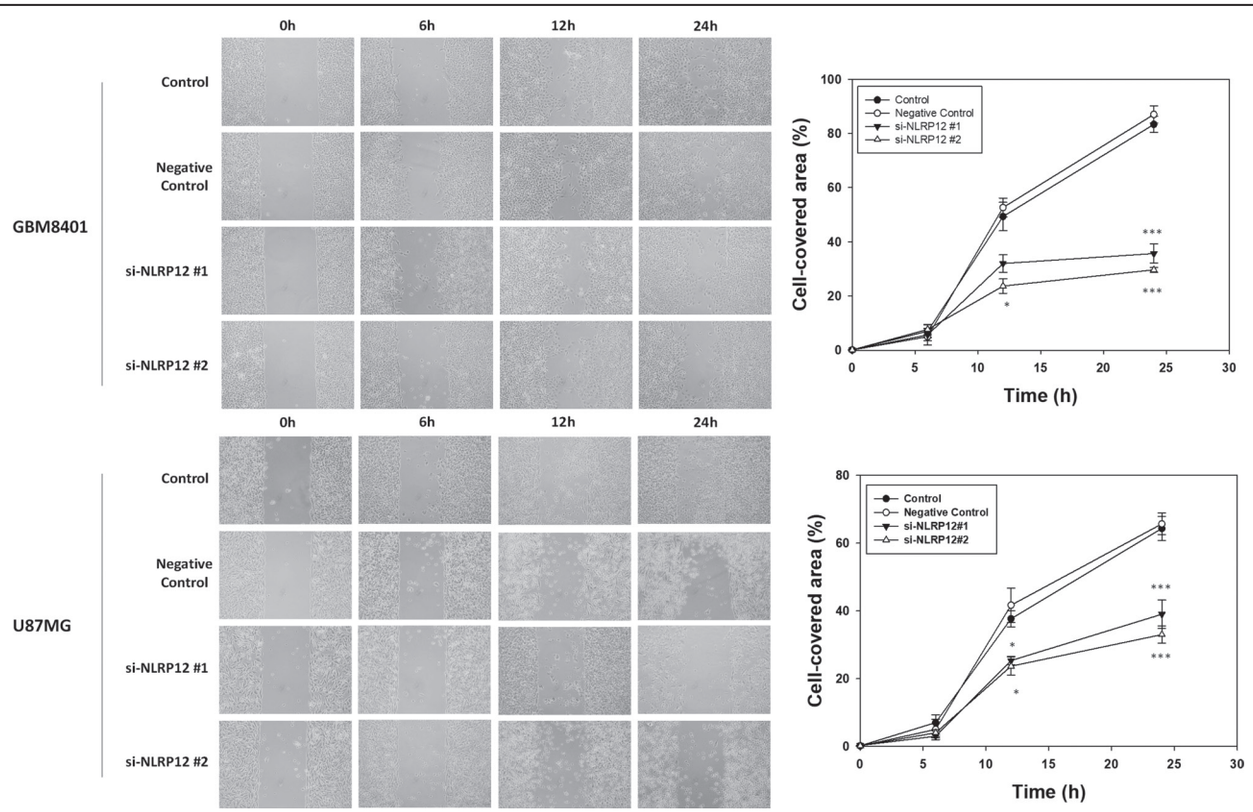

Fig. 6 Wound healing analysis following transfection with NLRP12 siRNA in GBM cells. The wound healing assay in GBM8401 and U87MG cells in the control, negative control, si-NLRP12 #1, and si-NLRP12#2 groups 0, 6, 12, and 24 h after transfection. *$p < 0.05$ and ***$p < 0.001$ compared with the control group at 12 and 24 h. GBM = glioblastoma multiforme.

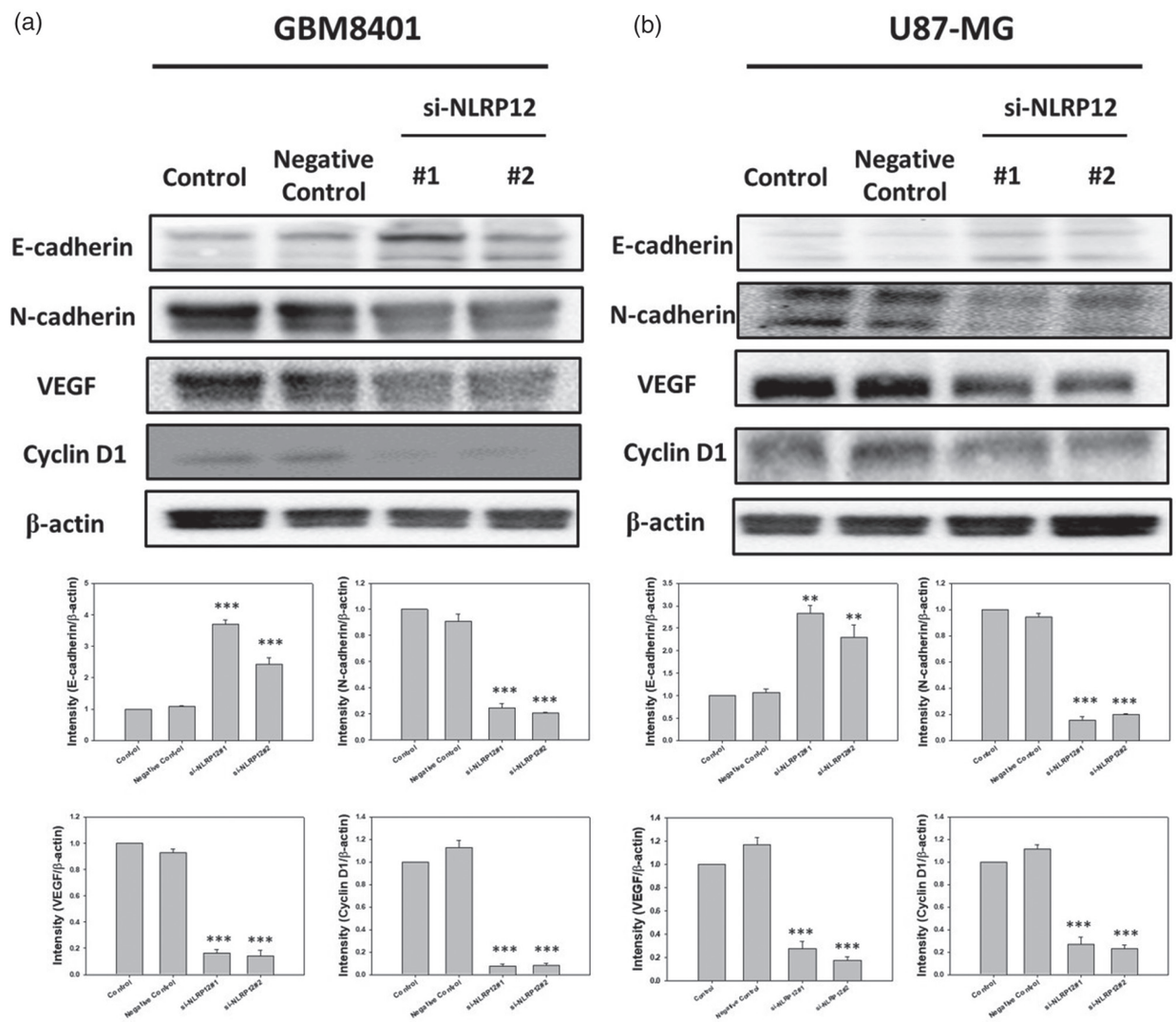

Fig. 7 Western blotting analysis of E-cadherin, N-cadherin, VEGF, and Cyclin D1 following transfection with NLRP12 siRNA in GBM cells. Following transfection with NLRP12 siRNA, the protein expression of E-cadherin, N-cadherin, VEGF, and Cyclin D1 were measured in the control, negative control, si-NLRP12 #1, and si-NLRP12#2 groups in (A) GBM8401 and (B) U87-MG. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the control group. GBM = glioblastoma multiforme.

3.8. Knockdown of NLRP12 attenuated the growth of GBM cells in vivo

An animal model with tumor injection was used to examine the *in vivo* function of NLRP12. The fluorescence intensity of mice injected with knockdown NLRP12 cells was significantly lower than the fluorescence intensity of mice in the control group (Fig. 8A). The survival time of mice injected with knockdown NLRP12 cells (26.83 + 3.00 days) was significantly longer than survival time in the control group (19.58 + 2.72 days) (p = 0.001) (Fig. 8B). Thus, NLRP12 play an important role in regulating tumor growth *in vivo*.

4. DISCUSSION

Intracranial gliomas, which are the most prevalent primary brain tumors, exhibit immense clinical heterogeneity and poor prognosis and survival. Gliomas are classified into four grades based on the degree of malignancy. Among these classifications, grade IV, also known as GBM, is the most lethal type of intracranial glioma.[7,9,17] This devastating tumor affects over 17 000 people in the US annually and accounts for more than 60% of primary adult brain tumors.[6] The average median survival time for patients with GBM is around 15 months, even when patients receive standard treatment.[7] Therefore, the development of new effective treatments, other than standard surgical resection, chemotherapy, and radiotherapy, is crucial.

The WHO established the classification and grading of glioma according to the pathohistological characteristics in 2007.[2] However, prognoses vary, even within the same tumor grade. In 2016, WHO included molecular parameters in addition to histology in brain tumor diagnoses.[18] Integrating phenotypic and genotypic parameters for the classification of CNS tumors have been proposed. Both genotype (ie, isocitrate dehydrogenase [IDH] mutation and 1p/19q co-deletion status) and phenotype may help determine whether the tumor is astrocytoma or oligodendroglioma. Patients with the IDH-mutant type have better outcomes and longer survival periods compared with outcomes in IDH-wild type patients.[19,20]

Identifying biomarkers for tumors has improved due to the application of molecular parameters. The use of biomarkers in basic and clinical research is an essential axis in clinical trials. Biomarkers can be objectively measured and evaluated as

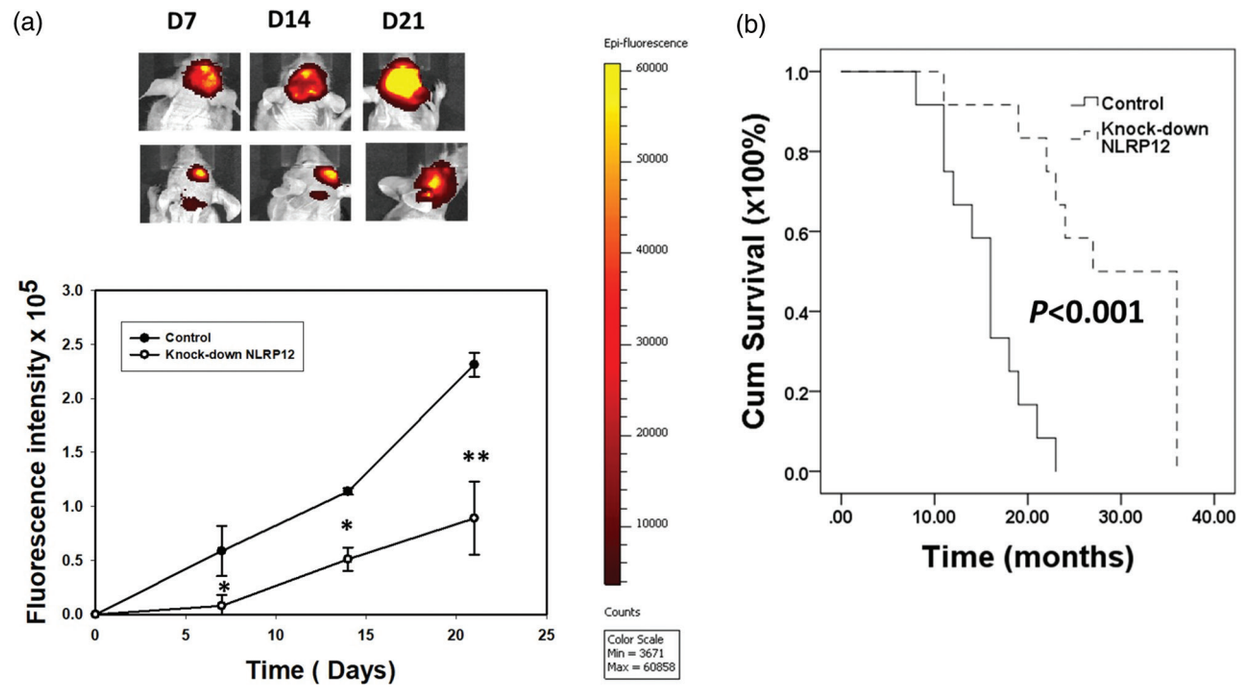

Fig. 8 NLRP12 effects on tumor progression of GBM cells in vivo. Following intracranial implantation of GBM8401 cells including fluorescent in the striatum, (A) the intensity and (B) survival times were compared between the NRLP12 knockdown and control groups. *$p < 0.05$ and **$p < 0.01$ compared with the control group. GBM = glioblastoma multiforme.

indicators of normal biological processes, pathogenic processes, and pharmacologic responses to therapeutic interventions. In the era of genomic medicine, next-generation sequencing is extensively used for detecting multiple changes in a single biological sample.[21] Research exploring potential biomarkers to predict outcomes and identify treatment targets is underway for the most aggressive and malignant glioma in humans, including glioblastoma, which has a dismal prognosis and poor outcome. In a previous study, the TCGA data indicate that NLRP12 expression is associated with survival in LGG and glioblastoma.[16] Thus, we inferred that NLRP12 might be an oncogene in gliomas. Overexpression of NLRP12 may cause malignant changes, resulting in tumor neogenesis, including proliferation, migration, and invasion.

The role of NLRP12 in gliomas is unclear; however, NLRP12 is considered a tumor suppressor gene in colon cancer and hepatocellular carcinoma.[7,14,22,23] NLRP12 influences human immune responses.[12,24] and modulates responses to injury, toxins, and invasion by microorganisms. In addition, some autoimmune diseases are attributed to the lack of NLRP12. Theoretically, immunosuppression can induce tumor neogenesis and further malignant changes; therefore, the lack of NLRP12 is a risk factor for tumors.

NRLP12 expression was high in most GBM cell lines, including GBM8401 and U87-MG, but not GBM8901 cells. We evaluated 95 patients diagnosed with intracranial glioma, who received chemotherapy or radiotherapy following surgical incision at Kaohsiung Medical Hospital between 2005 and 2015. NLRP12 was overexpressed in four types of cancers and is an independent predictor of survival in patients with these tumors. Chi-square analysis indicated that high expression of NLRP12 was significantly associated with advanced WHO grade. Kaplan–Meier analysis of intracranial glioma patients revealed a significantly longer survival period in patients with low NLRP12 expression compared with survival in patients with high NLRP12 expression. Multivariate analysis indicated that NLRP12 protein expression was significantly associated with prognosis in patients with intracranial glioma. Therefore, NLRP12 is an independent biomarker for glioma prognosis. In addition, silencing NLRP12 inhibited cell proliferation, migration, and invasion in GBM cells. However, NLRP12 is also present in neutrophils, eosinophils, monocytes, macrophages, and immature dendritic cells. Silencing NLRP12 in BV2 (microglia cells) cells increased colony formation, but silencing NLRP12 in LN18 (GBM cells) cells inhibited colony formation. In addition, lower NLRP12 expression in BV2 cells is associated with reduced migration toward glioma cells.[16] In this study, mice transfected with knockdown NLRP12 GBM cells survived longer than mice transfected with control cells. These results indicate that NLRP12 regulates both glioma cells and immune cells.

In conclusion, in this study, the expression of NLRP12 was higher in glioblastoma cells compared with expression in normal glial cells. In addition, increased NLRP12 expression was associated with higher grading in the WHO classification. Downregulation of NLRP12 using siRNA effectively inhibited tumor cell proliferation, invasion, and migration. Therefore, we conclude that NLRP12 is an oncogene in gliomas and the expression of NLRP12 is an independent prognostic factor for gliomas.

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