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The Role of The Ki-67 Labelling Index as an Independent Prognostic Factor in Indonesian Glioma Patients

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Abstract

Introduction: Gliomas are the most common type of brain tumor. However, interpreting glioma morphology is subjective, and identifying mitosis can be challenging. This can impact the determination of the patient's tumor grade, therapy, and prognosis. In addition, the Ki-67 expression level, which reflects the tumor cells' ability to proliferate, is closely related to the patient's survival. This study aims to find a correlation between Ki-67 expression and the overall survival (OS) of glioma patients in the Indonesian population.

Methods: Ninety-one glioma patients from Sardjito General Hospital were collected for formalin-fixed embedded paraffin (FFPE) samples, and the Ki-67 labeling index (LI) was calculated by determining the percentage of labeled nuclei per 1000 cells using a 40x objective lens in a randomized area (average method). The OS was calculated from the day of pathology diagnosis until death or the last follow-up (for censored cases). Kaplan-Meier survival analysis was used to analyze the OS.

Results: Individuals aged ≥60 with high-grade tumors, infratentorial gliomas, and a Ki-67 LI ≥10% had a shorter OS. The *p*-values associated with these factors were 0.001, 0.018, and 0.006, respectively. In multivariate analysis, age and tumor grade did not significantly correlate with OS.

Conclusion: Glioma patients with a Ki-67 LI ≥10% have a significantly shorter OS than those with a lower Ki-67 LI, indicating that Ki-67 LI is an independent prognostic factor in Indonesian glioma patients.

List of Abbreviations

CNS = central nervous system
EGFR = endothelial growth factor receptor
FFPE = formalin-fixed paraffin-embedded
GFAP = glial fibrillary acid protein
GTR = gross total resection
HE = hematoxylin-eosin staining
IDH = isocitrate dehydrogenase
LI = labeling index
MGMT= O(6)-methylguanine-DNA methyltransferase
OS = overall survival
PFS = progression-free survival
SPSS = Statistical Package of Social Sciences
WHO = World Health Organization

Introduction

Glioma is the most common brain tumor in the world (Arshad et al., 2010). However, glioma incidence in Southeast Asia is the lowest globally (2.55/100,000 population) (Leece et al., 2017). There is a need for precise glioma epidemiological data in Indonesia. Gliomas are classified into four grades (1 to 4) based on morphological features: cellularity, nuclear atypia, mitotic activity, pseudopalisading necrosis, and microvascular proliferation (Louis et al., 2016). Morphological interpretation can be very subjective, especially if the sample size is small or the histological appearance is difficult to assess (intricate histology). Mitosis, which reflects the tumor proliferation rate and is related to a patient's survival, will also be challenging to identify if it is only stained with Hematoxylin and Eosin (HE) (Skjulsvik et al., 2014).

Ki-67 is a non-histone protein expressed during mitosis in the cell cycle (G1, S, and G2) but not in the quiescent phase (G0) (Li et al., 2015; Sun and Kaufman, 2018; Menon et al., 2019). It is a reliable indicator for distinguishing tumor biological behavior and assessing tumor cell proliferation activity and is an independent predictor of survival and glioma responsiveness to therapy (Habberstad et al., 2011; Zeng et al., 2015). In addition, Ki-67 also serves to

distinguish high-grade and low-grade gliomas, both of which have fundamental therapeutic differences (Hsu et al., 2003; Nielsen et al., 2013).

According to several studies, the role of the Ki-67 proliferation index as a prognostic indicator of survival in glioma patients is still unclear (Fisher et al., 2002; Uematsu et al., 2005). Additionally, there is no established Ki-67 labeling index (LI) cutoff point for distinguishing the prognosis of gliomas. This study aims to determine the role of Ki-67 LI as a prognosis factor in glioma, especially in the Indonesian population.

Material and Methods

Samples and Data Collection

This is an analytical study with a retrospective cohort approach to assessing glioma patients' overall survival (OS) based on the Ki-67 LI. Other prognostic predictors such as age, sex, grading, and tumor location were also analyzed. From January 2010 to June 2023, ninety-one patients diagnosed with glioma in Dr. Sardjito General Hospital were enrolled, regardless of the grade of malignancy. Patients had to have undergone either biopsy or resection to be included in the study. Those with incomplete clinical data and insufficient paraffin blocks for immunohistochemical analysis were excluded from the study. The Ethics Committee approved this study. All FFPE samples were reviewed and reclassified based on the WHO 2021 CNS tumor classification. All samples were stained with GFAP (clone IHC484, GeneAb Monoclonal Rabbit Anti-Human) and IDH-1 (clone H09, Anti-Human IDH1 R132H, Mouse Monoclonal Antibody Dianova) immunostaining.

Immunohistochemistry Staining

Briefly, all FFPE samples were cut into 3 μm sections, deparaffinized using xylene, and hydrated before the antigen retrieval process using Tris EDTA buffer pH 8.0, followed by incubation with primary and secondary antibodies. Mouse monoclonal Ki-67/MIB-2 (Biocare Medical, USA, 1:100) was used as the primary antibody. 33'‑ diaminobenzidine was used as the chromogenic substrate and counterstained with Mayer Hematoxylin.

Evaluation of Ki-67 Immunostaining

Two observers evaluated the Ki-67 LI using the standard method, the photomicrograph counting technique, adapted from a previous study, where the maximum number of tumor cells counted in 1 photomicrograph is 500. The counting continued until the total number of tumor cells reached 1000. The average results of Ki-67 staining by two observers were the final results for the tumor proliferation index. The entire range of brown color intensity in tumor cell nuclei is interpreted as immunopositive (Leung et al., 2016).

Parameters studied

The parameters studied included patient demographics, such as age (≥60 / <60 years old), sex, tumor grade (Grade 1-4), location of the tumor (supratentorial/infratentorial), and Ki-67 LI. All parameters studied were analyzed regarding patients' OS.

Follow-up

Survival data were gathered in the outpatient clinic during patients' visits, in the ward during their hospitalization, and through phone calls or home visits. OS was calculated as the

time between the initial pathology diagnosis and either death or the last follow-up for cases where the outcome was not yet determined.

Statistical Analysis

All data were analyzed using SPSS version 22 (IBM Corp., Armonk, NY). Two observers' data from the measurement of Ki-67 were tested for Cohen's Kappa reliability to determine the consistency of measurements made by two assessors (Rater).

Survival time was estimated using the Kaplan-Meier method. Bivariate analysis was used to identify the relationship of Ki-67, age, sex, and tumor grade as a prognostic value of the OS of glioma patients using the log-rank test method (Mantel-Cox). Multivariate analysis was continued on parameters with a value of p <0.25 and analyzed using the Proportional Hazards (Cox Regression) models. A p-value <0.05 was considered significant.

Results

A total of 91 FFPE samples, were predominately male (56.0%), with ages ranging from 2 to 73 years but mostly under 60 years (83.5%), with an average age of 41.8 years. Based on WHO grading, 4.4% of the sample was grade 1, 31.9% grade 2, 18.7% grade 3, and 45.1% grade 4. The most common subtype of glioma was IDH-wildtype glioblastoma (40.7%). The most common glioma location was supratentorial (93.4%). Most cases had multiple brain lobes affected, with 52 patients (57.1%) showing involvement in various lobes. The characteristics of the patients based on clinicopathological factors are summarized in Table 1.

Measurement of Ki-67 LI

The expression of Ki-67 increased with tumor grading, ranging from 0.15% to 89.40%. Grade 1 had an average expression of 0.5%, grade 2 3.69%, grade 3 21.49%, and grade 4 26.11%. Only two low-grade glioma samples had Ki-67 ≥10%, while five high-grade glioma samples had Ki-67 <10%. Figure 1A-D illustrates the Ki-67 measurement for each grade.

Bivariate analysis

Until June 2023, 71.8% of patients survived, and 28.2% died. Age, glioma grade, and Ki-67 were factors influencing prognosis, according to Table 2. Kaplan-Meier was used to evaluate the impact of prognostic factors on survival (Figure 2A-E). Although females had a longer median OS, sex was not statistically related to survival, with a *p*-value of 0.809. In addition, patients aged 60 or older had shorter OS than those under 60 (mean OS of 13 months vs. 42.5 months, with *p*-value = 0.001).

The glioma grade was categorized into low (1, 2) and high-grade (3, 4). High-grade glioma results in significantly shorter survival compared with low-grade glioma. The median OS for a high-grade glioma was 20.7 and 42.5 for a low-grade glioma, with a log-rank score of 5.61 and a *p*-value of 0.018.

Infratentorial gliomas had a shorter survival time than supratentorial gliomas. The median OS for infratentorial gliomas was 13 months, with 28.2 months for supratentorial gliomas. However, these results were not statistically significant, with a *p*-value of 0.107.

Patients with an average Ki-67 LI of 10% or higher experienced shorter OS, with a median of 18.8 months compared with 42.5 months for individuals with a lower index. Statistical analysis showed a significant *p*-value of 0.006.

Multivariate analysis

A prognosis factor with p <0.25 was included in this analysis. In multivariate analysis, only Ki-67 ≥10% was statistically significant (p <0.005), being an independent predictor of survival in gliomas. Ki-67 ≥10% was associated with a higher risk of death. Specifically, those with infratentorial tumors had a 5.02 times higher risk of death, and those with Ki-67 ≥10% had a 3.81 times higher risk of death than those with Ki-67 <10% (see Table 3).

Discussion

The ratio of males and females in this study was 1.29:1, similar to the WHO 2016 publication, which stated that gliomas were more common in males with a ratio ranging from 1.2:1 to 2.3:1 depending on the glioma subtype (Louis et al. 2016). A study also stated that gliomas were 30-50% more frequent in males and increased with age (Leece et al., 2017; Ostrom et al., 2018a).

In this study, female patients had shorter survival than male patients (28.2 vs. 42.5 months). However, this difference was not statistically significant (p = 0.759). Therefore, it can be concluded that sex is not a prognostic factor for glioma patients in this study. This is in line with previous studies showing that sex cannot be an independent predictor of survival in glioma patients because of the loss of significance in the multivariate analysis (Abd El Atti et al., 2013; Wang et al., 2019). Nevertheless, some studies suggested that being female could be a marginally favorable prognosis factor. This was due to the higher number of IDH mutations and MGMT methylation in females (Ostrom et al., 2018b).

Age of onset has also been linked to the prognosis of glioma (Dahlrot, 2014). Previous studies showed that age ≥60 years increases the risk of death in patients with glioblastoma by 3.03 times compared with patients younger than 60 years (Reavey-Cantwell et al., 2001). It was also shown that, in grade 4 gliomas, age over 50 can be a negative prognostic factor (Deacu et al., 2022a). In contrast to previous studies, based on the bivariate analysis in this study, there were significant differences in the OS of patients aged < 60 and those ≥ 60. However, based on the multivariate analysis, age loses its significance, therefore, it cannot be an independent predictor of survival in gliomas.

High-grade gliomas (grades 3 and 4) exhibit more invasive growth than low-grade gliomas (grades 1 and 2) (Deacu et al., 2022a; Deacu et al., 2022b). The tumor grade is a crucial prognostic factor (Dahlrot, 2014), classified into high and low grades for distinct clinical approaches and outcomes (Hsu et al., 2003). Higher grades are more malignant with poorer prognoses and histologically distinct features (Walid, 2008). Variables like CDKN2A/2B and surgical resection type also influence tumor grade and survival (Deacu et al., 2022a; Deacu et al., 2022b). Some studies indicate a correlation between increased glioma grade and patient survival (Arshad et al., 2010). Glioma grade is an independent survival predictor in astrocytic gliomas (Abd El Atti et al., 2013). However, conflicting with prior research, the bivariate analysis in this study shows a significant difference ($p = 0.038$). However, the multivariate analysis did not ($p = 0.94$), challenging the independence of glioma grade as a prognostic factor. These findings align with a prior study (Wang et al., 2019) highlighting the reduced significance of tumor grade in determining survival.

Nearly 70% of adult gliomas were located in the supratentorial region, while in pediatric gliomas, 70% were in the infratentorial region (Hayat, 2010). The rarity of infratentorial gliomas

accounts for our limited knowledge regarding their characteristics and clinical behavior. It is known that low-grade cerebellar gliomas in adults frequently progress to high-grade tumors; however, in the pediatric population, this progression is rare and may even regress (Strauss et al., 2013). No studies have yet examined glioma patients' survival rates based on the location of the tumors—whether supratentorial or infratentorial. Nevertheless, this study showed better OS in the supratentorial group (28.2 vs. 13 months), with multivariate analyses yielding statistically significant results (p=0.022), underscoring tumor location as an independent predictor of glioma survival. This result is probably associated with the difficulty of performing resection in infratentorial tumors. Gliomas located in the infratentorial region and brain stem present challenges for total resection, leading to a poorer prognosis. The primary factor affecting the percentage of Gross Total Resection (GTR) is tumor location, representing challenges for complete resection in deep-seated tumors. Subtotal resection in supratentorial gliomas carries a mortality increase of 50% to 100%, similar to that in infratentorial locations (Blionas et al., 2018). Regrettably, in this study, data on tumor resection rates were unavailable, preventing further analysis.

The Ki-67 protein is expressed in all cell cycle phases except G0 and is a good proliferation marker. In a meta-analysis review, immunoreactive tumors with Ki-67 antibodies had far worse survival than tumors that did not express Ki-67. The mechanism underlying the influence of Ki-67 expression on tumor development and prognosis has yet to be established. However, it must be considered that the level of Ki-67 expression reflects the ability of tumor cells to continue to multiply after the tumor is resected. Indeed, several studies have suggested that the Ki-67 LI can be a potential prognostic indicator for glioma patients (Walid, 2008; Chen

et al., 2015). However, most previous studies were only performed in high-grade glioma patients (Agarwal et al., 2019; Abd El Atti et al., 2013; and Tavares et al., 2018).

Unlike previous studies, this study encompasses all glioma grades to determine the significance of the Ki-67 LI on OS. The method of measuring Ki-67 and the uniform field of view was expected to reduce variability. Statistical analysis using bivariate methods (Kaplan-Meier method and log-rank test) and multivariate analysis (Cox regression) shows that glioma patients with Ki-67 <10% have a significantly longer overall survival ($p<0.005$). This indicates that Ki-67 with a 10% cutoff is an independent predictor of survival for glioma patients. Ki-67 ≥ 10% increases the risk of death by 3.81 times compared with Ki-67 <10%. The results aligned with the study of Uematsu et al., however, other research showed contradictions. Ki-67 ≥22% (Wong et al., 2019) or > 27% (Bredel et al., 2002) in glioblastoma indicated extended survival, up to a 6:1 ratio for five-year survival. Varied outcomes were likely due to increased cell proliferation, affecting tumor susceptibility to chemoradiotherapy, as seen in lymphomas (Bredel et al., 2002).

This study has several limitations. First, several important variables that can affect OS, such as the extent of surgery and tumor recurrence, were not analyzed due to the unavailability of the data. Additionally, since it relies on retrospective data, it may be prone to biases and limitations associated with such an approach.

Conclusion

This study demonstrated that glioma patients with a Ki-67 LI ≥10% have a significantly shorter OS than those with a lower Ki-67 LI, indicating that Ki-67 serves as an independent prognostic factor in Indonesian glioma patients. This finding indicates the usefulness of

measuring Ki-67 LI in glioma as it helps the treating physician to understand the prognosis and develop appropriate treatment plans.

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Declaration of Conflicting Interest(s)

All the authors hereby declare that no conflicting interest(s) may affect the work and result of this paper.

Reference list. A) A period should be inserted after each of the first and middle names of the authors. B) The comma between the two last authors should be removed, the word "and" is enough between them. C) A period should be inserted after each abbreviated word of the Journal name. D) The issue number should be removed. E) the DOI number should be removed. You can get the instructions to authors in our web site. Please, follow them carefully.

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Tables

Table 1. Characteristics of patients based on clinicopathological factors

Variables	N (n=91) (%)	Mean	Median (SD)
Sex			
Male	51 (56.0)		
Female	40 (44.0)		
Age (years old)			
>60	15 (16.5)		
≤60	76 (83.5)		
Grade of Glioma			
Low Grade			
Grade 1	4 (4.4)		
Pilocytic astrocytoma	4 (4.4)		
Grade 2	29 (31.9)		
Diffuse astrocytoma, IDH-wildtype	14 (15.4)		
Diffuse astrocytoma, IDH-mutant	7 (7.7)		
Oligodendroglioma, IDH-mutant	6 (6.6)		
Ependymoma	2 (2.2)		
High Grade			
Grade 3	17 (18.7)		
Astrocytoma, IDH-wildtype	12 (13.2)		
Astrocytoma, IDH-mutant	1 (1.1)		
Oligodendroglioma, IDH- mutant	2 (2.2)		
Ependymoma	2 (2.2)		
Grade 4	41 (45.1)		
Glioblastoma, IDH-wildtype	37 (40.7)		
Astrocytoma, IDH-mutant	4 (4.4)		
Tumor location			
Supratentorial	85 (93.4)		
Infratentorial	6 (6.6)		
Lobe involvement			
Single lobe	39 (42.9)		
Multiple lobes	52 (57.1)		
Ki-67 LI		16.8	
Grade 1	4 (4.4)	0.5	
Grade 2	29 (31.9)	3.69	
Grade 3	17 (18.7)	21.49	
Grade 4	41 (45.1)	26.11	
<10%	41 (45.1)		
≥10%	50 (54.9)		
Overall survival (months)			
Ki-67 ≥10%			18.8 (4.7)
Ki-67 <10%			42.5 (16.2)

SD: standard deviation, OS: overall survival.

Table 2. The correlation of prognostic factors with overall survival in the bivariate analysis

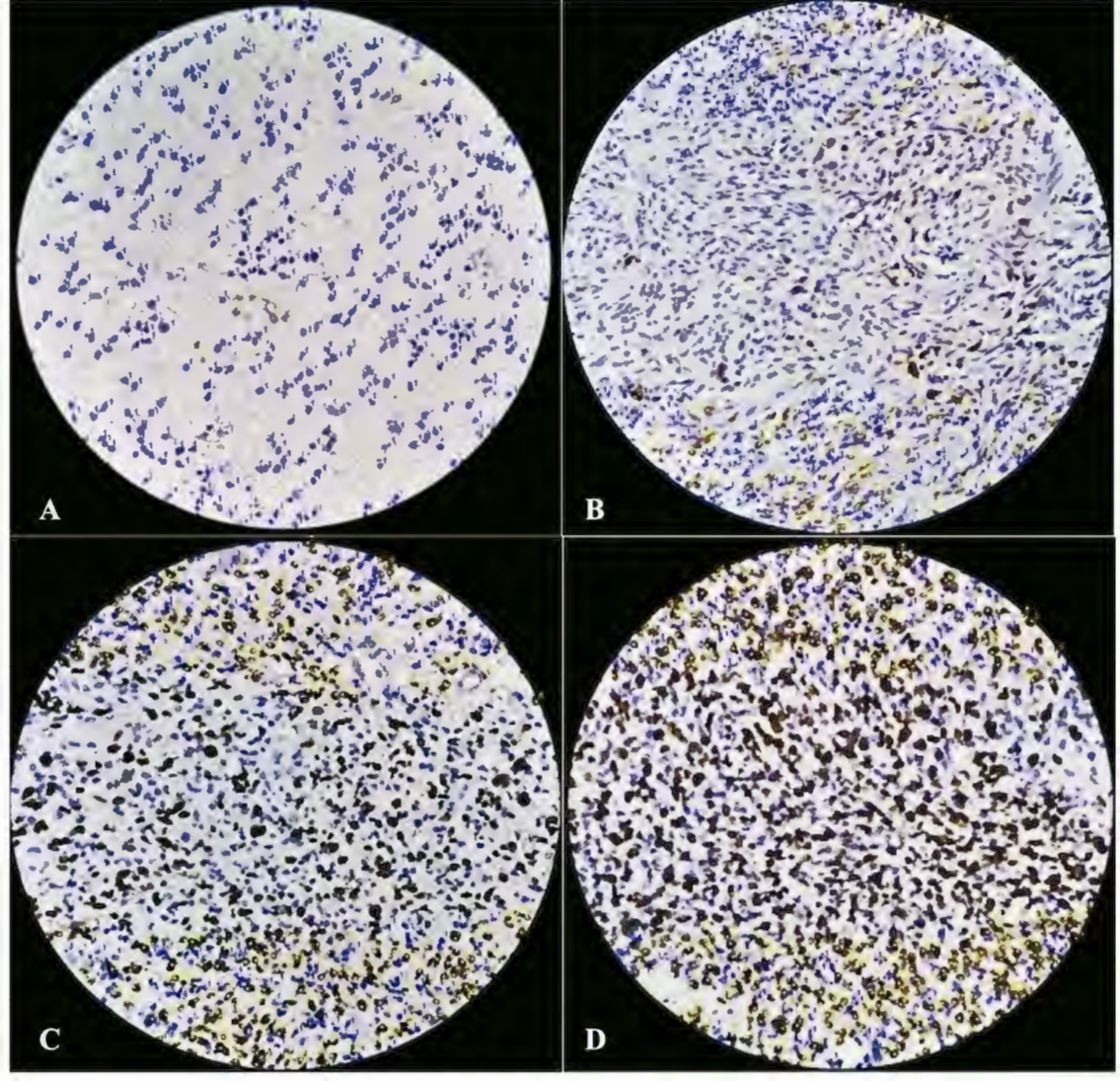
Variables	Median OS (SD) (months)	X^2	*Log Rank* *Sig.*
Sex		0.06	0.809
female	28.2 (4.8)		
male	42.5 (0)		
Age (year)		10.7	0.001*
≥60	13.0 (4.7)		
<60	42.5 (13.4)		
***Grade* Glioma**		5.61	0.018*
Low Grade	42.5 (16.5)		
High Grade	20.7 (4.6)		
Tumor location		2.6	0.107
Supratentorial	28.2 (9.7)		
Infratentorial	13.0 (8.2)		
Ki-67 LI *cutoff point* 10%		7.53	0.006*
≥10%	18.8 (4.7)		
<10%	42.5 (16.2)		

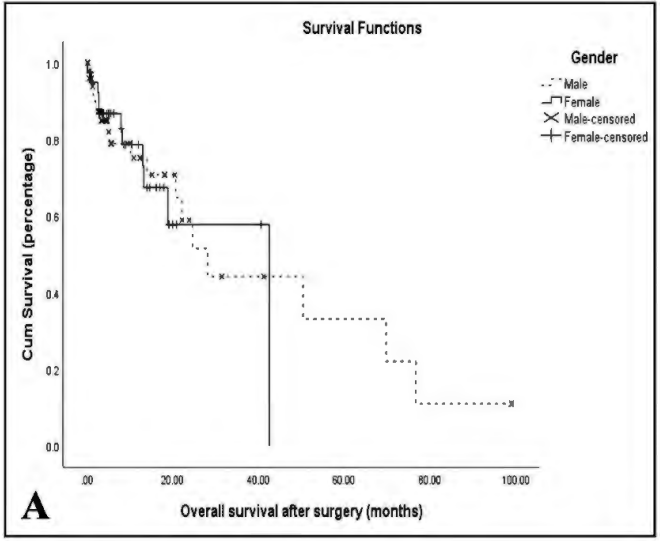
* Statistically significant (p <0.05). SD: standard deviation.

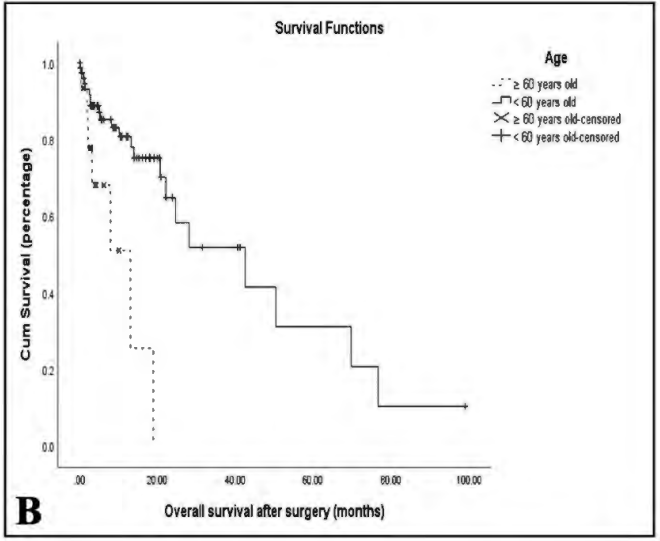
Table 3. Independent predictive factors in glioma based on multivariate analysis

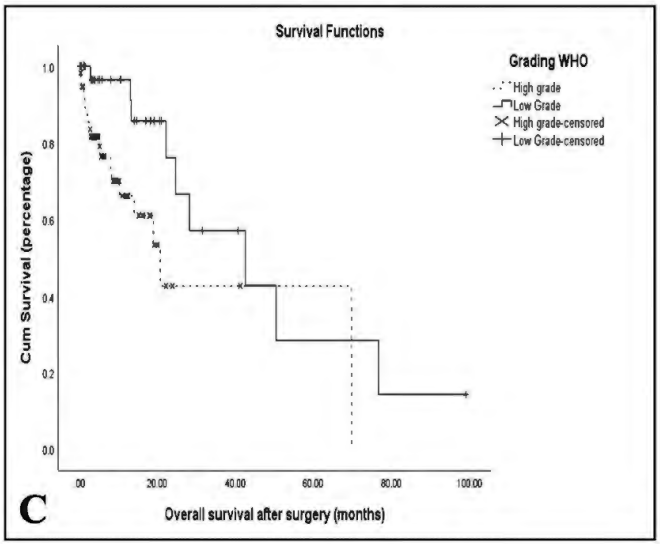
Prognostic factors		Unadjusted HR (95% CI)	*p*	Adjusted HR (95% CI)	*p*
Age	≥60	4.20 (1.65-10.67)	0.003*	2.51 (0.94-6.68)	0.065
Sex	Male	0.91 (0.41-2.01)	0.809	-	-
Grading	*High Grade*	2.76 (1.16-6.58)	0.022*	-	-
Location	Infratentorial	2.65 (0.77-9.06)	0.121	5.02 (1.26-19.97)	0.022*
Ki-67 LI	≥10%	3.29 (1.35-8.08)	0.009*	3.81 (1.39-10.41)	0.009*

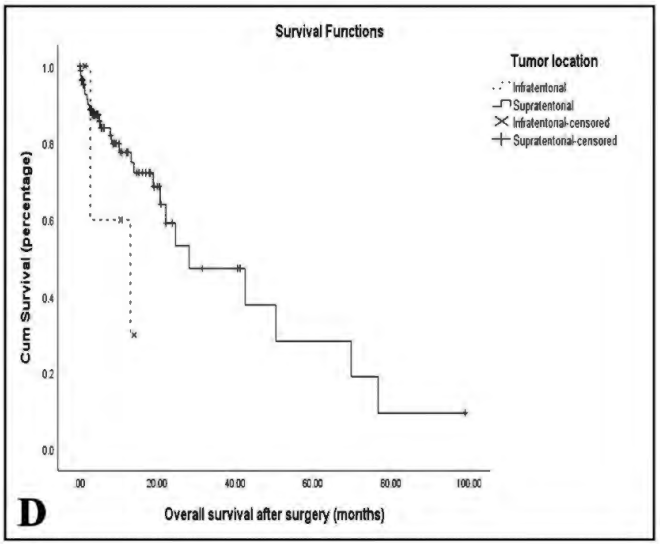
* Statistically significant (p <0.05) HR: Hazard ratio, CI: Confidence interval.

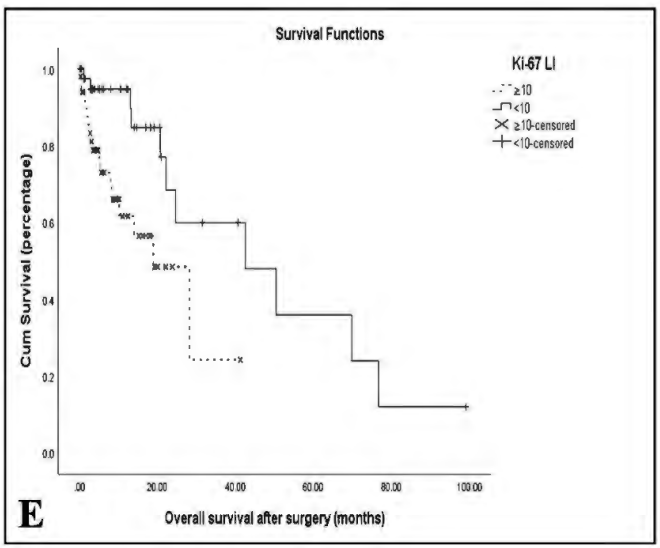

A
B
C
D


Survival Functions
Gender
Male
Female
Male-censored
Female-censored
Cum Survival (percentage)
1.0
0.8
0.6
0.4
0.2
0.0
.00
20.00
40.00
60.00
80.00
100.00
Overall survival after surgery (months)
A


Survival Functions
Age
≥ 60 years old
< 60 years old
≥ 60 years old-censored
< 60 years old-censored
Cum Survival (percentage)
1.0
0.8
0.6
0.4
0.2
0.0
.00
20.00
40.00
60.00
80.00
100.00
Overall survival after surgery (months)
B


Survival Functions
Grading WHO
High grade
Low Grade
High grade-censored
Low Grade-censored
Cum Survival (percentage)
1.0
0.8
0.6
0.4
0.2
0.0
.00
20.00
40.00
60.00
80.00
100.00
Overall survival after surgery (months)
C


Survival Functions
Tumor location
Infratentorial
Supratentorial
Infratentorial-censored
Supratentorial-censored
Cum Survival (percentage)
1.0
0.8
0.6
0.4
0.2
0.0
.00
20.00
40.00
60.00
80.00
100.00
Overall survival after surgery (months)
D


Survival Functions
Ki-67 LI
≥10
<10
≥10-censored
<10-censored
Cum Survival (percentage)
1.0
0.8
0.6
0.4
0.2
0.0
.00
20.00
40.00
60.00
80.00
100.00
Overall survival after surgery (months)
E