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RESEARCH ARTICLE

KI-67 IMMUNOHISTOCHEMICAL EXPRESSION IN LOW GRADE AND HIGH GRADE GLIOMAS

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ABSTRACT

Introduction: Cell proliferation is an important pre-requisite for the development of a neoplasm. Ki-67 is one of the most important cell proliferation markers. Its expression is correlated with the aggression of various gliomas to differentiated low grade and high grade. **Material and methods :** This case control study included 140 cases in which 62 cases of low grade glioma (23 Pilocytic Astrocytoma,12 Diffuse Astrocytoma, 13 Ependymoma, 5 Oligodendroglioma, 2 Diffuse Fibrillary Astrocytoma, 2 Gemistocytic Astrocytoma, 2 Myxopapillary Ependymoma, 2 subependymal giant cell Astrocytoma and 1 oligoastrocytoma) and 73 cases of high grade glioma (45 Glioblastoma, 9 Anaplastic Astrocytoma, 6 Gliosarcoma, 5 Anaplastic Ependymoma, 4 Anaplastic Oligodendroglioma, 3 Anaplastic oligoastrocytoma and 1 pilocytic Astrocytoma with anaplastic features) along with 5 normal brain specimen as control. **Aim:** The aim of this study to assess the expression of Ki-67 in various low grade and high grade gliomas and correlate immunohistochemistry status with the histomorphological grading and overall survival rate. **Results:** Majority of patients having Ki67 score 1 were low grade (68.67%) and rest were high grade while proportion of patients of high grade was higher as compared to low grade having Ki67 score 2 (60.00% vs. 40.00%) and Ki67 score 3 (97.62% vs. 2.38%). Association of Ki67 score and grade of glioma was found to be statistically significant ($p<0.001$). **Conclusion:** The conclusion can be made that Ki-67 can be used as a marker of for differentiate high grade and low grade glioma.

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INTRODUCTION

Gliomas are the most common primary malignant brain tumor in adults. They can occur anywhere in the central nervous system but primarily occur in the brain and arise in the glial tissue (Ostrom, 2013). Gliomas are either astrocytic, oligodendrocytic, ependymal or a mix of these 2 cell types (WHO, 2016) Astrocytic gliomas represent a heterogeneous group of malignancies which are classified into low grade astrocytomas (grade i–ii), anaplastic astrocytomas (grade iii) and glioblastoma (gB) (grade iV) (Louis *et al*., 2007). Histopathological classification and malignancy grading of human gliomas are based on criteria issued by the World Health Organization (WHO) (Louis *et al*., 2007). However, these criteria are encumbered with subjective interpretations, giving rise to inter- and intra-observer variability (Coons *et al*., 1997; Van Dent Bent, 2010). Because proliferation is a basic process in gliomagenesis, mitotic counting constitutes a cornerstone in the grading of these tumors. Since identification and counting of mitotic figures in haematoxylin-eosin stained sections can be difficult, glioma grading is imprecise and may unfavorably impact prognosis, treatment, and follow-up. Immunohistochemical determination of proliferative activity is a useful supplement for establishing the histopathological diagnosis of glioma. Ki-67/MIB-1 immunostaining is most commonly used and has been shown to correlate positively with tumor grade and prognosis (Prayson, 2005; Johannessen

et al., 2006). The current update (2016 CNS WHO) thus breaks with the century-old principle of diagnosis based entirely on microscopy by incorporating molecular parameters into the classification of CNS tumor entities. This system classifies gliomas into grade I to IV based on the combined phenotypic and genotypic classification and on the generation of integrated diagnosis (Coons *et al*., 1997). The Ki-67 also known as MKI67, is a protein that in humans is encoded by the MKI67 gene. Ki-67 is a nuclear protein that may be necessary for cellular proliferation. Ki-67 protein is expressed in all active phases of the cell cycle. The antigen expression is a measure of the proportion of cellular and, hence, biological aggressiveness in malignancy (Scott *et al*., 1991; Wilson *et al*., 1996). Ki-67 can be used as a marker to assess the growth portion of a given cell population, as this protein is present in all proliferating cells (normal and tumor) (Colozza *et al*., 2005). The aim of this study was to evaluate the Ki-67/MIB-1 proliferative indices (PIs) in a series of gliomas and correlate immunohistochemical status with the histopathological grading and overall survival rate

MATERIALS AND METHODS

Patients: This study includes a series of gliomas specimens from the Department of Neurosurgery, King George's Medical University, Lucknow were taken on the basis of clinicoradiological findings with adequate patient's clinical information and proper consent. Both the histopathological diagnosis (according to the WHO classification system) and determination of the Ki-67/MIB-1 PI were performed. Total 140 cases (Table 1) are studied which included 62 cases of low grade glioma (23 Pilocytic Astrocytoma,12 Diffuse Astrocytoma, 13 Ependymoma, 5 Oligodendroglioma, 2 Diffuse Fibrillary Astrocytoma, 2 Gemistocytic Astrocytoma, 2 Myxopapillary Ependymoma, 2 subependymal giant cell Astrocytoma and 1 oligoastrocytoma) and 73 cases of high grade glioma (45 Glioblastoma, 9 Anaplastic Astrocytoma, 6 Gliosarcoma, 5 Anaplastic Ependymoma, 4 Anaplastic Oligodendroglioma, 3 Anaplastic oligoastrocytoma and 1 pilocytic Astrocytoma with anaplastic features) along with 5 normal brain specimen as control.

Study Design: Prospective and Retrospective case control study**.**

Exclusion criteria: Brain tumors other than gliomas.

Case Definition: All diagnosed cases of brain glioma.

H and E Section histological diagnosis and grading: Histological types of gliomas were classified in accordance with World Health Organization guidelines and were divided into grade I, II, III and IV. The cases of Grade I and Grade II tumors were included in the category of low grade gliomas (Figure 1) and the cases with Grade III and Grade IV were included in the category of high garde gliomas (Figure 2).

Immunohistochemistry: Immunohistochemistry was performed with antibodies to Ki-67. Anti Ki-67 was manufactured by Biogenex (Pre-diluted ready–to-use Mouse monoclonal antibody to Ki-67 antigen).

Anti Ki-67 antigen (MIB-1) Positive control: Autopsy specimen of brain tissue showing unremarkable histology

The MIB-1 Index (Ki-67 labelling Index): It was calculated as the percentage of positively stained tumor cell nuclei out of the total tumor cells counted (n=1000) and the scoring was done as follows:

a.≤4% (Score 1).
b.5-10% (score 2).
c.≥10% (score 3).

Statistical analysis: The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical Analysis Software. The values were represented in Number (%) and Mean±SD. The Mann-Whitney U test was applied to estimate differences in the PIs between groups of tumors. $P < 0.05$ was considered significant.

All tumor samples were fixed in buffered formalin, usually for not more than 24 hours, and then embedded in paraffin. Paraffin sections (3-µm-thick) were cut and mounted on Superfrost glass slides, deparaffinized, and dehydrated. Different antigen retrieval methods were used during the study period, including pressure cooking, microwave oven and water bath. The Ki-67/MIB-1 antibody was supplied by Immunotech (Hamburg, Germany) and by DAKO (Glostrup, Denmark). The working dilution was 1:100 or 1:600 depending on the detection system used. The sections were incubated for 40 min at room temperature. Automatized immunohistostainers and detection systems were purveyed by DAKO (TechMate 500, Autostainer Plus, Autostainer Link 48). The staining procedures were performed according to the manufacturer's recommendations. Positive controls were used in each staining run. First, a standard streptavidin-biotin-peroxidase technique was used, and later the DAKO EnVision Flex+ System. Diaminobenzidine was used as the chromogene and haematoxylin as the counterstain.

Proliferation index evaluation: The immunostained sections were scanned using a 40× objective with an eye grid for the areas with the highest density of labeled tumor cells (hot spots). At least 1000 tumor cells, or alternatively three high power fields (HPF) were examined. Only immunoreactive tumor cell nuclei were counted. Necrotic areas and vascular endothelium were excluded. The Ki-67/ MIB-1 PI was defined as the percentage of immunoreactive tumor cell nuclei among the total number of cells.

RESULTS

The study was conducted in the Department of Pathology in collaboration with the Department of Neurosurgery, King George's Medical University, Lucknow to evaluate expression of Ki-67 in Low grade and High grade Gliomas and correlate immunohistochemistry status with the histomorphological grading and overall survival rate. Out of 135 cases of glioma, 62 (45.92%) were graded as Low grade and rest 73 (54.07%) as high grade glioma. Among low grade glioma most common diagnosis was Pilocytic astrocytoma (37.10%) followed by Ependymoma (20.97%) and Diffuse Astrocytoma (19.35%).
Out of 135 cases of glioma, two-third of cases were male (66.67%) and rest one-third cases were female (33.33%). In overall 62 low grade glioma cases (Table 2) the population with highest number of cases 31 (50.0%) is ≤ 20 yrs. In overall 73 high grade glioma cases (Table 3) the population with highest number of cases 32 (43.84%) is 40-60 yrs.

Association of age with histological diagnosis among High grade glioma cases was found to be statistically significant. Higher duration of symptoms was observed in cases of Low grade of glioma while lower duration of symptoms was observed in cases of High grade of glioma (Table 4). Mean duration of complaints among patients of Low grade glioma (8.78±10.08 months) was found to be higher as compared to High grade glioma (4.51±9.51 months). Majority of patients having Ki67 score 1 were low grade (68.67%) and rest were high grade while proportion of patients of high grade was higher as compared to low grade having Ki67 score 2 (60.00% vs. 40.00%) and Ki67 score 3 (97.62% vs. 2.38%). Association of Ki67 score (Table 5) and grade of glioma (was found to be statistically significant ($p<0.001$).

Survival: Proportion of Expiry was higher among High Grade of Glioma as compared to that in Low grade Glioma in all the Ki67 scores i.e. in Ki67 score 1 (55.00% vs. 14.63%), score 2 (100.00% vs. 33.33%) and score 3 (80.00% vs. 0.00%). Among Ki67 score 1 the majority of patients i.e. 44 (72.13%) survived; Among Ki67 score 2 the majority of patients i.e. 5 (71.43%) expired; Among Ki67 score 3 the majority of patients i.e. 24 (77.42%) expired.

DISCUSSION

Gliomas are the most common form of brain tumors, contributing to more than half of the incidence of brain tumors. Despite recent advances in imaging, surgical resection techniques and the development of novel adjuvant therapies, the long-term survival of patients suffering from malignant gliomas remains low (Strojnik, 2011). In this study the author included 140 cases and these cases were graded histomorphologically according to WHO Classification into Grade I to Garde IV. The cases of Grade I and Grade II tumors were included in the category of low grade gliomas and the cases with Grade III and Grade IV were included in the category of high garde gliomas. Based on these categorization 62 cases of Low grade Gliomas and 73 cases of high grade gliomas and 5 control cases (Autopsy Brain tissue).

Association of Ki67 with Grade of glioma: In this study the majority of cases with Ki-67 score 1 (<4% Li) were of low grade gliomas (Figure 3), while cases with Ki-67 score 2 (4-10% Li) have majority of cases of high grade glioma (Figure 4). The cases with Ki-67 score 3 (>10% Li) were mostly high grade gliomas. These data indicate that Ki67 score correlates with the grade of tumour ($p<0.001$). Thus, Ki-67/MIB-1 is useful for differentiating between high and low-grade gliomas, but categorizing score 4-10% is more problematic due to the overlap of values between the different tumor grades.

This overlap is a main limitation of this immunostaining. Our study is in concordance with the study of Anne J Skjulsvik *et al*. (2014) who have taken 267 gliomas cases in which 89 (33.3%) cases were of glioblastomas. They found that the Ki-67/MIB-1 PI correlated significantly with tumor grade for each glioma type. However, considerable overlap was observed between the malignancy groups which is in concurrence with our study. There was no significant difference in Ki-67 PI between glioma type of the same tumour grade. However anaplastic oligodendrogliomas and anaplastic oligoastrocytomas had indices comparable to glioblastomas. They found that indices for high-grade gliomas (grade III/IV) were significantly higher than in low-grade (grade I/II) tumors and concluded that Ki-67 is useful for differentiating between high and low grade glioma. Xinhua Hu *et al*. (2013) who found that the mean Ki-67 LI significantly increased with the glioma grade. Significant difference was identified in the Ki-67 LI between the various glioma grades ($P<0.05$), suggesting that the pathological grade was associated with the Ki-67 LI. Similarly the study done by Johan M. Kros *et al*. (1996) on 108 verified oligodendrogliomas found that immunohistochemistry for the Ki- 67 antigen provides prognostic information that is independent of histopathologic grading and tumor localization.

David W Ellison *et al*. (1995) studied 123 adult cerebral astrocytic tumours and found that an increasing median Ki-67 LI was present across the range of astrocytic tumours from fibrillary astrocytoma to glioblastoma. Similar results were found in the study done by Andreas H Habberstad *et al*. (2011) who determined proliferative activity in 27 cases of astrocytomas in which the proliferative activity determined by Ki-67 positively correlated to tumour grade. Another study done by Patrick Zuber *et al*. (1988) shows similar results in which they studied fifty-one frozen glioma specimens by staining with Ki-67 and found that mean Ki67 labelling indices increased from low grade astrocytomas to anaplastic astrocytomas and was highest for glioblastomas suggesting that the Ki-67 index of proliferating cells in human gliomas correlates with the usual histological classification of these tumors.

Comparison of survival with Ki67 score: In our study proportion of expiry is higher among high grade gliomas as compared to that in low grade gliomas in all the Ki-67 score. In cases with Ki-67 score 1, majority of patients survived. While in cases with Ki-67 score 2 and 3, majority of patients expired. This shows that with increasing Ki-67 score the survival of patients decreases and that there is a negative correlation between Ki-67 score and survival. However our study also proves that survival not only depends on Ki67 score but also on the histological grade. Hence Ki67 alone cannot be used as a single prognostic marker. Our study is in concordance with the study done by David W Ellison *et al*. [15] who found that there was difference in survival with astrocytic tumour showing a Ki-67 LI of < 2% and patients with astrocytic tumour showing a Ki-67 LI of >2%, the significance of which was lost after adjusting for other variables. They concluded that association between Ki-67 Li, tumour type, and prognosis makes Ki-67 immunohistochemistry a valuable adjunct to the histological diagnosis of astrocytic tumours. Our study is also in concordance with the study done by Tarik Tihan *et al*. (2000).

They analyzed the Ki-67 (MIB-1) labeling indexes in the stereotactic biopsy specimens from 11 pilocytic astrocytomas; 8 grade 2, 15 grade 3, and 16 grade 4 astrocytomas. The tumour with low Ki-67 Li (,15%) had better survival then tumours with high Ki67, and concluded that there was a strong correlation with poor outcome when Ki67 LI were higher than 15% in the same tumor for diffuse astrocytoma. Similar results were found in the study done by Fisher *et al*. (2002) who evaluated the expression of Ki-67 in 180 low-grade glioma tumor specimens immunohistochemically and correlated its expression with prognosis or tumour recurrence.

Table 1.Distribution of Cases according to Histological Diagnosis of Tumors

	No. of cases	% of low grade glioma (n=62)	Percentage Overall (n=135)
Low Grade Glioma			
Diffuse Astrocytoma	12	19.35	8.89
Diffuse fibrillary astrocytoma	2	3.23	1.48
Ependymoma	13	20.97	9.63
Gemistocytic Astrocytoma	2	3.23	1.48
Myxopapillary Ependymoma	2	3.23	1.48
Oligoastrocytoma	1	1.61	0.74
Oligodendroglioma	5	8.06	3.70
Pilocytic Astrocytoma	23	37.10	17.04
Subependymal giant cell astrocytoma	2	3.23	1.48
Total	62		45.92
High Grade Glioma		% of High grade glioma (n=73)	Percentage Overall (n=135)
Anaplastic Astrocytoma	9	12.33	6.67
Anaplastic Ependymoma	5	6.85	3.70
Anaplastic Oligodendroglioma	4	5.48	2.96
Anaplastic Oligoastrocytoma	3	4.11	2.22
Glioblastoma	45	61.64	33.33
Gliosarcoma	6	8.22	4.44
Pilocytic Astrocytoma with anaplastic features	1	1.37	0.74
Total	73		54.07

Table 2. Association of Age and Histological Diagnosis (Low Grade Glioma) (n=62)

Diagnosis	Total	≤20 yrs		21-40 yrs		41-60 yrs	
		No.	%	No.	%	No.	%
Diffuse Astrocytoma	12	3	25.00	8	66.67	1	8.33
Diffuse fibrillary astrocytoma	2	1	50.00	0	0.00	1	50.00
Ependymoma	13	9	69.23	3	23.08	1	7.69
Gemistocytic Astrocytoma	2	0	0.00	2	100.00	0	0.00
Myxopapillary ependymoma	2	1	50.00	1	50.00	0	0.00
Oligoastrocytoma	1	0	0.00	1	100.00	0	0.00
Oligodendroglioma	5	0	0.00	4	80.00	1	20.00
Pilocytic Astrocytoma	23	16	69.57	7	30.43	0	0.00
Subependymal giant cell astrocytoma	2	1	50.00	1	50.00	0	0.00
Total	62	31	50.00	27	43.55	4	6.45

χ^2=25.818 (df=16); p=0.057 (NS)
Min-Max (Median): 2-55 (20.50); 22.65±12.86 years

Table 3. Association of Age and Histological Diagnosis (High Grade Glioma) (n=73)

Diagnosis	Total	≤20 yrs		21-40 yrs		41-60 yrs		>60 yrs	
		No.	%	No.	%	No.	%	No.	%
Anaplastic astrocytoma	9	2	22.22	4	44.44	3	33.33	0	0.00
Anaplastic ependymoma	5	4	80.00	0	0.00	1	20.00	0	0.00
Anaplastic oilgodendroglioma	4	0	0.00	2	50.00	1	25.00	1	25.00
Anaplastic Oligoastrocytoma	3	0	0.00	1	33.33	2	66.67	0	0.00
Glioblastoma	45	2	4.44	16	35.56	21	46.67	6	13.33
Gliosarcoma	6	1	16.67	1	16.67	4	66.67	0	0.00
Pilocytic Astrocytoma with anaplastic features	1	1	100.00	0	0.00	0	0.00	0	0.00
Total	73	10	13.70	24	32.88	32	43.84	7	9.59

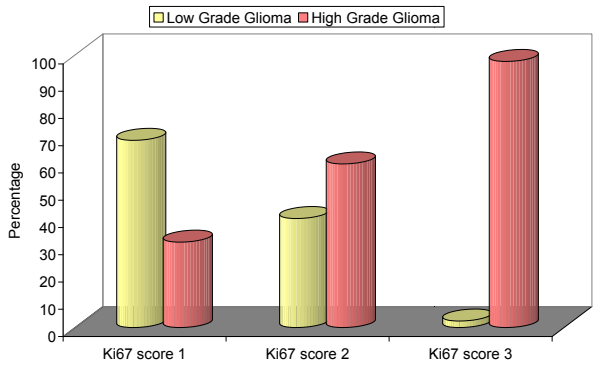
χ^2=35.783(df=18); p=0.008 (Sig)
Min-Max (Median): 4-70 (45.00); 40.30±15.83 years

Table 4. Comparison between Duration of Symptoms with grade of tumor

Duration of symptoms	Overall (n=135)		Low Grade Glioma (n=62)		High Grade Glioma(n=73)	
	No.	%	No.	%	No.	%
<3 m	70	51.85	22	35.48	48	65.75
3-6 m	35	25.93	18	29.03	17	23.29
6-12 m	11	8.15	9	14.52	2	2.74
>12 m	19	14.07	13	20.97	6	8.22
Total	135	100.00	62	100.00	73	100.00
			χ^2=15.929(df=3); p<0.001 (Sig)			
Min-Max (Median)	25 days -36 m (4 m)		6 days -68 m (2 m)		6 days -68 m (2 m)	
Mean±SD	6.47±9.97		8.78±10.08		4.51±9.51	

Table 5. Association of Ki67 score and Grade of Glioma

Ki67 Score	Low Grade Glioma		High Grade Glioma		Total	
	No.	%	No.	%	No.	%
Ki67 score 1	57	68.67	26	31.32	83	61.48
Ki67 score 2	4	40.00	6	60.00	10	7.41
Ki67 score 3	1	2.38	41	97.62	42	31.11
Total	62	45.93	73	54.07	135	100.00
				χ^2=49.506(df=2); p<0.001 (Sig)		



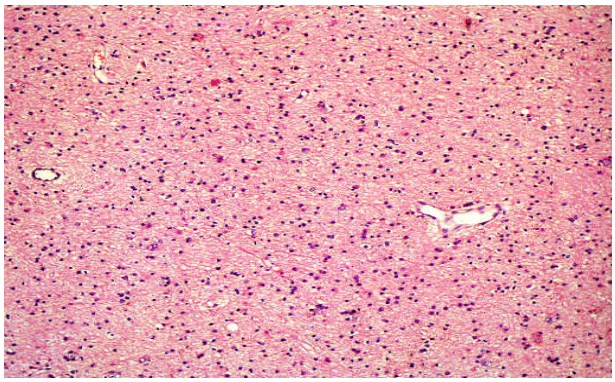


Figure 1. Histomicrophotograph of Hand E (10x) Low grade glioma (diffuse astrocytoma)

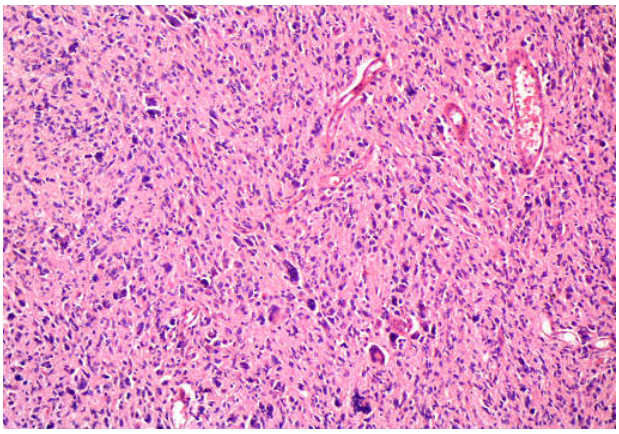


Figure 2. Histomicrophotograph of Hand E (10x) High grade glioma (Glioblastoma)

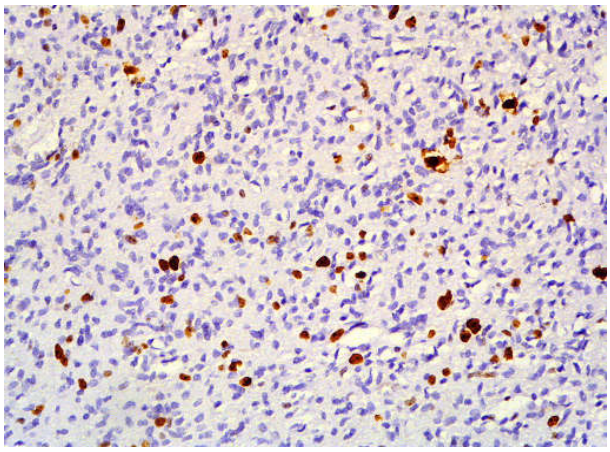


Figure 3. Immunohistochemistry of of Ki-67 showing less then 5% positive (low grade glioma)

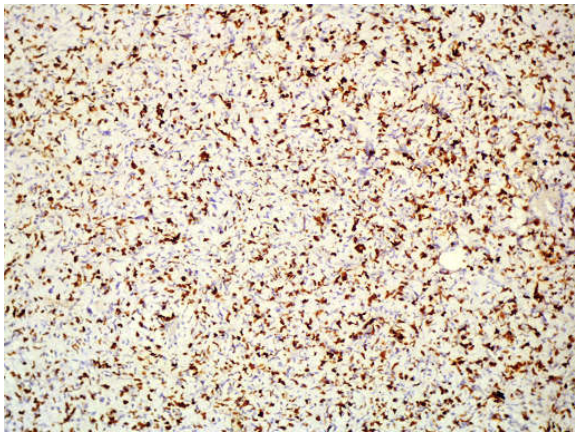


Figure 3. Immunohistochemistry of of Ki-67 showing more then 30% positive (high grade glioma)

They concluded that Ki-67 is a useful predictor of cause specific survival in low-grade gliomas; however, it is not independent of other prognostic factors, particularly age. Our study also in concordance with the study of Johan M. Kros el al. [14] who correlated expression of Ki-67 with survival time of patients in 108 cases of oligodendrogliomas. They found that the survival curve of the cases with the lowest MIB-1 LI was significantly different from the curve of the cases with the highest LI. They conclude that the MIB-1 LI may be considered an independent parameter of prognosis. Similar results was shown in the study done by Steffen Heegard *et al*. (1995) who investigated 32 pure supratentorial oligodendrogliomas and correlated Ki-67 immunohistochemical analyses with survival time. They concluded that a Ki-67 LI higher than 3% seem reliable as prognostic factors when investigating pure supratentorial oligodendrogliomas**.**

Conclusion

The author conclude that the duration of symptoms in case of high grade gliomas is usually < 6 months and Ki-67 scoring increased with increasing grade of glioma. The majority of patients with high grade glioma and higher Ki-67 score expired during the follow-up period. To improve the diagnostics for gliomas a battery of proliferation markers might be considered (Habberstad, 2011). Ki-67 should not be used as a diagnostic measure alone, but use in combination with other prognostic factors (Kanyilmaz, 2018).

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