IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 17, Issue 7 Ver. 16 (July. 2018), PP 11-17
www.iosrjournals.org

Role of Ki 67 Immunostaining as an Adjunct to Differentiate Low Grade and High Grade Gliomas

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Abstract: ***Background :****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 [6,7,8]. Despite its widespread use, the procedure still has many uncertain and limiting factors, including problematic overlap of indices between different glioma grades and inherent problems in the immunohistochemical analysis [5-11]. Thus, publishing data on Ki-67/MIB-1 immunostaining in human gliomas is still worthwhile in order to optimize this method, with the superior goal of achieving a standardized procedure. The aim of this study was to evaluate the Ki-67/MIB-1 proliferative indices (PIs) in a series of gliomas and critically evaluate the findings and procedure.*
Materials and Methods *: In the present study, a total of 54 patients with gliomas were included. Ki-67 LI was done on all cases and was compared in correlation with World Health Organization histological grading of astrocytomas.*
Results:*In our material we found that the Ki-67 LI correlated significantly with increasing tumor grade in all types of gliomas. The median Ki 67 LI was 1.5 (range 1-13%) in grade 1 gliomas, 3.5 (range 1-20%) in grade 2 gliomas, 23 (range 4-32%) in grade 3 gliomas and 26 (range 2-45%) in grade 4 gliomas. P values were significant in differentiating grade 2 from grade 3 and grade 4 gliomas.*
Conclusion*:Ki 67 is a simple, reliable and essential component in the evaluation of proliferative potential of glial lesions. Ki-67 cannot be used as a sole prognostic factor but can be a useful adjunct to other prognostic indicators like age, tumour location, tumour resection and genetic alterations.*
Keywords : *Glioma, astrocytoma, immunohistochemistry, ki 67*

Date of Submission: 15-07-2018 Date of acceptance: 30-07-2018

I. Introduction

Primary malignant brain tumours are rare. The annual global age standardized incidence of primary malignant brain tumours is approximately 3.7 per 100,000 for males and 2.6 per 100,000 for females.[1] Glial tumours represent 42% of all primary central nervous system (CNS) neoplasms of which over 75% are malignant.[2] Management of these tumours remains the greatest challenge in oncology.

Histopathological classification and malignancy grading of human gliomas are based on criteria issued by the World Health Organization (WHO).[3] However, these criteria are encumbered with subjective interpretations, giving rise to inter- and intra-observer variability.[4,5] 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 unfavourably impact prognosis, treatment, and follow-up.

IHC using monoclonal or polyclonal antibodies has greatly influenced the diagnosis of various neurological disorders. Using this technique, the presence of characteristic antigen can be precisely defined in a sensitive and reproducible manner, thereby providing a better tool for making an accurate diagnosis of brain tumors.[4]

Immunohistochemical determination of proliferative activity is a useful supplement for establishing the histopathological diagnosis of glioma. Ki-67 immunostaining is most commonly used and has been shown to correlate positively with tumor grade and prognosis.[6,7,8] Despite its widespread use, the procedure still has many uncertain and limiting factors, including problematic overlap of indices between different glioma grades and inherent problems in the immunohistochemical analysis.[5-11] Thus, publishing data on Ki-67 immunostaining in human gliomas is still worthwhile in order to optimize this method, with the superior goal of achieving a standardized procedure. The aim of this study is to evaluate the Ki-67 labelling indices (LIs) in a series of gliomas and critically evaluate the findings.

II. Materials and Methods

This is a prospective study over a period of 18 months (January 2016 – June 2017) conducted at Osmania General Hospital, Hyderabad

A total of 54 cases are taken up for study.The specimens were fixed in 10% buffered formalin and were manually processed. Gross features like size, shape, colour, consistency, cystic and necrotic changes were noted. The tissues were mostly in fragments/piecemeal. Wherever possible, the specimens were bisected longitudinally and a minimum of four bits each measuring 3-5 mm thickness was taken. After manual processing, sections of 3-5 µ thickness were cut and stained with routine hematoxylin and eosin (H and E).

Ki-67 immunostaining was done on all the 54 cases. Poly-L-lysine coated slides were dried overnight at room temperature/placed at 50-60°C in oven for 1 h. Representative blocks of formalin-fixed paraffin-embedded tissues were selected and 4-µm thick paraffin sections were floated onto slides previously coated with poly-L-lysine. The MIB-1 monoclonal antibody (monoclonal mouse anti-human Ki-67 antigen clone MIB-1) was used as the primary antibody for Ki-67 antigen detection.

During each batch of staining, appropriate positive control was used. Section from lymph node was included as a positive control. Sections were examined under high power field to observe for the immunoreactivity.

The immune stained sections were scanned using a 40× objective with an eye grid for the areas with the highest density of labelled 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 LI was defined as the percentage of immunoreactive tumor cell nuclei among the total number of cells.

All information collected in this study was recorded and analysed using SPSS 17 version software. These values were then compared with the Fisher exact test for proportions (p value), set to a 95% confidence interval.

Inclusion criteria : All neuro surgically excised specimens diagnosed as Gliomas on histomorphology.
Exclusion criteria: Left over squash specimens
Mixed neuronal-glial tumours

Results

In the present study a total of 54 cases have been diagnosed as gliomas (6 pilocyticastrocytomas, 14 diffuse astrocytomas, 2 gemistocyticastrocytomas, 1 oligoastrocytoma, 5 anaplastic astrocytomas, 1 anaplastic pleomorphic xanthoastrocytoma, 11 Glioblastomas, 7 oligodendrogliomas, 5 ependymomas and 2 anaplastic ependymomas) with a male:female ratio of 1.25:1.

Table 1 : Distribution of glial tumors :

Pilocytic astrocytoma	6 (11%)
Diffuse astrocytoma	14 (26%)
Gemistocytic astrocytoma	02 (4%)
Oligoastrocytoma	01 (2%)
Anaplastic astrocytoma	01 (2%)
Anaplastic PXA	01 (2%)
Glioblastoma	11 (20%)
Oligodendroglioma	07 (13%)
Ependymoma	05 (9%)
Anaplastic ependymoma	02 (4%)

Table 2 : Relationship between Ki-67 Labelling index (LI) and Grade of Glioma

Grade	Histomorphological Diagnosis	No. of Cases	KI-67 LI (in %) Range	Median KI-67 LI
Grade 1	**Pilocytic Astrocytoma**	**6**	**1-13**	**1.5**
Grade 2	**Diffuse Astrocytoma**	**14**	**1-20**	**3.5**
	Oligodendroglioma	**7**	**1-11**	**5.5**
	Oligoastrocytoma	**1**	**3**	**3**
	Gemistocytic Astrocytoma	**2**	**2-6**	**4**
	Ependymoma	**5**	**5-30**	**12**
Grade 3	**Anaplastic astrocytoma**	**5**	**4-32**	**23**
	Anaplastic PXA	**1**	**12**	**12**
	Anaplastic ependymoma	**2**	**10-19**	**13.5**
Grade 4	**Glioblastoma**	**11**	**2-45**	**26**

The table shows Ki 67 LI range in each grade of glioma and their respective median Ki 67 LI. The median Ki 67 LI increases in correlation with the grade of the tumour.

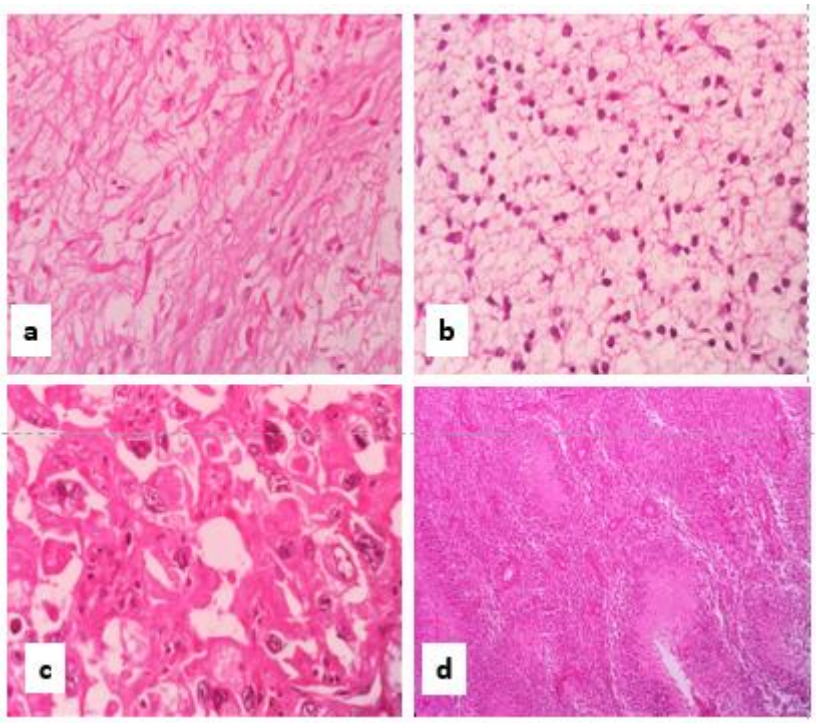

Figure 1: H and E (a) Grade I pilocytic astrocytoma, (b) Grade II diffuse astrocytoma, (c) Grade III anaplastic astrocytoma, and (d) Grade IV Glioblastoma

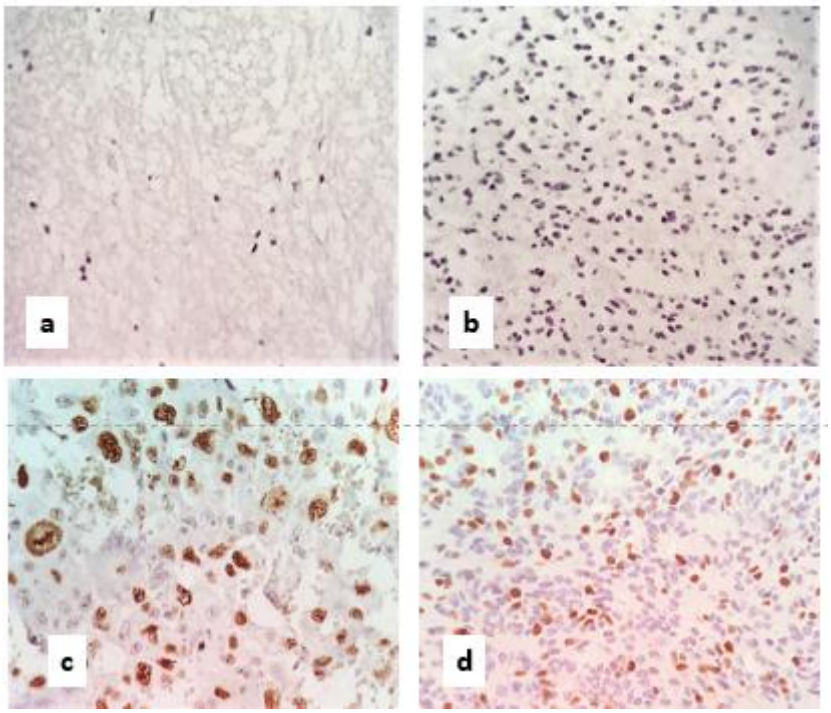

Figure 2: Ki-67 LI in (a) Grade I pilocytic astrocytoma, (b) Grade II diffuse astrocytoma, (c) Grade III anaplastic astrocytoma, and (d) Grade IV Glioblastoma

P value has been calculated between Ki 67 Labelling index and grade of glioma.
P value of < 0.05 is considered significant.

Table 3: p value between Ki 67 LI versus grade 1 and grade 2 gliomas

	Grade 1	Grade 2	p value
Ki-67 <= 6%	**02**	**21**	**0.7766**
Ki-67 >6%	**04**	**08**	

The relationship between grade 1 and grade 2 gliomas is not significant.

Table 4 :p value between Ki 67 LI versus grade 2 and grade 3 gliomas

	Grade 2	Grade 3	p value
Ki-67 <= 6%	**21**	**01**	**0.0025**
Ki-67 >6%	**08**	**07**	

The relationship between grade 2 and grade 3 gliomas is significant.

Table 5 :p value between Ki 67 LI versus grade 3 and grade 4 gliomas

	Grade 3	Grade 4	p value
Ki-67 <= 6%	**01**	**02**	**0.7373**
Ki-67 >6%	**07**	**09**	

The relationship between grade 3 and grade 4 gliomas is not significant.

Table 6 :p value between Ki 67 LI versus grade 2 and grade 4 gliomas

	Grade 2	Grade 4	p value
Ki-67 <= 6%	**21**	**02**	**0.0019**
Ki-67 >6%	**08**	**09**	

The relationship between grade 2 and grade 4 gliomas is significant.

III. Discussion

Conventional H and E staining is crucial for diagnostic neuropathology. The art of making a diagnosis in the practice of oncology has moved ahead from microscopic evaluation of the H and E stained slides, to the present era of the application of ancillary techniques in the form of immunohistochemistry and techniques of molecular biology. The use of these adjuvant modalities have not only helped in better understanding of the biological behaviour of malignancies, but have also opened doors to the development of specific targeted therapies.Astrocytic tumors have an inherited tendency to progress and recur. The accurate grading of astrocytic tumors is of prime importance because it is critical to the patient management and survival/outcome.

Although internationally accepted WHO grading system of CNS tumors is based on the histological features of H and E stained sections, there are cases where differentiation between Grade II and Grade III is difficult particularly when the biopsy is small.The labelling index derived from Ki-67 immunostaining has been found to be useful in the distinction between various grades of malignancy.Ki-67 LI is one of the most useful markers for evaluating cellular proliferation in various human neoplasms including intracranial tumors.

Counting procedures vary across studies. Usually counting is performed in areas with the highest immunoreactivity ("hot spots"), and approximately 1000 cells are counted using the 40× objective. The LI is calculated as the percentage of labelled tumor cell nuclei to the total number of tumor cells.[12,13]As the expression of the Ki-67 antigen changes during the cell cycle [14] ,the intensity of nuclear staining will vary; principally, all types of staining should be regarded as positive.[13]Counting can be done manually or by digitalized image analysis systems, but manual counting has turned out to be applicable for most diagnostic purposes.[12] Defining a cut-off value is also a topic of interest due to its impact on the determination of patients classified as "high Ki-67", which is indicative of a poorer outcome.

Generally, these patients will receive more aggressive treatment. However, the definition of threshold value is not straightforward mostly due to inter-/intra-observer variability and counting procedures.Accordingly, extrapolating values from other laboratories can be deceptive; thus, Ki-67 immunostaining should be interpreted in the context of one's own practice.[12] Each pathology department should regularly adjust its Ki-67

LIs by tumor grade and survival and develop its own in-house policy.Such a work-up will constitute an important part of a department's quality assurance and accreditation programs.[15] For astrocytomas, a cut-off of approximately 5 - 8% has appeared clinically feasible.[16] However, the predictive value of Ki-67 is ambiguous.[17,18]

In our material we found that the Ki-67 LIs correlated significantly with increasing tumor grade in all types of gliomas but an overlap occurred between the malignancy groups.The positive correlations between Ki-67 LI and tumor grade in our series of gliomas are in agreement with the literature.[19-23]

In the present study, Ki 67 LI showed a range of 1 – 13% in pilocytic astrocytoma with a median of 1.5%In case of diffuse astrocytoma Ki 67 LI ranged from 1-20% with a median of 3.5%.In anaplastic astrocytoma, median Ki 67 LI is 23% whereas glioblastoma had a labelling index of 26%.Oligodengrogliomas showed a median labelling index of 5.5% and ependymomas showed a median of 12%.The present study had 2 cases of anaplastic ependymoma and 1 case of anaplastic pleomorphic xanthoastrocytoma which showed Ki 67 Li of 13.5% and 12% respectively.

We found that indices were comparable between gliomas of similar malignancy grade, and indices for high-grade gliomas (grade III/IV) were significantly higher than in low-grade (grade I/II) tumors.In the present study, the p value between grade 1 and grade 2 tumours was 0.7766 which is not statistically significant.The p value between grade 2 and grade 3 tumours was 0.0025 and between grade 2 and grade 4 tumours was 0.0019 which showed a statistical significance.The present study, could not show any statistical significance between grade 3 and grade 4 tumours with a p value of 0.7373.

Thus, Ki-67 is useful for differentiating between high and low-grade gliomas, but differentiating between grade I and grade II or grade III and grade IV is more problematic due to the overlap of values between the different tumor grades.

This overlap is a main limitation of Ki 67 immunostaining and for this reason, Ki-67 should not be used alone as a marker of tumor grade but in conjunction with histological features.[24,25]

Pilocyticastrocytomas (Grade I) have distinct clinical, pathological, and prognostic characteristics when compared to diffuse astrocytomas (Grade II).[26]

A very low LI for pilocyticastrocytomas was noted and also no significant difference in LI between the pilocyticastrocytomas and diffuse astrocytomas was seen.[26-28] No significant prognostic role has been observed for Ki-67 LI in pilocyticastrocytomas, and there is a limited role for Ki-67 LI in determining the diagnosis in pilocytic astrocytoma.[26]

In the present study there were significant differences in Ki 67 LI between low grade (Grade II) and high grade (III-IV) gliomas and is in agreement with most of the other studies.[29-34]

Table 7 : Comparison of median Ki 67 LI between different grades of gliomas of present study with other studies

Authors	Number of Cases	Median Ki 67 LI
Ralte et al.[29]	Grade II – 30 Grade III - 11 Grade IV – 15	Grade II – 3.73 Grade III – 9.65 Grade IV – 10.33
Torp et al.[30]	Grade II – 22 Grade III - 10 Grade IV – 09	Grade II – 2.7 Grade III – 13.9 Grade IV – 12.1
Neder et al.[31]	Grade II – 10 Grade III - 05 Grade IV – 25	Grade II – 2.35 Grade III – 6.44 Grade IV – 12.28
Khalid et al.[32]	Grade II – 24 Grade III - 20 Grade IV – 33	Grade II – 1.78 Grade III – 13.47 Grade IV – 15.69
Wakimoto et al.[33]	Grade II – 19 Grade III - 25 Grade IV – 28	Grade II – 3.8 Grade III – 18.4 Grade IV – 31.6
Hsu et al.[34]	Grade II – 16 Grade III - 32 Grade IV – 33	Grade II – 0.8 Grade III – 8.75 Grade IV – 9.12
Present Study	Grade II – 29 Grade III - 8 Grade IV – 11	Grade II – 4.2 Grade III – 23 Grade IV – 26

Although few studies have found a significant difference in Ki-67 LI between Grade III and Grade IV astrocytomas[37] ,majority of them including the present study could not find a significant difference between them.[29-34].

In the present study, immunohistochemical results of three cases of astrocytomas did not show concordance with histopathological grade. Three cases (two cases of anaplastic astrocytomas and one case of gemistocytic astrocytoma Grade III) were histopathologically diagnosed as Grade II astrocytomas, which on Ki-67 immunostaining showed higher LI consistent with Grade III astrocytoma.

This implies that histological typing may under- or over-rate the actual biological behaviour of astrocytomas. The variations in the Ki-67 LI in various studies can be attributed to many factors such as fixative used, immunohistochemical procedures, especially antigen retrieval and interpretation of immunostaining.[35,36]

A low Ki-67 LI in high-grade astrocytoma could result from faulty sampling techniques and heterogeneity of the tumor. Retrieval of antigen can be better with hydrated autoclave treatment than with microwave treatment, which can result in higher Ki-67 LI possibly resulting from successful denaturation of formalin-fixed antigens.[35,36]

Ki-67 immunostaining to distinguish gliosis and low-grade gliomas should be interpreted with caution.[12] Normally, reactive astrocytes do not exhibit proliferative activity, but in some non-neoplastic conditions reactive astrocytes may have a proliferation rate of 1-5%.[38].In such cases, immunohistochemical analyses for mutated p53 and isocitrate dehydrogenase (IDH) proteins can be useful, though p53 immunoreactivity may occur in both settings, and there are gliomas without IDH mutation.[39-41].

The procedure for Ki-67 immunostaining is not standardized and has various analytical and clinical elements of uncertainty.[42]Nevertheless, the method is regarded as being robust[13] , which is also in accordance with our experience during several years with both clinical and experimental use.The recommended fixative is buffered formalin, and storage time, delay in fixation and fixation time does not seem to substantially affect the staining results.[43,44]

Loss of immunoreactivity has been described if cut sections are exposed to room air for some months.[45] A prerequisite for satisfactory immunostaining is adequate antigen retrieval.[14,43,44] Though Ki 67 LI has a few limitations, it serves as a useful supplement to the histopathological diagnosis of human gliomas.

IV. Conclusion

Immunohistochemical assessment of Ki 67 is an essential component in the evaluation of glial lesions.In our study, Ki-67 correlates well with the histomorphological grade with an occasional overlap between different grades and serves as an important tool in determining clinical course.Though, Ki-67 cannot be used as a sole prognostic factor it can be a useful adjunct to other prognostic indicators like age, tumour location, tumour resection and genetic alterations.With new concepts in genetic alterations introducing molecular markers will be key in targeted treatment and personalised care of glioma patients.

Acknowledgement

The authors would like to thank the technical staff of Osmania Medical College, Hyderabad, India

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Vikas Reddy Byreddy."Role of Ki 67 Immunostaining as an Adjunct to Differentiate Low Grade and High Grade Gliomas."IOSR Journal of Dental and Medical Sciences (IOSR-JDMS), vol. 17, no. 7, 2018, pp 11-17.