DOI: 10.7860/JCDR/2023/58640.17378

Original Article

Pathology Section

Evaluation of Nuclear Morphometry and Ki-67 Proliferative Marker in Astrocytomas: An Ambispective Study

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ABSTRACT

Introduction: Astrocytomas are the commonest primary Central Nervous System (CNS) tumours. Its diagnosis is based on histopathological criteria defined by the World Health Organisation (WHO) 2016 that grades astrocytoma into four grades. The subjective nature of WHO grading has prompted for more objective methods to evaluate nuclear features. Furthermore, Ki-67, a marker of cellular proliferation is a useful diagnostic tool that also helps in prognostic evaluation and to plan adjuvant therapy in astrocytomas.

Aim: To evaluate the nuclear morphometry and Ki-67 proliferative marker in astrocytomas and to assess the relationship of WHO grade with proliferative activity using Ki-67 immunostaining.

Materials and Methods: This ambispective study was conducted in the Department of Pathology, JSS Academy of Higher Education and Research, Mysuru, Karnataka, India, with a total duration of four years (October 2017 to September 2021). From 35 astrocytoma cases, Haematoxylin and Eosin (H&E) stained slides were retrieved and reviewed appropriately by two pathologists and graded using the WHO criteria. Nuclear morphometric analysis was performed on the H&E slides using Olympus BX-41 research microscope. The parameters evaluated were mean nuclear length, mean nuclear diameter, mean nuclear perimeter, mean nuclear area, Mean Nuclear Roundness Factor (MNRF) and mean nuclear ellipse form. The cases were then stained with Ki-67 antibody. The relationships between WHO grade of astrocytoma and nuclear morphometry and WHO grade with proliferative index was analysed.

Results: According to WHO grading, 4 (11.4%) cases were grade 1, 12 (34.3%) grade 2, six (17.1%) grade 3 and 13 (37.1%) grade 4. Significant correlation was seen between WHO grading and mean nuclear length, diameter, perimeter, area and Ki-67 with p-value <0.001.

Conclusion: Astrocytoma is an extremely heterogeneous disease with unpredictable outcome. The widely used WHO grading is subjective, while nuclear morphometry, using computer assisted image analysis, can ensure more objective assessment. The Ki-67 index could provide valuable information and may compliment the other parameters.

Keywords: Gliomas, Histopathological grading, Proliferative index

INTRODUCTION

Primary malignant Central Nervous System (CNS) tumour constitutes 2% of all malignancies, with relatively higher mortality and morbidity [1,2]. They are broadly classified as gliomas or non gliomas. Astrocytoma, a tumour of star shaped glial cells called astrocytes is the most common type of glioma [3]. The WHO grading system is being widely used and is based on histologic determinants like hypercellularity, mitosis, endothelial proliferation and necrosis. Grade 1 corresponds to pilocytic astrocytoma, grade 2 to diffuse astrocytoma, grade 3 to anaplastic astrocytoma and grade 4 as Glioblastoma Multiforme (GBM). The latest 2016 World Health Organisation (WHO) classification of astrocytoma has incorporated molecular characteristics and now the tumour is differentiated into Isocitrate Dehydrogenase (IDH)-mutant and IDH-wild type [3,4].

Histopathological examination although considered gold standard, however is not so reliable due to tumour heterogeneity, sampling error, poor reproducibility and the subjective nature of reporting [3]. Over the years, many newer diagnostic modalities have surfaced, like nuclear morphometry [5]. Nuclear morphometry aids in highly accurate measurements of the different nuclear facets of a tumour cell. Computer assisted image analysis has helped to calculate mean major axis, minor axis, nuclear area and nuclear perimeter, which relate to overall size of the nucleus and roundness of nucleus, which corresponds to nuclear shape [6,7]. Ki-67, a non histone nuclear protein which was first discovered by Gerdes J et al., is expressed in the proliferative phase of the cell cycle and is present in all phases except G0 phase [8,9]. Ki-67 Labelling Index refers to the percentage of cells that are positive for Ki-67 immunostaining [10].

The aim of the present study was to objectively assess the cases of astrocytomas and compare the results with conventional histologic examination. The objectives were to study nuclear morphometry of astrocytomas using computer assisted image analysis, correlate with histological grading and finally evaluate the relationship of histologic grade with proliferative activity using Ki-67 immunostaining.

MATERIALS AND METHODS

This ambispective study was conducted in the Department of Pathology, JSS Academy of Higher Education and Research, Mysuru, Karnataka, India, with a total duration of four years (October 2017 to September 2021). Ethical clearance from the Institutional Ethical Committee was obtained (ISS/MC/PG/5189/2019-20).

Inclusion and Exclsuion criteria: Thirty five cases included astrocytic tumours of varying grades. Mixed glioneuronal tumours and cases diagnosed as mixed oligoastrocytomas were excluded from the study.

Study Procedure

For retrospective cases, Haematoxylin and Eosin (H&E) stained slides were retrieved for morphometry and paraffin embedded blocks for Ki-67 Immunohistochemistry (IHC). For prospective cases, surgically removed brain tumour tissues were fixed in formalin, paraffin embedded and stained with H&E followed by immunostaining for Ki-67. All the cases were reviewed appropriately by two pathologists and graded using the WHO criteria published in 2016 [4]. Kappa statistics was used to measure the agreement in diagnosis amongst both pathologists. Finally, a consensus diagnosis was made by both the pathologists. For computer assisted nuclear morphometric

analysis, Olympus BX-41 research microscope connected with Jenoptix (Germany) progress Charge-Coupled Device (CCD) camera and progress capture pro imaging software was utilised. At 400X magnification, 100 nuclei were studied in each case. The software calculated the quantitative nuclear facets like nuclear length, nuclear diameter, nuclear perimeter and nuclear area. Other two parameters were calculated and included, Mean Nuclear Roundness Factor (MNRF)=perimeter 2/4 area and nuclear form ellipse (MNFe)=longest diameter/shortest diameter. Ki-67 IHC was performed on 3 µm thick sections on poly-lysine coated slides. Monoclonal antibody Ki-67 (Novocastra, code no. Ki-67-MMI-R7-C) was used by standard streptavidin-biotin technique using novostain universal detection kit (Novocastra, code no. RTU-Ki-67-MM1). Sections from reactive lymph node was used as positive control whereas sections treated with tris-buffer solution instead of primary antibody was used as negative control. Brown granular nuclear reactivity indicated positivity. LI was derived, which is the percentage of positively stained nuclei amongst 1000 cells noted under 400X magnification.

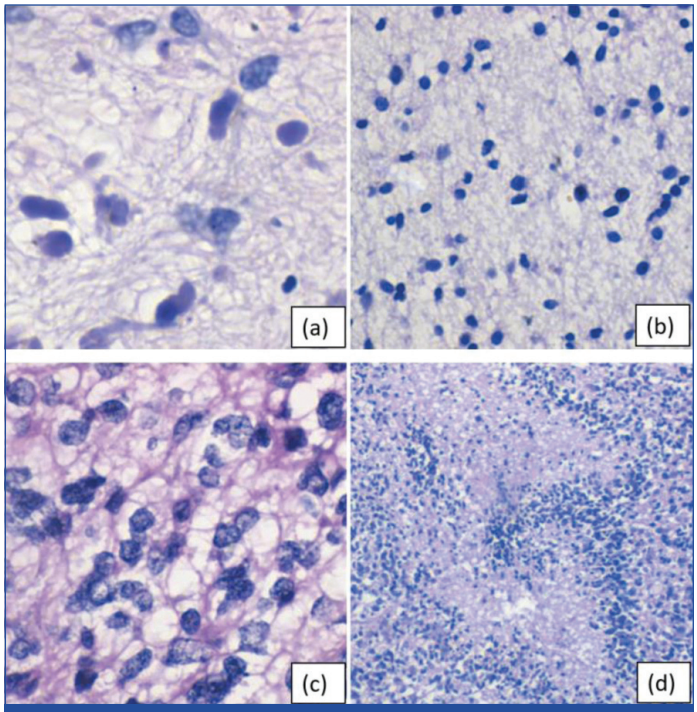
For optimal LI calculation, highly cellular area with evenly distributed cells having clear morphology and good staining was selected. Regions devoid of necrosis and endothelial proliferation were preferable.

STATISTICAL ANALYSIS

Kappa statistics was used to analyse the agreement and interobserver variability in diagnosis and grading of astrocytomas amongst the two pathologists. Statistical Package for Social Sciences (SPSS) version 13.0 was used to find the mean value of all parameters for each grade of astrocytoma, and Pearson's correlation coefficient was calculated. The p-value <0.05 was considered statistically significant.

RESULTS

After appropriate reviewing of H&E slides by two pathologists, 29 cases were in concordance to each other, whereas disagreement was seen in six cases. Kappa statistics was used to find out this agreement amongst both pathologists in WHO grading and was 74.7%, implying substantial agreement (fair agreement, κ=0.00 to 0.20; moderate agreement, κ=0.21 to 0.45; substantial agreement, κ=0.46 to 0.75; near perfect agreement, κ=0.76 to 0.99; perfect agreement, κ=1.00). Finally, both pathologists arrived to a consensus and there were four grade I cases [Table/Fig-1a], 12 grade II cases [Table/Fig-1b], six grade III [Table/Fig-1c] and 13 grade IV cases [Table/Fig-1d].



[Table/Fig-1]: Histomorphology of cases of different grades of astrocytoma. Pilocytic astrocytoma (biphasic cell population in a fibrillary background) (H&E, 400X); b) Fibrillary/diffuse astrocytoma (Round, monomorphic cells in a fibrillary background) (H&E, 100X); c) Anaplastic astrocytoma (Moderately pleomorphic cells, with vesicular to hyperchromatic nuclei) (H&E, 400X); d) GBM- Area of necrosis with peripheral palisading of cells (H&E, 100X).

Among these 35 cases, 12 were retrospective and 23 prospective cases with a male predominance and M:F ratio of 3:2. The age group ranged from 14-70 years with a mean age of 40.6 years. Age range for grade 1 tumour was 14-48 years, for grade 2 was 34-70 years, grade 3 was 18-60 years and for grade 4 was 17-68 years.

The correlation test was applied between WHO grade and nuclear morphometry among all 35 cases [Table/Fig-2] and the mean was calculated for each parameter in all cases followed by calculation of p-values and Pearson's coefficient [Table/Fig-3].

Case No.	Grade of tumour	Length (µm)	Diameter (µm)	Perimeter (µm)	Area (µm^2)	Nuclear roundness factor (Ratio)	Nuclear ellipse form (Ratio)	Ki-67 Labelling Index (LI) (in percentage)
1	1	6.545531	5.927146	20.151938	26.357797	1.30410	1.2269	2.70
2	1	6.999998	7.321766	22.942039	34.870223	1.28075	1.0425	4.30
3	1	6.058108	6.933218	20.918087	29.712637	1.20967	0.9167	3.00
4	1	9.725931	8.732005	29.606752	60.009287	1.22758	1.2052	2.80
5	2	4.848220	4.492627	15.058350	15.964833	1.15608	1.1202	20.00
6	2	6.180399	6.230701	20.001176	29.591187	1.09326	1.0108	5.50
7	2	6.645273	6.078057	20.546210	31.209278	1.09820	1.1164	3.50
8	2	6.348654	6.442323	20.754156	31.390523	1.15538	1.0382	5.20
9	2	6.019080	6.142237	19.637481	27.344894	1.16865	1.0252	5.40
10	2	7.485146	8.026600	24.954248	44.052454	1.18292	0.9894	4.90
11	2	6.506505	6.938421	21.574213	33.250331	1.16402	0.9770	11.50
12	2	6.509106	6.615784	21.033485	32.524221	1.13854	1.0135	2.60
13	2	6.134431	6.048568	19.582003	27.848382	1.13705	1.0577	2.00
14	2	5.444926	5.480485	17.625709	21.728488	1.16426	1.0511	5.50
15	2	4.523850	4.056374	13.667038	13.564408	1.13687	1.1474	2.80
16	2	7.616651	8.510841	25.901964	49.631771	1.13839	0.9198	6.50
17	3	7.058109	6.386816	21.725309	33.407923	1.15679	1.1369	1.00
18	3	7.657414	7.784908	25.144277	41.683062	1.25572	1.0347	7.80
19	3	6.452731	5.747615	19.655786	27.937819	1.57479	1.1674	3.50
20	3	6.451864	5.753686	19.475344	27.437451	1.11691	1.1346	12.50
21	3	7.270524	8.217544	24.949287	46.713526	1.11047	0.8973	8.20

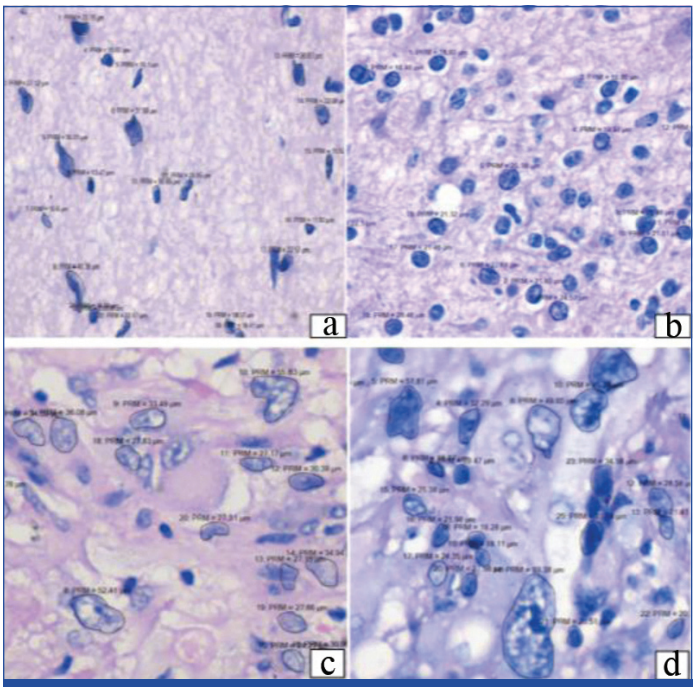
22	3	6.900259	7.439721	23.141562	39.822946	1.11599	0.9536	3.20
23	4	11.522978	10.294880	35.331472	107.632417	1.18118	1.1502	44.40
24	4	5.819601	5.725065	18.647659	23.422959	1.21864	1.0632	48.60
25	4	8.460535	8.554209	27.965140	49.518321	1.32736	1.0701	8.10
26	4	9.973110	9.179531	30.983291	70.437151	1.19905	1.1115	7.80
27	4	9.216824	8.675624	29.036720	63.001911	1.15656	1.0876	33.20
28	4	9.705984	9.723327	31.514357	70.803964	1.18525	1.0201	70.00
29	4	9.879128	9.279331	30.955399	63.968120	1.24702	1.1209	20.00
30	4	7.118819	7.007803	22.934761	35.997941	1.20560	1.0674	46.40
31	4	9.353860	10.270596	31.807881	70.823694	1.18484	0.9393	14.20
32	4	8.588028	8.371205	27.278572	50.831683	1.22004	1.0808	28.00
33	4	8.590628	8.525585	27.456342	51.568776	1.20110	1.0427	22.00
34	4	7.424110	7.758890	24.477657	42.450401	1.17483	1.0019	22.10
35	4	6.901125	6.982654	22.140039	35.680246	1.16636	1.0296	72.40

[Table/Fig-2]: Mean of each parameter for 100 nuclei calculated in each case and Ki-67 Labelling Index (LI).

WHO grade	Mean length (in µm)	Mean diameter (in µm)	Mean perimeter (in µm)	Mean area (in µm^2)	Mean nuclear roundness factor (Ratio)	Mean nuclear form ellipse (Ratio)
1	7.3324	7.2285	23.4047	37.7375	1.2555	1.0979
2	6.1885	6.2553	20.0280	29.8417	1.1445	1.0389
3	6.9652	6.8884	22.3486	36.1671	1.2218	1.0540
4	8.6581	8.4884	27.7330	56.6260	1.2052	1.0605
p-value	≤0.001	≤0.001	≤0.001	≤0.001	0.584	0.735
Pearson's coefficient	0.300	0.288	0.330	0.268	0.009	-0.006

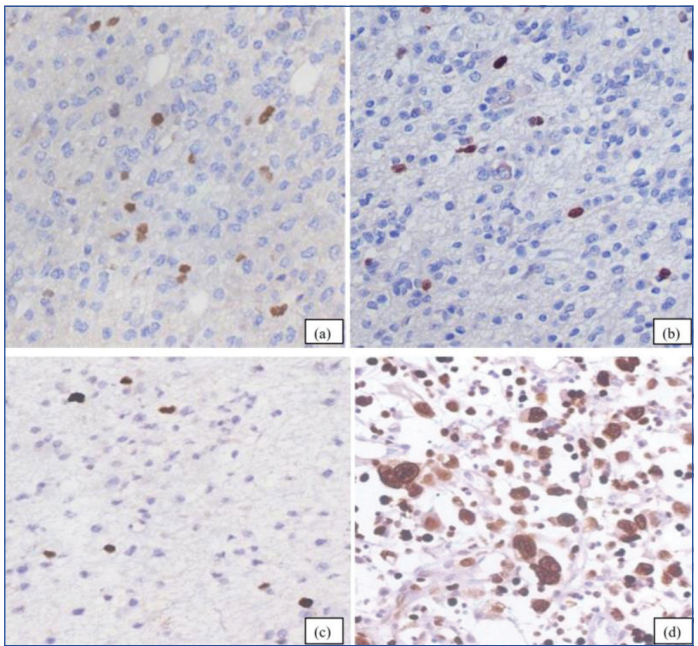
[Table/Fig-3]: Correlation of the six nuclear morphometric parameters of cases with each WHO grade.

Pearson's correlation was used to evaluate the relationship between WHO grade and nuclear morphometry and positive correlation was found between WHO grade and nuclear length, diameter, perimeter and area. Since, p-value of <0.05 is considered statistically significant, all the mean values of nuclear size morphometry in this study were statistically significant. However, nuclear roundness factor and nuclear form ellipse were not statistically significant [Table/Fig-3]. The photographs of nuclear morphometric analysis of each grade of tumour using research microscope are demonstrated in [Table/Fig-4].



[Table/Fig-4]: Nuclear morphometric analysis of each grade of tumour. Nuclear morphometry: a) Grade 1 tumour (H&E, 400X); b) Grade 2 tumour (H&E, 400X); c) Grade 3 tumour (H&E, 400X); d) Grade 4 tumour (H&E, 400X).

Ki-67 immunostaining: The Ki-67 LI was calculated based on the number of nuclei showing brown granular positivity for every 1000 nuclei counted in each case as shown in [Table/Fig-2,5]. The mean values of Ki-67 for the lower three grades were similar, whereas it was significantly higher for GBM [Table/Fig-6]. The Ki-67 results were correlated to consensus WHO grade using Pearson's correlation coefficient (p≤0.001). The coefficient was 0.643, which implies it positively correlated with WHO grade.



[Table/Fig-5]: Ki-67 immunostaining for each grade of astrocytoma. (a) Ki-67: Grade 1 astrocytoma, (x400); (b) Grade 2 astrocytoma, (x400); (c) Grade 3 astrocytoma, (x400); (d) Grade 4 astrocytoma, (x400).

WHO grade	Mean Ki-67 Labelling Index (LI) (in percentage)
1	3.2
2	6.2833
3	6.0333
4	33.6308

[Table/Fig-6]: Mean Ki-67 Labelling Index (LI) for each grade of astrocytoma.

DISCUSSION

Astrocytomas are heterogenous in their presentation. In this study, the age ranged from 14-70 years. Grade 1 astrocytoma is commonly seen in the paediatric age group and the youngest one in this study was 14 years and was histologically diagnosed with pilocytic astrocytoma [Table/Fig-1a] [11]. The variation in the age was also supported by Sukheeja D et al., [12]. This study showed a greater incidence of grade 2 and grade 4 tumours with lower prevalence of grade 1 and grade 3 tumours. A similar trend was seen by Ralte AM et al., [13]. Other studies like of Johannessen AL and Torp SH, and Akaishi K et al., showed grade 3 and 4 to be more common [14,15]. The studies conducted by Ralte AM et al., Johannessen AL and Torp SH, and Akaishi K et al., showed mean age of occurrence being directly proportional to the grade of astrocytoma, whereas this study saw the highest mean age for grade 2 tumour [Table/Fig-7] [13-15].

Various studies	Grade 1	Grade 2	Grade 3	Grade 4
Present study	4 (11.4%)	12 (34.3%)	6 (17.1%)	13 (37.1%)
Ralte AM et al., [13]	8 (12.5%)	30 (46.9%)	11 (17.1%)	15 (23.4%)
Johannessen AL and Torp SH [14]	10 (6.5%)	19 (12.3%)	36 (23.2%)	90 (58.0%)
Akaishi K et al., [15]	2 (5.6%)	6 (16.7%)	17 (47.2%)	11 (30.6)
Mean age (years)				
Present study	30.5	47	35.3	40.5
Ralte AM et al., study [13]	15.2	31	33.7	45.1
Johannessen AL and Torp SH [14]	26	39	42	49
Akaishi K et al., study [15]	6	39.8	52.8	52.8

[Table/Fig-7]: Incidence of astrocytoma among each grade with mean age of presentation in different studies.

Nuclear Morphometry

Even with advancement in WHO grading of CNS tumours, disconcordance among pathologists remain to certain extent.

Reason for this could be pathologists experience and the unclear criteria put forth by WHO. As grade of the tumour increases, cellularity and mitosis also increase. However, there is no defined cut-off for these criteria, which leads to subjective error in grading [4].

Aldape K et al., in their study found that in 457 astrocytoma cases, 23% had variable diagnosis, and 16% among them lead to patient mismanagement [16]. In the current study, there was disagreement in 6 (17%) out of 35 cases. Thus, histology alone cannot be sufficiently considered reliable to predict patient outcome. Newer methodologies like molecular genetics and Deoxyribonucleic Acid (DNA) ploidy are not always affordable by all patients, since they are expensive. Hence, nuclear morphometry is a simpler and cheaper tool to objectively differentiate between benign and a malignant tumour [6]. Till now, very few studies have been conducted on computer assisted morphometric analysis of CNS tumours. The variables used in a similar study conducted by Boruah D and Deb P, included major axis, minor axis, nuclear area, nuclear perimeter and nuclear roundness, very similar to variables used in this study, with addition of nuclear form ellipse [6]. The initial four parameters of this study assessed the nuclear size and the last two derived variables correspond to the nuclear shape of the tumour cells.

The study done by Boruah D and Deb P, on nuclear morphometry on gliomas showed negative correlation of roundness factor with WHO grade [6]. In the present study, there was a negative correlation for nuclear form ellipse. Both of these parameters pertain to nuclear shape. A technical error for this insignificant correlation may include missing out the details of nuclear shape irregularity while manually contouring on the research computer. A previous study done by Ikeguchi M et al., has investigated the interobserver variation in morphometry [17]. Other sources of error could be due to error in calibration and standardisation of the software [18].

The WHO grading is very subjective in which poor sampling, fixation and sectioning can lead to a false diagnosis [3]. Such limitations can be overcome with the evaluation of increased abnormality in nuclear size and shape. Tosi P et al., in their study stated that morphometric parameters like nuclear area are independent to tissue processing issues [19]. The best technique that could supplement grading are stereological factors like nuclear length, diameter, perimeter and area that predict the nuclear size.

Extensive work has been done on nuclear morphometry in tumours of other systems such as breast carcinoma, renal cell carcinoma, thyroid carcinoma [19-22]. A couple of studies have been devoted to conducting morphometric analysis on cytological smears [23]. Similar parameters have been used in the domain of morphometry in the study of these organ systems [20-22].

Ikeguchi M et al., studied computerised nuclear morphometry use in evaluating colorectal adenocarcinoma and their metastatic potential [17]. They inferred that the mean nuclear area increased as the case progressed from non neoplastic to an adenoma to colorectal adenocarcinoma. They also concluded that the capability of the tumour cells to metastasize into lymphovascular space and to distant organ depended on the nuclear area. Hence, the study stated that nuclear morphometry can be used as a screening program to assess the metastatic potential of the carcinoma.

Similar study conducted by Pienta KJ and Coffey DS, on breast carcinoma saw a steep increase in nuclear area among those cases that were node-metastasis positive [20]. Computer assisted image analysis in these studies support the fact that an aggressive tumour that gets graded into a higher category would expectedly have bigger nuclear area.

Ki-67 Labelling

The Ki-67 is a monoclonal antibody that targets protein expressed in a proliferating cell, except in the resting phase (G0 phase) of cell cycle [24]. It has already been used for breast tumours, lymphomas and other intracranial malignancies such as gliomas in general and meningioma [25-28]. The antibody can be utilised as a tool to supplement the diagnosis of astrocytoma.

In the present study, the mean LI of grade 1, grade 2 and grade 3 astrocytomas were within proximity to each other and were low. A similar observation was made by Sengupta S et al., [29]. There are a few studies which had differing results. The study conducted by Johannessen AL and Torp SH, showed a significant leap of the mean LI from grade 2 to grade 3 astrocytomas [14]. Ralte AM et al., also showed a similar finding [13]. The present study showed overlapping of Ki-67 LI amongst some of the grades of astrocytomas. A study conducted by Moskowitz SI et al., on Ki-67 proliferation on newly detected grade 4 GBM found it to range from 0-76.4% [30]. In a study, Raghavan R et al., described that the presence of well differentiated regions in higher grades of astrocytoma is responsible for giving an overlapping result [31].

A few studies like the ones conducted by Thotakura M et al., have shown Ki-67 correlation with histologic grade, much like the present study [32]. Significant correlation of the marker to astrocytoma grade was observed in Sengupta S et al., study, identical to the results of this study [29]. The issues that may occur with Ki-67 immunostaining range from type of products (manufacturing company), characteristic of the secondary antibody, fixative used and time for fixation and expertise in counting of Ki-67 positive cells [32].

Another issue encountered with calculation of the proliferative index is that an amount as huge as 1000 cells needs to be evaluated for Ki-67 positivity under a microscope. It is not impossible that sometimes pathologists do guess work to save time [33], and when its wrong, it alters the course of treatment. Therefore, newer automated computer assisted techniques are emerging for Ki-67 calculation [33].

Pleomorphic Xanthoastrocytoma (PXA) is a low-grade tumour with favourable prognosis [4]. The tumour exhibits moderate cellularity and high level of pleomorphism, however with no areas of necrosis [34]. Mitosis is also rare [35]. The nucleus is multilobulated and bizarre. A case like this may be misdiagnosed as GBM, if it shows any doubtful undifferentiated areas. It was difficult for the two pathologists to accurately differentiate both entities in cases of small biopsy which may not have been representative of the lesion. In the present study, there were two cases of PXA, both of which were subjected to interobserver variability prior to arriving at a consensus diagnosis. The benign nature of this tumour was supported by low Ki-67 LI. Similar finding was noted in a case reported by Ng WH et al., where an elderly female presented with an aggressive case of PXA with an initial over diagnosis of glioblastoma [36]. Despite having areas of necrosis on radiographic and histological examination, the LI of Ki-67 was surprisingly low.

Despite WHO grading being gold standard in diagnosis of astrocytomas, the present study demonstrated discordance in six out of 35 cases with significantly positive correlation for nuclear morphometric analysis of nuclear size and either statistically insignificant or a negative Pearson's coefficient for nuclear shape. Hence, nuclear morphometry which is relatively an easy tool can enhance the accuracy of diagnosis. In addition, Ki-67 LI showed overlapping results amongst some of the grades of astrocytomas. Hence, combined studies including WHO grading, nuclear morphometry and Ki- 67 LI in astrocytomas may help in arriving at correct diagnosis with better patient care.

Limitation(s)

In the present study, the mean values of all parameters in grade 2 tumours were lower than that of grade 1. This could be attributed to the fact that out of four grade 1 tumours, two were PXA. This tumour presented with large, hyperchromatic, multinucleated cells. Thus, its morphometric values were quite higher and have put the mean of grade 1 parameters slightly at a higher level than grade 2 tumours. The result might have altered if equal number of cases were assessed for each grade of astrocytomas.

CONCLUSION(S)

Astrocytomas are the most common glial tumour with varied characteristics. The WHO grading being the gold standard for diagnosis, accuracy is not guaranteed at all the times and nuclear morphometry is a relatively easy tool that can enhance the accuracy of diagnosis. It is mostly helpful as a supplementary tool on stereotactic small biopsies, which may not be representative of the tumour. In addition, Ki-67 is an immunomarker that is detected at any stage of the cell cycle except the quiescent phase. In the present study, grade 1 to grade 3 showed low Ki-67 labelling indices, whereas it was significantly increased for GBM with a positive correlation to WHO grade. As noted in the present study, both morphometry and IHC themselves are not sufficient to accurately diagnose astrocytoma, instead they make excellent supplementary techniques that complements the gold standard, histopathology.

Acknowledgement

The authors are extremely grateful to the laboratory technical staffs of the hospital for their contribution in this study. Authors are also thankful to the Head of the Department for immeasurable support and for providing great facility and opportunity.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jun 25, 2022
- Manual Googling: Nov 04, 2022
- iThenticate Software: Nov 22, 2022 (22%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. Yes

Date of Submission: **Jun 23, 2022**
Date of Peer Review: **Aug 20, 2022**
Date of Acceptance: **Dec 21, 2022**
Date of Publishing: **Jan 01, 2023**