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ARTICLE

A Correlative Study of Gliomas Using *In Vivo* Bromodeoxyuridine Labeling Index and Computer-aided Malignancy Grading

Suash SHARMA, Asis K KARAK, Rajvir SINGH,[1] VS MEHTA,[2] Chitra SARKAR, Horst P SCHMITT[3]

Departments of Pathology, [1]Biostatistics and [2]Neurosurgery, All India Institute of Medical Sciences, New Delhi, India; [3]Institute of Neuropathology, University of Heidelberg, Germany

An in vivo bromodeoxyuridine (BrdU) labeling index (LI) was estimated in 43 cases of astrocytic tumors and mixed gliomas by one hour intra-operative intravenous infusion at a dose of 200 mg/m^2 and correlated with (a) histological grading using a computer aided malignancy classifier TESTAST-268; and (b) histological typing using WHO classification. The lowest BrdU LI was seen in pilocytic and gemistocytic astrocytomas followed by astrocytomas, anaplastic astrocytomas and glioblastoma multiforme in that order. Mixed oligoastrocytomas followed the pattern of their astrocytic counterparts. Tumors of similar histological type showed different BrdU LI values especially amongst astrocytomas and glioblastomas. A statistically significant difference in the BrdU LI was also noted between the higher TESTAST grades of astrocytomas (T III and IV) versus the lower TESTAST grades (T II). Unlike earlier reports in literature, in the present study the category of BrdU LI of <1 contained no case of anaplastic astrocytoma or glioblastoma multiforme (TESTAST grades III and IV). Likewise, the category of BrdU LI >5 contained only anaplastic astrocytoma and glioblastoma multiforme (TESTAST grades III and IV). Maximum spread of cases was seen in the BrdU LI category of 1-5, not only in terms of histological types but also TESTAST grades. Thus there appeared to be a positive trend of increasing BrdU LI values both with histological types and increasing TESTAST grades. Further, an interesting observation was that by using a combination of TESTAST grades and BrdU LI, the histologically homogenous glioblastoma group could be further subdivided into 4 categories which showed a trend towards prognostic correlation. Thus, this study though preliminary with number of cases being small in some groups, highlights the possible usefulness of combined histological typing, TESTAST grading and in vivo BrdU LI for prognostication of gliomas especially glioblastoma multiforme. (Pathology Oncology Research Vol 5, No 2, 134–141, 1999)

Keywords: gliomas, in vivo BrdU LI, malignancy classification, histologic grading, computer aided grading

Introduction

Estimation of in vivo bromodeoxyuridine (BrdU) labeling index (LI) has been claimed to be an independent sensitive indicator of the degree of biological aggressiveness in gliomas irrespective of their histopathologic heterogeneity.[4,5,9-11,19-21] Hoshino et al.[9-11] have shown a correlation of in vivo BrdU LI with a 3-tiered malignancy grading of astrocytic tumors along with their respective survival periods. However, reports of studies attempting to find out any possible correlation between in vivo BrdU LI and Kernohan's 4-tiered grading system[15] are at present few in the literature. This is important especially because while Kernohan and Sayre[16] and Zulch[34] found a good correlation of their 4-tiered grading system with survival, many other workers failed to find any significant difference in survival between astrocytomas of Kernohan's grade1 and 2[2,3,7,22,23,33] and between grades 3 and 4.[2,3,22,25] Hence they proposed a 3-tiered grading system which they claimed showed better correlation with prognosis.

The main disadvantage of the Kernohan grading system has been its subjectivity resulting in high intra-and

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Correspondence: Prof Chitra SARKAR, Department of Pathology, All India Institute of Medical Sciences, New Delhi.-110029, India; Tel:+91-11-659 3371; fax: +91-11-686 2663;
e-mail: sarkarch@medinst.ernet.in / sarkarcs@hotmail.com

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inter-observer variability.[2,3,7,22-25,29,32,33] The TESTAST 268, a malignancy classifier system for astrocytic tumors and mixed gliomas, is a computer-aided semi-objectivised grading system which reproduces Kernohan's 4-step grading, either categorically or with the help of canonical values plotted on a standard reference plot. Schmitt[26] and Schmitt et al[27,28] proposed that this system provided reproducible observer independent results. In an earlier study,[29] we have also shown that TESTAST 268 can be recommended as an objectivised and highly reproducible tool to assess the malignancy of gliomas. Here, we report the correlation of in vivo BrdU LI in astrocytic tumors and mixed gliomas with (a) TESTAST 268 grading and (b) histological typing by the WHO classification.[17]

Materials and Methods

Forty-three astrocytic tumors and mixed gliomas from the Neurosurgery Department of All India Institute of Medical Sciences were studied without any selection bias. Age, sex and site of the tumors were recorded in each case.

BrdU was obtained from the Division of Cancer Treatment, National Cancer Institute, Maryland, USA. Permission to administer BrdU to the patients included in this study was obtained from the Ethics Committee of the Institute. Informed consent was obtained from the patients before administering the drug.

Each patient was given continuous intravenous infusion of BrdU slowly over a period of one hour at the time of surgery, so that the infusion finished a few minutes before the tumor was biopsied. The dose of BrdU used was 200 mg/m^2 of body surface area, reconstituted in 100 ml of sterile 5% dextrose. Constant monitoring for any side effects of BrdU was done.

Tissue was collected from 2 to 3 different sites of the tumor in a way that a minimum of 2 to 3 slides could be prepared per case. The tissue was divided into two portions: (a) One portion was fixed in 10% buffered formalin, routinely processed and paraffin embedded. Five micron thick sections were cut and stained with haematoxylin and eosin (H&E). (b) Another portion was immediately fixed in chilled 70% ethanol for 12 hours. This was then routinely processed, paraffin embedded and sections cut for immunohistochemical staining.

Histological typing

This was done on H&E stained sections of all the 43 cases using the WHO classification.[17] The gemistocytic variant was separated out from the rest of the astrocytoma group because it has been reported that this variant is often associated with an unfavourable prognosis.[1,24,31]

Grading using TESTAST 268

For grading tumors using this system, each case was evaluated for the 8 classification variables namely, 5 histologic and 3 clinical which include: mitoses (MITO), Rosenthal fibres (ROSE), necrosis (NECR), vascularization (VASC), vascular proliferation (VASP), age in years (AGE), localization-I (LOC-1) and localization-2 (LOC-2). The term vascularization is related to the number or density of blood vessels in the tissue section whereas vascular proliferation has been applied to the degree of endothelial cell proliferation in the vessels.

Mitoses were evaluated as; 0 = none; 1 = less than 1 in every 2nd high power field (hpf) (X 400); 2 = 1 in every 2nd to 1 in every hpf (X 400); 3 = more than 1 in every hpf (X 400). Rosenthal fibers, necrosis, vascularization and vascular proliferation were evaluated as: 0 = none (scanty: for vascularization); 1 = moderate to marked; 2 = rich, frequent, abundant, considerably increased. LOC-1 was defined as: 0 = Inferior (cerebellum, brainstem, spinal cord, suprasellar region, diencephalon, 3rd ventricle, optic nerve, pineal region); 1 = superior (hemispheres, basal ganglia, lateral ventricles). LOC-2, brainstem involvement was defined as 0 = no; and 1 = yes.

In each case, using the individual scores of the classification variables, the procedure "BAYTEST" was performed using a PC-XT and the TESTAST 268 program software. This test provided the following values for each malignancy class: (a) Squared-Mahalanobis distance (D^2); and (b) Posterior probability. The tumor was assigned the TESTAST grade (T I to IV) associated with the lowest squared-Mahalanobis distance or highest posterior probability value. The methodological and statistical details have been dealt within the original publications on the topic by Schmitt[26] and Schmitt et al.[27,28]

In vivo BrdU LI

Five micron thick unstained sections were cut from paraffin blocks of alcohol fixed tissues and subjected to immunohistochemistry using an avidin-biotin-conjugate (ABC) immunoperoxidase method. Briefly, sections were deparaffinized and hydrolyzed with 1N HCl prewarmed to 60°C for 8 minutes. The sections were, then reacted with the monoclonal anti-BrdU antibody in a dilution of 1:30 with overnight incubation at 4°C. After washing, they were treated with the biotinylated secondary antibody and subsequently with the ABC reagent. The colour reaction was developed with diaminobenzidine (DAB). Counter staining was done with haematoxylin.

Adequate positive and negative controls were used for each batch of slides stained. *Positive controls*: In-vivo intraperitoneal injection of BrdU at a dose of 10 mg/kg was given for a period of one hour to a rat weighing 250 g.

Table 1. **Distribution of 43 astrocytic tumors as per WHO histologic typing (1993)**

Histological typing	*Total number*	*Age range in yrs (median)*	*M:F*
Astrocytoma	11	23–46 (30)	1.2:1
Gemistocytic astrocytoma	2	32–35 (34)	1:1
Pilocytic astrocytoma	4	3–16 (11)	1:1
Anaplastic astrocytoma	3	35–62 (35)	1:2
Glioblastoma	20	7–72 (35)	2:1
Mixed oligoastrocytoma	1	50	1:0
Anaplastic oligoastrocytoma	2	28–49 (39)	0:2

After this period, the animals were sacrificed and the intestine was taken out, fixed in chilled 70% alcohol and processed routinely. Unstained sections were cut and used as postive controls. Large number of BrdU labeled nuclei were seen in the mucosa especially in the crypts. *Negative controls*: Two types of negative controls were used namely, (a) using an unrelated primary antibody like Pan-T; and (b) not using any primary antibody and then replacing this step by phosphate-buffered saline.

A total of 2000 nucleated tumor cells were counted under the oil immersion objective (x 100) in different microscopic fields randomly without any bias. The ratio of nuclei labeled by BrdU to the total number of tumor cells was expressed as a percentage which gave the in-vivo BrdU LI.

Statistical analysis

The range and mean ± standard deviation (S.D.) of the BrdU LI values against individual TESTAST 268 grades and WHO histologic types of tumor were calculated. To assess the statistical significance of the difference in BrdU LI values between any two grades or histologic type of the tumors, the Wilcoxon Rank-sum test was applied as follows:

BrdU LI and TESTAST 268 Grades: (a) TESTAST Grade I (T I) versus Grade II (T II); (b) TESTAST Grade II (T II) versus Grade III (T III); (c) TESTAST Grade III (T III) versus Grade IV (T IV) and (d) TESTAST Grade I (T I) versus Grades III and IV (T III & T IV).

BrdU LI and WHO histological types: Astrocytoma versus anaplastic astrocytoma versus glioblastoma multiforme.

The cases were further divided into three groups according to the BrdU LI values namely : those with LI less than 1 (<1), those with LI between 1 and 5 and those with LI more than 5 (>5). This LI distribution was then attempted for any correlation with the TESTAST 268 grades and the WHO histologic types.

Follow Up

The survival values of the glioblastoma patients along with their corresponding TESTAST 268 grades and BrdU LI values were categorised into 4 groups: Category A: Grade T III with LI<5; Category B: Grade T IV with LI>5; Category C: Grade T III with LI<5; Category D: Grade T IV with LI>5.

Survival curves were estimated by Kaplan-Meier method using BMDT Statistical Software release 7.0 (BMDP Statistical Software Inc., 1440 Sepulveda Blvd., Los Angeles, CA, USA). Log Rank Test was used to determine any difference in the survival curves between the following categories: (i) Categories A versus B and A versus C, (ii) Categories D versus B and D versus C, (iii) Categories A+B (LI < 5) versus Categories C+D (LI >5)

Results

The histological typing of 43 astrocytic tumors along with age range and sex distribution of the patients are shown in *Table 1*.

Correlation of BrdU LI and histological typing of tumors by WHO (1993) classification

From the results presented in *Table 2*, there appears to be a correlation between histological tumor type and BrdU LI. The lowest LIs were seen in gemistocytic and pilocytic astrocytomas whereas the highest was seen in glioblastoma multiforme.

The scattergram *(Figure 1)* shows the random distribution of BrdU LI values versus various histological types of gliomas. On applying Wilcoxon's Rank-Sum test, it was

Table 2. **Distribution of 43 astrocytic tumors belonging to different histological types (WHO, 1993) with the BrdU LI categories of <1, 1-5 and >5**

No.	*Histological type*	*BrdU LI values*		*Cases in BrdU LI Categories*			*Total cases*
		Range	*Mean+ S.D.*	*<1*	*1–5*	*>5*	
1.	A	0.2–3.9	1.6+1.3	5	6	0	11
2.	GA	0.4–0.5	0.5+0.2	2	0	0	2
3.	PA	0.3–1.4	0.6+0.5	3	1	0	2
4.	AA	0.1–5.4	4.8+0.6	0	2	1	3
5.	GBM	2.4–6.3	5.0+0.9	0	7	13	20
6.	MOA	0.9	–	1	0	0	1
7.	AOA	2.4–2.9	2.7+0.3	0	2	0	2

A – Astrocytoma; GA – Gemistocytic astrocytoma; PA – Pilocytic astrocytoma; AA – Anaplastic astrocytoma; GBM – Glioblastoma multiforme; MOA – Mixed oligoastrocytoma; AOA – Anaplastic oligoastrocytoma. (A vs. AA $p<0.05$; A vs. GBM $p<0.01$ and AA vs. GBM $p<0.05$).

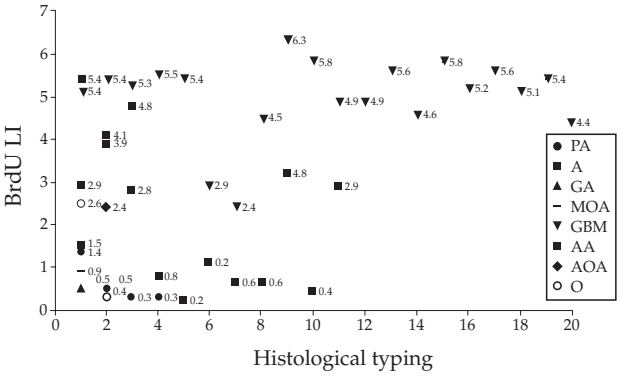


Figure 1. *Scattergram showing the distribution of BrdU LI values versus W.H.O histological types in 43 astrocytic tumors (A-Astrocytoma; GA-Glioblastoma multiforme; MOA-Mixed oligoastrocytoma; AOA-Anaplastic oligoastrocytoma).*

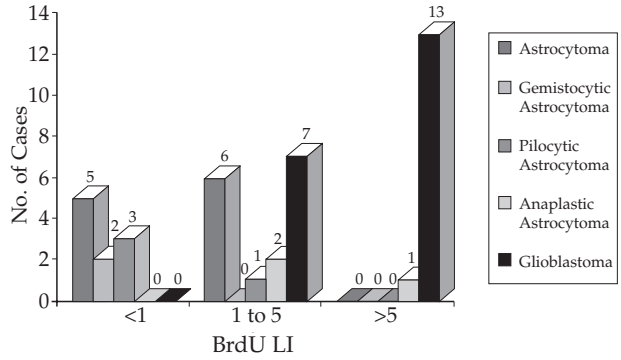


Figure 2. *Bar diagram showing the distribution of WHO histological types of 43 astrocytic tumors in three different categorised based on BrdU LI values.*

observed that there was statistically significant difference between the distribution of LI of astrocytomas and glioblastomas ($p<0.01$), astrocytomas versus anaplastic astrocytomas ($p<0.05$) and anaplastic astrocytomas versus glioblastomas ($p<0.05$). However, the number of cases of anaplastic astrocytoma are small and hence the statistical validation may not be unequivocal.

Table 3. **Distribution of 43 astrocytic tumors belonging to different TESTAST 268 grades within the categories based on BrdU LI values**

No.	*TESTST 268 type*	*BrdU LI values*		*Cases in BrdU LI Categories*			*Total cases*
		Range	*Mean+ S.D.*	*<1*	*1–5*	*>5*	
1.	T I	0.3–1.4	0.6+0.5	3	1	0	4
2.	T II	0.2–3.9	1.4+1.2	8	6	0	14
3.	T III	2.4–5.5	4.4+1.1	0	8	8	16
4.	T IV	4.6–6.3	5.4+0.5	0	3	6	9

(T II vs. T III $p<0.001$; T III and T IV $p<0.05$; T I vs. T II N.S.)

The bar diagram *(Figure 2)* and *Table 2* shows the distribution of these histological types within the three categories based on BrdU LI values. It can be made out that tumors with LI <1 included astrocytomas, pilocytic and gemistocytic astrocytomas and mixed oligoastrocytomas. Thirteen of 14 tumors (92%) with LI more than 5 were glioblastoma multiforme. The category of LI between 1 and 5 showed maximum overlap of different histological types in varying percentages.

Correlation of BrdU LI with TESTAST 268 grading

From the results presented in the *Table 3*, there appears to be a correlation between BrdU LI and TESTAST grading. The lowest LI was seen in grade T I tumors (0.6±0.5) and highest in grade T IV tumors (5.4±0.5).

The scattergram *(Figure 3)* shows random distribution of BrdU LI versus TESTAST grading. The differences of LI values between grades T III and T IV tumors and between grades T II and T III were found to be statistically significant ($p<0.05$ & $p<0.001$ respectively) while between grades T I and grades T II was not significant.

The bar diagram *(Figure 4)* and *Table 3* show the distribution of different grades in the three categories based on BrdU LI. Eleven tumors in the category showing LI <1 included only grades T I and grades T II tumors while the category showing LI>5 included only grades T III and grades T IV tumors. he category of LI between 1 and 5 was the most heterogene-ous overlapping group comprising tumors of all grades.

Survival

The cases were followed up over a variable period of 10–40 months. All grade T I patients (4/4) and 85.7% of grade T II patients (12/14) are alive at the time of the present communication. It was observed that using BrdU LI and TESTAST grading, the histological homogenous glioblastoma group could be split into 4 subcategories which interestingly showed a trend towards prognostic correla-

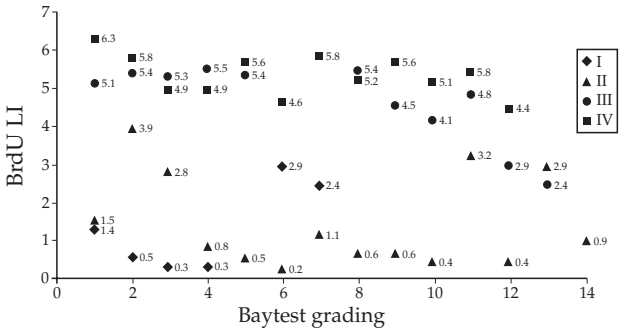


Figure 3. *Scattergram showing distribution of BrdU LI values versus TESTAST grades in 43 astrocytic tumors.*

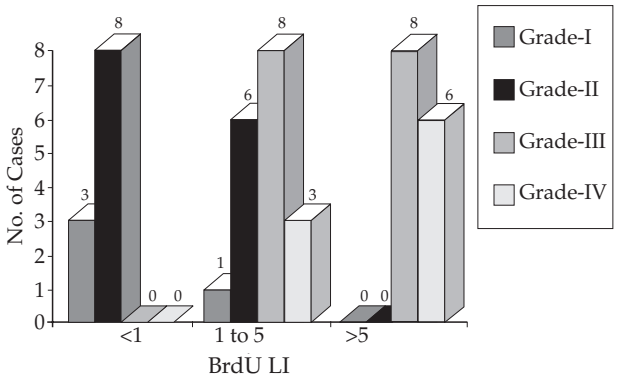


Figure 4. *Bar diagram showng the distribution of TESTAST grades of 43 astrocytic tumors in three different categories based on BrdU LI values.*

tion. The survival values for these glioblastoma subcategories along with their corresponding TESTAST 268 grades and BrdU LI values are summarized in *Table 4*. Using Log Rank test, there was a statistically significant difference in overall survival ($p<0.05$) between glioblastomas with BrdU LI of LI<5 (Categories A+B) as opposed to that of >5 (Categories C+D). It was also observed that the difference in the duration of overall survival in cases of Category A (i.e. glioblastomas with grade T III and BrdU LI value of <5) was longer as compared to the other three categories. Statistically, however, no significant difference was observed between the various categories, possibly because of the small number of cases in each of the categories.

Discussion

The precise determination of the biological behavior of tumors as heterogeneous as gliomas is faced with limitations not only because of limited surgical accessibility and potentially malignant clinical behavior by way of their site and pressure effect due to resistance offered by the rigid confines of the skull,[30] but also because their virtual rate of proliferation may be discordant with the proliferative potential reflected in the histopathological picture,[6,8-14] thereby justifying the continuing attempt at reclassification of these tumors.[17] It is therefore obvious that histological appearance alone may not be adequate to correctly prognosticate the overall behaviour of gliomas.[4,5,17,24] The present study addresses this issue by estimation of in vivo BrdU LI and correlating it with the WHO classification[17] and numerical 4-step grading by TESTAST 268.[26,27,29]

Correlation of BrdU LI with WHO histological types

It is evident from the results of the present study that there is a significant correlation between the histological tumor type and BrdU LI *(Table 2; Figure 1 and 2)*. This is possibly the first study showing correlation of BrdU LI with the new WHO classification.[17]

The lowest LI was seen in gemistocytic and pilocytic astrocytomas (mean 0.5±0.2 and 0.6±0.5 respectively). Astrocytomas showed a relatively higher mean value (1.6±1.3) followed by anaplastic astrocytomas (4.8±0.6) and glioblastoma multiforme showed the highest (5.0±0.9) LI values. Anaplastic oligoastrocytomas were more akin to anaplastic astrocytomas in terms of LI values (2.7±0.3). These findings are consistent with the earlier reports[9,10,19,20] wherein highest LI values had been noted in glioblastomas [7.3] and lowest in moderately anaplastic astrocytomas (MoAA-corresponding to astrocytoma of WHO classification) and intermediate values in highly anaplastic astrocytomas (HAA group).

It is also evident from the results of the present study that tumors of similar histological type can show wide variation in BrdU LI values, a finding which has also been observed by other workers.[4,5,11] This feature was particularly pronounced in the categories of astrocytoma and and glioblastoma multiforme in the present series, possibly because of the larger number of cases in these groups.

Hoshino et al[10] divided their cases into 3 groups based on BrdU LI values. They observed that more than 75% of

Tabel 4. **Follow up data of glioblastomas distributed in 4 categories based on BrdU LI values and TESTAST 268 grades**

Sr. No.	*BrdU LI category*	*BrdU LI value*	*TESTAST 268 grade*	*Follow up (months) Dead*	*Alive*
1.	<5	2.4	T III	–	18+
2.	<5	2.9		–	17+
3.	<5	4.4		–	12+
4.	<5	4.5		–	N.A.
5.	<5	4.6	T IV	–	12+
6.	<5	4.9		–	10+
7.	<5	4.9		5	–
8.	>5	5.1		12	–
9.	>5	5.1		12	–
10.	>5	5.3		–	12+
11.	>5	5.4		8	–
12.	>5	5.4		–	10+
13.	>5	5.4		–	N.A.
14.	>5	5.5		6	–
15.	>5	5.2	T IV	–	10+
16.	>5	5.6		12	–
17.	>5	5.6		8	–
18.	>5	5.8		10	–
19.	>5	5.8		2	–
20.	>5	6.3		5	–

N.A. = not available.

tumors with LI <1 were MoAA, more than 50% of tumors with LI between 1 and 5 were HAA and nearly 70% of those with LI >5 were glioblastoma. The different categories showed statistically significant survival values. They further postulated based on survival studies that BrdU LI appears to reflect proliferative potential better than histopathological typing. In this study, 65% [13 of 20] of glioblastomas had a LI >5, 67% of anaplastic astrocytomas had a LI between 1 and 5 while 45% of astrocytomas and all the gemistocytic and pilocytic astrocytomas except one had a LI of <1. These findings are concordant with the existing literature. It may however be noted that unlike the series of Hoshino et al[9,10] where upto 15% of HAA also had a LI of <1 along with MoAA, the present study did not have any case of glioblastoma, anaplastic astrocytoma or anaplastic oligoastrocytoma in this low LI category. Similarly, 13/14 (92%) of tumors with LI>5 were glioblastomas in this series unlike Hoshino's report (only 70%).[10] It thus seems that BrdU LI is a useful adjunct to histological typing in determining the biological behaviour of gliomas and is possibly a better predictor of survival with perhaps the following reservations: Firstly, 18 out of 43 (42%) tumors with LI between 1–5 in this series included various different histological types in varying percentages; a finding similar to earlier reports in the literature.[10] Secondly, Hoshino et al[10] have shown a significantly higher mean LI in MoAA and HAA of patients more than 50 yr old than those younger, a difference which was not observed in glioblastomas. Finally, juvenile pilocytic astrocytomas have sometimes been shown to have unusually high LI especially in young children with average range of 5.1 to 7.9%,[9,20] the explanation for which has varied from long-S phase, greater cell death and rapid proliferation to hamartomatous growth[9,20].

Correlation of BrdU LI with TESTAST grading

TESTAST-268 is a computer based mathematical model which provides numerical 4-step grading akin to Kernohan grading on the basis of 3 classifcation variables, 5 histologic and 3 clinical including age and site by means of linear discriminant functions using either Bayer minimum Mahalanobis distance classifier or canonical discriminant analysis. TESTAST-268 has been designed on a reference sample of 268 cases, in which evaluation of 23 nuclear followed by 15 histological parameters was improved with the help of multiple regression analysis; reclassification by Bayes classifier contest and inclusion of clinical parameters (age and site); thereafter, 4 empirically established malignancy classes (67 cases each) were fitted to a multivariate normal distribution in each class by Bayes classifier and new objects were identified. An object was assigned to a class based on minimum squared-Mahalanobis distance and maximum posterior probability. There were statistically significant differences in survival of patients with different TESTAST grades except between grades T I and T II.[26,27,28] This grading system has been found to be highly reproducible and when combined with quantitative morphometric evaluation of the histologic feature values, its reproducibility gets further enhanced.[29]

There are very few studies in the literature regarding correlation of histological grading with BrdU LI. In a study of low grade astrocytomas, of the 60% (29) tumors having BrdU LI of <1, only 3 died of recurrent tumor in a 3–1/2 years follow up, whereas 9 of the remaining 40% patients with LI >1 succumbed during the same period.[11] In a large study,[19] the range of BrdU LI in grade IV gliomas was higher (1.0–11.6) than either grade I and II (0.2–2.9) or grade III (0.4-2.2).

The present study shows a positive trend of increasing BrdU LI with increasing grade of the tumor. While only 1 of the 4 (25%) grade T I cases had BrdU LI >1, 6 of the 14 (43%) grade T II tumors showed this LI. Similarly, only 8 of 16 grade T III (50%) but 6 of 9 grade T IV cases (66.7%) had BrdU LI of >5. Further none of grade T I or T II cases had a LI of >5. Similarly, none of the grades T III or T IV tumors had LI of <1. The trend was further confirmed by the fact that the differences between BrdU LI values was statistically significant between grade T III and T IV and T II and T III but not between T I and T II. The latter could possibly be because of smaller number of cases of grade T I tumors *(Table 3)*.

The maximum spread of cases 18/43 (42%) was however still seen in the category of LI between 1 and 5 which included tumors of all the 4 different grades.

Contribution of TESTAST 268 grading to correlative prognostication of gliomas using histopathological typing and BrdU LI

It is evident from the literature that individually, there is a positive correlation between life expectancy and TESTAST 268 grading[26,28] on one hand and with the BrdU LI values on the other.[10] The latter also correlates with Ki-67 (growth fraction indicator) rather well.

It is apparent from the present study that the TESTAST 268 grading has taken care of at least some of the drawbacks of the combination of BrdU LI and histological typing. Firstly, the incorporation of age and location of the tumor in the program itself has eliminated their influence on the prognostication. Secondly, pilocytic astrocytomas has come out as a distinct category in this grading system, since presence of Rosenthal fibres in an astrocytoma invariably puts the tumor into grade T I category as is obvious in this study. This appears to explain the relatively indolent behavior of these tumors inspite of the spuriously high BrdU LI values reported in the literature.[9,20] Thirdly, it is evident that with the help of this grading system, the

glioblastomas can be further split up into 4 categories: (A) Grade T III with LI <5; (B) Grade T III with LI >5; (C) Grade T IV with LI <5; and (D) Grade T IV with LI >5. The most interesting point to note about this subcategorisation is its relationship to clinical outcome. There appears to be a trend towards increasing ominity in prognosis with increasing BrdU LI of a particular TESTAST grade *(Table 4)*. However, caution is to be exercised at this stage because the results of the survival analysis are preliminary and the number of observations in each of the categories is less. It is possibly for this reason that survival differences between the categories is statistically not significant except between Categories A+B with LI<5 vs. Categories C+D with LI>5 ($p<0.05$). It is also evident from the survival data in the present study, that a combination of a sensitive cell kinetic study such as BrdU LI and TESTAST grading can better define the relatively heterogenous category of glioblastoma multiforme than any one parameter individually. This finding further highlights the biological heterogeneity of the histologically homogenous glioblastomas and the recent concept of their subcategorisation into primary and secondary glioblastomas[18].

In summary, this integrated study is indicative that inclusion of BrdU LI and TESTAST 268 grading appears to serve as an improvement over the conventional WHO histological typing and that they are supplementary and synergistic to each other in more accurate prognostication. However, one important question which this study raises and which needs to be worked upon is how to further refine and subcategorize the group of cases with BrdU LI between 1 and 5 for purposes of better prognostication. In our opinion, the stage is ripe for the use of this reproducible integrated methodology on a larger scale at different centres backed up by long term follow up and survival analysis, in order to provide a definitive answer.

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