Indian Journal of Pathology and Oncology 2021;8(2):175–182

Content available at: https://www.ipinnovative.com/open-access-journals

Indian Journal of Pathology and Oncology

Journal homepage: www.ijpo.co.in

Original Research Article

# Comparison of mitosin and PHH3 indices with mitotic index and Ki-67 labelling index in diffusely infiltrating gliomas

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ARTICLE INFO

*Article history:*
Received 01-02-2021
Accepted 03-03-2021
Available online 19-05-2021

*Keywords:*
Tumors astrocytoma ki67 PHH3
mitosin Labelling index mitotic index

ABSTRACT

Astrocytomas are the most common primary brain tumor with prognosis highly dependent on Grading of the tumor. With the limited tissue available in stereotactic biopsy and often not representing the area of concern, development of new antibodies targeted against specific proteins in mitotically active cells is necessary. Ki67/MIB-1 LI has conventionally been used along with mitotic count to grade the astrocytomas. However Ki67 has limitation that it is expressed by cells through the cell cycle and it is difficult to differentiate mitotically active cells from apoptotic cells. In this study, thirty cases of various grades of astrocytomas were labelled with antibodies against Mitosin and PHH3 along with Ki67/MIB-1 and Mitosin Index and PHH3 Index were compared with Mitotic Index and MIB-1 LI. The present study depicted statistically significant difference of Mitosin Index and PHH3 Index amongst most of the grades of astrocytomas. Also it indicated a positive correlation amongst all four markers of proliferative index, the strongest being between PHH3 and Mitosin. Immunohistochemical determination of proliferative activity in astrocytomas using antibodies against PHH3 and Mitosin may assist in the histopathological diagnosis.



## 1. Introduction

Primary brain tumors are a heterogeneous group of benign and malignant tumors arising from the brain parenchyma and its surrounding structures. The median age at diagnosis for all primary brain and other CNS tumors is 59 years. Glioblastoma accounts for the majority of gliomas (56.1%). In India, CNS tumors constitute about 1.9% of all tumors. The most common primary CNS tumors are diffusely infiltrating astrocytomas. These account for more than 60% of all primary brain tumors in both sexes. Gliomas have a male predilection with GBM having a male-to-female ratio of 1.5:1.[1]

The genetic pathways included in the pathogenesis of primary glioblastomas include EGFR amplification, loss of heterozygosity of chromosome 10q, deletion of chromosome p16 and deletion of the phosphates and tension homologue on chromosome 10 (PTEN).[2] Secondary glioblastomas are seen in younger patients as low grade astrocytoma or anaplastic astrocytoma which gets transformed into over a span of several years. They carry a higher p53 mutation[3] along with TP53 mutation, platelets derived growth receptors (PDGFR) over expression, dysregulation of the p16 and retinoblastoma pathway. Loss of heterozygosity at 1p, 10p, 10q, 19q and 22q is usually observed[4,5] with heterozygosity at 10 occurring in 60 to 80% of the cases and is the most common genetic alteration in glioblastomas.[6,7]

The revised "WHO classification of CNS tumors" used molecular parameters in addition to histology to define many tumors.[8] This represents a shift from the traditional principle of using neuropathological diagnoses, which are

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https://doi.org/10.18231/j.ijpo.2021.035

primarily based on the microscopic features, to using molecularly-oriented diagnoses. Main molecular makers in gliomas are IDH, 1p19q deletion, MGMT, TERT, ATRX and p53 which are of diagnostic significance.

It is important to differentiate the low grade tumors from the high grade ones as prognosis and treatment modalities differ significantly. Histological grading is well accepted for the prognosis of patients with CNS tumors. However, the morphologic criteria are not always accurate in determining the grading and prognosis of tumor. This happens commonly in CNS tumors, as often small fragments of tissue are available from stereotactic biopsies. In order to better assess their classification many complementary methods have been introduced. And one of the convenient method to do so is to determine the mitotic figure count.[9] H&E picks up mitotic figures formed during prophase, metaphase, anaphase and telophase. Counting the mitotic figures is a time consuming method and is expressed as the number of mitoses observed per 10 high power fields (HPFs). Other drawback is that mitotic cells have distorted chromatin which appears similar to chromatin changes in apoptotic and pyknotic cells. Thus trained pathologist is required to differentiate apoptotic cells from mitotic figures.

Many robust antibodies have now been discovered that can be used in formalin- fixed, paraffin-embedded sections. The use of antibodies directed against the known elements of cell proliferation has become the new modality of assessing cell proliferation. IHC is a less subjective method. Thus, it is replacing the conventional mitotic cell counting techniques. In immunohistochemical staining, the commonly used method of reporting is the percentage of positively staining cells among a total of 1000 cells of interest. This percentage of positive cells is referred to as the labelling index. Generally, these antibodies target the nuclear proteins in the cells. Antibody against Ki-67 is the most commonly used modality to see proliferation activity in immunohistochemical studies in both CNS and non CNS tumors. Ki-67 protein is present during all phases of the cell cycle (G1, S, G2, mitosis) and is absent from resting cells (G0). This property makes it an excellent marker for determining the cells undergoing proliferation. Expression of the Ki-67 protein is regulated by a gene which is located on chromosome 10. Ki-67 is an $IgG_1$ class murine monoclonal antibody against a crude nuclear fraction of the Hodgkin's disease derived cell line.[10,11] The antibody was produced in Kiel, West Germany and grown in the 67th well of the tissue culture plate and hence named Ki67. The prognostic value of Ki-67 labelling index in determining survival has been proven in many univariate and multivariate analysis. The Ki67/MIB-1 adds prognostic information independent of patient's age, tumor site and grade.[12] Firstly, as Ki-67 is expressed by cells throughout the cell cycle, many Ki-67 positive nuclei may not survive the cell cycle and undergo apoptosis. In such cases, Ki-67 loses its prognostic value.[13] Secondly, many technical factors like section thickness and antigen-retrieval methods influence Ki-67 labelling index. Thus, Ki-67 index is subject to inter-laboratory variability and is difficult to standardize for the purpose of tumor grading. Thirdly, counting methods like choice of region for quantitative analysis and number of cells assessed also affect the final results.

Phosphohistone H3 is one of the major protein constituents of the chromatin in eukaryotic cells. The phosphorylation of serine residues at $10^{th}$ and $28^{th}$ position is minimum during interphase and reaches a peak during mitosis. The phosphorylated Histone H3 during cell division causes chromatin relaxation and ultimately activation of transcription in interphase cells. A true mitotic index can be estimated by labelling this protein by antibodies against it. PHH3 is a nuclear stain and imparts a brownish colour to the nucleus of mitotically active cells. It labels mitotic figures from late G2 through prophase, metaphase and anaphase. There is easy differentiation between mitotic cells and apoptotic cells and karyorrhectic debris as no phosphorylation of the histone H3 occurs during apoptosis. After adjusting for age, extent of resection and performance score, the PHH3 index remains an independent predictor of survival. It provides a simple and reliable method for quantifying proliferative potential and stratification of patients with diffuse astrocytomas.[14]

Mitosin, also called as p330d/CENP-F is a protein of gene assigned to human chromosome 1q32-41. It is a novel protein of 3,113 amino acids containing an internal tandem repeat of 177 amino acids. It was identified by direct binding to purified retinoblastoma protein in vitro. Its localization is dramatically reorganized from a rather homogeneous nuclear distribution in S phase to paired dots at the kinetochore /centromere region, to the spindle apparatus, and then to the midbody during M-phase progression. This spatial reorganization coincides closely with the temporal phosphorylation patterns of mitosin. Studies suggest that mitosin may play a crucial role in mitotic-phase progression. First, mitosin is expressed only during S and G2/M phases and it rapidly disappears upon the completion of mitosis.[15,16] Increased expression is associated with malignancy grade and survival in astrocytomas. It also displayed significant correlation to all of the proliferative marker, which is in accordance with studies on other malignancies.[17]

Many studies have shown that both mitotic index and Ki67/MIB-1 LI are significantly correlated with histologic grades and establishing cutoffs has been suggested.[18] These studies agree that the Ki67 index increases with increasing malignancy grade, and patients with high Ki67 index have poorer prognosis.[19,20] In an immunohistochemistry study consisting of 27 patients of different grades of glioma, a positive correlation between the proliferation markers Ki-67/ MIB1, mitosin, survivin, pHH3, and

DNA topoisomerase IIa was established.[21] As has been discussed, Mitosin and PHH3 seems to be more reliable and reproducible markers to identify mitosis and therefore for the grading of astrocytomas. The present study was undertaken to assess Mitosin and PHH3 as surrogate immunohistochemical markers for mitosis and to compare it with the conventional prognostic factors, viz. Mitotic Index and proliferation markers (Ki67/MIB-1 LI). Aim is to compare Mitosin and PHH3 Indices with Mitotic Index and Ki-67 Labelling Index in Diffusely infiltrating Gliomas.

## 2. Materials and Methods

The present study was carried at Department of Pathology of a tertiary care hospital. A total of 30 cases of astrocytic tumors diagnosed and managed at the hospital and willing to participate in the study were included. Relevant clinical details viz. age, sex were noted for all the cases in the study group. All non- astrocytic CNS tumors, recurrent astrocytic tumors, post radiation therapy cases of astrocytomas were excluded from the study.

The H&E (hematoxylin and eosin) stained slides along with relevant immunohistochemical stained slides of all the cases were reviewed and the original histopathological diagnoses, viz. tumor type and grade reconfirmed. For the microscopic analyses Dewinter was used. All immunostained sections were scanned for the most densely labelled areas, and calculations were done with the 40x objective, termed microscopic high power fields (HPF). Total number of mitotic figures were calculated in the consecutive 10 HPF (High Power field) in the area having highest mitotic activity on an H&E stained slide. It is expressed as mitoses per 10 HPF.

Immunohistochemistry is a technique in which exact location of antigens in cells is determined by using a specific antigen-antibody reaction. Positive controls were processed along with every batch of cases to determine the correctness of the procedure. The commercial antibodies used are shown in Table 1. All immunostained sections were scanned for the most densely labelled areas, and calculations were done with the 40x objective, termed microscopic high power fields (HPF). The antigens used are tabulated below [Table 1].

The labelling index (LI) was determined by counting at least 1000 tumor cells in one HPF. If this number of tumor cells could not be obtained, three HPFs were examined. The proliferative index was defined as the percentage of immunoreactive tumor cells out of the total number of cells. The variables included in the statistical analysis were the WHO grade, mitotic index, Ki67 /MIB-1 labelling index, mitosin and PHH3 index. All calculations were done using the SPSS software version 20.0 for windows. P value < 0.01 was considered significant.

## 3. Observations and Results

Thirty cases were distributed into 03 cases of grade I, 10 cases of grade II, 03 cases of grade III and 14 cases of grade IV according to WHO grading. All cases of grade I and grade II astrocytomas had a Ki67/MIB-1 LI < 5 and all cases of grade III and grade IV astrocytomas had a Ki67 LI > 5. The values of Ki67 LI, PHH3 LI and Mitosin LI increased linearly with tumour grade [Table 2].

### *3.1. Comparison of Mitosin and PHH3 index with Ki-67/MIB-1LI and Mitotic index*

Pearson correlation analysis was carried out for the four markers. All markers were strongly correlated amongst themselves. The strongest correlation was observed between PHH3 and Mitosin (r = 0.99). Fairly strong correlation was present between Mitotic Index and PHH3, Mitotic Index and Mitosin (r = 0.79). [Table 3]

There was a significant difference in means of Mitosin Index between Grade I and Grade III (p= 0.004), grade I and IV (p=0.000), Grade II and Grade III (p= 0.007), Grade II and Grade IV (p=0.000) while there is no significant difference between the remaining grades. [Table 5]

## 4. Discussion

To define a cutoff between the various grades of astrocytomas for diagnostic purpose, a proliferative marker with high reproducibility needs to be developed. In this study, new proliferative markers Mitosin and PHH3 were compared with conventional proliferative marker Ki67/MIB-1 and Mitotic count. In this study, all pilocytic astrocytoma and diffuse astrocytoma had a Mitotic Index (mitosis per 10 HPF) of ≤ 1 and all anaplastic astrocytoma and GBM had a Mitotic Index of >1. These above findings were consistent with study by Colman et al., where the Mitotic index cut off for classification of astrocytoma into high and low grade was 3 mitosis per 10 HPF.[22] The mean Ki67/MIB-1 LI for pilocytic astrocytoma, diffuse astrocytoma, anaplastic astrocytoma and GBM were 0.6, 1.6, 8 and 9.8 respectively. This was corroborated by the study of Karamitopoulou et al. that included a total of 59 cases of astrocytic tumors. Their study showed a mean Ki67/MIB-1LI of diffuse astrocytoma as 2.03, anaplastic astrocytoma as 12.8 and GBM as 14.57%.[23] Raghavan et al. also showed similar trend in Ki67/MIB-1LI.[24] Ki-67 LI rose linearly with the mitotic index. Additionally in our study, all garde I and Grade II had Ki67/MIB-1 < 5 and all grade III and grade IV had Ki67/MIB-LI of > 5%. The above findings were consistent with the study by Torp et al. that suggested a MIB-1 prognostication cut off between low and high grade astrocytoma as 5.2%.[25]

The present study depicted statistically significant difference of PPH3 index between grade I and grade III (p=0.002), between grade I and IV (p=0.000), between

**Table 1:** Antibodies and immunohistochemical staining

| Antibody | Source | Type | Clone | Positive Control | Dilution | Incubation Temperature |
|---|---|---|---|---|---|---|
| Ki 67/MIB-1 | Dako, Glostrup, DK | Monoclonal | Ki 67 Antigen | Germinal centre of lymph node | 1:100 | Room Temperature |
| PHH3 | Upstate Biotechnology. Millipore, Billerica, USA | Polyclonal | H3 (Ser10) | Germinal Centre of lymph node | 1:2000 | Room Temperature |
| Mitosin | Novus Biologicals, LTD, Cambridge, UK | Monoclonal | 14C10/1D8 | Germinal centre of lymph node | 1:500 | Room Temperature |

**Table 2:** Mean values of all markers in various grades of astrocytomas

| Antibody | WHO Gd I | WHO Gd II | WHO Gd III | WHO Gd IV |
|---|---|---|---|---|
| Mean Mitotic Index | 0 | 0.4 | 3.0 | 6.07 |
| Mean Ki67/MIB-1 LI | 0.67 | 1.62 | 8.0 | 9.86 |
| Mean PHH3 LI | 1.33 | 2.8 | 6.0 | 8.57 |
| Mean Mitosin LI | 2.4 | 4 | 6.8 | 9.4 |

**Table 3:** Correlation of various markers obtained in the study

| | Mitotic Index | Ki67/MIB-1 LI | PHH3 LI | Mitosin LI |
|---|---|---|---|---|
| Mitotic Index | 1 | 0.78 | 0.79 | 0.79 |
| Ki67/MIB-1 LI | 0.78 | 1 | 0.67 | 0.71 |
| PHH3 LI | 0.79 | 0.69 | 1 | 0.99 |
| Mitosin LI | 0.79 | 0.71 | 0.99 | 1 |

**Table 4:** Mean difference of PHH3 index between various grades

| Grade | Grade | Significance |
|---|---|---|
| I | II | 0.058 |
| | III | 0.002 |
| | IV | 0.000 |
| II | I | 0.058 |
| | III | 0.001 |
| | IV | 0.000 |
| III | I | 0.002 |
| | II | 0.001 |
| | IV | 0.137 |
| IV | I | 0.000 |
| | II | 0.000 |
| | III | 0.137 |

**Table 5:** Mean difference of Mitosin index between various grades

| Grade | Grade | Significance |
|---|---|---|
| I | II | 0.050 |
| | III | 0.004 |
| | IV | 0.000 |
| II | I | 0.050 |
| | III | 0.007 |
| | IV | 0.000 |
| III | I | 0.004 |
| | II | 0.007 |
| | IV | 0.056 |
| IV | I | 0.000 |
| | II | 0.000 |
| | III | 0.056 |

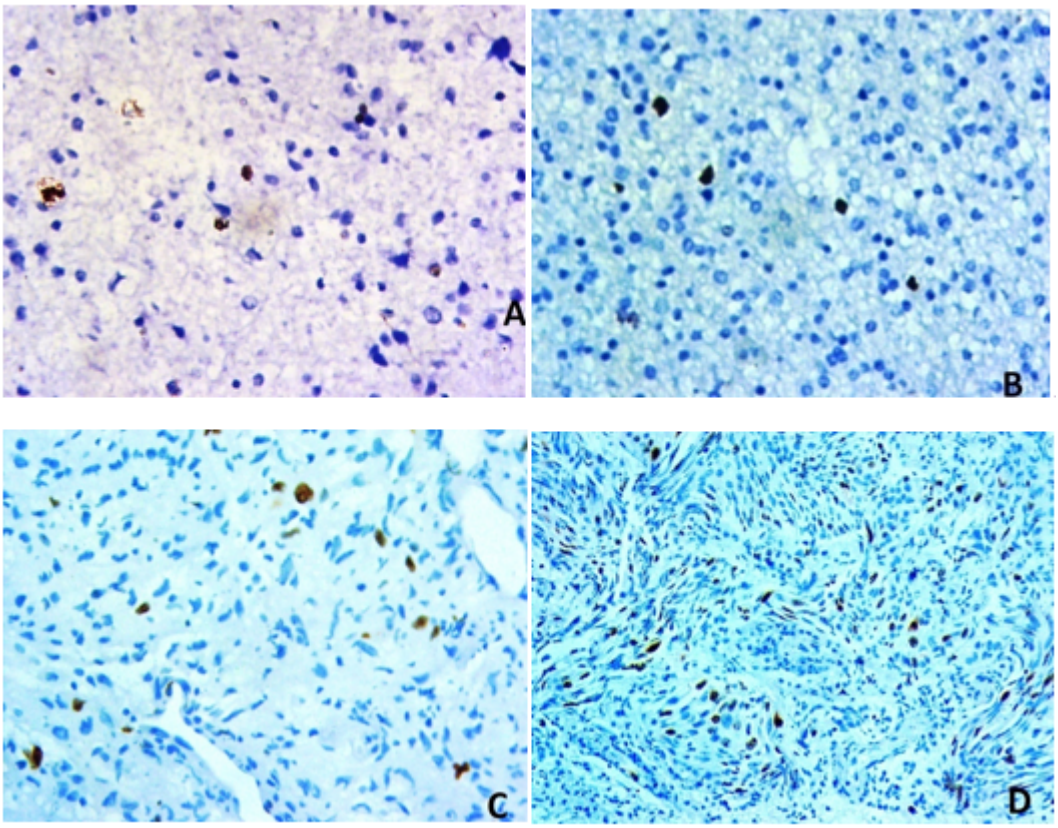


**Fig. 1:** Ki67/MIB-1 in various grades of Astrocytoma. Grade I (**A**), Grade II (**B**), Grade III (**C**), Grade IV (**D**)

grade II and III (p=0.001) and between grade II and grade IV (p=0.000). The p value obtained show that the use of PHH3 can help in definite identification of various grade of the tumor and making it easy to differentiate amongst the various grades. Mitosin also displayed significant correlations to all of the proliferative markers, which is in accordance with studies on other malignancies.

In our study we found that there is a significant difference in means of Mitosin Index between Grade I and Grade III (p= 0.004), grade I and IV (p=0.000), Grade II and Grade III (p= 0.007), Grade II and Grade IV (p=0.000). In a study done by Varughese et al. involving 59 and 33 infiltrative astrocytomas WHO grades II and III, respectively, immunostained with the proliferation markers mitosin and PHH3. Their results suggested that there is a significant difference in mitosin expression between WHO grade II and grade III astrocytomas[17] In our study, Ki-67/MIB-1 LI did not correlate well with PHH3, mitosin or mitotic index. While correlation between PPH3 and mitosin was statistically significant. In many other studies it has been established that Ki67 labelling index displayed a wide range of values that overlap with indices in both grade II and grade IV astrocytomas,[25] and is regarded as the main reason for Ki-67/MIB-1 not being included in the routine histopathological diagnosis of astrocytic tumors. Thus, this marker should not be used alone, but in combination with established histopathological criteria of malignancy. Both mitosin and PHH3 have been studied for prognostic value as a proliferative marker and has been compared with both mitotic index and Ki67/MIB-1 LI in CNS and non CNS malignancies. However there are only a few studies comparing mitosin, PHH3, Ki67 and mitotic index all together. Yoo- Jin et al. compared the PHH3 mitotic index with standard MF counts and the Ki-67 labeling index (LI) in a retrospective series of 265 meningiomas. The PHH3 staining method yielded greater sensitivity in the detection of MFs and facilitated MF counting. Mitotic thresholds of H&E Mitotic index of 4 or more per 10 high-power fields (HPF) and PHH3 MI of 6 or more per 10 HPF were found as the most appropriate prognostic cutoff values for the prediction of recurrence-free survival.[26] The study by Colman et al. in astrocytoma revealed the r value for PHH3 and MIB-1 as 0.74 and the r vale of PHH3 and MI as 0.76.[22]

In this immunohistochemical study, we demonstrated positive correlation between the proliferation markers Ki-67/MIB-1, PHH3 and Mitosin. Antibodies against proliferation-associated antigens are useful to obtain an optimal profile of the proliferative activity, especially in small brain tumor samples. In this study, PHH3 antibody provided reliable immunostaining with distinct

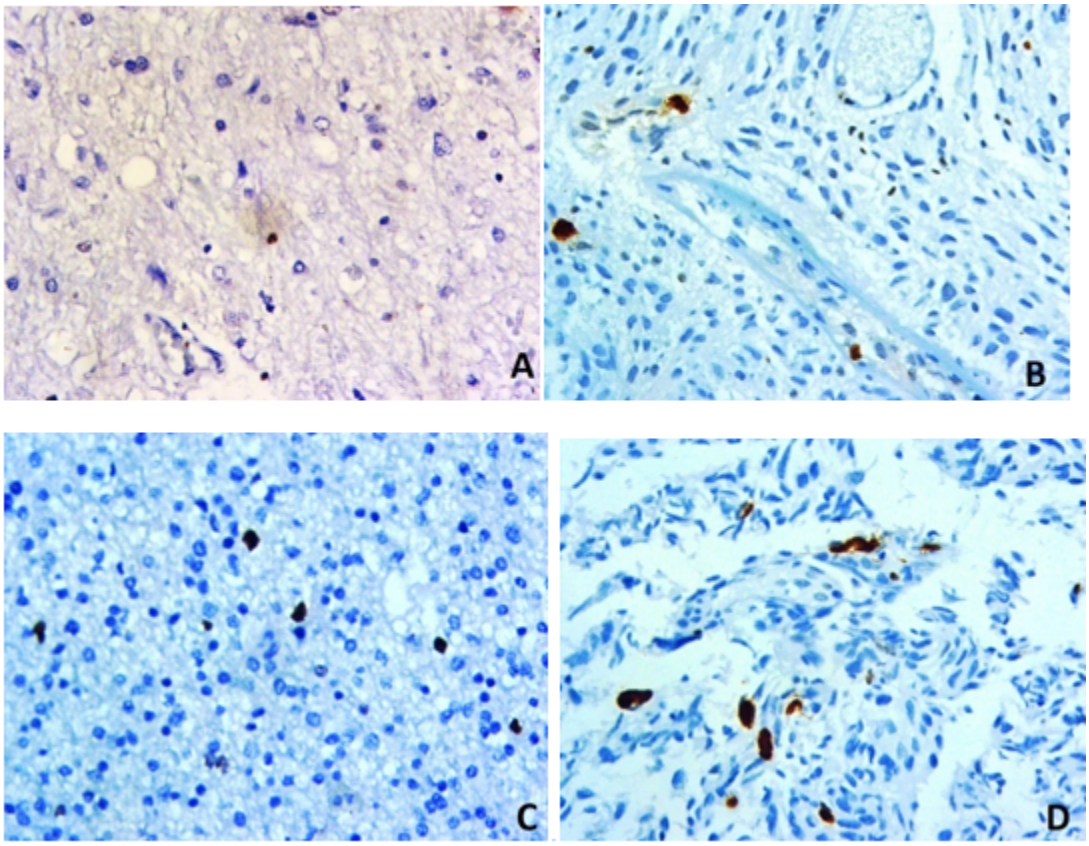


**Fig. 2:** PHH3 in various grades of astrocytoma. Grade I (**A**), Grade II (**B**), Grade III (**C**), Grade IV (**D**)

nuclear positivity in cells with mitotic morphology. Positive correlation was found with other proliferative markers including mitoses. Comparison between the number of mitoses and PHH3 positive nuclei, revealed the latter to be higher, in accordance with being a more sensitive marker for mitoses.[27–29] A major drawback of the PHH3 immunostaining seems to be positivity in non-mitotic cells.[28] Mitosin also displayed significant correlations to all of the proliferative markers, which is in accordance with studies on other malignancies.[15] Immunoreactive nuclei were easily identified and can be explained by the Mitosin expression in S-, G2-, and M phase, and its rapid degradation.[5,16] Thus, one should consider the possibility to introduce a panel of proliferation markers to identify more aggressive astrocytomas. The current WHO guidelines do not define any cut off to distinguish between grade II and grade III astrocytoma. As shown in our study, in addition to the various morphological parameters both Mitosin and PHH3 are promising markers in this regard. However because of a small sample size, further studies and meta-analysis involving PHH3 and Mitosin as markers for grading of astrocytic tumors are required for establishing such cut off.

## 5. Conclusion

The use of immunohistochemistry facilitates the correct categorization of many CNS malignancies. In astrocytomas, the use of proliferative markers like Ki67/MIB-1 serves the purpose. But owing to the high inter-observer variability and unequivocal cutoff values by various studies, there is a need for a marker which has a high sensitivity of picking up the proliferating cells and has a high reproducibility. With the above objective, we carried out the present study in thirty cases of astrocytic tumors and studied the various staining characteristics of Mitosin and PHH3. To achieve this objective, thirty biopsy proven cases of astrocytomas were studied with respect to histopathological features including the WHO grade and immunohistochemical markers using Ki67, PHH3 and Mitosin. The utility of Mitosin and PHH3 were studied, cutoffs between various grades were given and Mitosin Index and PHH3 Index were compared with Mitotic Index and MIB-1 LI. The present study depicted statistically significant difference of Mitosin Index and PHH3 Index amongst most of the grades of astrocytomas. Also it indicated a positive correlation amongst all four markers of proliferative index, the strongest being between PHH3 and Mitosin. In conclusion, immunohistochemical determination of proliferative activity in astrocytomas

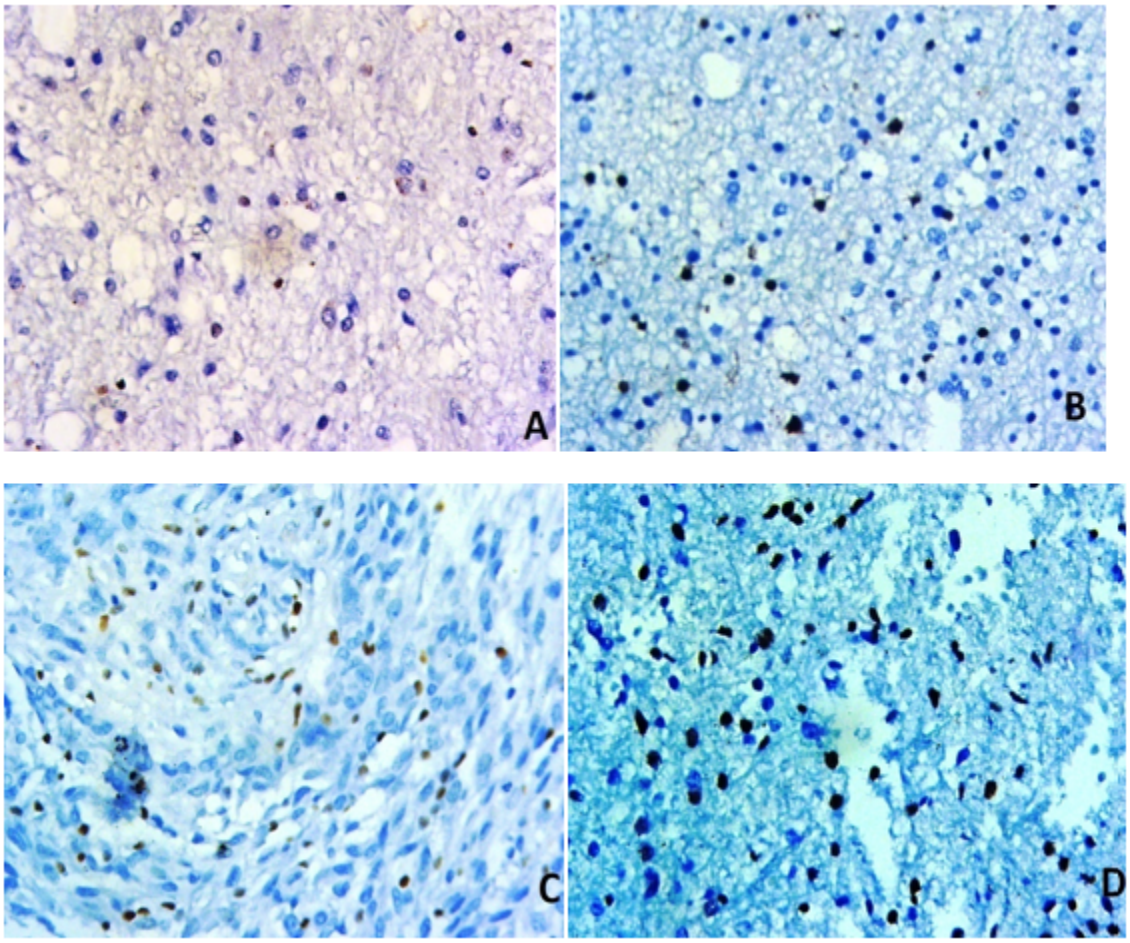


**Fig. 3:** Mitosin in various grades of astrocytoma. Grade I (**A**), Grade II (**B**), Grade III (**C**), Grade IV (**D**).

using antibodies against PHH3 and Mitosin may assist in the histopathological diagnosis. But in order to establish Mitosin and PHH3 as the markers of choice, more number of cases with various grades of astrocytoma need to be evaluated. Thus the prognostic value of these markers requires further investigation.

## 6. Source of Funding

Authors' independent work. No grants or funds taken.

## 7. Conflict of Interest

None.

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**Cite this article:** Bisht DS, Deb P, Sharma H, Bisht S. Comparison of mitosin and PHH3 indices with mitotic index and Ki-67 labelling index in diffusely infiltrating gliomas. *Indian J Pathol Oncol* 2021;8(2):175-182.