Expression of Ki-67 and AgNOR in Primary Central Nervous System (CNS) Tumours

*Nahar A,[1] Islam MN,[2] Kabir E[3]

Abstract

Background: Primary tumors in central Nervous System (CNS) include a large group of tumors. Ki-67 is usually used for determining proliferative activity, though it has some limitations. It is proved that the number or size of Argyrophilic Nucleolar Organizer Regions (AgNORs) of DNA closely correlates with cellular proliferating activities.

Objective: This study was conducted to find the correlation of expression of Ki-67 and AgNOR with different grades of primary CNS tumours.

Method: In this cross sectional study a total 85 cases with wide age range (2-70 years) were included based on selection criteria. Tissue fixation, processing, staining (hematoxylin and eosin stain, and AgNOR special stain) and immunohistochemistry were done according to the standard protocol followed by the pathology department of Sir Salimullah Medical College (SSMC) and National Institute of Neurosciences & Hospital (NINS), Dhaka.

Results: Ki-67 labeling index and mean AgNOR (mAgNOR) showed significant correlation with different grades of primary CNS tumours, as the grades increased both parameters were also increased. In intergrade comparisons, Ki-67 labeling index showed significant correlation among all the grades except between grade I & grade II and grade III & grade IV. Whereas, mean AgNOR showed significant difference among all the grades including the difference between grade I & II and grade III & IV. Positive significant correlation seen in between ki-67 Labeling Index (LI) and mean AgNOR, where the value of Spearman's correlation coefficient was 0.514 and p value was 0.001

Conclusion: Both Ki-67 expression and mAgNOR score were increased with the increasing grade of tumours from low to high grade and positive correlation between these two parameters were also found.

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1. **Dr. Asrafun Nahar, MBBS, MD(Pathology), Assistant Professor, Department of Pathology, National Institute of Laboratory Medicine and Referral Centre, Sher-E-Bangla Nagar, Dhaka-1207. dr.asrafunnahar@gmail.com.*
2. *Dr. Md. Nasimul Islam, MBBS, M. Phil (Pathology), Professor, Department of Pathology, Anwer Khan Modern Medical College, Dhanmondi, Dhaka.*
3. *Dr. Enamul Kabir, MBBS, M. Phil, M. Sc(Pathology), Professor and Head, Department of Pathology, Popular Medical College, Dhanmondi, Dhaka*

** For correspondence*

Introduction

Primary Central Nervous System (CNS) tumors are heterogeneous group of tumours arising either from the brain parenchyma, spinal cord, and/or surrounding structures which are associated with high morbidity and mortality.[1] The annual incidence of CNS tumours ranges from 10 to 17 per 100,000 persons for intracranial tumors and 1 to 2 per 100,000 persons for intraspinal tumors.[2] Glioblastomas are the common malignant tumors that represent about 15% of all primary brain tumors.[3] Meningioma is another common primary CNS tumor that is usually benign and arises from arachnoid cells or the arachnoid membrane covering the brain.[4] In children medulloblastoma is the common malignant brain tumor, accounting for about 20% of all primary CNS tumors, with a peak incidence at 5-7 years. Despite treatment, the 5-year survival rate of medulloblastoma is 60%.[5] CNS tumors have always been a cause of concern to pathologist due to the wide variety of their appearances. Grading of CNS tumors is fundamental for optimal prognostication and choice of therapy and is mainly based on certain morphologic criteria: cellularity, cytological atypia, mitotic activity, microvascular proliferation, and necrosis.[6] Measurement of proliferative activity is important in determining the tumor grade, recurrence span, and malignancy. Proliferative activity in tumors can be determined by mitotic counting, flow-cytometric determination of synthesis-phase fraction, and immunohistochemistry using antibodies reactive against various proliferating cellular antigens.[7] Counting mitotic figures in hematoxylin and eosin-stained sections can be extremely troublesome and time-consuming, particularly if the specimen is large or tissue preservation is poor.[8] Hence, there is a need to develop better prognostic markers to predict tumor behavior and one such complementary method is an assessment of the proliferative index of the tumor. The Ki-67/MIB-1 monoclonal antibody is commonly used and is reactive against the nuclear antigen Ki67 that is expressed during cell cycle phases G1, S, G2, and M, but is not found during G0.[9]

Most of the previous studies have shown a positive correlation of the Ki-67 Labeling Index with different grades of CNS tumors, and indices of ki-67 for high-grade gliomas (grade III/IV) were significantly higher than in low-grade (grade I/II) tumors. 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 as the difference of Ki-67 expression value between grade I & grade II and between grade III & grade IV are insignificant. This is the main limitation of Ki-67 immunostaining. For this reason, Ki-67 should not be used alone as a marker of tumor grade but in conjunction with histological features.[10] Some recent studies showed a close positive relationship between nucleolar organizer regions (NORs) and cell proliferation.[11] Nucleolar organizer regions (NORs) are loops of DNA containing ribosomal RNA genes that are transcribed to ribosomal RNA under the influence of RNA polymerase-I enzyme and in the presence of some argyrophilic, non-histone proteins called NOR-associated proteins.[12,13] These NOR-associated proteins have a strong affinity for silver, thus NORs can be demonstrated in tissue sections by staining their associated proteins with colloidal silver and these reaction products represent the Argyrophilic Nucleolar Organizer Regions (AgNORs) which appear as black dots of various sizes in the nucleolus and nucleus. The number or size of AgNORs not only closely correlates with cellular proliferating activities, but also with cellular differentiation; thus it can be used for detecting malignancy and grading the tumor.[12]Hence, it implies that rapidly proliferating high-grade malignant tumor cells

have higher AgNOR scores than less rapidly proliferating low-grade malignant tumor cells.[14] The use of Ki-67 and AgNOR could be valuable markers for cell proliferation to evaluate the mitotic activity of primary CNS tumors as well as to know the molecular behavior of these tumors. Over the past few years, AgNOR number and distribution in the nucleus were useful in the diagnosis and prognosis of some neoplasias, such as renal, bladder, pharyngeal carcinoma, multiple myeloma, and melanocytic lesions of the skin.[15]

So far there is no previous study on primary CNS tumors incorporating expression of Ki-67 and AgNOR in our country. That's why the present study was designated to find out the correlation of Ki-67 and AgNOR in primary CNS tumors and its relation with histological grading.

Methods

In this cross-sectional analytical study, 85 histopathologically diagnosed cases of primary CNS tumors irrespective of age and sex were included; and was carried out in the Department of Pathology, Sir Salimullah Medical College (SSMC) & Mitford Hospital and National Institute of Neurosciences & Hospital, Dhaka, Bangladesh from September 2017 to June 2019. Patients who were treated with radiotherapy or chemotherapy before surgical operation and secondary cases were excluded. Relevant clinical information was taken by using prescribed proforma. The Institutional Review Board (IRB) of SSMC gave the ethical clearance for this study. Tissue Fixation was done with 10% neutral buffered formalin. Tissue processing, paraffin embedding, block preparation, sectioning, and staining (hematoxylin and eosin stain, and AgNOR special stain) were done according to the standard protocol followed by the pathology department of SSMC. On the other hand, sections from each paraffin block were taken on the coated slide (DAKO, IHC Microscope slides, REF. K8020) for the immunohistochemical study of Ki-67 and other necessary markers like GFAP, EMA, S-100, LCA and Vimentin. At first classification and grading were done according to WHO (2007) CNS tumors classification.[16] Glial tumors were graded (from grade I to grade IV) by following the criteria like - cellularity, nuclear atypia, mitotic activity, microvascular proliferation, and necrosis.[6,17] The immunohistochemical staining for the Ki-67 antigen was performed by using the MIB-I antibody. In addition, some other immunohistochemical stains like GFAP, EMA, S-100, LCA, and Vimentin were done to differentiate different primary CNS tumors. Blocks were cut into 3-4 micrometer thin sections and the DAKO EnVisionTM FLEX system was used for visualizing the immunostained sections. For calculation of the Ki-67 Labeling Index at first, a hot spot (area with the highest density of immunostained nuclei) was selected and adjacent fields were counted to include 500 nuclei at a magnification of x400.[18] Distinct nuclear staining of the tumor cells was recorded as positive. Then Ki-67 Labeling Index was calculated as a percentage of positively stained tumor nuclei in 500 cells. The AgNOR-stained sections were observed under a light microscope by using an oil immersion objective (x1000). Hundred randomly selected cells were counted in each case. Individually discernible and separate black dots were recorded and an average number of dots were calculated in each nucleus. Where two or more dots were closely associated and not resolvable, they were counted as a single AgNOR dot. AgNOR dots were counted in 100 nuclei and then the mean was calculated (mAgNOR). It was expressed as arithmetic mean ± SD.[14] In both calculations, the Ki-67 Labeling Index and AgNOR count of vascular components, inflammatory cells, and necrotic, degenerated

& poorly preserved areas were excluded. Statistical analyses were done by the Statistical Packages for Social Sciences (SPSS-22) system. The results were calculated by using statistical formulas including ANOVA and Chi-square test and were presented in tables and figures. p-value <0.05 was considered as significant.

Results

In the current study involving 85 cases, 20% of the patients were in the pediatric age group (≤18 years), while the remaining were adults, with ages ranging from 2 to 70 years. The data indicated a male predominance, with a male-to-female ratio of 1.36:1. The frontal lobe was observed as the most frequent site of involvement and was found in 20 (23.5%) cases followed by the posterior fossa in 13 (15.3%) cases, the spinal in 11 (12.9%) cases, and the fronto-parietal lobe in 10 (11.8%) cases.

Among the studied cases, the most common histopathologically diagnosed tumor was astrocytoma (32.94%) and among this group, the commonest was glioblastoma (14.12%). The second most histopathological diagnosis was meningioma (22.35%), in which meningothelial meningioma (7.06%) was most prevalent. In the pediatric age group, the commonest observed primary CNS tumor was medulloblastoma (41.2%) followed by ependymal tumors (23.5%), and astrocytic tumors (17.6%).

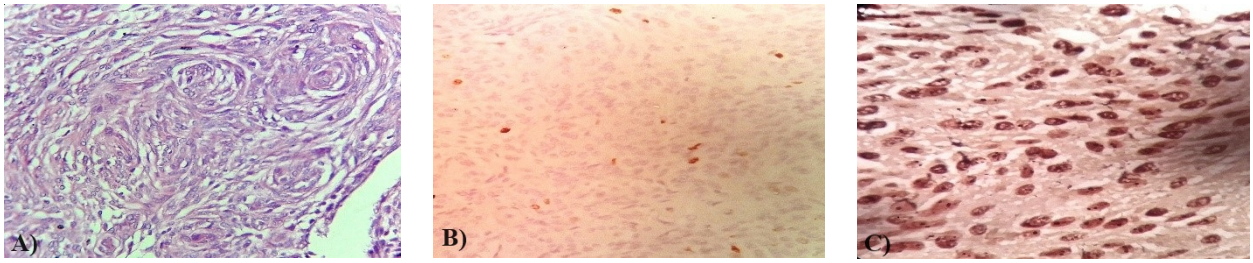

Figure 1. Photomicrograph shows meningothelial meningioma (WHO grade I); A) H & E stain x400, B) Ki-67 immuno-stain x400, C) AgNOR stain x1000

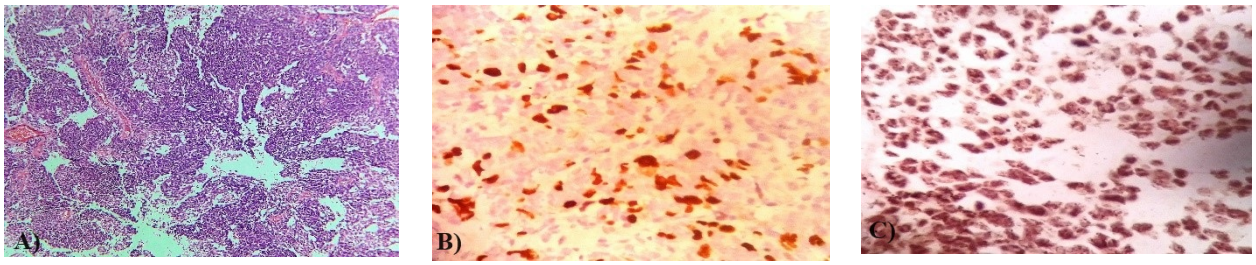

Figure 2. Photomicrograph shows medulloblastoma (WHO grade IV); A) H & E stain x100, B) Ki-67 immuno-stain x400, C) AgNOR stain x1000

In the adult age group, the most common tumor was astrocytoma (36.8%) followed by meningioma (27.9%). It was observed that most of the CNS tumors belonged to WHO grade I (40%), followed by grade IV tumors (27%), grade II tumors (21.2%), and grade III CNS tumors (11.8%). Among the studied cases, the mean of the Ki-67 Labeling Index and the mAgNOR score were much higher in WHO grade IV in comparison to lower grades (Figure 4-7). The differences in both parameters were found statistically significant

($p<0.05$) (Table I). Comparisons of the Ki-67 Labeling Index among different WHO grades of CNS tumors (n=85) were shown in Table II and there was found significant correlation of Ki-67 Labeling Index (LI) among all grades of tumors except between grades I & II and between grade III & IV primary CNS tumors. Table III shows the comparison of mAgNOR among different grades of CNS tumors and it was found a significant correlation of mAgNOR among all grades of tumors.

Table I: Association of Ki-67 Labeling Index and mAgNOR score among different WHO grades of CNS tumors (n=85)

WHO Grade		Ki-67 Labeling Index (LI)				m AgNOR			
	n	Mean	±SD	Min	-max	Mean	±SD	Min	-max
Grade I	34	3.68	±1.18	0.8	-6	1.43	±0.24	1.00	-1.90
Grade II	18	6.04	±3.75	0.2	-14.6	1.97	±0.46	1.24	-2.92
Grade III	10	28.92	±10.86	12	-45	2.69	±0.39	2.08	-3.52
Grade IV	23	36.19	±11.43	21	-62.6	3.46	±0.52	2.90	-4.56
P value		0.001[s]				0.001[s]			

s= significant
p-value reached from the ANOVA test

Table II: Comparison of Ki-67 Labeling Index (LI) among different WHO grades of CNS tumors (n=85)

WHO Grading of the CNS tumors		Level of significance
Grade I	Grade II	1.000[ns]
	Grade III	0.001[s]
	Grade IV	0.001[s]
Grade II	Grade III	0.001[s]
	Grade IV	0.001[s]
Grade III	Grade IV	0.057[ns]

s= significant,
ns= not significant
ANOVA test followed by the Boneferroni test was done to measure the level at significance among the groups.

Table III: Comparison of mAgNOR among different WHO grades of CNS tumors (n=85)

WHO Grading of the study		Level of significance
Grade I	Grade II	0.001[s]
	Grade III	0.001[s]
	Grade IV	0.001[s]
Grade II	Grade III	0.001[s]
	Grade IV	0.001[s]
Grade III	Grade IV	0.001[s]

s= significant. ANOVA test followed by the Boneferroni test was done to measure the level of significance among the groups.

In Figure 3, the Ki-67 value was expressed in the study population as % and mAgNOR value as mean. The value of Spearman's correlation coefficient was 0.514 and the p-value was 0.001. So, it can be concluded that there was a positive significant correlation between ki-67 Labeling Index (LI) with mAgNOR in the studied population.

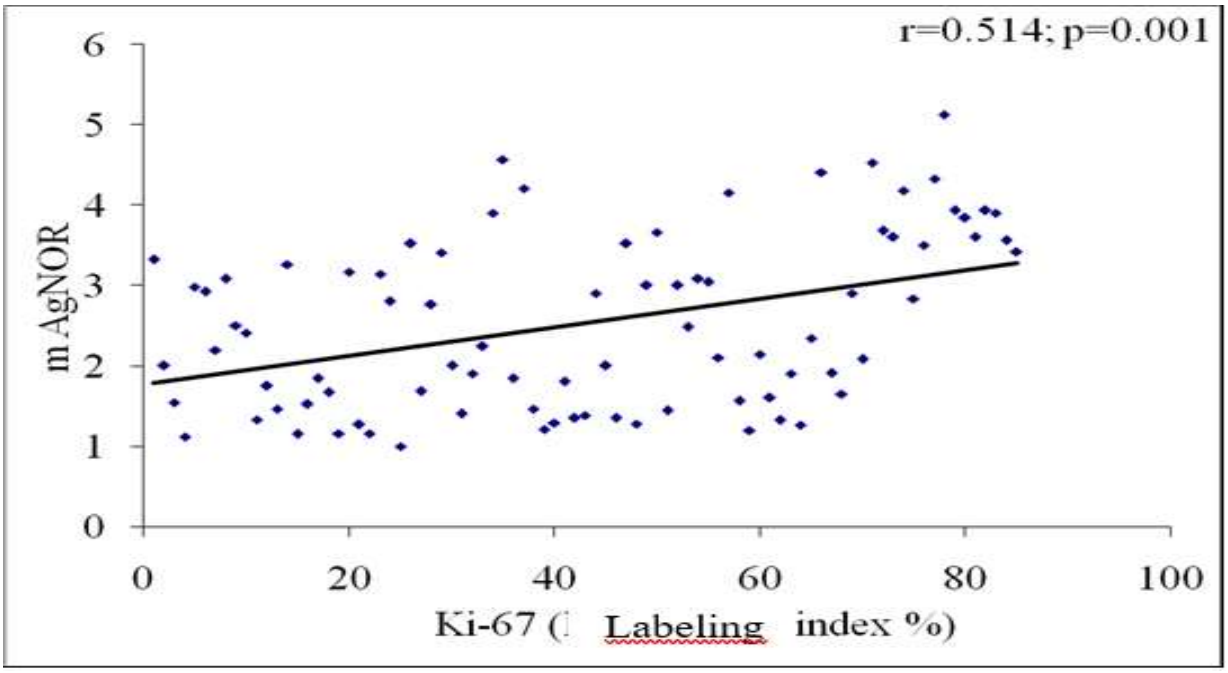

Figure 3. Scatter diagram showing Spearman's positive significant correlation (r=0.514; p=0.001) between the ki-67 (LI) with mAgNOR

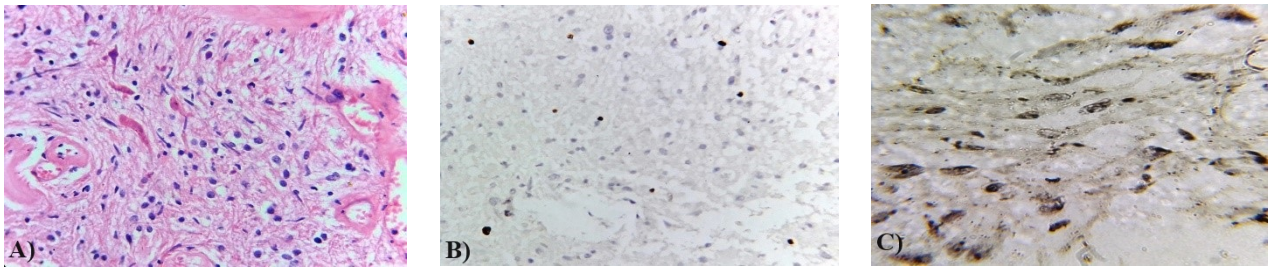

Figure 4. Photomicrograph shows pilocytic astrocytoma (WHO grade I); A) H & E stain x400, B) Ki-67 immuno-stain x400, C) AgNOR stain x1000

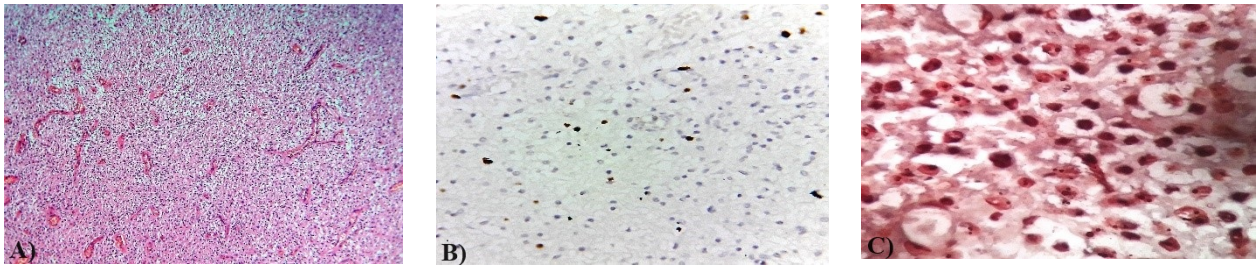

Figure 5. Photomicrograph shows oligodendroglioma (WHO grade II); A) H & E stain x100, B) Ki-67 immuno-stain x400, C) AgNOR stainx1000

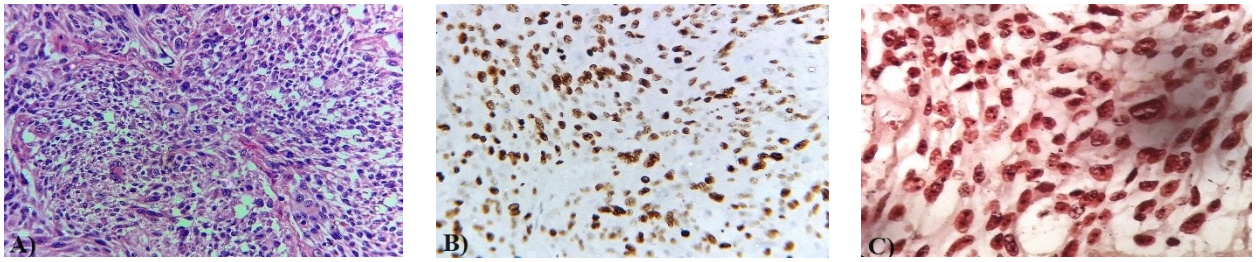

Figure 6. Photomicrograph shows anaplastic astrocytoma (WHO grade III); A) H & E stain x400, B) Ki-67 immuno-stain x400, C) AgNOR stain x1000

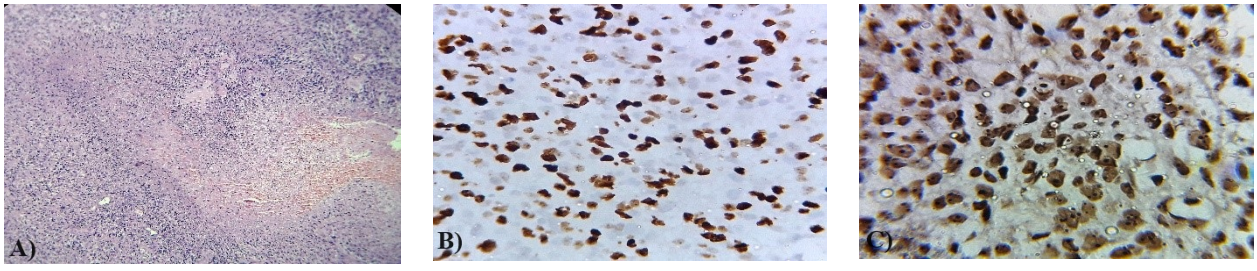

Figure 7. Photomicrograph shows glioblastoma (WHO grade IV);A) H & E stain x100, B) Ki-67 immuno-stain x400, C) AgNOR stain x1000

Discussion

This present cross sectional study was aimed to evaluate AgNOR and Ki-67 in primary CNS tumours of different grades and to compare AgNOR stain with Ki-67 immunostaining. It was observed that majority of cases (41.18%) were seen in 41-60 years age group which is similar to the study of Thambi et al.[19]

Frontal lobe was the commonest site and astrocytoma (32.94%) was the most common variant of CNS tumours in the present study. This finding was also supported by some previous studies.[19,20] However, meningioma made up the largest subgroup in some recent studies.[19,21] WHO grade I tumors were the most frequently observed, making up 40% of the cases in this study, consistent with findings from previous research.[22]

In the present study Ki-67 expression of primary CNS tumours had a significant correlation with histological grading. Ki-67 Labeling Index in WHO grade I, II, III and IV CNS tumours in present study were 3.68%, 6.04%, 28.92% and 36.19% respectively (Table I). As the tumour grade increased the mean Ki-67 Labeling Index also increased. Previous studies also showed significant increase in Ki-67 LI in different grades of CNS tumours, like glioma and meningioma.[14,23] When the intergrade comparison of Ki-67 expression was done, no significant difference was observed between grade I and grade II and between grade III and grade IV. However, other intergrade comparisons showed significant changes (Table-II). Shivaprasad et al.[24] also didn't find a significant difference of Ki-67 LI between grade I and II as well as between grade III and grade IV tumours.

Mean AgNOR scores also found to be significantly correlated with histological grading of CNS tumours in this study. The mAgNOR in WHO grade I, II, III and IV CNS tumours in the present study were 1.43, 1.97, 2.69 and 3.46 respectively. Significant difference was observed in the mAgNOR among the different grades (Table I). Similar to Ki-67, as the tumour grade increased the mAgNOR also increased (Fig: 4-7).

Furthermore, significant difference was also observed between grade I & II and between grade III & IV when intergrade comparison of mAgNOR was done. Previous study also showed significant difference of mAgNOR between grade I & II and between grade III & IV tumours.[25]In the present study, there was positive significant correlation between ki-67 Labeling Index (LI) and mAgNOR, where the value of Spearman's correlation coefficient was 0.514 and p value was 0.001 (Fig 3). The similar significant positive correlation between ki-67 with mAgNOR was also found in previous study.[26] This correlation indicates effectiveness of both Ki-67 LI and mAgNOR score in gradation of primary CNS tumours.

Conclusion

Both Ki-67 expression and mAgNOR score were increased with the increasing grade of tumours from low to high grade and positive correlation between Ki-67 and AgNOR was also found.

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