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IMMUNOHISTOCHEMICAL EXPRESSION OF NESTIN – A NEURAL STEM/PROGENITOR CELL MARKER IN GLIAL TUMORS

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ABSTRACT

Objective: Glial tumors as a group constitute one of the important tumors of brain both in India and in the world. The present study was done with the aim to study immunohistochemical expression of nestin in glial tumors and to evaluate expression of nestin in different grades of Gliomas according to WHO grading. **Material and Methods:** A total of 40 cases of glial tumors were included in the study. Glial tumors were graded histomorphologically as Pilocytic astrocytoma, Fibrillar Astrocytoma (Grade-II), Anaplastic Astrocytoma (Grade-III), Glioblastoma, Oligodendroglioma (Grade-III) and Oligoastrocytoma (Grade-II). Nestin expression was assessed and correlated with histopathological grade (WHO classification) of glial tumors. Furthermore, immunochemistry for GFAP, S-100 and VEGF was applied and correlation between nestin expression and GFAP, S-100 and VEGF was studied. **Results:** Out of 40 cases, most of the cases were Glioblastoma Multiforme; WHO grade IV(42.5%) followed by astrocytoma; WHO grade II (22.5%) and anaplastic astrocytoma; who grade III(12.5%). Nestin expression was assessed and correlated with histopathological grade (WHO classification) of glial tumors. Nestin immunoexpression was present in 35 cases (89.5%) out of 40 cases. Out of 40 cases of glial tumors, GBM constitute 17 cases. Out of which 16 cases showed maximum nestin grading (3+). In lower grade gliomas (WHO grade I & II) immunoexpression of nestin was weak. Statistically significant correlation was noted in the immunoexpression of VEGF and Nestin, (p value 0.008). **Conclusion:** There is increase in immunoexpression of nestin with increase in grade of malignancy from WHO grade I to IV. Nestin expression may be a potential indicator of biological aggressiveness of the tumors and may be considered as marker of tumor burden and recurrence in human gliomas.

KEYWORDS: glial tumors, astrocytoma, oligodendroglioma and nestin.

INTRODUCTION

Tumors of Central Nervous System accounts for less than 2% of all malignancies (about 175,000 cases per year worldwide). Brain metastasis is three times more common than all primary brain tumors. Gliomas are the most common primary neoplasms of the brain that originates from supporting cells, called glial cells. The main types of gliomas include astrocytomas, oligodendrogliomas, ependymomas and mixed gliomas.[1] World Health Organisation (WHO) classified astrocytomas into four prognostic grades based on histologic features: Grade I (pilocytic astrocytomas, subependymal giant cells astrocytomas); Grade II (diffuse astrocytomas); Grade III (anaplastic astrocytomas) and Grade IV (glioblastoma). Grade I and II are considered low grade, and grades III and IV high grade, astrocytomas. Oligodendrogliomas, accounting for 15-20% of gliomas, are infiltrating gliomas, comprised of cells that resemble oligodendrocytes. They are classified by the WHO into well differentiated oligodendrogliomas (Grade II) and anaplastic oligodendrogliomas (Grade III). Ependymomas are tumors derived from ependymal cells that line the ventricular surface. They account for approximately 5% of childhood tumors and frequently arise from the wall of fourth ventricle in posterior fossa. In adults, the spinal cord is the most common location; tumors in this site are particularly frequent in setting of neurofibromatosis type 2 (NF2).[2]

Although, radiological studies like CT scan and MRI are used to establish the diagnosis of glial tumors, the definitive diagnosis is made by histological examination of biopsy. Haematoxylin and Eosin staining is routinely used for this purpose. CNS tumors show diverge differentiation towards glial, glioneural, and other lineages. Immunohistochemistry (IHC) has important role in making differential diagnosis of CNS tumor.

Numerous immunohistochemical markers are used routinely namely Synaptophysin, GFAP, S-100, and EMA, CK, transthyretin, Ki-67, Nestin etc.[3]

Nestin is a class VI intermediate filament that is produced in neural stem/progenitor cells in the mammalian central nervous system during development. When differentiation starts, cells that exit the cell cycle down regulate Nestin and subsequently up-regulate alternative intermediate filaments (IFS). Down regulated Nestin may be re-expressed in the adult, under certain pathological conditions such as brain injury, ischemia, inflammation and neoplastic transformation.[4,5] Nestin expression has been detected in brain tumors and tumors derived from CNS tissues, such as neurocytomas, gangliogliomas, ependymomas, pilocytic astrocytomas, malignant gliomas including glioblastoma, primitive neuroectodermal tumor (PNETs), medulloblastomas and medulloeptitheliomas.[6] So, immunohistochemical expression of nestin may serve as useful tool for classification and accurate grading of human malignancies. Glial fibrillary Acid Protein (GFAP) is strongly positive in reactive astrocytes and used as marker for glial cells.S-100 Protein is a general "glial" marker, stains both oligodendrocytes and astrocytes. Vascular Endothelial Growth Factor (VEGF) is a signal protein produced by cells that stimulates blood vessels formation. VEGF is highly expressed in acute and sub-acute stages of CNS injury.[3]

Histology remains the mainstay for definitive diagnosis of glial tumors. Numerous ancillary techniques have been introduced along with routine histology for early and accurate diagnosis. Various immunohistochemical markers used in diagnosis of glial tumors are, GFAP, Ki-67, S-100, Oct4, Nestin etc. In view of its ease and reproducibility this techniques can be routinely used, avoiding need for repeated biopsies, delay in treatment and unnecessary radical treatment.[1]

MATERIAL AND METHOD

Present study was conducted in Department of Pathology in collaboration with Department of Neurosurgery, Pt.B.D.Sharma PGIMS Rohtak with the aim to study expression of nestin in glial tumors. Haematoxylin & Eosin staining and Immunohistochemical markers were used to evaluate different grades of Gliomas according to WHO grading. A total of 40 cases of glial tumors were included in the study.

Specimen was fixed in buffered formalin and the tissue block was sectioned at 4-5µm. Diagnosis was established on routine haematoxylin and eosin stain.[7] Tumor grade was assigned based on the World Health Organization criteria for Glial tumors. One representative section from tumor block was subjected to immunohistochemical staining for Nestin, GFAP, S-100 and VEGF. Paraffin sections measuring 3-5 µm in thickness on slides coated with suitable tissue adhesive were deparaffinization and hydrated. Endogenous peroxidase was inactivated with 3% hydrogen peroxidase for 20 minutes; the sections underwent antigen retrieval with microwave oven heating for 30 minutes using citrate or tris EDTA. Sections then incubated with the monoclonal antibody (prediluted) (DAKO) overnight at 4°C. Then sections were rinsed with TBS solution. This was followed by incubation with the secondary antibodies The reaction was visualized using DAB (3,3′-Diaminobenzidine), and nuclei were lightly counterstained with hematoxylin.[8]

Positive and negative controls were run with each batch of IHC stain. Positive control for Nestin was skeletal muscles /tonsils and tongue. Negative control was obtained by substituting the primary antibody with an antibody of non-specific positivity.

Interpretation of result

Representative sections subjected to immunostaining for Nestin were considered as positive if cytoplasm of tumor cells show immunoreactivity. The score of Nestin was evaluated as[9]

Negative -0: When no positive cell observed within the tumor.

Weak-1+: When <30 % of the tumor cells were positive.

Moderate-2+: When 30-60 % of the tumor cells were positive.

Strong- 3+: When >60 % of the tumor cells were positive.

Expression of Nestin was assessed and compared in different grades of glial tumors and expression of GFAP, S-100 and VEGF was analysed in glial tumors.

Statistical Analysis

A descriptive study was carried out for all the variables included in the study. The whole data was entered in Microsoft excel master sheet and analysed using SPSSv20 software. The results obtained were interpreted and descriptive statistics (mean, standard deviation, range, percentages) were applied wherever appropriate. Where the data was qualitative Chi-square test and Spearman correlation coefficient was calculated to assess the association between these parameters. A value of $p < 0.05$ was taken as significant.

RESULTS

A total of 40 cases of glial tumors were included in the study. Patients of glial tumors were between 6 months and 65 years with mean age of 38.86 years. It was observed that maximum numbers of cases were in the age group of 21-30 years and 41-50 years forming 25 % and 22.5% of the study group respectively. The main presenting symptom was headache (25%), followed by vomiting (20%). Out of 40 cases, most of the cases were Glioblastoma Multiforme; WHO grade IV(42.5%) followed by astrocytoma; WHO grade II (22.5%) and anaplastic astrocytoma; WHO grade III (TABLE 1). Nestin expression was assessed and correlated with histopathological grade (WHO classification) of glial tumors (TABLE 2). Nestin immunoexpression was present in 35 cases (89.5%) out of 40 cases. Out of 40

cases of glial tumors, GBM constitute 17 cases. Out of which 16 cases showed maximum nestin grading (3+). In lower grade gliomas (WHO grade I & II) immunoexpression of nestin was weak. Oligodendroglioma (WHO grade III) showed high immunoexpression of nestin (3+). Furthermore, immunochemistry for GFAP, S-100 and VEGF was applied and correlation between nestin expression and GFAP, S-100 and VEGF was studied (TABLE 3). Statistically significant correlation was noted in the immunoexpression of VEGF and Nestin, (p value 0.008). No significant correlation between S-100, GFAP and Nestin immunoexpression was noted in our study. Also there is increase in immunoexpression of nestin with increase in grade of malignancy from WHO grade I to IV (FIG1&2).

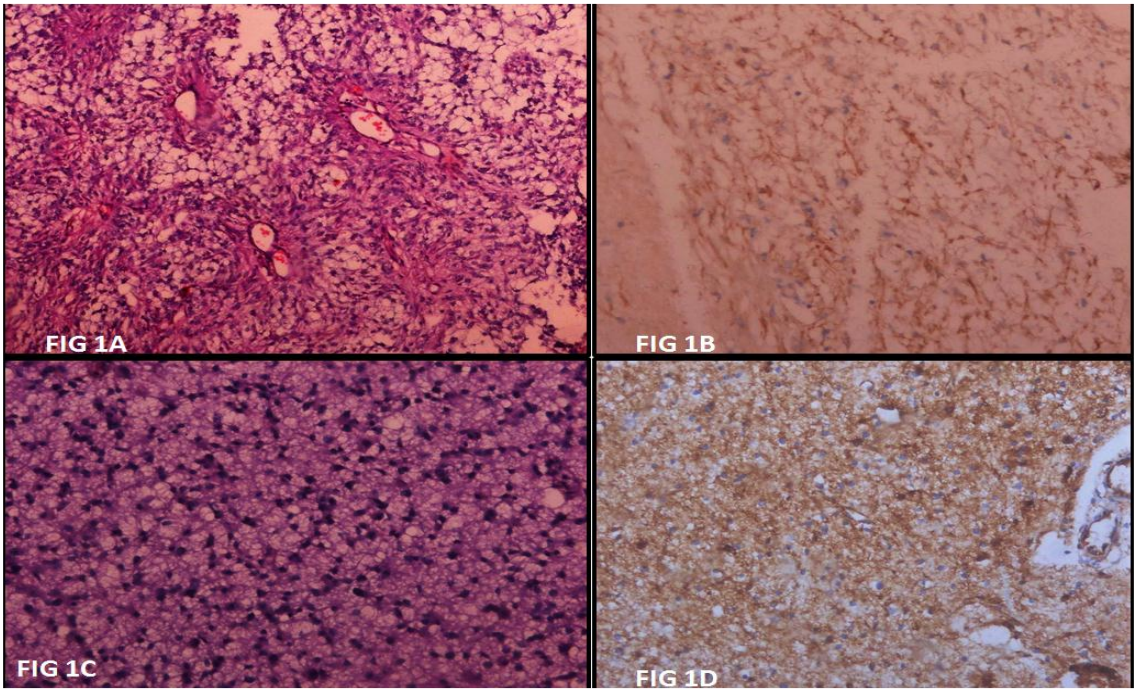

Fig 1 A & B: Case of Pilocytic Astrocytoma on H&E (10x) And Nestin Stain (10x).
Fig 1 C & D: Photomicrograph Showing Grade 2 Astrocytoma (H&E And Nestin Stain, 10x).

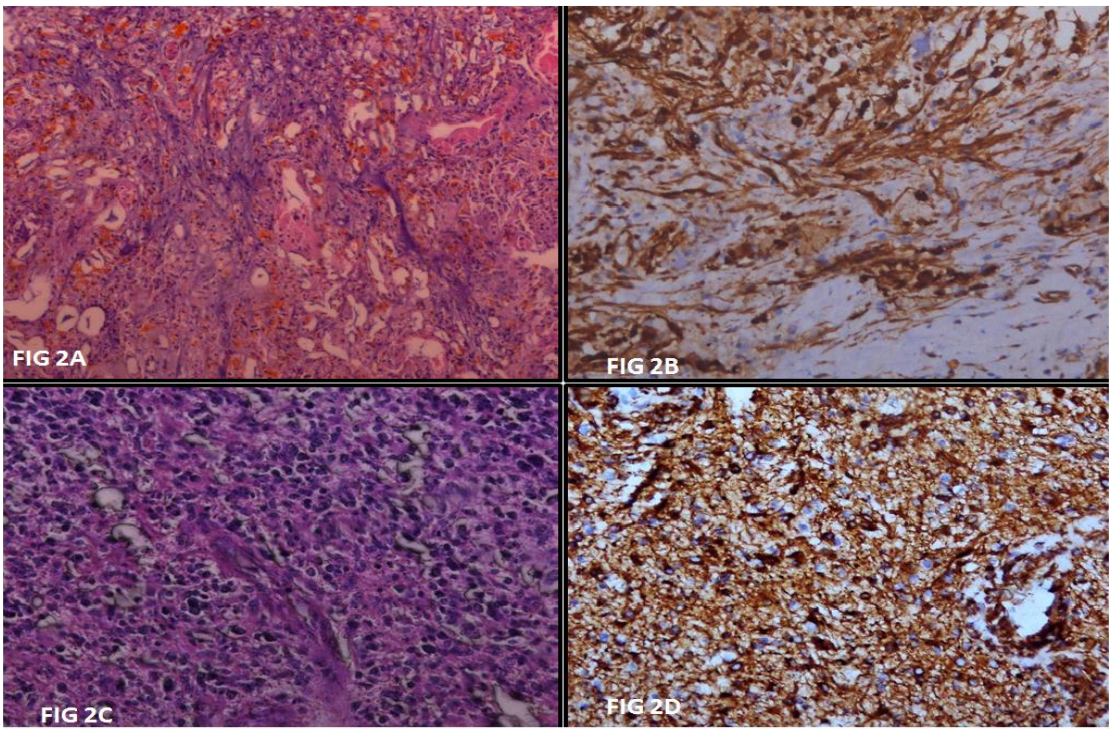

Fig 2 A & B: Case of Anaplastic Astrocytoma On H&E (10x) And Nestin Stain (20x).
Fig 2 C & D: Photomicrograph Showing Glioblastoma Multiforme (H&E And Nestin Stain, 10x).

Table 1: Case Distribution According To Histological Type and Grade of Glial Tumors.

Histological Type	TOTAL NO. OF CASES	PERCENTAGE
Pilocytic Astrocytoma	3	7.5%
Astrocytoma (Gr Ii)	9	22.5%
Anaplastic Astrocytoma	5	12.5%
Gbm	17	42.5%
Oligodendroglioma (Gr Iii)	2	5.0%
Astrocytoma (Gemistocytic)	2	5.0%
Oligoastrocytoma (Gr Ii)	2	5.0%

Table 2: Correlation Between Nestin Grading and Who Grade of Glial Tumors.

	PILOCYTIC ASTROCYTOMA	ASTROCYTOMA	ANAPLASTIC ASTROCYTOMA	GBM	OLIGODENDROGLIOMA	ASTROCYTOMA	OLIGOASTROCYTOMA	P VALUE	X^2
	GR -1	GR- 2	GR- 3	GR- 4	GR- 3	GEMISTOCYTIC	GR-2		
NEG (0)	0	4	0	0	0	0	1		
1+	2	2	1	1	0	0	1	0.008	35.39
2+	1	1	1	0	0	0	0		
3+	0	2	3	16	2	2	0		

Table 3: Correlation of Immunoexpression of Nestin, Gfap, S 100 And Vegf.

		NESTIN
NESTIN	SPEARMEAN CORRELATION COEFFICIENT	1.000
	P VALUE	
GFAP	SPEARMEAN CORRELATION COEFFICIENT	0.12
	P VALUE	0.4
S 100	SPEARMEAN CORRELATION COEFFICIENT	-0.08
	P VALUE	0.6
VEGF	SPEARMEAN CORRELATION COEFFICIENT	-0.2
	P VALUE	0.008

Table 4: Comparison of Expression of Nestin of Previous Studies With Present Study.

S N0	STUDY	EXPRESSION	P VALUE
1.	Dahlstrand et al[12]	Positive – 100% Negative – nil	< 0.002
2.	Sugawaraet al[13]	Positive – 90.0% Negative- 10%	<0.001
3.	Ehrmann et al[14]	Positive – 85.7% Negative – 14.3%	<0.004
4.	Rani et al[15]	Positive – 75.4% Negative – 24.5%	>0.05
5.	Present study	Positive – 87.5% Negative- 12.5%	<0.05

DISCUSSION

The diagnosis of glial tumors is based on spectrum of clinical, laboratory features, histomorphology, and radiological imaging. Histological assessment is necessary for diagnosis of glial tumors and other brain tumors. Immunohistochemistry helps in making correct diagnosis and grading of glial tumors.

In our study group comprising of 40 cases of glial tumors, the mean age of diagnosis was 38.86 years. It was in concordance with the study of Hashmi et al[10], in which the mean age of patients for glial tumors was 38.26 years whereas in a study by Ahmed et al[11], the mean age of glial tumors was slightly higher (43.01 years).

Applying WHO grading system maximum number of cases were of Glioblastoma Multiforme -WHO grade IV (n=17,42.5%) followed by Astrocytoma - WHO grade II (n=9,22.5%), Anaplastic Astrocytoma –WHO grade III (n=5, 12.5%) and Pilocytic Astrocytoma- WHO grade I (n=3, 7.0%). Similar observations were seen in a large study conducted by Agakhan University Hospital, Pakistan over a period of five years in which Astrocytomas comprised the largest group and the majority was of high grade III and IV.

Nestin immunoreactivity was studied in different grades of glial tumors. Representative sections subjected to immunostaining for Nestin were considered positive if cytoplasm of tumor cells showed immunoreactivity ranging from negative to weak (+), moderate (2+) and strong (3+). Sixteen of the 17 cases of Glioblastoma Multiforme showed strong positivity for Nestin, which was in agreement with the studies conducted by Dahlstrand et al[12] and Sugawara et al[13] in which high grade gliomas (grade IV) showed strong positivity for Nestin. In our study low grade glial tumors (grade I) show negative or weak Nestin positivity, whereas grade II and grade III glial tumors showed weak to moderate Nestin positivity. There was a significant association between Nestin grade and grade of malignancy with a p value <0.05 (TABLE 4).

However, the study by Rani et al[15] was in discordance with our results and noted that both low and high grade brain tumors showed Nestin immunoreactivity and did not parallel the malignant grade of tumor. This was explained on the fact that there exist heterogeneicity in degree of differentiation of cell population.

Correlation of immunoexpression of Nestin with VEGF was statistically significant with p value of <0.05. VEGF is implicated in the angiogenesis of glial tumors. A study by Chaudhary et al[16] supported this fact and showed that Vascular Endothelial Growth Factor (VEGF) expression correlates with tumor grade and vascularity of tumor. Blocking the activity of this factor (VEGF) may offer a novel therapeutic approach to the treatment of glial tumors.

Correlation of Nestin expression with S-100 and GFAP in different grades of glial tumors in our study did not show any significant correlation. However, study by Schiffer et al[17] noticed that GFAP expression progressively increased as that of Nestin decreases.

A study of Zalal and Zalal[18] showed an increased relation of GFAP expression with grade of glial tumors. The GFAP expression decreases in high grade glial tumors as the tumors loose the differentiation features.

CONCLUSION

From the study it is concluded that there is up regulation for expression of nestin as there is increase in grade of glial tumors from WHO grade I to IV i.e. from Pilocytic astrocytoma (WHO grade I) to Glioblastoma Multiforme (WHO grade I) and which was found to be statistically significant p value 0.008 i.e. p value <0.05.

Nestin expression may be a potential indicator of biological aggressiveness of the tumors and may be considered as marker of tumor burden and recurrence in human gliomas. The presence of different expression levels of Nestin indicate a possible role as prognostic marker of clinical outcome and provide information about new possible therapeutic targets.

A significant correlation between the high grade of glial tumors, Nestin expression and Vascular Endothelial Growth Factor may help to innovate novel therapeutic approach to the treatment of glial tumors.

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