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Expression of nestin - a stem cell associated intermediate filament in human CNS tumours

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***Background & objectives*: Nestin is an intermediate filament protein expressed in undifferentiated cells during the development of brain and is considered as a marker for neuroepithelial stem cells. Expression of this protein in various CNS tumour cells suggests the possibility of existence of tumour stem cell modulating the evolution. We carried out an immunohistochemical study to demonstrate the expression of nestin and its co-expression with neuronal and glial intermediate filament and correlate with the grade of malignancy.**

***Methods*: Formalin fixed, paraffin processed sections from two human foetuses, 16 brain tumours of both neuronal and glial lineage and two metastatic tumours were immunostained with polyclonal antibody to nestin. Serial sections from primary brain tumours were also stained with monoclonal antibody to neurofilament (NF) and glial fibrillary acidic protein (GFAP). Fluorescent double labeling was carried out on four cases using laser confocal microscopy, to document co-localization of nestin with other intermediate filaments in the tumour cells.**

***Results*: Nestin expression was observed along the paraventricular zone of human foetuses and in brain tumours of both glial and neuronal lineage, of both high and low grades of malignancy. In addition, mature dysplastic spinal motor neurons adjacent to tumour and cerebellar Purkinje cells also expressed nestin along with neurofilament.**

***Interpretation & conclusion*: Nestin expression was noted in both low and high grade brain tumours and dysplastic neurons and did not parallel the malignant grade of the tumour. The expression of nestin in tumour cells and dysplastic neurons suggests aberrant expression of antigenically primitive proteins in cells to facilitate remodelling of the cell and migration. More studies are needed to elucidate the concept.**

Key words CNS tumours - GFAP - immunochemistry - intermediate filament - nestin - neurofilament - stem cell

During foetal development and origin and progression of neoplasia, certain ontological cellular events are probably shared to facilitate growth and differentiation by temporal expression of various cellular proteins. To account for hierarchical expression of these genetic events, the stem cell concept is evoked. The stem cell is considered to be the primitive undifferentiated mother cell, capable of continuous yet controlled cell division, self renewal and differentiation in predetermined patterns to form organs. Tumourigenesis, on the other hand, is considered to be promoted by sequential mutations regulating the biological pathways, allowing the cells to divide unchecked evading apoptosis, respond abnormally to various trophic factors, receive aberrant blood supply (angiogenesis) and to migrate to farther locations (metastasis and invasiveness).

Bailey and Cushing[1] suggested that brain tumours evolved from undifferentiated blast cell- a progenitor stem cell of varying levels of commitment to cell lineages. There is overwhelming evidence in haematological malignancies like leukaemias, where, among the population of leukaemic cells, only a few stem cells have unlimited proliferative potential, facilitating the progression of the neoplastic process, and these cells can be fractionated and studied[2,3]. Similar phenomenon could be operational in central nervous system (CNS) neoplasia as well. Nests of undifferentiated cells, which normally form granule cells of cerebellum are considered to transform to primitive, malignant neurectodermal tumour (PNET), the medulloblastoma[4] and radial glia which facilitate neuronal migration, can lead to the evolution of pilocytic astrocytomas. During the evolution of neoplasia and tissue repair, some cellular and molecular events are called upon to facilitate the process. Nestin is a gene encoding a class of intermediate filaments (IF) specific to neural stem cell, sharing structural homology of varying grades with other IF, but preserving hierarchical and sequential expression of events during the development of brain[5,6]. During CNS development the intermediate filament component of the cytoskeleton undergoes significant remodeling. Initially nestin is expressed in CNS stem cells[5] followed by downregulation and shift to expression of glial fibrillary acidic protein (GFAP) and neurofilament (NF) in differentiated astrocytes and neurons respectively[7,8] in addition to peripherin and internexin in some subset of neuronal population[9,10]. These events reflect temporal and spatial control of intermediate filament expression facilitating changes in shape and migratory potential[7]. As the expression of intermediate filaments is used as a marker of cell lineage in tumour pathology, we studied the expression of stem cell IF marker, nestin in brain tumours and attempted to correlate with other appropriate lineage specific IF expression, and the grade of malignancy. As the antibody used was specific to human foetal brain, sections from human foetal brain were stained to confirm the specificity of the antibody labeling.

Material & Methods

Sixteen brain tumour specimens of varying grades of malignancy (WHO grade I - IV), and two metastatic tumours were derived from the surgical biopsies submitted to Department of Neuropathology, National Institute of Mental Health & Neurosciences (NIMHANS), Bangalore during July 2000 to December 2004 for diagnosis. In addition, similarly processed two paraffin blocks of human foetal brains (well preserved human foetal brains of 14 and 24 wk of gestation from the collection of Human Brain Tissue Repository, a National Research Facility at NIMHANS, Bangalore, collected following informed consent from the parents to use the material for research purposes and approved by the

Institutional Ethics Committee, were also studied. Six micron thick paraffin sections were collected on poly L-lysine coated glass slides for immunohistochemistry.

Antibodies: (*i*) Rabbit polyclonal anti-human nestin, (150 amino acid fragment of human nestin obtained from cloned nestin cDNA from human foetal brain, a gift from Dr Mahendra Rao, Laboratory of Nervous System, National Institute of Ageing, NIH, Bethesda, MD, USA, 1:5000); (*ii*) Neurofilament (Monoclonal, Isotype IgG, 1:50, DAKO, USA); (*iii*) Glial fibrillary acidic protein, GFAP (Monoclonal, Isotype IgG, 1:50 DAKO, USA).

The antigen retrieval was carried out by microwaving in citrate buffer (*p*H 6.0) and the sections were stained by immunoperoxidase method, using diaminobenzidine/hydrogen peroxide (DAB/H_2O_2) as the chromogen[11]. Slides were counterstained with Meyer's haematoxylin (Sigma Chemicals, USA). One case of medulloblastoma, which was found to contain nestin positive cells in the pilot study was used as a positive control. Sections from a case of gamistocytic astrocytoma for GFAP and normal cerebellum for neurofilament were used as normal controls, both of which exhibited strong immunolabeling for the respective intermediate filaments. Identically processed sections skipping the primary antibody in immunostaining procedure served as negative controls.

The sections were screened for (*i*) numerical density and phenotype character of tumour cells expressing nestin/GFAP/NF and were visually graded from 0 to +++, blind to the type of antigen labelled and then analysed. (0- no labeling of cells ; + : 20% of cells ; ++ : 20-50% of cells; +++ > 50% cells); (*ii*) spatial co-localization of nestin with GFAP/ neurofilament in sections.

Double labeling for co-localization of nestin and GFAP or nestin and neurofilament was carried out in four selected tumours (medulloblastoma, dysplastic gangliocytoma of cerebellum with associated medulloblastoma, subependymal giant cell astrocytoma and giant cell glioblastoma - one case each). The rabbit polyclonal nestin antibody (1:5000) was visualized by goat anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC) flurochrome (1:200, green fluorescence) and monoclonal antibodies to GFAP or NF (1:50) were highlighted by rabbit anti-mouse secondary antibody conjugated with Cy 3 (1:200, red fluorescence Sigma Chemicals, USA). Briefly, the sections were equilibrated in phosphate buffer saline with 0.1 per cent triton × 100 (0.1 M PBST, *p*H 7.4) for 10 min. After blocking the non-specific binding sites with 5 per cent delipidated milk powder and bovine serum albumin (BSA) for one hour, the sections from medulloblastoma, dysplastic gangliocytoma of cerebellum, both tumours of neuronal lineage were incubated with a mixture of primary antibodies, anti-nestin (polyclonal, 1:5000) and anti-NF (monoclonal 1:50); and subependymal giant cell astrocytoma and giant cell glioblastoma with a cocktail of antibodies GFAP (1:50) and nestin (1:5000) for 48 h at 4°C. After washing in buffer the slides were viewed under Laser confocal scanning microscope (DMIRE)-TCS from Leica, Germany, with laser illumination at 488 nm for FITC and 514 nm for Cy3, respectively. The fluorescence emitted by the cells in the same field (green-nestin, red-NF/GFAP) and also compound images of co-localization by the two fluorochromes representing different antigens, nestin-neurofilament and nestin-GFAP expression were recorded.

Results

The human foetal forebrains of both the ages (14 and 24 wk) revealed nestin labeling along periventricular zones, especially the temporal horn, while other diencephalic nuclear clusters were not

labeled. The immunolabeling revealed a gradient, with high expression in ependymal layer and sub-ependymal progenitor cells, and gradually receeding with increasing distance from the ventricle (figures not shown). The metastatic epithelial tumour cells were not labeled for nestin while the reactive glia and adjacent neurons were stained light.

Among the tumours studied (Table), pilocytic astrocytomas (Fig. 1A) of both juvenile and adult type (WHO-grade I) were stained by nestin and GFAP. The piloid cells especially those floating in myxoid matrix of the cystic areas were strongly labeled for nestin (Fig. 1B), while perivascular foot processes and subpial glial cells were labeled light and variable. The gliotic neuropil was labeled lightly by nestin, but intensely by GFAP (Fig. 1C).

In subependymal giant cell astrocytoma (Fig. 2A), majority of large ganglioid cells were strongly labeled by nestin, the label extending along the cell processes (Fig. 2B) while the perivascular foot processes were not labeled. GFAP stained variably the tumour cells, the perivascular foot processes and gliotic neuropil. Only a few tumour cells revealed co-labeling, strongly with nestin, GFAP (Fig. 2C) and faintly with NF antibody (Fig. 2D) in serial sections visualized by immunoperoxidase technique, suggesting divergent antigenic expression of these cells. In lobar ependymoma and neurocytoma, both relatively differentiated

Table. Expression profile of nestin, glial fibrillary acidic protein (GFAP) and neurofilament (NF) in CNS tumour of different grades

Case no.	Type	Age/sex	WHO grade	Location	Nestin	GFAP	NF
1	Pilocytic astrocyma	26/M	I	Brain stem	+++	++	-
2	Juvenile pilocytic astrocytoma	12/M	I	Cerebellum	++	+++	-
3	Subependymal giant cell astrocytoma		I	Lateral ventricle	+++	+	+
4	Ependymoma	25/F	II	Right postfrontal	+	+	-
5	Central nerocytoma	20/F	II	Lateral ventricle	+	-	-
6	Dysplastic ganglioma with medulloblastoma	9/M	I & IV	Cerebellum	DYS N ++ M+	-	DYS N+M+
7	Anaplastic astrocytoma	28/F	III	Left frontal lobe	+	++	-
8	Anaplastic astrocytoma	33/F	III	Corpus collosum	++	++	-
9	Anaplastic oligodendroglioma	43/F	III	Right forntal	+	+	-
10	Desmoplastic medulloblastoma	5/M	IV	Cerebellum	+++	-	-
11	Medulloblastoma	20/M	IV	Cerebellum	+++		+
12	Medulloblastoma (nodular)	8/F	IV	Cerebellum	++	-	+
13	Neuroblastoma	3/M	IV	Spinal cord	+++	+	+
14	Small cell glioblastoma	62/M	IV	Left frontal	++	+	-
15	Glioblastoma-focal anaplastic oligodendroglial zones	26/M	IV	Left frontal	+++	++	-
16	Pleomorphic glioblastoma	6/M	IV	Left temporal	++	+	-

DYS N, Dysplastic neurons; M, medulloblastoma
- Absence of labelling

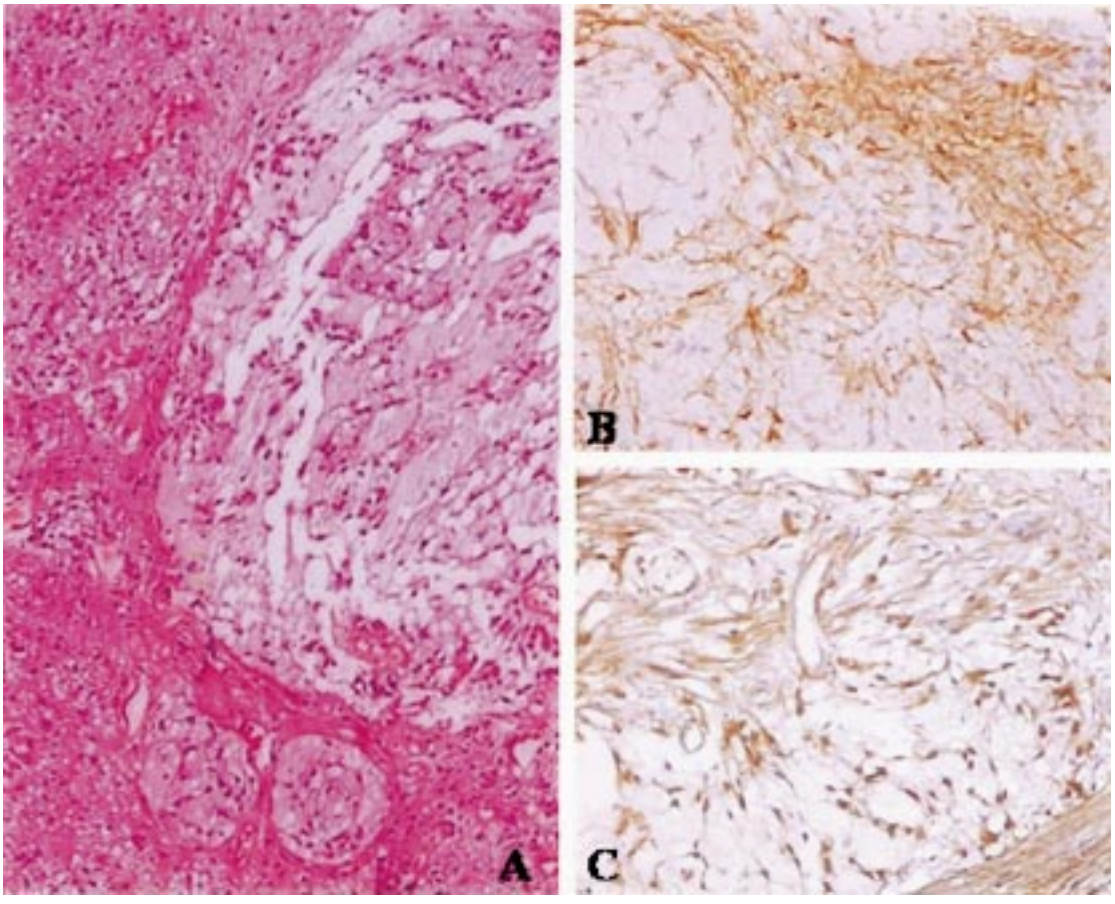

Fig. 1. Pilocytic astrocytoma (WHO grade I), thin piloid fibres floating in pools of myxoid stroma (**A**). GFAP labeled the tumour cells light and perivascular glial fibres dark (**B**), while nestin stained the tumour cells intensely (**C**). A: Haematoxyclin eosin (HE) x 240; B, C-Immunoperoxidase x 240.

benign tumours, nestin labeled cells lightly and they were randomly distributed and close to vessels.

In anaplastic astrocytoma and oligoastrocytoma (Fig. 3) occasional small round tumour cells with short process and the neuropil around were intensely labeled by nestin (Fig. 3, inset), which probably represented anaplastic or primitive oligodendroglia. Large stretches of fibrillated, GFAP labeled glial cells did not express nestin. Segments of axonal tracts traversing across were stained by NF antibody, but none of the tumour cells were labeled. In small cell glioblastoma, both GFAP and nestin stained small islands of tumour cells, where the expression of nestin was less intense in contrast to GFAP. In cases of giant cell glioblastoma (Fig. 4) and glioblastoma with focal anaplastic oligodendroglial zones, the tumour giant cells located essentially close to blood vessels and in the infiltrating front were strongly labeled by nestin and GFAP as revealed by co-localizing fluorescence in laser scanning confocal microscopy (Fig. 4 A, B). Similarly, the tumour cells floating in microcystic space in anaplastic astrocytomas, expressed nestin strongly. GFAP on the other hand labelled large zones, both in the tumour around the blood vessels and the infiltrating front only focally, corresponding to nestin stained areas. This is in contrast to diffuse labeling observed in low grade astrocytomas.

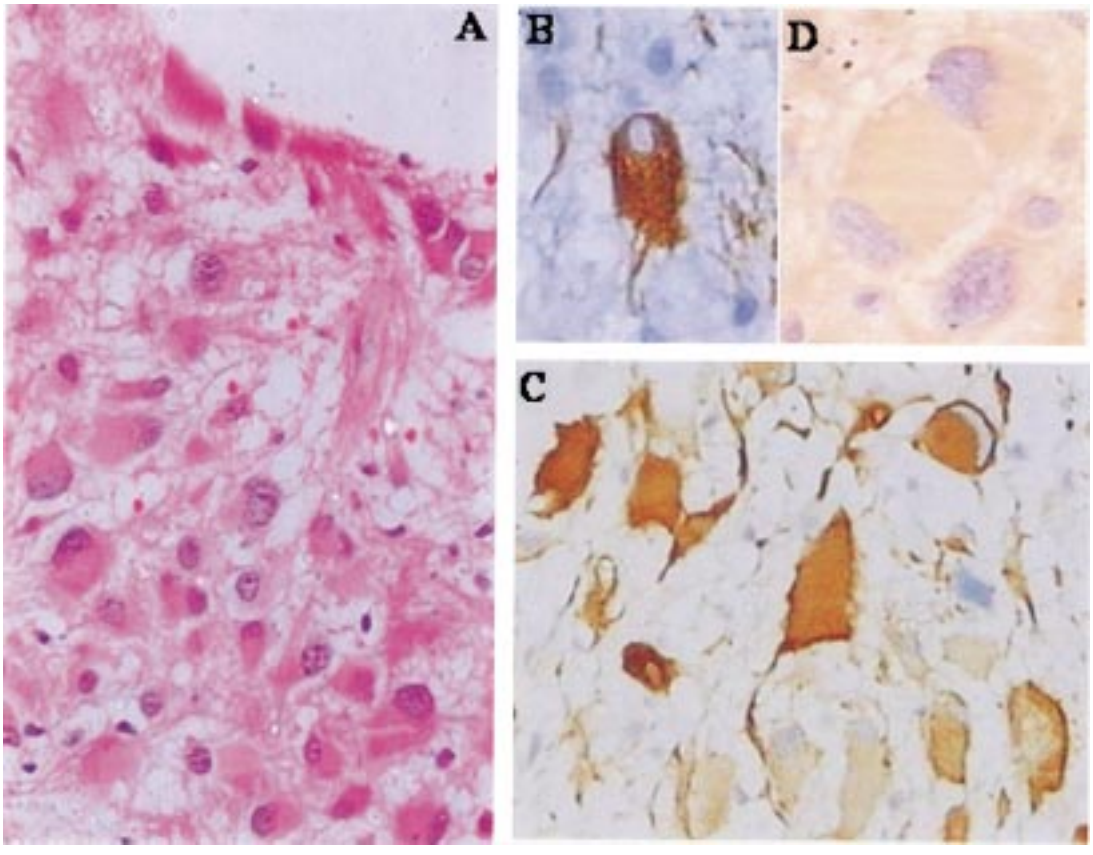

Fig. 2. Subependymal giant cell astrocytoma (WHO grade I) - many large gangliod cells in a fibrillated glial stroma (**A**). The gangliod cells are labeled intensely with nestin (**B**) and express GFAP (**C**) and neurofilament (**D**) variably. A: HE x 240; B, C, D-Immunoperoxidase x 480.

In medulloblastomas (Fig. 5A), large areas of tumour cells were stained by nestin. In Homer-Wright rosettes both the cells and the central fibrillated zones were labeled by nestin, reflecting the primitive nature (Fig. 5A, inset). On confocal microscopy, the nestin labeling was found to be more intense in the primitive tumour cells than NF, but co-localizing reflecting dual expression (Fig. 5 B, C, D). In nodular zones, the margin of the nodules had many nestin positive cells, suggesting the presence of primitive cells in the spreading front. GFAP failed to label the centrally located pale cells in the nodular areas in medulloblastoma, but a few cells stained with NF.

In one case of dysplastic gangliocytoma with co-existing medulloblastoma, (Fig. 6A) a band of pale, large, dysplastic ganglion cells occupied the molecular layer of cerebellar folium, depleting the small granular neurons. Medulloblastoma, a PNET, was found infiltrating the folia and spreading along the surface. Majority of the large dysplastic pale neurons were strongly labeled by nestin and NF (Fig. 6B, C). Co-expression of nestin/NF was noted in a few mature neurons. The islands of medulloblastoma were focally labeled by nestin and NF, but not by GFAP, while GFAP labeling was noted in reactive astrocytes and gliotic zones. The reactive astrocytes were found labeled by nestin in 7 of the 17 tumours examined, representing heterogeneity in the degree of differentiation of the cell population. In addition to tumour cells and dysplastic neurons, a few of the cerebellar Purkinje cells and anterior horn cells of the spinal cord

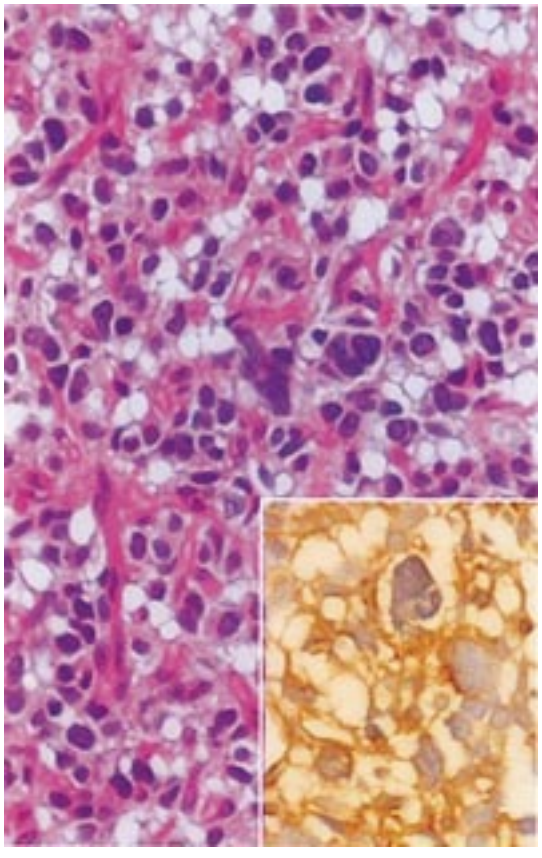

Fig. 3. Anaplastic oligodendroglioma (WHO grade III) with chicken wire vascular channels and anaplastic tumour cells. HE x 240. Inset: Cytoplasmic labeling of tumour cells by nestin. Immunoperoxidase x 300.

distant to the primitive neuroectodermal tumour expressed nestin co-localizing with NF (Fig. 7 A, B), suggesting aberrant expression of stem cell related IF in mature cells.

Fourteen of the 18 tumours analysed had nestin labeling of capillary endothelial cells. Nestin immunoreactivity was most intense in proliferating endothelial glomeruli of malignant glioma (Fig. 8), found in both the tumour and adjacent normal brain. A few of the capillaries in the tumour and endothelium lining thick hyalinized large vessels were nestin negative, suggesting differential expression. In fragments of normal brain resected along with tumour, NF expressing neurons and GFAP expressing glia, were observed, but none were nestin positive, except for the capillary endothelium widely.

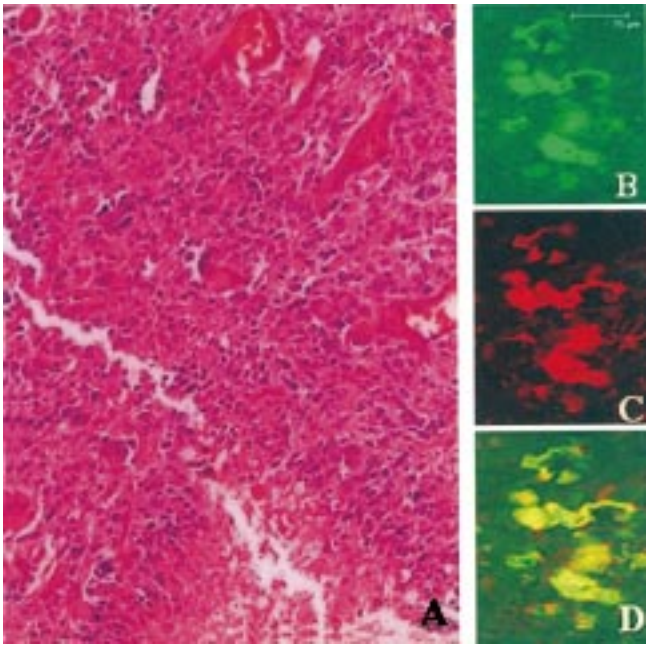

Fig. 4. A Giant cell glioblastomas (WHO grade IV) with numerous pleomorphic tumour giant cells adjacent to zone of necrosis. HEx240B. **B-D.** Tumour giant cell labeled for nestin (greenish yellow-FITC channel) and GFAP (Reddish-CY3 channel/and co-expression (yellow-orange) for nestin and GFAP respectively (Bar 75 μm).

Discussion

In mammalian nervous system subependymal cells lining the ventricles were identified as stem cells with an ability to generate new neurons and also proliferate and migrate in response to injury and to differentiate to glial lineage at the site of the lesion[12,13]. In the human embryo, vimetin and nestin expression was restricted to primitive neuroepithelial cells and radial glial cells of spinal cord at 6-11 wk of gestation and the ependyma and germinal matrix of the telencephalic walls at 17-20 wk of gestation[14], similar to labeling feature noted in the present study. The immunolabeling showed a gradient with high expression in ependymal cells and subventricular progenitor cells and gradually receding with increasing distance from the ventricular wall, similar to earlier studies[14]. We did not detect GFAP in the telencephalic wall. This indicates the ontogenic and spatial regulation of the stem cell associated

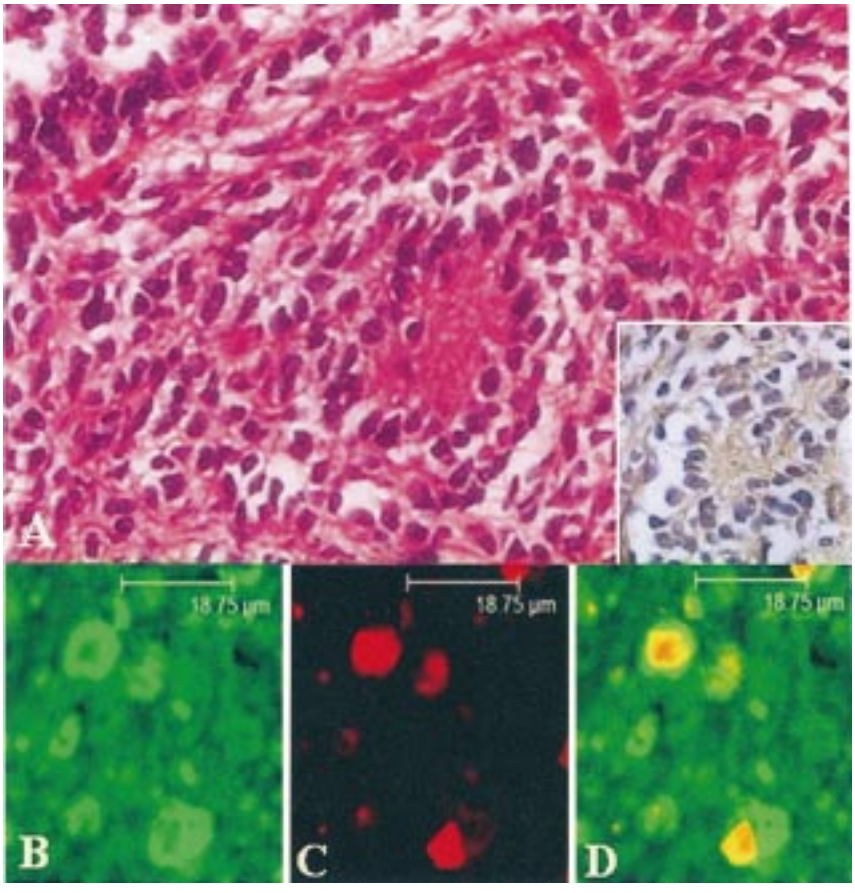

Fig. 5. Medulloblastoma (WHO grade IV) with central Homer-Wright rosette. HEx300. Inset: Nestin labeling of the primitive tumour cells and fibrillary zone within the rosette. Immunoperoxidase x 300. Laser confocal labeling of tumour cells **B**-Nestin (Greenish yellow, FITC), **C**- Neurofilament (Red-CY3), **D**-colocalisation of nestin and NF in tumour cells. (Bar 18.75 µm).

intermediate filament, nestin expression in human foetal brain.

Among the tumours, in pilocytic astrocytomas, the piloid cells, especially those floating in the myxoid matrix of the cystic zones were strongly labeled for nestin, while the perivascular foot processes, subpial glial cells, and piloid tumour cells in compact zones labeled light and variable, indicating their origin from radial glial population. Frisen *et al*[16] have observed that astrocytes showed high levels of nestin when grown at low cellular density and a weaker nestin immunoreactivity with increased cell density causing crowding. Similar feature is reflected by the strong labeling for nestin in the cells floating in cystic spaces, and low level of labeling in the compact zones. This variable nestin expression modulated by cell density could indicate remodeling of cytoskeletal framework to facilitate migration and self-renewal. The quiescent gliotic neuropil was labeled light by nestin, while reactive astrocytes were stained moderately strong both by nestin and GFAP reflecting transient shift in the intermediate filament expression to ontologically early phase in response to proximate tumour/injury. Contrary to the observations and inferences of Almiquist *et al*[17], the number of piloid cells labeled strong in our study were high, thus not correlating with the degree of malignancy.

The subependymal giant cell astrocytoma (SEGA), though considered hamartomatous in origin,

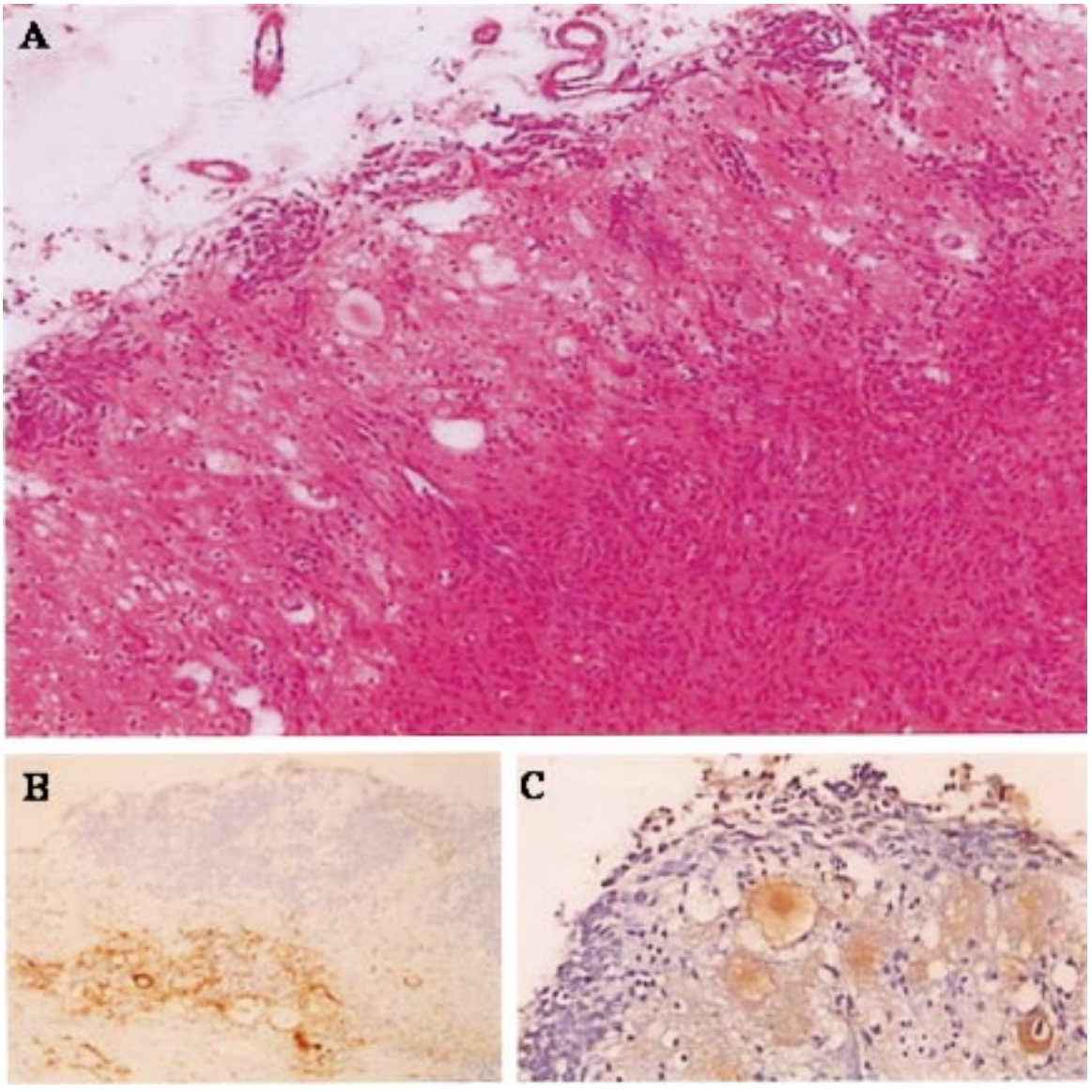

Fig. 6. Gangliocytoma of cerebellum (WHO grade I) widening the cerebellar folia by large abnormal dysplastic neurons occupying the molecular layer and depleting the internal granular layer and co-existing medulloblastoma above (WHO grade IV) (**A**) Antibody to neurofilament (**B**) labeled the large dysplastic neurons and its processes. Nestin (**C**) co-labelled the large dysplastic neuronal soma and a few medulloblastoma cells on the surface. **A**: HE x 240; **B** - Immunoperoxidase x 150; **C** Immunoperoxidase x 350.

is now recognized to be a benign tumour, the tumour cells having a potential to express dual lineage of IFs, both neuronal and glial, in the same cell. In the present case under study, majority of the cells in SEGA expressed nestin of varying intensity, while nearly one fifth of cells in the lesion expressed neurofilament and GFAP. The expression of nestin in majority though suggests relatively primitive stage, the phenotypic features of the cells were ganglioid. The persistence of bipotential (neuronal and glial) expression of IF protein reflects aberrant differentiation of the cell lineage. The cells in intermediate grade neoplasms (WHO grade II), the ependymoma and neurocytoma, one of glial and the other neuronal lineage expressed nestin in small focal areas, indicating persistence of cells of stem cell potential. The cells expressing nestin, morphologically were small, with globular cell soma

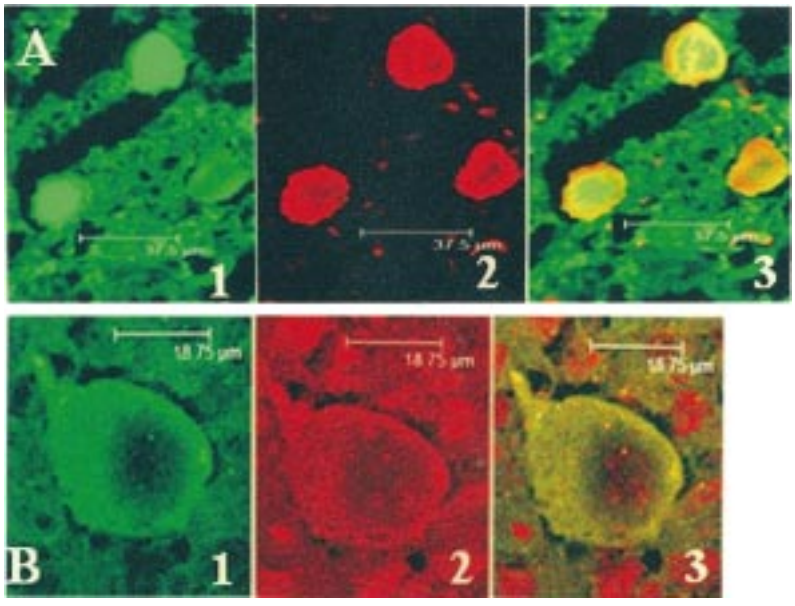

Fig. 7. Laser confocal microscopy. **A**-spinal motor neurons, (37.5 mm) **B**-cerebellar Purkinje cells (Bar 18.75μm) away from the lesion in a case of ganglioglioma. 1. Nestin (yellow-green-FITC channel); 2. Neurofilament (Red CY3 channel); and 3. Co-localization of nestin and NF (yellow-orange) channel.

and small, stubby processes, resembling Golgi type II neurons or oligodendroglia in Golgi silver staining as also reported by others[17,18] on ependymomas.

In the present study, case 6 had a dysplastic gangliocytoma of cerebellum, the large dysplastic neurons expressing nestin in the cell soma and co-localized with neurofilament. Unusually this case also had a co-existing medulloblastoma, a PNET of cerebellum, focally expressing nestin and NF. This case demonstrated the persistence of stem cell marker, both in primitive tumour cells and mature neurons as a continuum. This lends support to the hypothesis that focal cortical dysplasia is not just arrested neuronal migration, but also failure of maturation and hierarchical intermediate filament shift to early stages[19]. This accounts for the round ballooned nature of dysplastic neurons with absent or stunted dendritic arborisation. The expression of nestin and phosphorylated NF in cerebellar Purkinje cells and spinal motor neurons away from the neoplastic zone reflects reactivation of developmental programme in mature neurons for survival under pathological conditions, as suggested in cases of Creutzfeldt-Jakob disease[20].

The malignant glial neoplasms, anaplastic astrocytomas and glioblastomas expressed nestin in variable number of tumour cells, especially close to vessels present as clusters or randomly distributed. In medulloblastoma especially in the nodular and desmoplastic zones, expression of nestin along the margin of the nodular areas and in inter-nodular zones, suggests centrifugal spread of stem cells along the advancing front, the central area having differentiated cells. The anaplastic astrocytoma and glioblastoma had cells expressing both nestin and GFAP and negative for NF, indicating the presence of only glial progenitor cell. The percentage of nestin positive cells in medulloblastoma and glioblastoma are essentially similar to those in benign subependymal giant cell astrocytoma. This observation highlighted two features. (*i*) Nestin expressing stem cells/progenitor cells do exist both in low and high grade tumour as clonal clusters or randomly distributed, (*ii*) Nestin

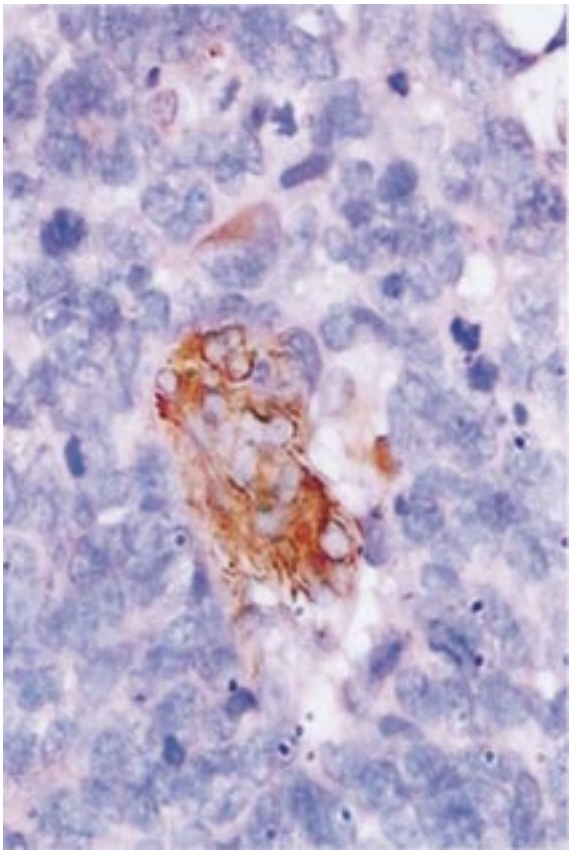

Fig. 8. Strong immunolabelling of vascular endothelial tufts in glioblastoma by antibody to nestin. Immunoperoxidase x 240.

expression does not run parallel to the malignant grade of the tumour.

These results are in contrast to the earlier observations[14,17] though the cause is not evident. The mature oligodendroglial component and anaplastic cells, both expressed nestin, though of different degrees, indicating preservation of foetal characters in the tumours cells.

The expression of nestin in neoplastic cells, the reactive astrocytes, normal cerebellar Purkinje cells and spinal motor neurons adjacent to the neoplastic lesion and dysplastic neurons, address different issues. In the scar tissue and the edge of the pathological lesion, the cells revert ontogenically expressing nestin to facilitate remodeling of cytoskeletal framework of the cells, thus facilitating migration and remodeling of the injured area[12]. On the contrary, the potential of a progenitor cell, nestin expression in neoplastic cells probably facilitate self renewal and migration along the interstitial space to reach the vessels. This multipotent progenitor character is further exemplified by the new group of glioneuronal tumours recognized recently expressing both the phenotypes of glia and neurons[21]. In dysplastic neurons, nestin expression could be an aberrant event or failure of "switching off" the hierarchically early forms of cytoskeletal protein.

The ubiquitous expression of nestin in the vascular endothelium either normal or glomeruloid structures in glioblastoma multiforme (GBM) probably refers to perpetual self renewal potential which is probably essential to replenish the endothelial cells subjected to continuous shearing haemodynamic flow and facilitate neurogenesis from stem cells as well[22]. This may not be a reflection of neoplasia.

The definitive evidence for the concept of tumour stem cell is identifying the clones of premature cells and demonstrating their *in vitro* tumourogenesis. Singh *et al*[23] have evolved primary tumour sphere cultures and isolated 'CD-133 expressing stem cell population'. These cells in culture transformed to tumour cells, phenotypically resembling the parent tumour in the patient. It seems that normal stem cells in the nervous system and cancer cells share similar phenotype and functional properties, yet differing in some unique molecular mechanisms to take divergent pathways. More studies are needed to further elucidate the concept and the biology of tumour stem cells.

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