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Pathology

NESTIN IN HUMAN CEREBELLAR DEVELOPMENT AND TUMORIGENESIS OF MEDULLOBLASTOMA

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ABSTRACT

Background & Objectives: The aim of this study was to see the localization and migration of neural stem cells (NSC's) in developing human cerebellum and neuroectodermal tumors of cerebellum by the expression pattern of Nestin, a neuroepithelial stem cell marker. It was observed that the stem cells decreased during the progressive maturation of the progeny, still some persisted postnatally. In our study the higher Nestin expression was observed in desmoplastic medulloblastomas as compared to classic medulloblatosmas which provided a credible evidence that this tumor originates from these neuroectodermal stem cells.

Methods: Nestin immunohistochemistry (IHC) was conducted and quantitatively analysed on

a) Eighteen human fetal cerebellum ranging from 17 to 36 weeks of gestational age (GA) – three fetuses each in age groups of 14-17, 18-21, 22-25, 26-29, 30-33 & 34-36 weeks

b) Forty cases of childhood medulloblastomas (34 cases of classical and 6 of desmoplastic variants)

Results: The results tabulated reflected that

a) With the increase in gestational age, Nestin expression decreased in external germinal layer (EGL), periventricular zone (PVZ) and internal granular layer (IGL) while increased in ventricular layer (VL). However, still Nestin positivity persisted in small percentages in each layer.

b) The tumor cells showed perivascular and diffuse (<50%) Nestin staining in classical variant as compared to focal nodular and diffuse (>50%) in desmoplastic variant.

Interpretation & Conclusions: The careful per-lustration indicated that there was a significant reduction in the neural progenitor cells and increase of these cells in the VL. The Nestin positive cells present in EGL, PVZ, VL and IGL suggested the origin of medulloblastoma from these neural stem cells. The desmoplastic variant of medulloblastomas showing higher Nestin expression in comparison to classical variant points that the desmoplastic variant is more primitive and hence with poor prognosis.

KEYWORDS : Fetal cerebellar cortex, Medulloblastoma, Nestin, Periventricular zone

INTRODUCTION:

The development of human fetal cerebellum extends from the early embryonic period till the first postnatal year. The cerebellar progenitor cells are derived from two major germinal zones i.e. periventricular germinal matrix and external granular layer (EGL). Two zones can be distinguished in the EGL. The outer zone cells of EGL differentiate towards granular, stellate and basket neurons. The inner zone cells differentiate and start to migrate inwards to form the IGL. The periventricular germinal matrix in 3rd to 8th weeks of gestation forms three layers-ventricular (VL), intermediate and marginal layer. The VL further differentiates to form Purkinje cells and deep cerebellar nuclei. [1-4]

The NSC's decline in number with gestational age, though some still continue to persist. Persistent nests of these neuronal stem cells in the any of above layers may lead to genesis of medulloblastoma, a prototypical embryonal tumor which includes a constellation of neoplasm's that most commonly occur during childhood. [5,6]

In present study we used Nestin as an immunomarker to identify these NSC's in different layers of developing cerebellum as well as the subtypes of medulloblastoma. Nestin, an intermediate filament protein has long been known not only a specific marker of cells but also as an indicator of the degree of neural stem cell differentiation. This study aims in understanding the localization and migration of stem cells in developing human cerebellum and in tumorigenesis of medulloblastoma.

SUBJECTS AND METHODS:

Human fetuses

Eighteen human fetuses with gestational age of 17to 36 weeks , including three fetuses in each age group of 14-17, 18-21, 22-25, 26-29, 30-33 & 34-36 weeks were included in the study. Fresh still births with no detectable CNS malformation on antenatal ultrasound were collected. The age of each fetus was based on a combination of post ovulatory age and early ultrasonographic gestational age estimation. The written consent was obtained from the parents after explaining the purpose of the study. The study was performed within the guidelines of Institutional Ethics Committee.

Medulloblastoma

Forty cases of medulloblastomas (34 classic and 6 desmoplastic cases). The mean age was 7.62±4.35 years and M: F ratio =7: 3.

Immunohistochemical Staining

Serial tissue sections of 4µm thickness were obtained. Paraffin-embedded sections were heated at 58~60°C for 30 min. Then baked slides were deparaffinized in xylene and rehydrated in a graded series of alcohols. Blocking was done by using 3% H_2O_2 in methanol. The antigen retrieval was performed by heat-induced in citrate puffer, PH 6.0. Sections were incubated overnight at 4°C with primary polyclonal antibody (Nestin, Santa Cruz, USA) in humid chamber. TBS was used in place of the primary antibody as negative control. In the following day, sections were stained using labeled streptavidin biotin kit (LSAB kit) (Diagnostic Biosystems, USA) with modified timings for 2 hours followed by conjugated streptavidin horseradish peroxidase complex for 1 hour. The bound peroxidase was revealed using 3.3'-diaminobenzidine peroxidase in TBS. Finally, sections were counter-stained with hematoxylin, dehydrated and mounted in a neutral mounting medium.

Evaluation of immunohistochemistry

1. The sections were screened for the density of Nestin positive cells in each layer and were visually graded from 0 to +++ and results were recorded. (0- No expression, +- 20% of cells showing expression ,++- 20-50% of cells showing expression, +++- >50% of cells showing expression)
2. The percentage of Nestin positive field area in full cerebellar cortex at different range of gestational age was determined morphometrically (Biovis Image analysis software, Expert vision, Mumbai, India). Mean positive field area in each group was taken out.

3. The patterns (perivascular, diffuse<50%, diffuse≥50%, focal nodular) of Nestin staining in medulloblastoma cases were recorded.
4. We analysed our data quantitatively in different layers of the cerebellum as well as full cerebellar cortex at different weeks of gestation.

RESULTS:

The Nestin expression decreases in EGL, PVZ, and IGL while it increases in VL with advancing gestational age in the human fetus. The mean percentage field area of Nestin positive cells decrease in cerebellar cortex with advancing gestational age. The expression in individual layers of whole cerebellar cortex indicates that there is reduction in number of neural progenitor cells to a greater extent in all the layers than the relative increase of these cells in the VL. The cerebellar purkinje cells are Nestin positive throughout the fetal life. **(Table1, Figure 1)**. In medulloblastomas, Nestin expression was observed in all cases with 50% showing diffuse, 45% perivascular and 5% focal (nodular) pattern. **(Table2, Figure 2)**. The diffuse pattern of Nestin was more frequent in children of higher age group and in desmoplastic subtype. The endothelial cells are immunopositive during tumorigenesis.

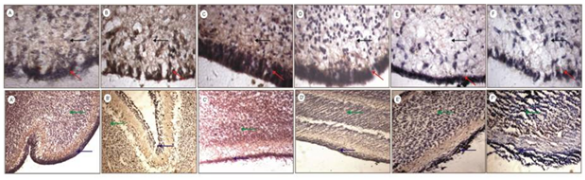

Figure1:Nestin expression in ventricular (red arrows) and periventricular (black arrows) layers (A-F) and external (blue arrows) and internal (green arrows), granular layers (A`-F`) of developing human cerebellum in different gestational ages. (A,A`)17weeks; (B,B`)20weeks; (C,C`)24weeks; (D,D`)28weeks; (E,E`) 32weeks; (F,F`) 36weeks. The nestin expression decreased in external germinal layer (EGL), periventricular zone (PVZ) and internal granular layer (IGL) while increased in ventricular layer (VL).

Table 1: Mean Percentage of Nestin Positivity in Cerebellar Cortex in Relation to Gestational Age

Gestational age groups (in weeks)	Grading of nestin positive cells (Manual analysis)				Mean percentage field area of nestin positive cells (Morphometric analysis)
	EGL	IGL	PVZ	VL	Cerebellar Cortex
14-17	+++	+++	++	+	11.49
18-21	++	++	++	+	9.71
22-25	++	++	++	++	6.32
26-29	++	++	+	++	5.50
30-33	+	+	+	++	3.23
34-36	+	+	+	++	2.57

EGL external granular layer, IGL Internal granular layer: PVZ Periventricular

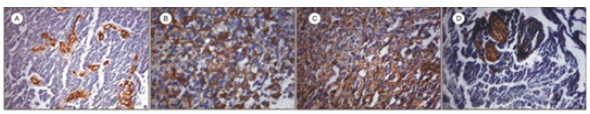

Figure2: Nestin expression in classic vs. desmoplastic /Nodular medulloblastomas. Perivascular (A) and diffuse pattern (B) is seen classic medulloblastomas while diffuse (C) and focal nodular (D) in desmoplastic medulloblastomas.

Table 2: Nestin expression in Classic vs. Desmoplastic/Nodular Medulloblastomas with relation to age

Histological Types of Medulloblastomas	Age Groups (Mean±S.D.)	Perivascular	Diffuse ≤ 50%	Diffuse > 50%	Focal (Nodular)	Total
Classic Medulloblastoma	<10years (4.92±1.51)	16	8	0	0	24
	≥10years (13.2±3.27)	2	8	0	0	10
Desmoplastic/Nodular Medulloblastoma	<10years (3years)	0	0	2	0	2
	≥10years (11.2±2.12)	0	0	2	2	4
Total	7.62± 4.35	18	16	4	2	40

DISCUSSION:

We analysed Nestin expression in human cerebellar embryogenesis in detail. Nestin, an intermediate filament is a well-established marker for neuronal stem. In our study Nestin expression decreased in EGL, IGL, and PVZ while increased in ventricular layer with the increase in the gestational age. Our findings were in agreement to the findings in the study conducted by Hockfield et al stated that during embryogenesis; CNS stem cells differentiate into neurons and glia on stereotyped schedule in different regions of the brain.[7] Few other studies have documented Nestin expression in the cerebellum, in both NSCs and radial (Bergman) glia.[8,9] With the decrease in progenitor stem cells, Nestin looses expression and there is a transition from Nestin to neural markers of mature elements i.e. vimentin, glial fibrillary acidic protein (GFAP), and S-100.[10] It has been documented that the cells of the EGL differentiate to form the granule cells, basket cells and stellate neuron.[11] In our study though there was decrease in Nestin positive cells in EGL with advancing gestational age; still some Nestin positive cells persisted at 36 weeks of gestation. This was in contrast to previous study by Yachnis et al reported that in human fetal cerebellum at 15 & 20 weeks of gestation the EGL were Nestin negative. These EGL cells give rise to cerebellar interneurons. We observed that there was increase in Nestin positive cells in VL. The findings were in agreement to the study by Yachnis et al which states that the ventricular germinal matrix progenitor's gives rise to Purkinje cells, dentate & Golgi Type II neurons and expressed Nestin positivity.[10]We observed Nestin positivity in cerebellar Purkinje cells of all the gestational age groups. Our findings were consistent with the previous studies which suggest Nestin expression in somatodendritic domain of cerebellar purkinje cells at 20 weeks of gestation continued to be expressed in the adults and in cerebellar Purkinje cells of tumor areas of medulloblastomas. [12, 13] We found Nestin gradually decreasing in internal granular layer with increase in GA. This is due to migration of neural progenitor cells and differentiating granule cells of EGL inwards beneath the Purkinje layer to form IGL. The previous studies on human fetal cerebellum do not comment on this layer. This study indicates increase in Nestin positive cells in ventricular layer. The study by Maller *et al* showed Nestin expression in ventricular progenitor cells of 15 and 20 weeks fetuses, in addition to cells close to the ventricle throughout the CNS of zebra fish.[14] Our study was performed on human fetuses and with increase in GA revealed increase Nestin positive cells in ventricular layer of cerebellum. Triulzi F et al reported the periventricular region of temporal horn of 14 & 24 weeks fetuses and the ependymal lining of the spinal cord expressing some Nestin positive cells.[1]

The histomorphometric data analysis reveals decrease in Nestin positive cells in the cerebellar cortex with advancing gestational age. To the best of our knowledge no other similar studies mentioning the morphometric analysis has been reported for comparison.

Some reports of post-mortem studies on human neonatal cerebellum are available in support of our results. These document the existence of Nestin positive cells at two sites up to fourth month of extrauterine life, namely, small foci of embryonal cells in proximity to germinal zone of the posterior medullary vellum[13] and nests of primitive germinal cells within the deep cerebellar nuclei.[15] Furthermore, these cells appear to be derived from periventricular germinal matrix.

Several studies on cell lines indicate the oncogenic targets of medulloblastoma are progenitor stem cells of the cerebellar EGL. In addition, Nestin expression observed in human medulloblastoma supports neuroectodermal stem cell origin of this tumour. In our study perivascular pattern of staining was observed in classic medulloblastoma which could be due to proliferating endothelial cells or astrocytic cells in the vicinity of blood vessels undergoing dedifferentiation. Nestin showed diverse staining pattern that varied from strong homogeneous positivity in most malignant cells to very focal staining in a small subset of tumour cells. On high magnification granular and fibrillary staining was seen. The perivascular pattern (62%) of Nestin immunostaining was dominant in patients of age group < 10 years while diffuse pattern (71.43%) in patients of age group ≥10 years**.** As Nestin is a marker of immature cells the pattern of staining suggests that with the increasing age the primitive cells predominate. Further most cases (52.94%) of classic medulloblastoma showed perivascular pattern of staining while the desmoplastic showed predominantly diffuse pattern of staining (66.7% cases). In Homer-Wright rosettes of classical medulloblastomas both cells and central fibrillated areas were Nestin positive reflecting the primitive nature of this neoplasm which is consistent with the previous study by Sandhya Rani et al. [16]

CONCLUSION:

In the whole cerebellar cortex there was a significant reduction in the

number of neural progenitor cells allegorized to an increase of these cells in the VL. The Nestin positive cells present in EGL, PVZ, VL and IGL suggested the origin of medulloblastomas from these NSC's.

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