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Original Research Article

Immunohistochemical evaluation of neuronal dysfunction in paediatric patients with Hirschsprung's disease and allied disorder

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ABSTRACT

Background: Neonatal bowel obstruction may result due to defect in the intestine wall which may be classified as neuropathic, myopathic or idiopathic types according to the pathological changes observed. The present study was conducted between September 2014 to December 2015 with the aim to study histomorphological changes and evaluate the role of various IHC markers (calretinin, S-100, CD117) in Hirschsprung's disease (HD) to assess neuronal dysfunction in these patients.
Methods: Thirty cases with clinical suspicion of HD were included in our study. The tissue sections were processed and wax blocks were prepared. Histopathological diagnosis was established on routine H and E. Representative sections were further subjected to IHC staining with calretinin, CD117 and S-100 protein. A descriptive study was carried out. Chi-square was used with P-value less than 0.05 accepted as statistically significant.
Results: Out of 30 cases with clinical suspicion of HD, 13 cases were diagnosed as HD, 10 as Non-HD motility disorder whereas 7 were without any definitive diagnosis. All the cases were subjected to IHC staining using calretinin. Out of 13 cases diagnosed as HD, 1 case showed presence of ganglion cell using calretinin. All 7 equivocal cases were accurately diagnosed by calretinin. Thus 12 cases were confirmed HD while 18 were diagnosed as Non HD motility disorder. On statistical analysis, sensitivity (92.3%) of calretinin was lower than specificity (100%). Nerve bundle hypertrophy was observed in 11 cases of HD and 9 cases of Non-HD motility disorder using S-100 as an IHC marker. CD117 was used to demonstrate altered density and distribution of ICCs was statistically significant in cases of Non-HD motility disorder.
Conclusions: IHC is being widely used as a reliable adjunctive test in evaluation of motility disorders of bowel. In view of its ease and reproducibility, it can be routinely used, avoiding need for repeated biopsies, and delay in treatment.

Keywords: Calretinin, CD117, Equivocal, Hirschsprung's disease, S100

INTRODUCTION

According to World Health Organization (WHO) report, about 3 million fetuses and infants are born each year with congenital defects.[1] Prevalence of congenital anomalies varies from country to country due to variation in social, racial, ecological and economic factors. In India birth defects prevalence varies from 61 to 69.9/1000 live births.[2] Gastrointestinal system along with central

nervous system represent two most commonly affected systems.[3]

Most of the congenital anomalies involving large intestine result in obstruction. The incidence of neonatal bowel obstruction (NBO) is about 1 in 2000 live births. It may occur due to a variety of conditions namely intestinal atresia, stenosis, malrotation, volvulus, meconium ileus, Hirschsprung's disease (HD), colonic atresia, anal atresia and anorectal malformations (ARM).[4]

Intestinal pseudo-obstruction (IPO) are group of disorders characterized by the signs and symptoms of intestinal obstruction without any organic lesion in the intestinal lumen. It has been classified into neuropathic, myopathic and idiopathic types according to the causative pathological changes. The neuropathic type is associated with various abnormalities of the enteric nervous system and has been subclassified as aganglionosis (Hirschsprung's disease), intestinal neuronal dysplasia (IND) types A and B, hypoganglionosis, immaturity of ganglion cells and internal anal sphincter neurogenic achalasia. In addition to abnormalities of ganglion cells defective innervations of the neuromuscular junction and abnormal distribution of c-kit-positive ICCs is also seen. Hirschsprung's disease (HD) or congenital megacolon is characterized by absence of ganglion cells in meissner and myentric plexus.[5]

Although anorectal manometry and radiologic studies are used to establish the diagnosis neonatal bowel obstruction, the definite diagnosis is made by histological demonstration of neuronal and myopathic changes. Diagnosis and Management of Hirschsprung's disease (HD) depends on identification of ganglion cell. Documenting aganglionosis is often difficult and needs evaluation of several sections on routine H and E stain. Pathologist should also be alert in newborns and preterms where ganglion cells are immature and difficult to identify. Rectal suction biopsy is increasingly becoming the procedure of choice for obtaining specimens for the initial diagnosis of HD. Diagnosis of HD on the basis of rectal biopsy is difficult due to absence of criteria to define how much area of submucosa that must be scanned before the absence of ganglion cells can be confirmed as well as the relative difficulty of accurately identifying smaller and sparse submucosal ganglion cells by comparison with the more compact ganglion cells of the myenteric plexus.[6-8] A number of ancillary methods have been introduced to facilitate the diagnosis. Numerous IHC markers are used routinely namely calretinin, CD117, S-100, etc to diagnose and differentiate various cause of neonatal intestinal obstruction.

Calretinin is a vitamin D dependent calcium-binding protein and plays an important role in the organization and functioning of the enteric nervous system. Both nuclear and cytoplasmic immunoreactivity is present in ganglion cells and also there is positive staining of nerve fibers of lamina propria, submucosa and muscularis mucosa of normal bowel and ganglionic bowel of HD, whereas in aganglionic segments of HD a lack of calretinin expression is observed, which may serve as a diagnostic tool in suction biopsies.[9,10] S-100 a nerve sheath marker is used to identify the presence or absence of nerve hypertrophy in various cases of intestinal obstruction. The interstitial cells of Cajal (ICCs) are considered to be the pacemaker cells of the gut which control and coordinates gut muscular activity. Recent report suggested that ICCs express the tyrosine kinase receptor c-kit and that disruption of the c-kit signalling pathway inhibits differentiation of subpopulations of ICCs. IHC staining using an anti c-kit antibody provides a sensitive technique for the identification of ICCs which is also positive in mast cells. CD117 staining is typically cytoplasmic, with stronger accentuation along the cell membrane.[11] Abnormal distribution of ICCs has been reported in bowel affected by HD, allied Hirschsprung's disease, infantile pyloric stenosis and chronic idiopathic IPO. In the aganglionic segment of HD bowel, the c-kit positive ICCs usually sparses and localised mainly around the nerve trunks between the circular and longitudinal muscle layers. In the transitional zone c-kit positive ICCs were higher in number than in aganglionic bowel but less than in normal bowel.[12,13]

METHODS

Patients

Cases with clinical suspicion of HD (n=30) were included in the study. Full length resected specimens (n=12) or full thickness biopsies (n=18) were submitted from the children admitted with intestinal obstruction for evaluation of the cause. Specimens were fixed in 10% formalin. A minimum of 4 representative pieces were passed from the specimen. The tissue was processed for paraffin embedding and wax block was prepared for routine histological technique and IHC stains. Histopathological diagnosis was established on routine H and E stain.[14]

Representative sections were also subjected to IHC staining[15] for calretinin, CD117 and S-100 protein. Paraffin sections measuring 3-5µm 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. 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. Positive and negative controls were run with each batch of IHC stain. Positive control for calretinin and S-100 were sectioned from colonic tissue and for CD117 from

gastrointestinal stromal tumor tissue. For number and distribution of ICCs, sections from normal colon were studied. Negative control was obtained by substituting the primary antibody with an antibody of non specific positivity.

Interpretation of result

Resected segments with representative tissue were examined for presence or absence of ganglion cells using calretinin. It was considered as positive if ganglion cells showed nuclear and cytoplasmic positivity either within the submucosal nerve plexus or in the muscularis mucosae or in the lamina propria. Immunostaining was considered positive for S-100 in nerve fibres, if the schwann cells showed cytoplasmic positivity and the ganglion cells were negatively stained. Density and distribution pattern of ICCs using CD117 was also examined in the sections. The cells were counted in 10 HPF (X400) in the longitudinal and circular muscles as well as myentric plexus separately. As mast cells are also CD117 positive but are round and contain cytoplasmic granules, only cells with cytoplasmic processes were included. The density and distribution were compared to the control group. It was considered abnormal if relatively reduced number of positive cells or total loss of immunostaining was observed. The positive cells were semi-quantitatively estimated: a) Negative-none of the positive cells at 40X magnification, b) Weak positive (+) -visible cells only at 40X magnification or occasional weakly positive, c) Moderate positive (++) - easy distinguished cells at low magnification 10X, and d) Intense positive (+++) - cells intense positive at 10X magnification.[16]

Statistical analysis

A descriptive study was carried out for all the variables included in the study. Chi-square test was used for statistically analysis. P-value less than 0.05 was accepted as statistically significant.

RESULTS

Eighteen cases of full thickness biopsy and 12 cases full length resected segment comprising of dilated segment and narrow segment were examined using H and E stain. Dilated portion of the segment showed presence of ganglion cells in all the 12 cases (Figure 1A). Narrow portion showed presence of ganglion cell in only 3 (25%) out of 12 cases. Six cases showed absence of ganglion cell (Figure 1B and 1C) whereas no definite comment was possible in 3 (25%) cases. Aganglionosis was observed in 7 (39%) cases in full thickness biopsy. Seven cases (39%) showed presence of ganglion cell whereas no definite comment was possible in 4 (22%) cases. On H and E, out of 30 cases clinically diagnosed as HD, 13 (43%) cases showed absence of ganglion cell and were diagnosed as HD. Ten (33%) cases showed presence of ganglion cell and were diagnosed as Non-HD motility disorder, whereas 7 (24%) were suspicious cases without any definitive diagnosis. All the cases were subjected to IHC staining using calretinin for further confirmation of presence/absence of ganglion cell. All 10 cases diagnosed as Non-HD motility disorder histologically on H and E, showed presence of ganglion cell using calretinin (Figure 2A). Out of 13 cases diagnosed as HD histologically on H and E, 12 cases showed absence of ganglion cell (Figure 2B) whereas 1 case showed presence of ganglion cell using calretinin. All 7 cases initially considered as suspicious for HD using H and E were accurately diagnosed by calretinin immunohistochemistry and showed presence of ganglion cell.

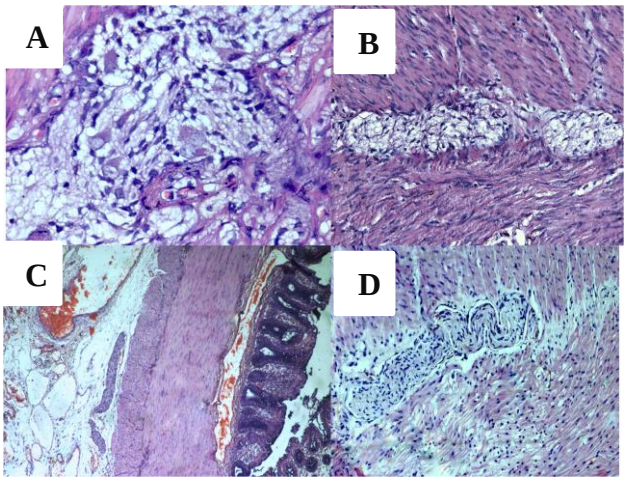

Figure 1. A) ganglion cells in myenteric plexus in a case of non-hirschsprung's disease. (H&E stain, x400), B) absence of ganglion cell in myentric plexus in hirschsprung's disease. (H&E, x200), C) full wall thickness section of a case of hirschsprung's disease showing ill defined intermuscular plane and absence of ganglion cell. (H&E, x40), and D) nerve bundle hypertrophy with absence of ganglion cell in a hirschsprung's disease. (H&E, X200).

Twelve out of 30 cases were diagnosed as HD whereas 18 cases showed presence of ganglion cells and diagnosed as Non-HD motility disorder. Calretinin showed high specificity (100%) and positive predictive value (PPV) of 100% with a sensitivity of 92.3% and negative predictive value (NPV) of 99.9%. Thus, we observed calretinin is extremely useful in solving the suspicious cases of HD and it can serve as a valuable cost-effective diagnostic aid (Table 1).

Age range of the patients was 2 day- 12 years in cases of HD (n=12) with mean and median of 1.8 years and 0.62 year respectively. Maximum numbers of cases were between age group of 1 month-1 year (42%). Range of age in Non-HD cases was 2 day-11 years. Mean and median age was 1.95 year and 0.78 year respectively. Sixty six percent of patients were below the age group of 1 year. Males were predominantly affected in both HD and Non-HD motility disorder with M:F ratio of 5:1.

In addition to aganglionosis nerve hypertrophy is an important histological marker of HD (Figure 1D).

In our study 11 cases of HD (92%) and 9 cases of Non-HD motility disorder (50%) showed presence of nerve bundle hypertrophy using S-100 as an IHC marker (Figure 2C and 2D).

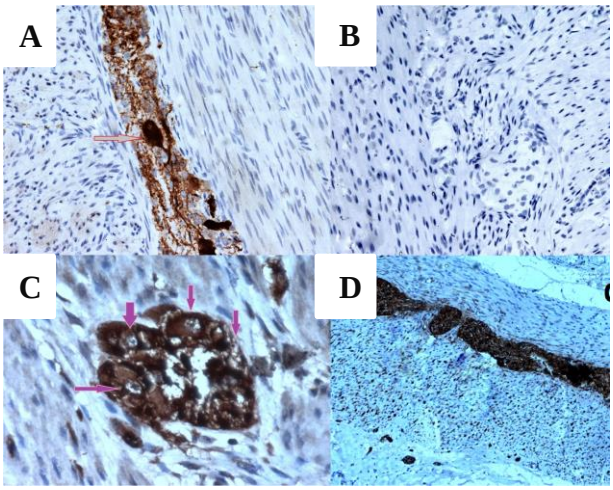

Figure 2: A) Non-Hirschsprung's disease showing calretinin expression in ganglion cells (arrow) and nerve fibers. (Calretinin, X200), B) Total absence of calretinin immunostain in Hirschsprung's disease. (Calretinin, X200), C) Non-Hirschsprung's disease showing calretinin expression in ganglion cells (arrow) and nerve fibers. (Calretinin, X200), and D) Nerve fibers in myentric plexus with S-100 immunostain. Ganglion cell are negatively stained (arrow) in Non-Hirschsprung's Disease. (S-100, X400).

Table 1: Comparison of presence of ganglion cell on routine H&E and calretinin in cases of hirschsprung's disease (HD).

H&E diagnosis		Calretinin	
		Positive	Negative
Non-HD motility disorder	10	10	00
HD	13	01	12
Suspicious	07	07	00

All the cases were divided into four grades according to the density and distribution of ICCs using CD117 as IHC marker (Figure 3). Majority of cases had grade 2 (n=7, 58%) followed by Grade1+ (n=5, 42%) intensity in HD. Maximum number of cases belong to grade 2+ (n=11, 61%) followed by grade 1+ (n=6, 33%) and grade 3+ (n=1, 6 %) in cases of Non-HD motility disorder (Figure 3).

Density and distribution of ICCs was altered in cases of HD and Non-HD motility disorder. It was statistically significant in cases of Non-HD motility disorder (p=0.016) only (Table 2).

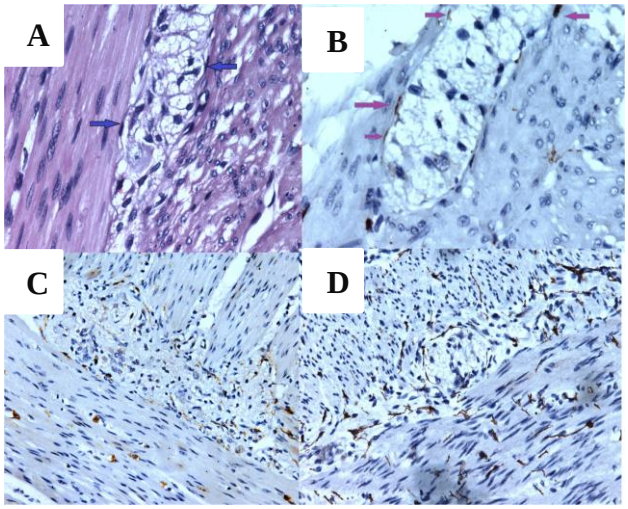

Figure 3- A) Interstitial cells of Cajal (ICCS) (arrow) around the myentric plexus (H&E, x400), B) grade 1 immunostained interstitial cells of Cajal in non-HD motility disorder-a few weakly positive cells (arrow). (CD117 stain, x400), C) grade 2 immunostained interstitial cells of cajal in non-HD motility disorder - easily distinguished cells. (CD117, x200), and D) grade 3 immunostained interstitial cells of cajal in non-HD motility disorder-intense positive cells. (CD117, X200).

TABLE 2: Distribution of cases according to grading of interstitial cells of cajal (ICCS) using CD117 immunostain.

Grading	HD			Non-HD motility disorder		
	No. of cases	%	P-value	No. of cases	%	P-value
0	-	-		-	-	
1+	5	42%		6	33%	
2+	7	58%	0.564	11	61%	0.016
3+	-	-		1	06%	
Total cases	12	100%		18	100%	

Chi- Square test, degree of freedom =1 (HD) and 2 (Non-HD motility disorder)

DISCUSSION

The histological diagnosis of HD and allied disorder on H and E sections is challenging, requiring the expertise of a senior pathologist. Moreover, difficulties in analysis may arise in several situations: (1) when the site of biopsy is too distal, because of the physiological paucity of ganglion cells; (2) when the sample is too superficial with not enough submucosa; and (3) when there is difficulty in identifying ganglion cells with confidence, particularly in neonates. This makes the diagnosis of HD difficult causing a delay in the treatment of the child. Numerous IHC markers are used routinely namely calretinin (ganglion cells), CD117 (ICCs), S-100 (nerve fibre), etc to diagnose and differentiate various cause of neonatal intestinal obstruction.

In the present study we tried to study histomorphological changes and evaluate the role of various IHC markers (calretinin, S-100, CD117) in HD and allied disorders to assess neuronal dysfunction in these patients. As stated by various studies in the past, calretinin solved all cases which were reported inconclusively on H and E. Out of 13 cases which were diagnosed as HD on H and E, one case showed presence of ganglion cell using calretinin. All 7 equivocal cases showed presence of ganglion cell. Thus, proving to be extremely useful in solving the suspicious cases of HD which was compatible with the observations made by previous studies namely Sameul et al, Torre et al and Hiradfar et al (Table 3).[17-19]

Table 3: Comparison of H&E with calretinin in HD within various studies.

Study	No. of cases with clinical suspicious of HD	H & E		Calretinin	
		Diagnosis	No. Of cases	Positive	Negative
Sameul et al[17]	131	HD	42	0	42
Torre et al[18]	12	Non-HD	73	0	73
		Suspicious	16	4	12
		HD	8	0	8
Hiradfar et al[19]	30	Non-HD	4	4	0
		HD	30	2	28
Kannaiyan et al[20]	60	HD	43	2	41
Yadav et al[21]	33	Non-HD	2	2	0
		Suspicious	15	3	12
		HD	26	0	26
Anbardar et al[22]	55	Non-HD	17	17	0
		HD	27	0	27
Present study	30	Non-HD	28	26	2
		HD	13	1	12
		Non-HD	10	10	0
		Suspicious	7	7	0

Table 4: Comparison of distributions of ICCS on CD117 with other studies.

STUDY	Type of disorder	No of cases	ICCs on CD117
Jain et al[24]	Non-HD	6	Total absence
Streuker et al[25]	Non-HD	30	Markedly decrease
Anatol et al[26]	HD	10	Markedly decrease
Barshack et al[27]	Non-HD	8	Markedly decrease
Becheanu et al[16]	Non-HD	2	Markedly decrease
Geramizadeh et al[28]	HD	29	Decrease
Present study	HD	12	Decrease
	Non-HD	18	Decrease

The inability to observe the ganglion cells in the H and E stained sections in suspicious cases may be attributed to the presence of immature ganglion cell which are difficult to perceive on H and E stain. Immature ganglion cell have smaller nuclei without a recognizable nucleoli. Although immaturity of ganglion cells has been mostly seen in neonates and premature infants and often improved with maturity it may however persist.[23] Calretinin IHC is accurate in detecting the presence or absence of ganglion cells and holds several advantages such as follows:

- It can be carried out on paraffin-embedded tissue sections;
- Staining pattern is simple;
- Binary pattern of interpretation (negative or positive);
- It is cost effective.

In addition to aganglionosis nerve hypertrophy is an important histological marker. This finding can be observed on routine H and E stain. Sometimes this finding is difficult to define with H and E by inexperienced pathologist. Thus S-100 has been proposed as an IHC stain to identify hypertrophic nerve fibres. In our study 11 cases of HD (92%) and 9 cases of Non-HD motility disorder (50%) showed presence of nerve bundle hypertrophy using S-100 as an IHC marker. Torre et al

observed that S-100 had low sensitivity (41.7%) but a specificity of 100% in diagnosing nerve hypertrophy as it also stains different cells like glia and Schwann cell.[18]

Recent studies have demonstrated variable results regarding role of ICC in neonatal bowel obstruction. Jain et al and Streuker et al had shown complete absence of ICC using CD117 whereas as stated by Anatol et al and Barshack et al.[24-27] We observed altered density and distribution of ICCs in cases of HD and Non-HD motility disorder which was statistically significant in cases of Non-HD disorder (p=0.016) (Table 4).

The underlying mechanism responsible for decrease in ICCs in cases of HD and Non-HD motility disorder is not clear. Two alternative interpretations have been proposed: either that the ENS is required for full differentiation of ICCs or there is a common mechanism acting that affects both migration of neural crest derivatives and differentiation of ICCs.[27] The median age at which children are diagnosed with HD and allied disorders has progressively decreased over the past decades with greater awareness of disease but some children do not present during neonatal period and present later in infancy most commonly around weaning. The male predominance in HD and allied disorders was in concordance with the literature studied. The reason for this ratio is still unclear as no X linked loci has been described.[28,29]

CONCLUSION

Full thickness biopsy is equivalent to the resected segment in the interpretation for the ganglion cell. All these cases on IHC stain with calretinin showed presence of ganglion cells indicating calretinin to be highly sensitive and specific marker for diagnosis of HD. It can be extremely useful in studying the suspicious cases of HD, can serve as a valuable cost effective diagnostic aid and should be included along with H and E stain for diagnosis of HD routinely. Non-HD motility disorder showed a significant alteration in density and distribution of ICCs with loss of immunoreactivity for CD117. These findings explain large bowel motor abnormalities known to occur in Non-HD motility disorders. However further studies including contractile function, electrophysiology, IHC and biochemical assay involving more number of cases are required for better interpretation and management of the problems.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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