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ORIGINAL ARTICLE

Is nerve bundle hypertrophy a reliable criteria for diagnosing Hirschsprung disease? A case control study using Calretinin as an adjunct tool for confirming Hirschsprung disease.

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ABSTRACT… Objective: To determine the frequency of nerve bundle hypertrophy in aganglionic segments in relation to the site of biopsy, along with the assessment of value of Calretinin immunostaining in the diagnosis of suspected cases of Hirschsprung disease. **Study Design:** Cross Sectional study. **Setting:** Department of Pathology, Pakistan Institute of Medical Sciences (PIMS) Islamabad. **Period:** September 2018 to March 2019. **Material & Methods:** After routine tissue processing colonic biopsies were examined for presence of ganglion cells and hypertrophic nerve presence or absence. Diagnosis of Hirschsprung disease was based on the absence of ganglion cells in submucosal and myenteric plexus, the presence or absence of hypertrophic nerves (more than 4 nerves >30 μm thick/×200 field or more than 2 nerves >40 μm thick/×200 field) was also noted in all cases of Hirschsprung disease (aganglionic segments). Calretinin immunostaining was applied to all the cases and controls and findings were recorded as positive or negative staining. Data was analyzed using SPSS version 23. Qualitative data was calculated as frequencies and percentages. Pearson Chi square test was used to establish the association of nerve bundle hypertrophy with the site of biopsy. **Results:** Total biopsies were 60; 30 each from ganglionic and aganglionic segments. Calretinin sensitivity in our study was 90%, specificity 83.3%. In 30 cases of aganglionosis hypertrophic nerves were present in 13(21.7%) and they were absent in 17 (28.3%). No significant association (p value= 0.447) was seen in nerve bundle hypertrophy and site of biopsy. **Conclusion:** Calretinin immunohistochemistry can be used as a reliable ancillary technique in the diagnosis of HD. Aganglionosis may not always be associated with submucosal nerve hypertrophy which alone should not be used as a criteria for HD diagnosis but instead adjunct methods like Calretinin immunostaining must be utilized to confirm presence or absence of ganglion cells. There is no association of nerve hypertrophy with site of biopsy.

Key words: Ganglion Cells, Immunohistochemistry, Hirschsprung disease, Calretinin.

INTRODUCTION

Hirschsprung disease (HD) is a congenital disease which results in absence of ganglion cells in submucosal plexus and Myenteric plexus in the distal part of large gut. The disease prevalence is estimated to be almost 1:5000 live births. HD is more common in male and the male: female ratio is reported around 4: 1.[1]

Hirschsprung's disease is classified according to the length of aganglionic section as: short aganglionic segment (disease involves distal part of the sigmoid colon and rectum), long aganglionic segment (ganglion cells are absent in rectum, sigmoid colon and proximally extend up to the splenic flexure), total colonic aganglionosis and an ultrashort segment type in which the aganglionic section is very small and restricted to anal canal above the pectinate line.[2]

The gold standard for diagnosis of Hirschsprung disease is biopsy. The accurate diagnosis of HD depends on a number of factors including the quality of biopsy which is influenced by the site of biopsy, number of specimens, age of the patient and type of biopsy. Pathologist's skill and

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experience in this regard is also an important factor. The histologic criterion of HD diagnosis is the absence of ganglion cells in the submucosal and intramuscular nerve plexus of the intestinal wall with the presence of hypertrophic nerve fibres and trunks.[3] Suction biopsy is the routine procedure used for this purpose.[4] Problem arise in the identification of ganglion cells when they are immature, overly superficial, when the patient is very young or there is associated inflammation and also in technically damaged specimens. The presence of various artefacts from the technical processing during Eosin & Haematoxylin (H&E) staining also causes interpretation problems.[4] One then relies on the presence of hypertrophic nerves to reach a diagnosis. As HD is a potentially treatable disease, an accurate diagnosis has an immense effect on the future recovery and morbidity of the patient. For this purpose many ancillary methods have emerged to confirm the presence absence of ganglion cells beyond doubt and Calretinin immunostaining is one of them; which has proven to be quite sensitive and a specific marker in this regard.[5]

The present study aims to determine the frequency of nerve bundle hypertrophy in aganglionic segments in relation to the site of biopsy, along with the assessment of the value of Calretinin immunostaining in the diagnosis of suspected cases of Hirschsprung disease. This will aid in a reliable diagnosis of HD.

MATERIAL & METHODS

This Cross Sectional study was conducted at Pathology Department of Pakistan Institute of Medical Sciences (PIMS) Hospital of Shaheed Zulfiqar Ali Bhutto Medical University, Islamabad for 6 months from September 2018 to March 2019 after approval of the ethic review board of the university.

The sample size was 60 cases, 30 of ganglionic segments and 30 aganglionic segments of intestine selected by using sampling technique of Consecutive sampling.

Inclusion Criteria

Clinically suspicious cases of Hirschsprung disese.

Exclusion Criteria

Inadequate biopsies showing only superficial mucosa without submucosa and muscularis propria.

Following approval from the hospital ethics committee, the formalin fixed colonic biopsies were routinely processed in the pathology department, PIMS hospital, Islamabad for histological and immunohistochemical evaluation. Demographic data was collected along with clinical presentation of all the patients. Calretinin immunostaining was recorded as either positive or negative. Diagnosis of Hirschsprung disease was based on the absence of ganglion cells in submucosal and myenteric plexus, the presence or absence of hypertrophic nerves (more than 4 nerves >30 μm thick/×200 field or more than 2 nerves >40 μm thick/×200 field)[6] was also noted in all cases of Hirschsprung disease (aganglionic segments). Calretinin immunostaining was applied to all the cases and controls and findings were recorded as positive or negative staining.

Data Analysis

Data was analyzed using SPSS version 23. Variables as age, sex, H&E diagnosis, IHC diagnosis and presence and absence of hypertrophic nerves was calculated as frequencies and percentages. Sensitivity, specificity, positive predictive value and negative predictive value were calculated by 2x2 tables. Pearson Chi square test was used to establish the association of nerve bundle hypertrophy with the site of biopsy. p value <0.05 was considered as significant.

RESULTS

Total number of biopsies was 60. 30 biopsies were from ganglionic segments and 30 from aganglionic segments. The age distribution is shown in Table-I.

	Frequency (%)
New born to 11 months	31 (51.7%)
1-5 years	24 (40%)
>5 years	5 (8.3%)
Total	60 (100%)

Table-I. Age distribution of patients (n=60)

Male to female ratio was 3.2:1 and the most common symptom was constipation (59%) which was followed by abdominal distension. 15 (25%) biopsies were from rectal wall and 45 (75%) were from sites proximal to rectum which included rectosigmoid, sigmoid, descending, ascending colon and one biopsy was from small intestine. Ganglion cells were absent in 30 cases and were present in 30 controls. Results of Calretinin IHC in all the 60 biopsies (n=60) is as shown in Table-II.

		Eosin & Hematoxylin Stain	
		Ganglion Cell Seen	Ganglion Cell Not Seen
Calretinin	Positive	27	5
	Negative	3	25
Total		17	30

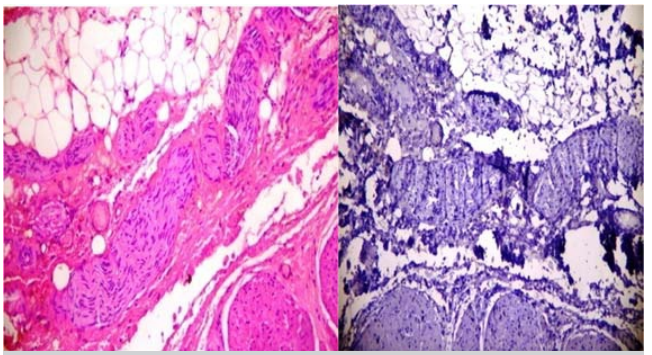
Table-II. Comparison of Calretinin and H&E staining in 60 biopsies (n=60)

In cases of aganglionosis (n=30 cases diagnosed as HD) hypertrophic nerves were present in 13(21.7%) and they were absent in 17 (28.3%) cases. The frequency of nerve bundle hypertrophy as seen in 30 cases of Hirschsprung disease (absent ganglion cells) and its relation to biopsy site is shown in Table-III

		Nerve Bundle Hypertrophy	
		Absent	Present
Site of biopsy	Rectum	6 (20%)	5(16.66%)
	Proximal to rectum	13(43.33%)	6(20%)
	Total	19(63.33%)	11(36.66%)

Table-III. Frequency of nerve bundle hypertrophy and relation with biopsy site in aganglionic segments (n=30)

No significant association (p value= 0.447) was seen in nerve bundle hypertrophy and site of biopsy but observation of frequency shows that more proximal the biopsy site in HD, it is less likely that nerve bundle hypertrophy is present (absent in 63.33% cases). Figures A & B show nerve bundle hypertrophy and negative Calretinin immunostaining. Figures C & D show ganglion cells and positive Calretinin immunostaining.



A: H & E showing nerve bundle hyperplasia (100X)
B: Calretinin negativity of hyperplastic nerves (100X)

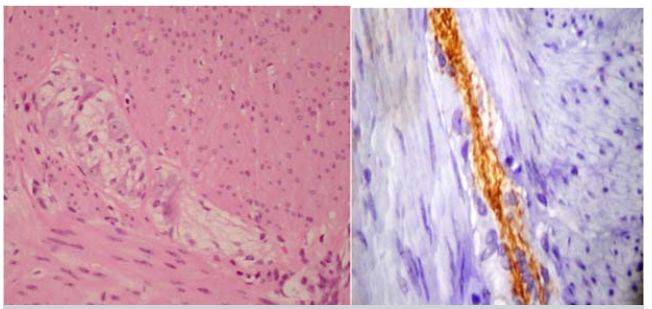


Figure C: H & E Myenteric plexus showing ganglion cells. (100X)
Figure D: Calretinin immunopositivity of ganglionic segment. (100X)

DISCUSSION

To establish a diagnosis of Hirschsprung disease one need to consider and correlate clinical presentation, ultrasound findings and colonic biopsy which must prove the absence of ganglion cells and presence of hypertrophic nerve bundle fibres. To facilitate the diagnosis Hirschsprung Disease, ancillary methods like Calretinin immunohistochemistry is very useful. Keeping in mind the importance of histopathological diagnosis of HD and the fact that HD is a potentially treatable surgical disease, it will reduce the interobserver and intraobserver variability in diagnosis.[7]

In the present study the patient age ranges from two months to 10 years with male: female ratio of 3.2:1 and the major presenting complaint was constipation (94.5%). RM Jankovic conducted a study in Serbia and also quoted a male predominance of the disease and similar distribution of presenting complaints.[8]

Calretinin gave positive result in all controls and ganglionic segments of all cases except three cases. Calretinin sensitivity in our study was 90%, specificity 83.3%, positive predictive value of 84% and negative predictive value of 89%. Singh SK. et al performed a similar study on 41 cases of HD and used Calretinin to confirm diagnosis in 9 doubtful cases. They reported sensitivity of calretinin as 100%, specificity as 68.75%, and positive and negative predictive values as 85.33% and 100%, respectively.[9]

Under light microscopy the ganglion cells are identified by their classic cytological features. They have sufficient cytoplasm, nucleus is to one side and it is round and have a prominent nucleolus. There is a perinuclear pallor. Ganglion cells are mostly present in groups in ganglia and/or alongside neuropil (neurites and glia). The cytologic features of immature ganglion are; less cytoplasm, stippled chromatin and indistinct nucleolus. In our study there was one case in a neonate with very small biopsy and two cases in which inflammation was so severe as to mask the ganglion cells but immunohistochemistry aided in locating them. The use of calretinin IHC in our study thus helped in locating ganglion cells and avoiding a wrong diagnosis. Szylberg L et al also showed that anal biopsy has 95% accuracy in HD diagnosis but with the use of ancillary techniques like IHC, the sensitivity of correct diagnosis is very high, that is up to 99.7%.[10]

In the present study there were 30 aganglionic segments, 13 cases of which showed nerve bundle hypertrophy while 17 cases did not show any hypertrophy. All these cases of aganglionosis were confirmed with negative staining of calretinin for ganglion cells and also the hypertrophic nerves (>40um). This is in contrast with Heena N et al. findings whose study in Ayub Medical college, Abbottabad showed that in the ganglionic segments, hypertrophic nerves were present in 1 out of 13 patients ($p=0.002$) and in HD group, thickened nerves were significantly more common as in 43 (84.3%) out of a total of 51 had thickened nerve fibres ($p<0.001$).[11] Barberi J. et al emphasized that hypertrophic nerves are not always necessarily present when ganglion cells are absent. They further added that if the biopsy shows the presence of occasional small, slack nerve fibers when the ganglion cells are absent then it is best to consider other diagnosis like long-segment aganglionosis or total colonic aganglionosis.[12]

Findings of our study agrees with that of Russo P. According to Russo P. the presence of nerve trunks > 40 microns in diameter in the submucosa in the absence of ganglion cells is a diagnostic feature of classic HD which is the short segment type, but hypertrophic nerves my not be observed in long segment HD. Long segment HD has thickened nerve trunks only in rectosigmoid colon, and these trunks are absent as we move proximally where an occasional thick nerve trunk can be present in the myenteric plexus but there are no hypertrophic nerve trunks identified in the submucosa.[13]

Narayanan SK. studied 92 cases of HD and concluded that the absence of nerve fibre hypertrophy in the presence of aganglionosis on rectal biopsy specimens is frequently reported and they suggested that this feature is predictive of long-segment HD. However, they also mention that hypertrophic nerves may not be present in some types like long-segment HD, very young ages (premature) and total colonic aganglionosis.[14]

Kapur RP et al. states that in the pathogenesis of HD, gene expression is highly variable. As a result the length of affected segment is also variable and there are types with very limited involvement so that they can be easily missed on biopsy. There is a very short-segment aganglionosis, which only involves distal 1 to 2 cm of rectum, and also there are patients without a true aganglionic segment. In clinically suspicious cases of HD the submucosal ganglion cells status (presence or absence) in a rectal biopsy may or may not be associated with submucosal nerve hypertrophy and the only diagnostic clue is the presence of abnormal cholinergic innervation of the mucosa.[15]

Nerve hypertrophy is not always associated with HD and it may also be present in other disorders

of intestinal innervation like intestinal neuronal dysplasia as shown by Terra S et al.[16]

The hypertrophic nerves in HD arise from the associated autonomic ganglia. In Hirschsprung disease, especially the short segment disease, submucosal nerve hypertrophy is not only present in the aganglionic segment but also in the adjacent ganglionic bowel which actually is abnormal neuroanatomically and is temed, transition zone. Kapur P showed that the majority of Hirschsprung patients exhibit thickened submucosal nerves which are >40 µm in diameter in their post-pull-through specimens even when innervation (ganglion cells are present) of the proximal margins of their initial resections was normal thus they insisted that ganglion cells should be sought for and for this purpose ancillary techniques maybe used so as to avoid a wrong diagnosis.[17]

CONCLUSION

Calretinin immunohistochemistry can be used as a reliable ancillary technique in the diagnosis of HD. Aganglionosis may not always be associated with submucosal nerve hypertrophy which alone should not be used as a criteria for HD diagnosis but instead adjunct methods like Calretinin immunostaining must be utilized to confirm presence or absence of ganglion cells.

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AUTHORSHIP AND CONTRIBUTION DECLARATION

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2	Maryam Qaiser	Review of histopathology slides and compilation of result.	
3	Aisha Akbar	Collection of data and review of slides for diagnosis.	
4	Muhammad Ashraf	Statistical analysis of data.	
5	Tehseen Rafaqat	Statistical analysis, Collection of data.	
6	Humera Javed	Data analysis and proof reading.