ORIGINAL ARTICLE

Diagnostic Utility of Calretinin Immunohistochemistry for the Diagnosis of Hirschsprung's Disease

Maryam Qaiser[1], Saira Javeed[2], Rabiya Fawad[3], Ayesha Sarwar[4], Iram Kehkashan Khurshid[5], Abrar Ul Haq Satti[6]

ABSTRACT

Objective: The aim of this study was to evaluate the diagnostic utility of calretinin immunostaining in colonic biopsy specimens in patients having a clinical suspicion of Hirschsprung's disease.

Study Design: This study was a Cross sectional validation study.

Place and Duration of Study: The study was conducted at Pakistan institute of medical sciences, Shaheed Zulfiqar Ali Bhutto Medical University (SZABMU), Islamabad, for a period of 11 months (December 2018 to November 2019).

Materials and Methods: Sixty specimens from patients including multiple colonic biopsies and biopsies from rectum for doubted Hirschsprung's disease were evaluated. The biopsies were processed in Histopathology lab and Hematoxylin and eosin slides were examined. 30 ganglionic segments and 30 aganglionic segments along with control specimens were further stained with calretinin immunohistochemical marker and evaluated.

Results: On Hematoxylin and eosin staining, 30 biopsies showed ganglion cells and 30 biopsies showed absence of ganglion cells. Calretinin immunohistochemistry was then evaluated on these ganglionic and aganglionic segments. In this study, sensitivity was found to be 91.4 % and a 100% of specificity was established. The positive predictive value and negative predictive value was found to be 100% and 89.2% respectively.

Conclusion: Calretinin immunohistochemical marker can be applied on colonic and rectal biopsy specimens as a dependable and adjunctive tool for diagnosing Hirschsprung's disease, as it is easy and reliable to be used in daily practice.

Key Words: *Calretinin, Hirschsprung's Disease, Immunohistochemistry.*

Introduction

Obstruction of large gut in infants is commonly caused by Hirschsprung's disease (HD). The disease occurrence is about 1 per 5000 live births, with a predicted disease frequency of 1.4 per 5000 live births in Asian populations.[1]

The etiology of HD is explained by the disruption of migration of neural crest cells, resulting in congenital absence of ganglion cells in the colon, along with their disrupted proliferation and differentiation. Two types of HD are identified: Type 1 or short-segment disease that is identified in 60% to 85% of patients with HD. It is characterized by aganglionosis which is limited to the rectum, reaching up to the large gut distal to the splenic flexure. Type 2 is labeled as long-segment disease, that is found in 15% to 25% of the patients having widespread aganglionosis of the rectum and colon. Aganglionosis in the gastrointestinal tract is demonstrated by the lack of the ganglion cells in the two plexuses of gut wall, the submucosal and myenteric plexuses.[2,3]

A combination of clinical symptoms help in diagnosing the HD including, contrast enema, anorectal manometry and histologic findings.[1] Histopathological absence of ganglion cells and nerve plexus of submucosa and muscularis propria showing hypertrophy and hyperplasia constitute the diagnostic criteria for HD. Hematoxylin and eosin (H & E) staining is conventionally used for the diagnosis of Hirschsprung's disease but it requires serial sectioning and also depends on observer's capability

[1,4]*Department of Histopathology,*
HBS Medical and Dental College, Islamabad
[2]*Department of Histopathology,*
Islam Medical and Dental College, Sialkot
[3]*Department of Histopathology,*
Quetta Institute of Medical Sciences, Quetta
[5]*Department of Histopathology,*
CMH Medical College, Khariyan Cantt
[6]*Department of Pediatrics*
Rawal Institute of Health Sciences, Islamabad

Correspondence:
Dr. Maryam Qaiser
Assistant Professor Histopathology
Department of Pathology,
HBS Medical and Dental College, Islamabad
E-mail: maryamsherazi110@gmail.com

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to evaluate the biopsy specimen.[3] Immunohistochemistry (IHC) in the recent years has been proven to be an additional tool in the diagnosis of HD, with increased diagnostic yield. [4] Calretinin is one of the many markers used for diagnosing HD. It is a calcium binding protein which is vitamin D dependent and richly expressed in neurons, steroid producing cells, testicular cells, and neuroendocrine cells etc. [4, 5] Ganglion cells in submucosal and myenteric plexus of a normal gut show a positive calretinin expression whereas aganglionic regions of diseased gut show absence of calretinin expression.[6,7]

In contrast to routine H & E, calretinin IHC staining has become frequently applied immune-marker to identify ganglion cells in colonic biopsies with suspicion of Hirschsprung's disease.

Calretinin carries several advantages in this regard. It is easily performed on paraffin-embedded sections, staining pattern is simple, pattern of interpretation is easy, and it is cost effective.

Many studies in literature demonstrated a high sensitivity (96%) and specificity (100%) for Calretinin.[8] Calretinin highlights ganglion cells which are not very apparent on H & E-stained sections, thereby preventing unnecessary surgeries. It is found to be a cost effective and valuable diagnostic support in HD.[9]

The study was carried out to determine the diagnostic utility of calretinin immunohistochemical marker for the diagnosis of Hirschsprung's disease in order to benefit the clinicians for the management of the patients.

Materials and Methods

The place of study was Pathology department, conducted at the Pakistan institute of medical sciences, Shaheed Zulfiqar Ali Bhutto Medical University (SZABMU), Islamabad, Pakistan. Duration of the study was 11 months, from December 2018 to November 2019. It was a cross sectional validation study. Multiple colonic and rectal biopsies from 60 suspected patients of Hirschsprung's disease were evaluated. The biopsies were processed in Histopathology lab and Hematoxylin and eosin slides were examined. Biopsies including 30 ganglionic segments, 30 aganglionic segments and control specimens were stained with calretinin immunohistochemical marker. Calretinin showed a granulated staining of neuronal fibers in lamina propria and submucosal layer of ganglionic segments, whereas ganglion cells showed a dark nuclear and cytoplasmic staining which was seen in the ganglionic segments in both Meissner and myenteric plexuses. Negative staining shown by neuronal fibers and ganglion cells in all the gut layers was considered as negative for calretinin immunohistochemical maker.

Inclusion criteria: All colonic biopsies with full thickness sections with suspicion of Hirschsprung's disease were included.

Exclusion criteria: Inadequate biopsies which showed only the superficial mucosa without submucosa and muscularis propria were exempted from the study.

Statistical package for social sciences (SPSS16) Software was applied for performing the statistical analysis. Different variables including sex, H&E staining patterns and immunohistochemical results were calculated as frequency and percentages. Sensitivity, Specificity, Positive and Negative Predictive values were considered by means of 2x2 tables.

- Sensitivity = True Positives / True Positives + False Negatives X 100
- Specificity = True Negatives / True Negatives + FP X 100
- Positive Predictive Value = True Positives / True Positives + FP X 100
- Negative Predictive Value = True Negatives / True Negatives + FN X 100

Results

Colonic biopsies were taken from 60 patients showing an age range from two months to 10 years. Male to female ratio was 3.2 to 1. Most common presenting complaint was constipation (93%) while some of them presented with abdominal distention and delayed passage of meconium.

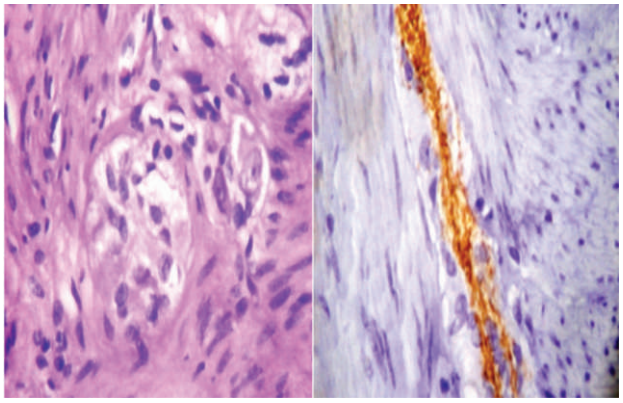
Out of total 60 biopsies (n=60), fifteen biopsies (25%) were from rectosigmoid junction and fourteen (23%) were rectal wall biopsies. On H & E, 30 biopsies showed ganglion cells and the other 30 biopsies showed that ganglion cells were absent. In 16 cases (26.7%), ganglion cells were present in myenteric plexus and 14 (23.3%) cases showed the presence of ganglion cells in both messeiner's and myenteric plexus. Out of 30 aganglionic segments, 13 cases

(21.7%) showed nerve bundle hyperplasia. Calretinin IHC was then applied on all ganglionic and aganglionic biopsies. Out of the 30 ganglionic segments, calretinin immunoexpression was seen in 27 segments but 3 ganglionic segments were negative for calretinin despite the existence of ganglion cells on routine H&E staining (10%). Out of 30 aganglionic segments calretinin immunoexpression was seen in five cases (16.6%). All the other 25 cases were negative for calretinin. (Photomicrograph showing Calretinin Immunostaining)

Therefore 32 cases {27 cases (90%) having ganglion cells on both H & E and calretinin and 5 (16.6 %) cases which showed ganglion cells positive based on calretinin, but H & E declared it negative} were true positives. 25 (83.3%) cases were true negatives showing no ganglion cells on H & E and calretinin. 3 (10%) cases are false negatives which are negative on calretinin but having ganglion cells on H & E. So, this method showed a sensitivity of 91.4 % and specificity was found to be 100%. The positive predictive value was found to be 100% and negative predictive value was 89.2 %. (Table I)

Table I: Utility of Calretinin Compared to H & E

		H&E		Total
		Ganglion Cells Seen	Ganglion Cells Not Seen	
Calretinin	Positive	27(90%)	5(16.6%)	32
	Negative	3(10%)	25(83.3%)	28
Total		30	30	60



Photomicrograph:

A: H & E Showing Ganglion Cells in Myenteric Plexus (400x) arrow points ganglion cell

B: Calretinin iImmunopositivity of Ganglionic Segment. (400X)

Discussion

As diagnosis of HD depends upon the demonstration of the aganglionosis in the colonic biopsy specimens, sometimes it becomes very difficult and tiresome; necessitating furthermore serial cut sections on H&E.[10] To overcome these problems calretinin IHC is now widely used in the diagnosis of HD.[5]

The age range of the patients in the current study (n=60) was 2 months to 10 years. Rakhshani et al in their study showed that age range of patients with HD was, one day to 12 yr.[11] Similarly Anbardar et al demonstrated that age of patients suffering from the disease was ranging from 2 days to 10 years.[5] The current study showed a male predominance (75%). Collins et al in their study showed that 82% of the patients were male.[12] The study conducted by Rakhshani et al also pointed male predominance (67.1%).[11] Joseph et al in his study showed that constipation was the most common complaint (94.5%).[13] This was comparable to the current study in which constipation (93%) was most common presenting symptom. In present study out of the total 30 ganglionic segments (which showed ganglion cells on H&E), 27 cases (90%) showed staining of ganglion cells by calretinin. Out of the 30 aganglionic segments calretinin IHC was able to pick 5 (16.6 %) cases which were negative on H & E. This study indicated a sensitivity of 91.4 % and specificity was found to be 100%. The results were comparable to the study conducted by Mukhopadhyay et al (n=80) and it was shown that Calretinin IHC marker had sensitivity equals to 96% and a specificity of value 100%. The positive predictive value was found to be 100% and negative predictive value was 92.59%.[14] Musa et al and Kazemi et al in their studies concluded that calretinin immunostaining, is a very sensitive (100%) and specific (100%) diagnostic tool to the histopathological analysis in the suspected cases of HD.[15,16] In adequate biopsy specimens calretinin IHC is confirmatory, but in case of inadequate biopsies it is decisive, and diagnostic as compared to H&E.[17] In present study, out of 13 cases of nerve bundle hyperplasia, not a single case showed positivity of calretinin. Similar was the finding observed by Gabal et al in their study that the nerve fibers showing calretinin positivity alone was not sufficient to consider the colonic biopsies as positive for innervation to exclude Hirschsprung's disease.[18]

3(10%) cases in the current study were classified as false negative which were positive for ganglion cells on H & E but did not show immunoreactivity with calretinin. Many studies conducted describe the reasons for false negative results of IHC in the laboratories.[19]

Human inaccuracy leading to false negative results comprise “wrong on slide control,” “no on-slide control,” and “wrong protocol run,”. It is predicted that the explanations for unsuccessful IHC staining, and their incidences are inconstant among variable laboratories based on test list, platforms, regularity of use, conservation, and obviously the value of the control materials in place.[19,20]

Many researchers applied calretinin along with other markers as ancillary techniques on rectal biopsies to rule out aganglionosis. Study conducted by Takawira et al has demonstrated that calretinin is superior to acetylcholinesterase (AChE) as an ancillary technique because it is readily available, can be utilized on paraffin embedded tissue and helpful in improving the diagnostic accuracy.[3] Zani et al observed in the survey study that as the loss of calretinin staining is a marker of ganglion cell absence in the colon of HD patients and can be easily utilized on paraffin embedded tissue, it has widely replaced AChE staining which is applied on frozen sections.[21] Similar observation was made by Kovach et al showing more significance of calretinin to AChE.[22] Chisholm KM et al also reported that calretinin is easier to accomplish and understand and increases the investigative accurateness in patients of HD.[23] There are few limitations in the study including: small sample size, anatomical subtyping of the lesion into short or long segment or total colonic aganglionosis was not done and the data regarding clinical follow-up was lacking. Further studies with large sample size are suggested. Research should be directed to describe the calretinin immunostaining configurations of the variable gut portions including transitional zone, rare types of Hirschsprung's disease, total hypoganglionosis patients, and the anorectal junction. Genetic studies for mutational analysis in cases of HD should also be carried out.

Conclusion

Calretinin immunohistochemistry is an important and reliable diagnostic tool showing high sensitivity and specificity that aids histopathological examination in suspected HD. Hence based on sensitivity and specificity we strongly recommend the use of calretinin stain especially in cases where ganglion cells are not seen on routine Hematoxylin and Eosin staining.

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CONFLICT OF INTEREST

Authors declared no conflicts of Interest.

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DATA SHARING STATMENT

The data that support the findings of this study are available from the corresponding author upon request.