ORIGINAL RESEARCH PAPER

Volume-8 | Issue-3 | March-2019 | PRINT ISSN No 2277 - 8179

INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

# EVALUATION OF CALRETININ EXPRESSION IN DIAGNOSIS OF HIRSCHSPRUNG'S DISEASE: AS AN ADJUNCT TO HISTOPATHOLOGICAL DIAGNOSIS

Pathology

**Madhumita Mukhopadhyay** Professor , Department of pathology IPGME&R

**Chhanda Das*** Assistant Professor, Dept of pathology IPGME&R *Corresponding Author

**Madhu Kumari** PGT 3rd year, Dept of pathology, IPGME&R

**Biswanath Mukhopadhyay** Professor ,Dept of paediatric surgery Apollo Gleneagles Hospital, Kolkata

**Ashis Kumar Saha** Associate Professor , Department of Surgery, CNMC&H

## ABSTRACT

**BACKGROUND:** Hirschsprung's disease (HD) is the major cause of pediatric intestinal obstruction. It occurs due to failure of migration of ganglion cells from neural crest, leads to development of aganglionic segments which varies from ultra short to total intestinal involvement.

**AIMS & OBJECTIVES:** The aim of the study was to describe the characteristics of Calretinin immunostaining pattern in both ganglionic and aganglionic segments of colon in patients with Hirschsprung's disease and to evaluate the role of Calretinin immunohistochemistry in the diagnosis of Hirschsprung's disease.

**MATERIALS & METHODS:** A prospective study was done in 80 cases. Patients with suspected and follow-up cases of Hirschsprung's disease were taken who came for definitive surgery. Hematoxylin and eosin (H and E) staining was done for detection of ganglion cells followed by Immunohistochemical staining for Calretinin.

**RESULTS:** During histopathological examination of the spastic segment by H&E staining, out of total 80 cases, Ganglion cells were seen in 14(17.5%) cases, absent in 50(62.5%) cases and 16 (20%) cases were suspicious for Ganglion cells. Calretinin immunoexpression was negative in all cases of aganglionic segments. 2(2.5%) cases that were reported as positive for Ganglion cells in H&E staining, were reported as negative by Calretinin IHC. So Calretinin had sensitivity of 96% and specificity of 100% in our study. The positive predictive value was 100% and negative predictive value was 92.59%.

**CONCLUSION:** Calretinin IHC is accurate in detecting ganglion cells. It is extremely useful in solving the doubtful cases of HD. It can serve as a valuable and cost-effective diagnostic aid in Hirschsprung's disease.

## KEYWORDS

Calretinin, Ganglion cell, Neonate

## INTRODUCTION

Hirschsprung's disease (HD) is a genetic disorder with a complex pattern of inheritance. It is the major cause of pediatric intestinal obstruction [1]. It occurs due to failure of migration of ganglion cells from neural crest, leads to development of aganglionic segments which varies from ultra short to total intestinal involvement [2]. Significant racial variation is seen in the incidence of the disease occurring in 1 in 5,000 live births, with the highest incidence in Asian populations (2.8 in 10,000) [3]. Hirschsprung's disease (HD) is diagnosed by qualitative and morphological evaluation of ganglion cells and analysis of hypertrophy and hyperplasia of nerves in submucosa and muscularis propria[4]. Additional laboratory methods like enzyme histochemistry and immunohistochemistry using neuronal markers are used as an adjunct to histopathological diagnosis Qualitative evaluation of ganglion cells by calretinin IHC helps in diagnosis of HD. Calretinin is a calcium binding protein, expresses in ganglionic cells within the submucosal and myenteric plexus of normal bowel whereas aganglion regions of Hirschsprung's disease lack Calretinin expression[5].

## AIMS AND OBJECTIVES:

**GENERAL OBJECTIVES-1.)** To detect the presence or absence of Ganglion cells by histopathological examination using Haematoxylin and Eosin stain

**SPECIFIC OBJECTIVES-2.)** To confirm the cases of HD by immunohistochemistry stain Calretinin.

## MATERIALS AND METHODS

It is type of prospective and observational study. After obtaining approval from ethical commitee the study was conducted in the Department of Pathology in association with Department of Pediatric Surgery, Institute of Post Graduate Medical Education and Research, Kolkata Kolkata from January 2015 to June 2016 with a total of 80 patients. Patients presenting with symptoms of delayed passage of meconiun, abdominal distension, chronic severe constipation and diagnosed radiologically as Hirschsprung's disease underwent subsequent full thickness bowel biopsy at Pediatric surgery Department were taken. **(Table/Fig 1)**

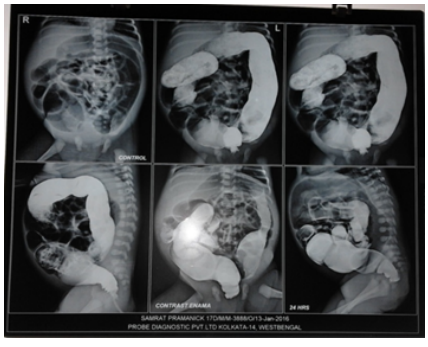


**Table /Fig1: Radiograph of Barium Enema showing Proximal Dilated Segment and Distal Spastic Segment**

At first, Hematoxylin and eosin (H and E) staining was done for detection of ganglion cells. Immunohistochemical staining was done for Calretinin [monoclonal mouse antihuman antibody (DAKO)]. Immunohistochemistry (IHC) staining was done on all paraffin embedded blocks of spastic segment. The antibody used predominantly stained cytoplasm and nucleus of ganglion cells. Calretinin was considered as positive if any specific staining was present either within the sub mucosal or the myenetric plexus. Calretinin stained benign mesothelial cells were used as positive control. Previously diagnosed HD were taken as negative control. Histopathology is considered as gold standard in diagnosis of HD.

## STATISTICAL ANALYSIS

All statistical tests were performed and analysed by Graph Pad Prism version 5 [San Diego, California: Graph Pad Software Inc., 2007]. Chi-square test was used to assess the correlation between H&E and

Calretinin Staining.(p-value<0.05 was taken as significant)

## RESULTS

During the study period of one and half year a total of 80 patients were selected out of which, 20 (25%) were of follow-up HD who underwent definitive pull through surgery, and 60 (75%) were of suspected HD who underwent full thickness bowel biopsy among the patients attending the Pediatric surgery OPD . Patient's age ranges from 2 days to 6 years with median age being 19 days. Mean age was 10 months. Most of patients 51(63.75%) were in neonatal age group, followed by 10(12.5%) infants, followed by 19(23.75%) in age group above 1 year. 55 patients were male (68.75%) and rest 25 patients (31.25%) were female. Out of total 80 cases 39(48.75%) were Hindu, 40(50%) were Muslim and 1(1.25%) was Christian. Abdominal distension was the main diagnostic symptom seen in 53(66.25 %) cases, followed by delayed passage of meconium in 31(38.75%), followed by bilious vomiting in 28(35%), followed by intestinal perforation in 4(5%) and chronic severe constipation in 3(3.75%). Total 53 cases were diagnosed as HD. Forty one (77.36%) cases were of short segment disease 7(13.21%) cases were of long segment disease, and 5(9.43%) cases were of total colonic aganglionosis. Forty three(81.13%) cases were sporadic, 8(15.09%) cases were familial and 2(3.78%) cases were syndromic . 8(15.09%) cases had family history of HD of which 6 had similar history in siblings and 2 cases had similar history in sibling and father also. 2 cases were syndromic associated with Down syndrome. (Table /Fig 2)

**Table/Fig2: Types of HD according to segment affected**

| Type of HD | Total number of cases |
|---|---|
| Short segment Disease | 41(77.36%) |
| Long Segment Disease | 7(13.21%) |
| Total colonic aganglionosis | 5(9.43%) |

During histopathological examination of the spastic segment by H&E staining, out of total 80 cases, Ganglion cells were seen in 14(17.5%) cases, absent in 50(62.5%) and 16 (20%) were suspicious for Ganglion cells**.( Table/Fig 3)**

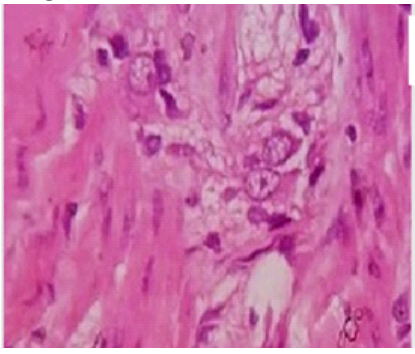
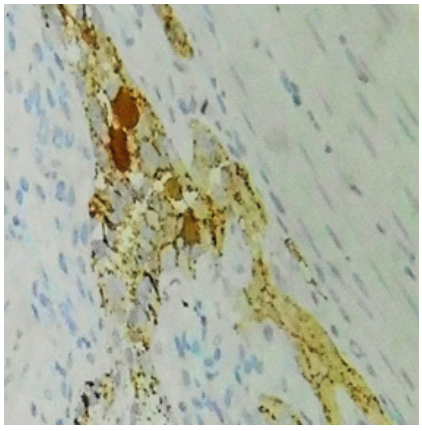


**Table Fig3 Photomicrograph showing Ganglion Cells in Myenteric Plexus in H&E Staining (x400)**

Calretinin immunostaining was done in all of these cases. Out of total 80 cases Calretinin showed presence of Ganglion cells in 25(31.25%) cases and absent in 55(68.75%) cases. (Table /Fig 4)



**Table/Fig 4 Photomicrograph of Calretinin IHC showing Ganglion cells in Myenteric Plexus(x400)**

Out of 50 (62.5%) cases reported negative for Ganglion cells through H&E staining, 44(55%) were reported negative, and 6(7.5%) were reported positive for Ganglion cells through Calretinin IHC of which 2 cases are of neonates did not have muscularis propria layer in sections. In 16(20%) patients, H&E sections were suspicious for Ganglion cells. Calretinin IHC showed immunopositivity in 7(8.75%) cases of which 3 cases are of neonates did not have muscularis propria layer, whereas 9(11.25%) cases were negative for Ganglion cells. Therefore, Calretinin is useful in detection of ganglion cells even in absence of muscle layer.

Calretinin immunoexpression was negative in all cases of aganglionic segments. 2(2.5%) cases that were reported as positive for Ganglion cells in H&E staining, were reported as negative by Calretinin IHC. So Calretinin had sensitivity of 96% and specificity of 100% .The positive predictive value was 100% and negative predictive value was 92.59%. Diagnosis of Hirschsprung's was done with the help of two tests H&E staining and Calretinin IHC. Diagnosis of HD & NHD was correlated with Calretinin by Chi-square test and the p- value was <0.0001 that is highly significant**. (Table/Fig 5)**

**Table/FIG5; Calretinin IHC Staining Pattern In HD & NHD**

| | Calretinin –ve | Calretinin +ve | Total |
|---|---|---|---|
| **HD** | 53(66%) | 0(0%) | 53(66%) |
| **NHD** | 2(3%) | 25(31%) | 27(34%) |
| **Total** | 55(69%) | 25(31%) | 80(100%) |

Diagnosis of HD was done with the help of two tests H&E staining and Calretinin IHC. Diagnosis of HD & NHD was correlated with Calretinin by Chi-square test (with Yate's correction=67.163), degree of freedom=1, and the p- value was <0.0001 that is highly significant.

## DISCUSSION

It is the major cause of pediatric intestinal obstruction so priority is given to early diagnosis. Diagnostic modalities are -1) clinical examination, 2) radiological examination and 3) histopathological examination of the biopsy specimens. In conventional haematoxylin & eosin stain, NHD and HD are diagnosed depending on the presence and absence of ganglion cells in one or more tissue sections [6]. False positive interpretation of HD occurs due to following factors -1) Minimally appreciable submucosa, 2) inappreciable and infrequent ganglion cells in submucosa, 3) difficulty in identification of immature ganglion cells specially in neonates and 4) observers error due to lack of experience. False positive cases are minimized by other anicilliary investigations like enzyme histochemistry and immunohistochemistry . We have selected Calretinin, in adjunct to routine H&E stain because of its avalability in our department and its Qualitative evaluation of Calretinin immunostaining is a simple and efficient tool for detection of ganglion cells [7]. Negative immunohistochemical expression of Calretinin in both submucosal and myenteric plexus of spastic segment is diagnostic of HD [7].

Our study was a single-institution based study over a span of one and half years, we found total 80 patients, of which 53 turned out to be HD. S Alexandrescu et al. conducted similar study in USA on 28 cases [8]. In a 15 year study conducted in France, Samuel et al published 131 cases [9] . In India, L Kannaiyan et al conducted a study with 60 cases[10].

In our study, Patient's age ranges from 2 days to 6 years (mean 10 months), most of patients 51(63.75%) were in neonatal age group, followed by 10(12.5%) infants, followed by 19(23.75%) patients in age group above 1 year. In a study conducted by Samuel et al patients were aged from 3 days–9 years (mean 1.3 years). [9] In another study conducted by L Kannaiyan age of patients ranged from 1 day to 14 years (mean 8.2 months). 63.8% presented at less than 1 month of age, 19.5% patients presented between 1 month and 1 year of age, 16.7% patients presented after the age of 1 [10]. In a study conducted by S Alexandrescu et al.[8] age of the patients ranged from 15 days to 8 years.

In our study 37(69.81%) patients were male and 16(30.19%) were female. Male to female ratio was 2.31. In M Hiradfar et al.[11] study they found patient's age varied from 2 days to 12 years (mean, 17±4 months) and male-to female ratio was 2.75. In another study conducted by L Kannaiyan male-to female ratio was 3.2-3.3:1 [10].

In our study we found 41(77.36%) cases were of SSD ,7(13.21%) were

of LSD, and 5(9.43%) were of TCA. In a study done by V Zuikova et al. 12] they found 75% cases of short segment disease, 16.7% cases of long, 8.3% of total colonic aganglionosis which is similar to our study. In another study conducted by L Kannaiyan et al.[10] 81.7% had short segment disease, 8.3% had long segment disease, 10% had total colonic aganglionosis.

In our study out of total 80 cases, 20(25%) cases were follow up of HD who underwent definitive pull- through surgery and 60(75%) cases were of suspected HD who underwent full thickness colonic/rectal biopsy. In a similar study conducted by L Kannaiyan et al. specimens from 60 patients were evaluated , 36(60%) were full-thickness rectal biopsies (for suspected HD) and 24(40%) were bowel segments resected during the definitive pull-through surgery. [10]

In our study during histopathological examination of the spastic segment by H&E staining out of total 80 cases ganglion cells were seen in 14(17.5%) cases, absent in 50(62.5%) cases and 16(20%) cases were suspicious for ganglion cells. In a similar study conducted by L Kannaiyan et al. [10] H&E staining revealed absence of ganglion cells (negative) in 52.7%, presence of ganglion cells (positive) in 5.56%, and suspected presence of ganglion cells in 41.67%. In our study out of 50(62.5%) cases reported negative for ganglion cells through H&E staining, 44(55%) were reported negative and 6(7.5%) were reported positive for ganglion cells through Calretinin IHC. In a similar study conducted by L Kannaiyan et al. of the 19 cases reported negative through H&E staining, 47.22% were reported negative, and 5.56% positive through Calretinin IHC. In our study in 16(20%) patients , H&E sections were suspicious for ganglion cells. Calretinin IHC showed immunopositivity in 7(8.75%) cases, whereas 9(11.25%) cases were negative for ganglion cells. The relatively undifferentiated and non-neuronal appearance of "immature" ganglion cells in the sub-mucosa of neonates, particularly in premature infants, is associated with difficulty in diagnosis of Hirschsprung's disease by H& E.

In our study we found that Calretinin immunoexpression was negative in all cases of aganglionic segments. 2(2.5%) cases that were reported as positive for ganglion cells in H&E staining, were reported as negative in Calretinin IHC, so Calretinin had sensitivity of 96% and specificity of 100% in diagnosis of HD. The positive predictive value was 100% and negative predictive value was 92.59%. In another study by M Hiradfar et al. they found, this method had sensitivity of 93.3% and specificity of 100% in diagnosis of HD in full thickness specimens of intestinal wall. [11]The positive predictive value was 100% and negative predictive value was 93.8% which was similar to our study. In a study done by V Zuikova et al. they found Calretinin as the most valuable immunohistochemical marker of the three evaluated Calretinin, Chromogranin A and synaptophysin, and Calretinin had sensitivity of 90.5% and specificity of 92.9% for diagnosis of HD. The positive predictive value was 90.5% and negative predictive value was 92.9%. [12] In a study by L Kannaiyan et al. Calretinin is proven to be highly sensitive for the presence of ganglion cells. [10] For Kapur RP et al. an ideal immunohistochemical marker would be easy to use and interpret, it works with paraffin section and circumvents the need to analyze large numbers of histological hematein–eosin levels.[13] Calretinin seems to achieve all these properties when combined with a limited analysis of hematein– eosin levels to confirm the absence of ganglion cells. I Barshack et al. study showed that Calretinin was not expressed in aganglionic segments of HD , whereas in ganglionic HD segments and in normal colon ganglion cells were immunopositive as in our study. [7] In a study by S Alexandrescu et al.[8]they concluded that Calretinin IHC offered additional diagnostic value in the specimens with inadequate amount of submucosa and rarely seen ganglion cells. Ayper Kacar et al. demonstrated that Calretinin immunohistochemistry is a very sensitive and specific method for detecting aganglionic tissue, may eliminate the need for repeat biopsy and may lower the need for excessive sectioning. [14]

Guinard-Samuel, et al. have studied the largest number of suction biopsies using Calretinin. They have concluded that Calretinin is accurate in proving the absence of ganglion cells, it is easy to interpret, and can replace AChE to diagnose HD. They however, recommended that it should be used as an aid along with H and E examination, especially in ultrashort segment disease and transition zone specimens. [9] In our study, we found that Calretinin correlated with H and E examination in both rectal biopsies and the resected bowel specimens. In the rectal biopsy specimens, Calretinin also aided in the diagnosis of HD with ambiguous findings in H&E staining.

We have done diagnosis of HD with the help of two tests H&E staining and Calretinin immunohistochemistry . Diagnosis of HD was correlated with Calretinin IHC with the help of chi-square test and it was statistically significant. (p value<0.001).

## CONCLUSION.

Calretinin IHC is accurate in detecting ganglion cells. It is extremely useful in solving the doubtful cases of HD. It can serve as a valuable and cost-effective diagnostic aid in Hirschsprung's disease.

## LIMITATIONS

As it is a single institute based study and the study period being very short, the number of case is small. So it is not sufficient to give generalised result to comment on whole population.

## REFERENCES


1. Dasgupta R, Langer JC. Hirschsprung disease. Curr Probl Surg 2004;41(12):942–988.
2. Newgreen D, Young HM. Enteric nervous system: development and developmental disturbances – part 2. Pediatr Dev Pathol 2002;5(4):329–349.
3. Amiel J, et al. Hirschsprung's disease, associated syndromes and genetics: a review. J Med Genet 2008;45(1):1–14.
4. Moore SW, Rode H et al. Familial aspects of Hirschsprung's Disease. Eur J Pediatr Surg. 1991;1:97-101.
5. Holland SK, R P,R Podolsky et al. Calretinin immunostaining as an adjunct in the diagnosis of Hirschsprung's disease. Ann Diag Path . 2011;15(5):323-328.
6. Kini U, Babu MK, Yadav L, Mohanty S, Divya P, Saleem KM, et al. Role of syanptophysin in leveling circumferential full thickness (doughnut) bowel. Indian J Pathol Microbiol 2012;55:s10.
7. Barshack I, Fridman E, Goldberg I, Chowers Y, Kopolovic J. The loss of calretinin expression indicates aganglionosis in Hirschsprung's disease. J Clin Pathol 2004;57(7):712–716.
8. S Alexandrescu, H Rosenberg, N Tatevian. Role of calretinin immunohistochemical stain in evaluation of Hirschsprung disease: an institutional experience Int J Clin Exp Pathol 2013;6(12):2955-2961.
9. V Guinard-Samuel, A Bonnard, Pascal De Lagausie, P Philippe-Chomette, C Alberti, et al. Calretinin immunohistochemistry: a simple and efficient tool to diagnose Hirschsprung disease Modern Pathology 2009; 22:1379–1384.
10. L Kannaiyan, S Madabhushi, R Malleboyina, N Kumar Are, K. Ramesh Reddy, B Rao. Calretinin immunohistochemistry: A new cost-effective and easy method for diagnosis of Hirschsprung's disease. J Ind Asso. Ped Surg 2013;18(2):66-68.
11. M Hiradfar, N Sharifi, M Khajedaluee, N Zabolinejad, S Taraz Jamshidi. Calretinin Immunohistochemistery: An Aid in the Diagnosis of Hirschsprung's Disease Iran J Basic Med Sci 2012;15(5)1053-1059.
12. V Zuikova, I Franckevica, I Strumfa, I. Melderis Immunohistochemical Diagnosis of Hirschsprung's Disease and Allied Disorders. Acta Chirjurgica Latviensis 2015;15(1):50-57.
13. Kapur RP. Can we stop looking? Immunohistochemistry and the diagnosis of Hirschsprung disease. Am J Clin Pathol 2006;126(1):9–12.
14. A Kacar, A Türker Arikok, M Nur Azili, G Ekberl. Calretinin immunohistochemistry in Hirschsprung's disease: An adjunct to formalin-based diagnosis. Turk J Gastroenterol 2012; 23 (3): 226-233.