

Comparison of Haematoxylin and Eosin Staining and Calretinin Immunohistochemistry in Clinically Suspected Hirschsprung Disease to Evaluate the Diagnostic Utility of Calretinin in Hirschsprung Disease

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ABSTRACT

BACKGROUND

Hirschsprung disease is a congenital disorder that results from lack of coordinated propulsive movement of distal portion of large bowel due to the absence of Meissner's and Auerbach's plexus. It is usually diagnosed by histopathological examination of biopsy specimens and acetyl cholinesterase histochemistry. But acetyl cholinesterase histochemistry requires frozen section and the interpretation of results show subjective variation. So, recently calretinin immunohistochemistry has replaced this, which is technically less demanding and can be performed on formalin fixed paraffin embedded specimens. It can also be used in cases with paucity of ganglion cells, immature ganglion cells, sub optimal amount of sub mucosa and in instances where inter muscular zone is not properly visualised. We wanted to assess the histomorphological features of Hirschsprung disease and study the expression of calretinin in Hirschsprung disease.

METHODS

53 cases of clinically suspected Hirschsprung disease, during a two-year period were included in the study. Calretinin immunohistochemistry was done in all the 53 cases. Staining of either nucleus and/or cytoplasm of ganglion cells in submucosa or inter muscular zone or staining of nerve fibers in lamina propria were taken as positive. Hematoxylin and eosin stained sections and calretinin stained sections were compared. Data analysis was performed using SPSS version 16. Kappa coefficient was applied for determining the level of agreement between hematoxylin and eosin stained sections and calretinin stained sections.

RESULTS

Haematoxylin and eosin stained sections and calretinin stained sections were compared in all the 53 cases studied. 49 cases showed concordant results and 4 cases showed discordant results which were studied in detail. The number of observed agreements between them were 49 (92% of observations). Kappa coefficient was 0.625. The strength of agreement was moderate according to this calculation.

CONCLUSIONS

Our study concludes that calretinin can serve as a very reliable adjunctive in the evaluation of Hirschsprung disease. It can reduce the need for repeated biopsies and serial sectioning of blocks and serves as a very useful, valuable, sensitive and specific technique for detecting aganglionosis in patients with suspected Hirschsprung disease.

KEY WORDS

Hirschsprung Disease, Ganglion, Acetyl Cholinesterase Histochemistry, Calretinin Immunohistochemistry

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BACKGROUND

Hirschsprung disease, also known as congenital megacolon or intestinal aganglionosis is a neurocristopathy, in which there is congenital absence of neurons in a portion of the intestinal tract, due to a disruption of normal neural crest cell migration, proliferation, differentiation, survival and/or apoptosis.¹ It is inherited in a complex, often non-Mendelian pattern, and characterized by variable penetrance.¹ Patients who present with Hirschsprung disease are usually newborns or infants and is one of the frequent causes of intestinal obstruction in newborns.²

The diagnosis rests on clinical data, imaging studies and histological features.³ The investigations that will aid in diagnosis include anorectal manometry and barium enema, but definite diagnosis is made by histologic demonstration of aganglionosis in intestine.^{4,5,6} Several histological techniques are there for evaluating rectal biopsies for the presence of ganglion cells. One of the most available and commonly used methods is haematoxylin-eosin (H&E) stained sections. But it has got several limitations in terms of examining submucosal area, where the ganglion cells are small and irregularly distributed, requirement of several serial sections to establish a diagnosis and also in identifying immature ganglion cells in neonates. A skilled and experienced pathologist is required for interpretation of H&E section and it is very time consuming because of the necessity to interpret many sections before a biopsy can be confirmed negative.^{7,8,9,10}

Another method is acetylcholinesterase histochemistry, which demonstrates the acetylcholinesterase positive cholinergic nerve fibres in the lamina propria and thus providing supportive evidence for a diagnosis of Hirschsprung disease.¹¹ But the problem with acetylcholinesterase staining is that the changes may not be apparent in a patient before 6 months of age. In addition to that, frozen section is required for this technique and the staining procedure must be followed precisely with freshly prepared reagents.¹² Over the years, many immunohistochemical markers have been tried for the diagnosis of Hirschsprung disease as they have the advantage of using formalin fixed paraffin embedded sections and thus eliminating the need for frozen section. Although, most of these have been proved to be diagnostically insignificant, some, like calretinin are shown to be of diagnostic value.¹³

Calretinin is a vitamin D-dependent calcium-binding protein which is involved in the physiological buffering of excess cytosolic calcium ions; calcium transport and protection against calcium-ion overload.¹⁴ It is normally expressed in enteric nervous system, and when stained, can be seen as a distinct granular network of fibres in lamina propria between the crypts, submucosa and muscularis propria which is an indirect evidence of ganglionosis. Calretinin also stains ganglion cells strongly. So, absence of calretinin staining indicates aganglionosis and thus a diagnosis of Hirschsprung disease can be made.¹⁵ The main advantage of calretinin is that it is technically less demanding and can be done on paraffin embedded sections. The interpretation is also very easy with less interobserver variation.^{8,16} It is also useful in diagnosing Hirschsprung disease in sections with paucity of ganglion cells, immature

ganglion cells, sub optimal amount of submucosa and when the intermuscular zone is not properly visualised.

This study attempts to assess the usefulness of calretinin in histological diagnosis of clinically suspected Hirschsprung disease.

METHODS

The present observational study was conducted in the Department of Pathology, Government Medical College, Thiruvananthapuram. All suction biopsy, sero muscular biopsy, full thickness biopsy and excision biopsy specimens and surgical resection specimens of clinically suspected cases of Hirschsprung disease received in the Department of Pathology, Government Medical college, Thiruvananthapuram during the period June 2015-May 2017 were included in the study. A total of 53 cases were studied. Sample size was taken based on the convenience of the study. Got approval from IEC and informed consent was obtained. The specimens were fixed in 10% neutral buffered formalin. After cutting, bits were taken from representative areas. Paraffin embedded blocks were made and 4-5 μ thick sections were taken. Haematoxylin and eosin staining was done and examined for the presence or absence of ganglion cells in the intermuscular zone or hypertrophied nerve bundles in intermuscular zone. Unstained slides from paraffin blocks were made and immunohistochemical staining with anti-calretinin antibody was done by peroxidase anti-peroxidase method. Staining of either nucleus or cytoplasm or both of ganglion cells in submucosa or inter muscular zone or staining of nerve fibers in lamina propria were taken as positive.

Statistical Analysis

All collected data were entered in Microsoft Excel and was analysed using the statistical software SSPS version 16.0.

RESULTS

A total of 53 cases were studied. The age group ranged from 1 day to 6 years, with majority of cases being less than one month of age. (refer table 1). 66% of the cases were males (refer Table 2). Most common clinical features were failure to pass meconium and abdominal distention (refer Table 3). Barium enema was done in 23 patients, of which 15 were suggestive of Hirschsprung disease with definite transition zone at different levels depending on the type of Hirschsprung disease. There was no evidence of transition zone in two cases, of which one showed ganglion cells in the intermuscular zone and was calretinin positive, thus ruling out a diagnosis of Hirschsprung disease and the other case was diagnosed as classical Hirschsprung disease on histopathological examination. Barium enema was inconclusive in 5 cases which were turned out to be Hirschsprung disease on histopathological examination. One case with barium enema suspicious of Hirschsprung disease was not Hirschsprung disease. Most of the specimens received were of seromuscular biopsy from transition zone.

(Refer Table 4). Out of the 4 resection specimens, two cases were of total colonic aganglionosis, and two were long segment disease. The proximal segment was dilated with a distal narrow segment. 12 specimens were of distal end of pull through bowel. All of these 12 cases were previously diagnosed as Hirschsprung disease. Majority of the cases were classical Hirschsprung disease (38%) in which aganglionic segment involved the distal colorectum and a considerable distance in the adjoining proximal dilated bowel. Out of the 8 long segment diseases, transition zone was in the splenic flexure in 6 cases and it was in the mid transverse colon in the remaining 2 cases. Two ultrashort segment disease were diagnosed, correlating with the barium enema finding, which showed a small transition zone in the distal rectum. (Refer Table 5).

Out of the 53 cases studied, 10 case showed both ganglion cells and hypertrophied nerve bundles in the intermuscular zone, consistent with transition zone. In 3 cases, there was absence of ganglion cells, and presence of hypertrophied nerve bundles in the intermuscular zone which was consistent with a diagnosis of Hirschsprung disease, and 3 cases showed absence of ganglion cells, without hypertrophied nerve bundles. Intermuscular zone was not properly visualised in one case. All the remaining cases showed ganglion cells in the biopsies received during the study period, most of which were previously diagnosed as Hirschsprung disease.

Age	Frequency	Percentage
Less than one month	31	58
1-6 months	12	23
6 months-1 year	7	13
More than 1 year	3	6
Total	53	100

Table 1. Age Distribution

Sex	Frequency	Percentage
Males	35	66
Females	18	44
Total	53	100

Table 2. Sex Distribution

Clinical Feature	Frequency	Percentage
Failure to pass meconium + abdominal distention	24	45
Abdominal distention	10	19
Failure to pass meconium	8	15
Abdominal distention+ vomiting	8	15
Chronic constipation	3	6
Total	53	100

Table 3. Clinical Features

Type of Specimen	Frequency	Percentage
Seromuscular biopsy	34	64
Pull through bowel	12	23
Resection specimen	4	7
Rectal biopsy	3	6
Total	53	100

Table 4. Type of Specimen

Type of Hirschsprung Disease	Frequency	Percentage
Classical Hirschsprung disease	20	38
Short segment Hirschsprung disease	13	25
Long segment Hirschsprung disease	8	15
Total colonic aganglionosis	4	7
Ultra-short segment Hirschsprung disease	2	4
Non Hirschsprung disease	6	11
Total	53	100

Table 5. Type of Hirschsprung Disease

	H & E	Calretinin	Number of Cases
GC	+	+	45
GC	-	-	4
GC	-	+	3
GC	+	-	1
Total			53

Table 6. Comparative Analysis of H&E and Calretinin Immunohistochemistry

Calretinin immunohistochemistry was done in all the 53 cases and were compared with corresponding haematoxylin and eosin stained sections. Cytoplasmic and/or nuclear staining of ganglion cells with calretinin, in intermuscular zone or submucosa or staining of nerve fibres in lamina propria were considered as positive. Ganglion cells showed granular positivity of cytoplasm and/or nucleus, with calretinin, and were seen in the intermuscular zone and/or submucosa. Nerve fibres in the lamina propria were highlighted with calretinin as distinct granular network between the crypts. 46 cases showed presence of ganglion cells in haematoxylin and eosin stained sections. Of these 46 cases, 45 cases were calretinin positive and one case was calretinin negative. 7 cases showed absence of ganglion cells in haematoxylin and eosin stained sections, of which 4 cases were calretinin negative and 3 cases were calretinin positive. (Refer Table 6).

Data analysis was performed using SPSS version 16. Kappa coefficient was applied for determining the level of agreement between haematoxylin and eosin stained sections and calretinin stained sections. The number of cases showing observed agreement between them were 49 (92% of observations). Kappa coefficient was 0.625 showing moderate strength of agreement.

DISCUSSION

Diagnosis of Hirschsprung disease is a stressful practice for pathologists especially if they infrequently encounter the condition. Limitations associated with hematoxylin and eosin-based sections in diagnosing Hirschsprung disease and technical difficulties associated with acetylcholinesterase histochemistry made us to study the utility of calretinin immunohistochemistry in diagnosis of Hirschsprung disease.

The age and sex distribution of present study was comparable with the studies by Nasser Rakhshani et al and Mehran Hiradfar et al. Most common presenting symptom in the present study was failure to pass meconium and abdominal distension (44%). In a study by Mohammad Hossein Anbardar et al, most common presenting symptoms were delayed meconium passing, chronic constipation, abdominal distention and vomiting. In a study conducted by Menezes M, Corbally M and Puri P in 259 cases of Hirschsprung disease, intestinal obstruction was the presenting feature in 147 patients (56.8%). Most common type of Hirschsprung disease was classical type in the present study, which was similar to the studies conducted by Hiradfar M et al and Anbardar M H et al

Haematoxylin and eosin stained sections of all the 53 cases were studied. Sections from transition zone showed presence of ganglion cells and hypertrophied nerve bundles in the intermuscular zone. Absence of ganglion cells and

presence of hypertrophied nerve bundles, which is diagnostic of Hirschsprung disease was observed in three cases. Three cases showed absence of ganglion cells without hypertrophied nerve bundles.

These findings were comparable with the observations in the studies by Sanda Alexandrescu et al and I Barshack et al. Our aim was to study the diagnostic utility of calretinin in Hirschsprung disease. Haematoxylin and eosin stained sections and calretinin stained sections were compared in all the 53 cases studied. Ganglion cells showed cytoplasmic and/or nuclear granular positivity with calretinin and were seen in intermuscular zone or submucosa. Nerve fibres in lamina propria were seen as distinct granular network with calretinin. Irregularly scattered ganglion cells in the submucosa and immature ganglion cells in neonates which were difficult to be made out with haematoxylin and eosin staining were also highlighted by calretinin. This pattern of staining was observed in various studies reviewed in the literature. In a study by Mehran Hiradfar et al, there was positive immunostaining of nerve fibers in the lamina propria, submucosa and muscularis propria in control and patient group. There was also nuclear and cytoplasmic staining of ganglion cells in submucosa and intermuscular zone in all specimens of both control group (100%) and ganglionic segments (100%). These observations were similar to the studies by David Hernandez Gonzalo et al, Sanda Alexandrescu et al and Lokendra Yadav et al.

49 cases showed concordant results; and 4 cases showed discordant results which were studied in detail. Among concordant cases, 45 cases showed ganglion cells in both haematoxylin and eosin stained sections and calretinin stained sections, and 4 cases showed absence of ganglion cells in both. Out of the 4 discordant cases, two cases showed absence of ganglion cells in the inter muscular zone in haematoxylin and eosin stained sections, but calretinin staining picked out ganglion cells in the intermuscular zone which thus ruled out a diagnosis of Hirschsprung disease. In the third case the intermuscular zone was not properly visualised, but calretinin positive nerve fibres were observed, thus excluding Hirschsprung disease. In the fourth case, ganglion cells were seen in haematoxylin and eosin stained sections, but calretinin was negative. Repeated attempts to rule out any technical errors also showed negative results. In one of the concordant cases, it was difficult to identify ganglion cells in the intermuscular zone in haematoxylin and eosin stained sections as they were immature. But the cells were positive for calretinin.

CONCLUSIONS

Calretinin can serve as a very reliable adjunctive in the evaluation of Hirschsprung disease. In addition to the fact that it is technically less demanding and easy to interpret, compared to acetylcholinesterase histochemistry, calretinin is particularly useful in diagnosing Hirschsprung disease in sections with paucity of ganglion cells, immature ganglion cells, suboptimal amount of submucosa and when the intermuscular zone is not properly visualised. Thus, calretinin immunohistochemistry can reduce the need for

repeated biopsies and serial sectioning of blocks and serve as a very useful, valuable, sensitive and specific technique for detecting aganglionosis in patients with suspected Hirschsprung disease.

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