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TO STUDY THE EXPRESSION OF CALRETININ IN HIRSCHSPRUNG'S DISEASE:AN INSTITUTIONAL STUDY

Pathology

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ABSTRACT

Background/Aim: The use of calretinin immunostain(IHC) in evaluation of rectal suction biopsy for Hirschsprung Disease(HD).The goal of this study is to report our institutional experience with use of calretinin in specimen for evaluation of HD.The second goal is to describe the pattern of expression of calretinin in the ganglionic to aganglionic segment of specimen of patient with previous diagnosis of HD on suction rectal biopsy.**Material&Method:**For this study 40 rectal suction biopsy specimen were taken 22 from HD patient and 18 from non HD patient .The specimen were selected from pathology department,RIMS ,Ranchi over a period of 12 months.According to calretinin immunohistochemistry expression two groups were divided (1)HD group:C-ve (2)non HD group:C+ve .**Results:**Calretinin positivity stained ganglion cells in the submucosal plexus of ganglionic bowel but not aganglionic bowel.**Conclusion:**The pattern of expression of calretinin in rectal biopsy in HD & non HD patients coincides with the one's previously described in the literature .Calretinin IHC offered addition diagnostic value in the specimens for diagnosis of Hirschsprung disease.

KEYWORDS

Hirschsprung Disease,Calretinin,Suction or mucosal rectal biopsy.

INTRODUCTION

Hirschsprung disease (HD) is a condition of large bowel that cause difficulty in passing stool. It is congenital disorder(1 in 5000 newborn) ,characterized by absence of parasympathetic ganglion cell from intramural and submural plexuses[1] .Identifying ganglion cells by rectal suction or mucosal rectal biopsy are now typically done to confirm the diagnosis of Hirschsprung's disease (HD). Although this procedure is highly accurate,it requires technical skills and general anesthesia and may leads to complications[2]. The ganglion cells of the submucosal plexus are smaller and more irregularly distributed than those located intermuscularly,and their identification requires expertise,patience,and the performance of serial sections in the biopsy obtained[2].The aim of this study was to evaluate the usefulness of calretinin immunohistochemistry in the diagnosis of HD.It is diagnosed during the first year of life in most patients, but may present later, occasionally even in adulthood[3].The early recognition and the prompt implementation of surgical procedures obviously protect infants affected with HD from potential life-threatening conditions, including enterocolitis and debilitating constipation. Hirschsprung disease is currently regard as a genetic disorder with a complex pattern of inheritance[1].

MATERIALS AND METHODS

For this retrospective study, 40 rectal suction biopsy specimens (22 from 11 HD patients and 18 from 9 non-HD patients) were selected from the pathology department, RIMS over a period of 12 months. for ganglion cells with calretinin immunohistochemistry. According to calretinin immunohistochemistry performed on all paraffin-embedded rectal biopsies, we defined two HD groups: C-ve; showing an absence of any staining within mucosa, muscularis mucosae and submucosa and non HD group: C+ve; showing an absence of staining within the mucosa and muscularis mucosae, but a positivity of some submucosal hypertrophic nerves. Criteria of diagnosis based on our standard method (table 1).

Calretinin immunohistochemistry

Immunostaining was performed on paraffin embedded archival tissue following the Avidin-Biotin peroxidase technique. In brief, sections measuring 3-5micron thick were cut, air dried for 15 minute, heat-fixed at 42degree Celsius and then air dried overnight at room temperature. After deparaffinization with xylene, endogenous peroxidase activity was eliminate by treating the slides with AOH/HO for 30 min at room temperature. Then the slides were incubated with the diluted primary antibody (clone: DAK-calret 1, monoclonal mouse anti-human, dako, Denmark,1/100 dilution) at 40 degree Celsius in a humidified chamber for 60 minute. Biotinylated anti mouse igG and avidin-biotin peroxidase complex were added in sequence. The section were then incubated with DAB for 10 min for visualization of the peroxidase reaction. After being washed in water for few minutes, the sections were counter stained with hematoxylin, dehydrated in alcohol,cleared in xylene and mounted.

Calretinin immunoreactivity pattern of staining for ganglion cells(nuclear and cytoplasmic)and also nerve fibers in different layer of bowel(lamina propria,muscularis mucosa,submucosa and muscularis propria)was evaluated in IHC stained slides.

Immunohistochemistry analysis

Calretinin was considerd as positive if any specific staining was present either within the submucosa's nerve plexus ,or in muscularis mucosae or in lamina propria(figure 1)and negative if any specific staining was not present either within the submucosa's nerve plexus,or in muscularis mucosae or in lamina propria(figure 2) .On the basis of result of the calretinin immunohistochemistry,an algorithm was constructed and patients were classified as having HD or non-HD.

STATISTICAL ANALYSIS

Statistical calculation were carried out using SPSS version 11.5.for description of result in each group, method of descriptive analysis such as P-value, Sensitivity,Specificity,Positive predictive value,Negative predictive value,kappa value were used as table(table 2).

RESULTS

Calretinin positively stained ganglion cells in the submucosal plexus of the ganglionic bowel but not aganglionic bowel(figure 1 & 2). Calretinin usually stained ganglion cell cytoplasm and nuclei more intensely .9/20 patients (45 %) belonged to the C+ve group and 11/20 (55 %) patients were within the C-ve group.

Table 1:Criteria of diagnosis based on our standard method

	Pathological finding
Typical Hirschsprung disease	No ganglion cell
	Nerve hypertrophy
	No calretinin staining
Non Hirschsprung disease	Ganglion cell
	No nerve hypertrophy
	Normal calretinin staining

Table 2 :it shows final diagnosis (%) HD:Hirschsprung disease

	HD	Non HD	P	Sensitivity,%	Specificity,%	Postive predictive value,%	Negative predictive value,%	Kappa index

Calretinin								
Negative	22 (100)	0(0)						
Positive	0(0)	18 (100)	<0.001	100	100	100	100	1
Total	22	18						

From:calretinin immunohistochemistry: a simple and efficient tool to diagnose Hirschsprung disease

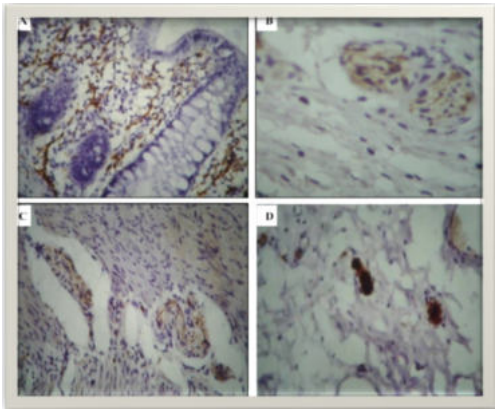

Figure1:positive calretinin immunostaining of nerve fibers in lamina propria(A),submucosa(B),and muscularis mucosa (C).Nuclear and cytoplasmic calretinin immunostaining of ganglion cells(D).

1

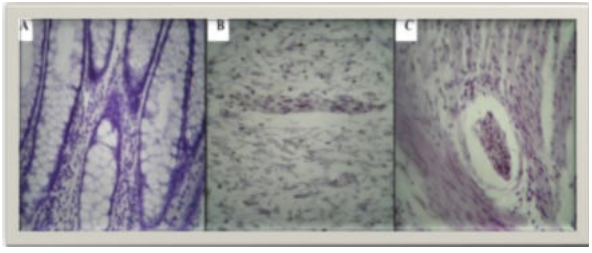

Figure 2:Total absence of staining after calretinin immunoh istochemistry in the aganlionic segment in lamina propria (A), submucosa(B) and muscularis propria ©

DISCUSSION

Lack of ganglion cells in colonic neural plexus is required for the pathological diagnosis of HD. Immunohistochemical staining of calretinin assist in the detection of small immature ganglion cells through intense staining of ganglia, facilitating the recognition of small immature ganglion cells. Nevertheless, the assessment of many cases is still difficult, thus requiring demanding repeated deeper sections.[4]

In the present study, all HD cases showed negative expression of calretinin in small nerve fibers of the lamina propria, musularis mucosae, and submucosa. On the other hand, all non-HD cases (diagnosed histopathologically by the demonstration of ganglionic cells) revealed positive expression of calretinin.

In agreement to these results, Barshack *et al*. summarized that aganglionic segments revealed absence of calretinin expression in ganglion cells and in the nerve fiber in HD, and conversely calretinin expression was positive in both ganglion cells and nerve fibers in ganglionic areas of HD and normal colon.[4]

Likewise, Małdyk *et al*. reported a study in 2014 including results that are concordant with the present one, showing that expression of calretinin was positive in all rectal biopsies with ganglionic cells while negative expression was noticed in all aganglionic segments, thus concluding that immunohistochemical staining of calretinin is a valuable adjunct to histopathology in the diagnosis of HD.[5]

Lim *et al*. recorded two false negative results out of the 27 patients with HD, caused by technical overstaining and punctate immunoreactivity of deep submucosal hypertrophied nerves. They concluded that immunostaining with calretinin is a reliable ancillary technique in the investigation of HD.[6]

Another study performed by Guinard-Samuel *et al*. demonstrated that difficulties faced using combined histopathological examination and staining with acetylcholinesterase can be bypassed through immunostaining with calretinin, and that all HD can be diagnosed accurately without false positive results.[7]

In an institutional experience, Alexandrescu *et al*. affirmed in their work published in 2013 that calretinin immunohistochemical test is a dependable diagnostic method for the pathologist when used in combination with histopathological examination, particularly in cases with sparse or immature ganglion cells in colonic submucosa.[8]

Hiradfar *et al*. investigated the expression of calretinin in colonic sections of HD in comparison to control cases and found that, in both HD patients and control cases, calretinin immunostaining was positive in the nerve fibers of the lamina propria, submucosa, and muscularis propria. Ganglion cells in submucosa and muscularis propria revealed positive calretinin expression in all specimens of both control group and ganglionic segments of HD cases. Immunohistochemical expression of calretinin was negative in all but 2 cases in the muscularis propria nerve fibers of the aganglionic segments. Sensitivity and specificity of this method for the diagnosis of HD in full thickness specimens of intestinal wall were 93.3% and 100%, respectively, with a positive predictive value of 100% and negative predictive value of 93.8%.[9]

In the same manner, Mukhopadhyay *et al*. reported that sensitivity of calretinin immunohistochemistry for ganglion cells detection was 100% and that the specificity was 97.44%, with positive and negative predictive value of 84.62% and 100%, respectively. Another study showed that the sensitivity of calretinin reactivity in ganglion cells was 90.5% and specificity was 92.9%.[10]

Results of Kaçar *et al*., which are concordant with the present work, revealed a great equivalence between histopathological assessment and calretinin immunostaining, concluding that immunohistochemical testing of calretinin has high sensitivity and specificity for the diagnosis of HD, reducing the requirement for repetitive biopsies and unnecessary sectioning for suction biopsy, full thickness biopsy, as well as resection specimen.[11]

Similarly, Gonzalo *et al*. found in their retrospective study that all patients without HD had positive immunohistochemical expression of nerve fibers in lamina propria or muscularis mucosae, and all Hirschsprung patients showed negative calretinin expression concluding that immunohistochemical testing of calretinin is quite supportive in triaging additional workup based on clinical suspicion.[12]

CONCLUSION

As many other institution had done research on calretinin as IHC marker for evalution of suction rectal biopsy specimens to diagnose Hirschsprungs's disease.we also looked into this additional methods of diagnosis to assist us in the evalution of suction rectal biopsy specimens.we found calretinin to be a reliable additional diagnostic tool,when correlated clinically. It helps in the diagnosis of cases with paucity of ganglion cells,immature ganglion cells and suboptimal amount of submucosa. These complementary methods could ameliorate the diagnostic difficulties associated with HD.

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