Original Article

Role of calretinin immunohistochemical stain in evaluation of Hirschsprung disease: an institutional experience

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Abstract: Background: The use of calretinin immunostain (IHC) in the evaluation of rectal suction biopsies for Hirschsprung disease (HD) has been reported by Kapur et al. and others. The first goal of this article is to report our institutional experience with the use of calretinin in specimens for evaluation of HD. The second goal is to describe the pattern of expression of calretinin in the junction of ganglionic-to-aganglionic segment of pull through specimens of patients with a previous diagnosis of HD on suction rectal biopsy. Material and methods: Three pathologists at University of Texas at Houston evaluated 28 rectal biopsy specimens from 2010-2011. The patients’ age ranged from 15 days to 8 years. Twenty-three cases were suction biopsies, and five were rectal full thickness biopsies. Hematoxylin-eosin (H&E) stain was performed on at least 80 levels for the suction biopsy specimens. Calretinin immunohistochemical stain was performed on levels 40-42 in all cases, with adequate controls. The H&E slides of nine pull through specimens with a diagnosis of HD on a suction rectal biopsy that was evaluated in this study, were evaluated. Calretinin IHC was performed on the slide(s) showing the junction of aganglionic-to-normal rectum, along with adequate controls. Results: The presence of ganglion cells consistently correlated with calretinin-positive thin nerve fibrils in the lamina propria, muscularis mucosae and superficial submucosa. These nerve fibrils were absent in the aganglionic segments of bowel and in the areas without ganglion cells from the junction of normal with diseased rectum. Calretinin was strongly expressed in the submucosal and subserosal nerve trunks in the ganglionic segment. It had faint expression in the thick nerve trunks from the areas without ganglion cells 1.6-2.5 cm proximal to the normal rectum. No calretinin expression was seen in the nerve trunks in the rest of the aganglionic segment. Conclusion: The pattern of expression of calretinin in rectal suction biopsies in HD and normal rectum coincide with the ones previously described in the literature. Calretinin IHC offered additional diagnostic value in the specimens with inadequate amount of submucosa and rarely seen ganglion cells. The pattern of expression of calretinin in HD pull-through specimens correlates with the rectal biopsy ones. Faint positivity of the thick submucosal and subserosal nerves in the absence of ganglion cells and calretinin positive nerve fibrils, is characteristic of the junction of the aganglionic-to-normal rectum. We are the first ones to document this finding.

Keywords: Hirschsprung, calretinin, pull-through specimen

Introduction

Hirschsprung’s disease is a congenital defect characterized by aganglionosis involving a segment of rectum and proximal bowel. It occurs in approximately 1/5000 birth, and the pattern of inheritance is complex, often non-Mendelian and shows variable penetrance [1]. The majority of children present in early infancy, with inability of passing meconium or constipation, accompanied by abdominal distension [2, 3]. But presentation later in life, with ongoing constipation, is not infrequent.

The diagnosis of Hirschsprung’s disease is rendered when no ganglion cells are seen in suction rectal biopsies. Hypertrophied submucosal nerve trunks, with a diameter greater than 40 microns, might be seen, too [4]. Many times, examination of tens of levels, if not of the entire block, is necessary for the finding of ganglion cells, or for giving an accurate diagnosis of “no
ganglions are seen". Some institutions use acetylcholinesterase histochemical stain to help guide the pathologists in their diagnosis [5]. Acetylcholinesterase will show abnormally coarse and dense nerve fibrils in the muscularis mucosae in aganglionosis, and it is the only auxiliary diagnostic method that is positive in HD. The drawbacks of acetylcholinesterase stain have been previously mentioned in the literature [6, 7]; it necessitates frozen tissue, it is a difficult histochemical stain that is performed only for suction rectal biopsies, and it needs a quantitative and qualitative assessment, and therefore a degree of subjectivity exists in its interpretation. Many institutions started to build their experience with calretinin immunohistochemical stain, as an additional diagnostic tool for HD [6, 8-10].

The goal of this article is to unfold the experience that the pathologists in the Department of Pathology at University of Texas at Houston accumulated over the past three years, to compare their findings with the ones reported in the literature, and to address the calretinin immunohistochemical findings at the junction of aganglionic-to-normal rectum in pull through specimens from patients with HD.

Material and methods

Case and control selection

In January 2010, three pathologists in the Department of Pathology at University of Texas at Houston started evaluating calretinin immunohistochemical stain on levels 40 and 41 of each rectal biopsy for evaluation of HD that was received in pathology. Twenty-eight cases accumulated within 2010-2011-time period. No effort was made to select biopsies in which the diagnosis was challenging or the specimen was suboptimal. All biopsies received for evaluation of HD were included. The age of the patients ranged from 15 days to 8 years. Twenty-three of the 28 cases (23/28) were suction rectal biopsies, and 5 were rectal full thickness biopsies. At least 80 H&E levels cut without waste were examined for each case; for some cases with suboptimal amount of mucosa or no ganglion cells seen, levels without waste were cut through the block.

Also, H&E sections of nine pull-through specimens corresponding to nine aganglionic suction rectal biopsies presented here were included in the study in order to characterize the pattern of calretinin immunostain at the junction of aganglionic-to-normal rectum in HD. The pull-through specimens were received fresh, oriented distally. They were opened longitudinally, and an entire longitudinal section was submitted for each case, with the orientation maintained from proximal-distal. Additionally, a cross section of the distal margin was submitted en face for each specimen. All sections for H&E and calretinin were cut 4 microns thick.

Two of the observers were pediatric pathologists with lengthy experience in evaluation of rectal biopsies for HD. The third observer was a senior resident with a specific career interest in pediatric pathology, who examined HD specimens for two years. All three observers had limited experience with calretinin IHC in the context of HD prior to 2010. The knowledge in this area was gathered from the study of the existing literature and prior occasional calretinin IHC for the evaluation of HD. Acetylcholinesterase stain is not currently performed in the Department of Pathology at University of Texas at Houston, due to technical reasons.

Calretinin immunohistochemical stain in rectal biopsy specimens and pull-through specimens

Calretinin immunohistochemical stain (monoclonal mouse anti-calretinin antibody, clone Z11-E3, 1:1000, Invitrogen, Camarillo, CA) was performed on levels 40 and 41 of each rectal biopsy. A negative control was performed on level 42 of each rectal biopsy. A calretinin immunostain was performed on each block representing the junction from aganglionic-to-normal rectum of the nine pull through specimens. Also, a negative control was performed for each of these latter cases.

The rectal biopsies in which ganglion cells were identified, as well as the ganglionic segments of the pull through specimens, were used as intra-study controls for calretinin immunohistochemical stain.

Results

Hematoxylin-eosin diagnosis of the rectal biopsies

Thirteen of the twenty-eight (13/28) rectal biopsies included in this study had no ganglion
Figure 1. A: Calretinin IHC x400: Section shows rectal biopsy lamina propria with calretinin positive thin, linear, granular nerve fibrils, in a non-HD case. **Figure 1A** inset: Calretinin IHC x400: The submucosa of the same case shows positive ganglion cells and expression of calretinin in the nerve trunks. B: Calretinin IHC x400: In another non-HD case, lamina propria shows linear nerve fibrils with a granular pattern of reactivity on calretinin IHC. C: Calretinin IHC x40: Suction rectal biopsy specimen with suboptimal amount of submucosa and very few ganglion cells seen at cutting through the block. There are a few submucosal cells that express calretinin, possibly mast cells. D: Calretinin IHC x400: This is a section of the same case represented in **Figure 1C**. Even if the amount of submucosa was suboptimal, and the ganglion cells were difficult to find when cutting through the block, the lamina propria shows the linear, granular pattern of reactivity that was mentioned above. E: Calretinin IHC, x400: Section shows the lamina propria of an HD specimen. No nerve fibrils are present in the lamina propria. There were no nerve fibrils present in the superficial submucosa and muscularis mucosae. There is non-specific pale stain in the crypts. F: Calretinin IHC, x400: In a limited number of suction rectal biopsies with a diagnosis of HD, the submucosal hypertrophied nerve trunks showed axonal expression of calretinin in a faint, granular pattern. This finding correlated with the junction of aganglionic-to-ganglionic segment of bowel in pull-through specimens, and extended 1.5-2.5 cm proximal to the aganglionic segment.
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cells found after the examination of the entire block. Hypertrophied submucosal nerve trunks were seen in these biopsies. These findings were consistent with aganglionosis. Fourteen of the 28 cases (14/28) had ganglion cells seen either easily or after additional H&E levels through the block, excluding a diagnosis of HD. One case (1/28) had no submucosa. Rebiopsy was suggested for this case. Ten of the cases (10/28) had suboptimal amount of submucosa per published standards [11], and ganglion cells were seen on H&E examination on eight (8/10) of these cases. Rebiopsy was suggested in the other two cases (2/10).

Hematoxylin-eosin evaluation of the nine pull-through specimens, corresponding to nine HD rectal suction biopsies that had prior evaluation and were included in this study, showed an abrupt debut of ganglion cells in the normal segment of bowel. Hypertrophied submucosal nerve trunks persisted very close (within approximately 1.5 centimeters) to the beginning of the ganglinated segment.

Calretinin pattern of expression in normal rectum

Calretinin immunohistochemical stain highlighted the ganglion cells, and the submucosal and subserosal nerve trunks in normal rectum (Figure 1A inset). Also, it was expressed in a linear, granular pattern, in the nerve fibrils of the superficial submucosa, muscularis mucosae and lamina propria (Figure 1A and 1B, lamina propria shown). A few submucosal cells, probably mast cells, were highlighted by calretinin IHC, too (Figure 1C). These findings were common to normal suction rectal biopsies, full thickness biopsies and ganglionic segments of pull-through specimens. In the normal section rectal biopsies, the linear thin nerve fibrils were seen in the lamina propria, muscularis mucosae and superficial submucosa, even in the levels that did not show ganglion cells in the submucosa. Two of the ten (2/10) suction rectal biopsies with suboptimal amount of submucosa did not show ganglion cells at leveling through the block (Figure 1C). But calretinin IHC showed nerve fibrils in the lamina propria and muscularis mucosae, even in the absence of ganglion cells (Figure 1D). Re-biopsy was suggested for these two cases.

Calretinin pattern of expression in aganglionosis

Calretinin immunohistochemical stain was negative in the aganglionic segments of bowel examined. (Figure 1E) There was no lamina propria, muscularis mucosae or superficial submucosa nerve fibrils seen. Occasional cells, possible mast cells, expressed calretinin in the submucosa. Eleven (11/13) of the rectal biopsies with HD showed no calretinin reactivity in the submucosa nerve trunks, negative finding that was previously described in the literature [6]. Two of the HD rectal biopsies (2/13) showed no ganglion cells, no calretinin positive nerve fibrils in the lamina propria, muscularis mucosae or superficial submucosa, but, unexpectedly, there was faint, granular, axonal reactivity in the submucosal hypertrophied nerve trunks. (Figure 1F). These two cases were reported with a diagnosis of HD.

Calretinin immunohistochemical stain performed on the junction from aganglionic-to-ganglionic segment of nine pull-through specimens showed the patterns described above for the ganglionic and aganglionic segments. However, the aganglionic segment within 1.5-2.5 centimeters to the junction showed faint, granular expression in the axons of the submucosal and subserosal still enlarged nerve trunks, in the absence of ganglion cells or granular, linear staining in the nerve fibrils of the superficial submucosa, muscularis mucosae or lamina propria. These findings were identical with the ones observed in the two HD rectal biopsies described in the paragraph above, and they are unique to the 1.5-2.5 centimeters in which the transition is made from the aganglionic segment to normal rectum.

Discussion

Hirschsprung disease has a complex pathogenesis that, to date, is not completely understood. Significant progress has been done for a better molecular characterization; there is a very strong body of literature pointing to specific genes being involved in the developments and migration of the enteric nervous system [12]. For example mutations in RET on chromosome 10q11.2 was shown to be responsible of approximately 40% of the sporadic cases of HD. But HD can be familial or associated with
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other syndromes and chromosomal anomalies [13-16] and in many of these situations, multiple other genes are involved in pathogenesis. Most likely, these genetic and molecular findings have strong potential to become additional diagnostic tools in the future.

On clinical service, the most utilized method of diagnosis of HD is histopathologic. The first encounter that a pathologist has with a specimen submitted for evaluation of HD, is in the form of a suction rectal biopsy. The gastroenterologists and pediatric surgeons are trained to provide biopsies taken more than 2 cm proximal to the pectinate line, and the biopsy needs to have an adequate amount of submucosa, for the evaluation of enteric nervous system. Although this is always the goal of the procedure, absolute precision is difficult to achieve. Not infrequently, the pathologic specimen consists of a small fragment of bowel mucosa with limited amount of submucosa, and the distance to the pectinate line is not always completely known. Most pathologists examine multiple H&E levels, and sometimes the entire block of tissue on light microscopy. Few institutions are experienced with performance and interpretation of acetylcholinesterase histochemical stain. It is the only histologic technique that shows positive findings in HD (a meshwork of coarse, thick, disorganized nerve fibrils in the muscularis mucosae and superficial submucosa). Acetylcholinesterase is performed on frozen tissue, and requires a quantitative and qualitative interpretation. It is a histochemical stain that is performed only for evaluation of HD, and the success of the staining is greatly dependent on the experience of the histotechnician. Because it is not performed on the same fragment of tissue as the H&E stain, correlation of the microscopic findings is not absolute. During the past last years, more and more institutions started to build their experience with calretinin immunohistochemical stain, as an auxiliary method of diagnosis of HD. In 2009, Kapur et al., in a comparative study of calretinin and acetylcholinesterase stain performed on a total of fifty-three cases, showed that there was better diagnostic accuracy in the diagnosis of HD when compared with acetylcholinesterase. Also, there was less interobserver variability in the interpretation of the immunohistochemical stain.

Additionally, four of the five observers in the study did not have prior experience with interpretation of calretinin immunohistochemical stain in the context of HD. After a short tutorial, they were able to interpret the stain accurately, and their interpretation was used in the study.

In the past, in the Department of Pathology at University of Texas at Houston, the evaluation of suction rectal biopsies was done exclusively on H&E. Absence of ganglion cells, accompanied by hypertrophic nerve fibers, in a specimen with adequate amount of submucosa that was not taken from the physiologic hypoganglionic zone, was given a diagnosis of HD if the clinical presentation fit. But not infrequently, a final diagnosis cannot be issued because of quality of tissue limitations, or because there is paucity of ganglion cells, and/or they are immature. Because the majority of these patients are young infants, increasing the chance to a definite diagnosis is crucial. In January 2010, we installed a protocol for evaluation of rectal biopsies, in which calretinin is performed on levels 40 and 41 for each case submitted for evaluation of HD.

Our microscopic observations for H&E stain and calretinin immunohistochemical stain in normal rectum coincide with the ones reported in the past and current literature: calretinin highlighted the ganglion cells in the submucosa, and the nerve fibrils in the lamina propria, muscularis mucosae and superficial submucosa, in a linear, granular pattern. Because of a non-specific background in the submucosa in some cases, the observers of this study felt most comfortable with interpretation of positive nerve fibrils in the lamina propria and muscularis mucosae. The submucosal nerve trunks showed axonal staining. In rectal biopsies in which a diagnosis of HS was issued, there was no expression of calretinin in the lamina propria and muscularis mucosae. There were no ganglion cells highlighted by calretinin. Eleven (11/13) of the rectal biopsies with a diagnosis of HD, showed no axonal calretinin reactivity in the hypertrophied nerve trunks. Two of these biopsies (2/13) showed no ganglion cells and no calretinin immunoreactivity in any of the compartments, except faint granular axonal positivity in the submucosal hypertrophied nerve trunks. These same findings were observed in the nine pull through specimens in which calretinin was performed on the sections.
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showing the transition form affected-to-unaffected bowel; the 1.5-2.5 centimeters proximal to the junction with normal rectum showed no ganglion cells, and no calretinin positive thin nerve fibrils. However, there was faint axonal positivity in the submucosal and subserosal hypertrophied nerve trunks. The authors of this manuscript do not have an explanation for this pattern. Such finding, if in a suction rectal biopsy, should prompt a diagnosis of HD, as it translates in a location of the biopsy in the aganglionic segment, and most likely within the 2.5 centimeters proximal to the junction with the unaffected colon.

Conclusions

Like many other institutions, we looked into additional methods of diagnosis to assist us in the evaluation of suction rectal biopsy specimens. We found calretinin to be a reliable additional diagnostic tool, when correlated with the H&E microscopic findings. It can assist the pathologist in the diagnosis of cases with paucity of ganglion cells, immature ganglion cells and suboptimal amount of submucosa.

The finding of calretinin reactivity in the nerve trunks, in the absence of ganglion cells and calretinin positive nerve fibrils in the lamina propria, muscularis mucosae and superficial submucosa, might lead the pathologist away from the diagnosis of HD, and such finding it is important to be recognized, and taken into consideration. This finding spans throughout 1.5-2.5 centimeters of colon.

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Disclosure of conflict of interest

None.

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