EVALUATION OF CALRETININ IMMUNOHISTOCHEMISTRY AS AN ADDITIONAL TOOL IN CONFIRMING THE DIAGNOSIS OF HIRSCHSPRUNG DISEASE

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Hirschsprung disease (HD) is a congenital malformation defined as the absence of myenteric and submucosal ganglion cells (GCs) in the distal rectum and variable length of the contiguous bowel. The aim of this study was to assess the utility of calretinin immunochemistry in comparison with that of standard histology complemented with acetylcholinesterase (AChE) histochemistry routinely employed at our institution to evaluate rectal biopsies carried out for suspicion of HD. Twenty-one rectal biopsies were reviewed, including 14 from patients with suspected HD, 6 from infants with necrotizing enterocolitis (NEC), and 1 from a patient diagnosed with spontaneous intestinal perforation (SIP). Sections stained with hematoxylin-eosin (HE) revealed absence of ganglion cells in 13 cases which included 11 patients with HD and 2 patients with NEC. Among 13 cases of aganglionosis the AChE reaction pattern was consistent with HD in 2 patients. Calretinin positivity was observed in all rectal biopsies showing the presence of GC, and the staining was consistently absent in all cases of aganglionosis. In 6 rectal biopsies in which abnormal acetylcholinesterase (AChE) staining was not seen, loss of calretinin immunoreactivity helped establish the diagnosis of HD.

Key words: Hirschsprung disease, calretinin, acetylcholinesterase, ganglion cells.

Introduction

Hirschsprung disease (HD) is a common congenital disorder (1 in 5000 newborns) characterized by a lack of ganglion cells, resulting in bowel obstruction secondary to a distal narrow aperistaltic hypertonic segment, which is aganglionic, and a dilated proximal segment caused by obstruction. In the most common form (75% to 80% of cases), known as short-segment disease or classic Hirschsprung disease, the aganglionic segment begins in the rectum and extends for a variable distance in the adjoining proximal dilated bowel. In a smaller number of cases (10-20%), the disease extends proximally beyond the splenic flexure (long-segment disease). In rare instances (5%) the disease involves most or all of the large bowel and occasionally extends even to the small bowel (total bowel aganglionosis). Approximately 90% of patients present in infancy with constipation, abdominal distention, vomiting, and delay of meconium passage. Diarrhea may occur, and some infants can be affected by life-threatening enterocolitis and toxic megacolon. Hirschsprung disease is an important clinical differential diagnosis in infants and children presenting with severe constipation [1]. The diagnosis of HD is based on histological features [2]. Microscopically, the hallmark of the disease is the absence of ganglion cells (aganglionosis) in both submucosal and myenteric plexuses of the affected segment of bowel. This is often associated with the presence...
of hypertrophied (> 40 µm) nerve fibers in the aganglionic segment. In routine practice, a biopsy that samples the mucosa and submucosa is considered the method of choice to establish a pre-operative diagnosis. The biopsy should include an adequate amount of submucosa, e.g. submucosa equal in thickness to the mucosa. However, in some cases, the diagnosis of aganglionosis may be difficult on routine hematoxylin-eosin (HE) stained histologic sections alone. 

Hirschsprung disease remains a challenging diagnosis, especially among general surgical pathologists who evaluate these cases infrequently and lack sufficient experience. In the neonatal period, submucosal GCs may not be easily recognizable because they are typically small and undifferentiated. Characteristic neuronal nuclear and cytoplasmic features may not be present. Many pathologists prefer to examine frozen section slides stained for acetylcholinesterase (AChE) in addition to standard HE-stained sections. In Hirschsprung disease, examination using the acetylcholinesterase stain demonstrates increased acetylcholinesterase-positive nerve fibers in the lamina propria and muscularis mucosae. The utility of this technique as an essential component in the accurate diagnosis of aganglionosis is debated. Acetylcholinesterase histochemistry requires fresh frozen tissue, the method demands superior technical expertise of the laboratory, and false-positive, as well as false-negative reactions have been reported. Acetylcholinesterase staining has been associated with relatively high rates of interobserver variability in the interpretation. The use of this method appears to be a matter of personal preference. Meier-Ruge et al. have demonstrated that the absence of ganglion cells in Meissner’s plexus, accompanied by increased AChE activity, is highly specific for HD [3]. Despite a high degree of accuracy, results are not always uniform, and reaction patterns may be different depending on the variant of the disease and patient’s age. In the ultra-short segment form of HD, increased AChE activity is demonstrable only in nerve fibers of the muscularis mucosa and in the submucosa, but not in the lamina propria [4]. False negative AChE results related to patient’s age present additional diagnostic difficulty. An absence of characteristic AChE reaction does not exclude HD in neonates within the first 3 weeks of life [4, 5]. Immunohistochemical (IHC) techniques provide additional helpful tools for the diagnosis of HD. In recent years, the use of several markers was attempted to aid the diagnosis of this disorder, including S-100 protein [7, 8], neuron specific enolase (NSE) [8], glial fibrillary acid protein (GFAP) [9, 10], glucose transporter 1 (GLUT-1) [11], microtubule associated protein 5 (MAP-5) [12], and others. None of the above stains has been widely adopted. In 2004, Barshack et al. demonstrated that the absence of calretinin expression in nerve fibers correlated with aganglionosis in patients with HD [13]. Calretinin is a vitamin D dependent calcium binding protein. The anti-calretinin monoclonal antibody stains 80% of ganglion cells as well as small intrinsic nerve fibers of the submucosal and myenteric plexus in normal colon [14]. Loss of calretinin-immunoreactive nerves has been documented to be characteristic of HD. The stain has only recently gained broader recognition and has become the most common adjunct immunohistochemical marker used to facilitate the diagnosis of HD. The stain is easy to interpret and works with paraffin embedded material [13-16]. Numerous studies comparing the results of calretinin immunohistochemistry with those of AChE histochemistry demonstrate that calretinin may be superior to AChE assay [1, 17-19].

We evaluated the patterns of calretinin expression in rectal biopsies obtained from HD and non-HD patients diagnosed at our institution. We compared
the utility of calretinin stain with that of traditional AChE histochemistry used in routine clinical practice by our laboratory in conjunction with histologic examination of HE-stained preparations.

**Material and methods**

The rectal biopsy samples were obtained from 21 patients ranging from 5 days to 17 years of age. Tissues were obtained from 14 patients who presented with chronic constipation and underwent diagnostic workup to exclude HD. The study group also included 6 patients diagnosed with necrotizing enterocolitis (NEC) and 1 patient with spontaneous intestinal perforation (SIP). Paraffin-embedded, 4 μm thick sections were stained with HE. Additional unstained paraffin-embedded sections were used for calretinin IHC. Calretinin staining was performed with an automated immunostainer from Dako, using mouse anti-human calretinin from Dako (Calret 1, Dako, Denmark). For AChE histochemistry, frozen sections were stained according to a modified Karnovsky-Roots method. The immunostained slides were evaluated by a pediatric pathologist who had no prior knowledge of the histologic diagnosis or clinical outcome. Calretinin immunostaining was scored as either positive or negative. Calretinin stain was considered positive if any staining was present either within the submucosal plexus or in the lamina propria. Positive calretinin reaction in mast cells served as an additional, internal positive control. Subsequent review of the biopsy material was performed in conjunction with pathologic evaluation of surgical resection specimens and final clinical diagnoses.

**Results**

Ganglion cells were absent in 13 of 21 preoperative rectal biopsies. This group of 13 cases in which histologic evaluation confirmed aganglionosis included 11 patients clinically suspected to have HD and 2 patients diagnosed with NEC. Acetylcholinesterase reaction pattern fully consistent with HD was observed only in 2 aganglionic biopsies, and focal increase in AChE activity was observed in 1 case. Neg-
ative AChE staining excluded the diagnosis of HD. In the remaining 8 biopsies ganglion cells were identified by HE, and this finding was associated with diffusely increased AChE activity in 1 case while a focally positive pattern was seen in 3 biopsies. The presence or absence of calretinin expression was assessed concurrently in all biopsy samples by immunohistochemistry. Calretinin positivity was consistently present in all biopsies where the presence of ganglion cells was documented by HE. Loss of calretinin-immunoreactive nerves characteristic of HD was observed in 8 of 13 cases of histologically confirmed aganglionosis. In 5 biopsy specimens devoid of ganglion cells on HE stained sections, calretinin positivity was present, and the diagnosis of HD was ruled out (Table I). Pre-operative diagnoses of HD were subsequently confirmed in all cases by intraoperative seromuscular biopsies and examination of surgical resection specimens (Figs. 1, 2).

Discussion

Histopathologic analysis of suction rectal biopsies that sample mucosa and underlying submucosa remains the mainstay for diagnosis of HD. The diagnostic approaches vary among institutions as there are no established strict practice parameters [20]. Some centers base the diagnosis mainly on morphologic examination of serial sections of rectal biopsy specimens stained with HE; others use histochemical stains on frozen sections as their primary diagnostic tool, and still others rely on AChE as an adjunct to routine histology [21]. Examination of serial HE-stained sections from paraffin-embedded mucosal biopsies is the most common approach. However, detection of ganglion cells based solely on their HE appearance is not always straightforward. The density of submucosal ganglion cells decreases in the terminal rectum, and the first centimeters of the anal canal are normally hypoganglionic. To avoid a potential sampling problem it is recommended that the biopsy be obtained at least 2-3 cm above the dentate line. An additional obstacle may arise when the biopsy is suboptimal due to a paucity of submucosa. An adequate amount of submucosa is essential for accurate assessment of the presence or absence of ganglion cells. Hypertrophy of submucosal nerves is a useful diagnostic clue. However, this finding is not present in all cases and by itself is not considered sufficient evidence to establish the diagnosis of HD. Submucosal ganglia are relatively sparse and are considered to be most abundant along the internal layer of the muscularis propria, in the deep portion of the submucosa that is not always sampled well by the suction biopsy technique [23]. Pathologists occasionally encounter suboptimal biopsy specimens that can be very difficult to evaluate or simply insufficient for the diagnosis, requiring patients to undergo repeat biopsy procedures. Identification of ganglion cells in neonates and premature infants can also be a challenging task because neurons are often undifferentiated and confused with endothelial cells, histiocytes or lymphoid cells. Supportive evidence can be obtained with the use of ancillary methods such as histochemistry or immunohistochemistry in problematic cases. Numerous published reports state that AChE histochemistry is essential for a definitive diagnosis of HD in rectal mucosal biopsies [6]. A characteristic increase in AChE activity is associated with hypertrophied extrinsic nerve fibers of the aganglionic segment in HD [23]. However, Hamoudi et al., who evaluated 131 specimens using AChE histochemistry, demonstrated false-negative reactions in 29% of cases. The same authors report that an abnormal pattern of AChE is impossible.
diagnostic, whereas a normal pattern does not exclude HD [24–26]. Difficulty in interpretation of the stain is common among pathologists with insufficient experience in interpretation of AChE histochemistry in rectal biopsies. Lack of sensitivity in neonates and in cases of long segment variant of HD are also among the known problems associated with AChE-based diagnosis. The AChE staining protocol requires frozen sections, and a separate biopsy is typically needed, posing additional disadvantage. Since 1988, at least 10 immunohistochemical stains have been reported to facilitate the diagnosis of HD by highlighting ganglion cells. However, none of them offers a significant advantage over histologic examination of HE stained preparations, and in current practice the majority of them are rarely useful [7-12]. In 1994 McConalogue et al. demonstrated that the calretinin antibody highlights different neuronal populations in the large intestine. Since then a number of comparative studies have been published on the expression of calretinin in HD and its utility as a marker for assisting in the diagnosis of HD. Calretinin belongs to the EF-hand family of calcium-binding proteins, which comprises over 150 members. The calretinin antibody is often used in IHC panels to assist in the diagnosis of mesotheliomas. Calretinin has been shown to have a broad tissue distribution, which also includes strong expression in neural elements [27]. Barshack et al. investigated calretinin immunoreactivity in ten large bowel specimens from patients with classic rectosigmoid HD and documented loss of calretinin expression in aganglionic segments in both the submucosal and myenteric plexus. Both ganglion cells and nerve fibers expressed calretinin in ganglionic segments of HD specimens and in normal controls [13]. In a 2009 study by Kapur et al., the patterns of calretinin expression in suction rectal biopsies were evaluated, and the diagnostic value of the stain was compared with a still relatively widely used rapid AChE method. Interobserver disagreements were reported to be common occurrences in cases of AChE interpretation. Calretinin IHC eliminates this problem and appears to be a reasonable and potentially superior alternative to AChE as an adjunctive diagnostic method [19]. Similar observations were published by Guinard-Samuel et al., who investigated a large series of rectal suction biopsies from patients with suspected HD, and demonstrated that calretinin is more accurate than AChE. However, they also showed that in short-segment HD a slight calretinin positivity can be observed [23]. In our study, calretinin positivity was observed in all biopsies in which ganglion cells were documented by histology. The stain was also noted to be positive in five aganglionic biopsies. However, these aganglionic biopsies were also negative for AChE, and the application of calretinin proved to be helpful in excluding diagnosis of HD. Characteristic loss of calretinin expression was observed in 8 biopsies where ganglion cells were not identified. Six aganglionic rectal biopsies lacked hypertrophic nerve fibers. Negative calretinin reaction pattern in this subset aided the final diagnosis of HD. Our study adds to the growing evidence that calretinin immunohistochemistry is a valuable adjunct during workup of problematic cases, potentially reducing the need for repeat biopsies or full thickness rectal biopsies. Calretinin immunohistochemistry technique also holds several practical advantages over the traditional AChE histochemistry. It is carried out on a permanent, formalin-fixed, paraffin-embedded tissue. The staining pattern is simple and distinct, and equivocal or misleading results are rare, as the stain is either positive or negative.

In conclusion, our study further emphasizes the value of calretinin immunohistochemistry as a diagnostic aid in histopathologic evaluation of rectal biopsies for HD.

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References


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