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Original Article

Calretinin immunohistochemistry for the diagnosis of Hirschprung disease in rectal biopsies

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ABSTRACT

In this study we aimed to evaluate the usability of calretinin staining in the diagnosis and exclusion of HD in 36 rectal biopsies. Through immunohistochemical examination, in of a total of 21 pediatric patients in whom ganglion cells were detected in first rectal biopsies and in re-biopsies, ganglion cells were seen through nuclear and cytoplasmic staining. In the lamina propria and superficial submucosa, staining of nerve fibers was detected in a granular pattern in varying intensities. Out of a total of 5 biopsies (including one re-biopsy) of non-HD patients, where ganglion cells could not be seen, the nerve fibers were all stained. On the other hand, in 10 HD patients, diagnosed by a colon pull through operation, calretinin staining was not detected in any area of the rectal biopsies except for the mast cells. We conclude that calretinin immunostaining for the diagnosis of HD is an easy and reliable method for use in daily practice.

Introduction

The question of whether or not there is a ganglion cell in small biopsies has always troubled pathologists in their practice of pathology. During intraoperative evaluation, the stress begins even before the sample reaches the laboratory. There are many reasons for this: lack of experience (pathology laboratories on the whole do not see enough of these samples to gain experience); small and inadequate samples; contusion artifacts; difficult tissue orientation; presence of immature ganglion cells in the neonatal period; resemblance of lymphocytes, stromal and endothelial cells to ganglion cells, etc.

Most of the time, the clinician takes a biopsy with the initial diagnosis of Hirschprung disease (HD), or to exclude that diagnosis, in patients presenting with an inability to produce stools and abdominal distension. As is well known, HD refers to the congenital absence of ganglion cells in the submucosal and myenteric plexus of the colon, and on many occasions, this is accompanied by hypertrophic nerve fibers. A rectal biopsy is the standard approach in diagnosis, however there is no specific standard for pathological evaluation. In institutes that specialize in the area of pediatric pathology, histochemical staining with acetylcholinesterase (ACE) is applied to frozen sections, in addition to routine hematoxylin and eosin (H&E) to serial sections. In some centers in Europe and Japan, only a histochemical staining panel is applied, and a paraffin section is not utilized [1,2]. However in many pathology laboratories around the world, only serial sections stained with H&E are used, as ACE requires a frozen section, furthermore, it is difficult to comment on it as it relies on experience, and also presents with a low sensitivity (85%) [3,4]. For instance, with ACE staining, prominent hypertrophy in nerves cannot be seen, even in cases with total colon aganglionosis [1]. However, too few studies have been reported in the literature to prove the superiority of either method [2].

When immunohistochemical methods that may be helpful in the diagnosis of HD have been considered, various antibodies such as S-100 [5], neuron-specific enolase (NSE) [5], glial fibrillary acidic protein (GFAP) [6], peripherine [7], c-kit [8,9], Bcl-2 [10], microtubule-associated protein-5 (MAP-5) [11] have all been used for the detection of ganglion cells and hypertrophic nerve fibers; while a nerve growth factor receptor (NGFR) [12], synaptic vesicle protein-38 (SVP-38) [13], syntaxin 1A [14], neuronal nitric oxide syntase (nNOS) [9,15], and growth-associated protein-43 (GAP-43) [16] have been used for the detection of intrinsic nerve fibers only. However, of all these methods, none have been shown to have the edge over ACE in the diagnosis of HD.

Taking a different approach from these other studies, in 2004 Barshack et al. showed, for the first time, that calretinin is expressed only in ganglionic segments from the large bowel and not in aganglionic segments [10]. They suggested that this method could be

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Table 1
H&E and calretinin staining of the rectal biopsy from 14 patients.

Case	Rectal biopsy	Rebiopsy	Operation	Diagnosis
1	Ganglion cell(−)/calretinin(−)	N/A	Pull through	HD
2	Ganglion cell(−)/calretinin(+)Only lamina propria	Ganglion cell(+)/calretinin(+)	N/A	Non-HD
3	Ganglion cell(−)/calretinin(−)	N/A	Pull through	HD
4	Ganglion cell(−)/calretinin(−)	N/A	Pull through	HD
5	Ganglion cell(−)/calretinin(−)	N/A	Pull through	HD
6	Ganglion cell(−)/calretinin(+) Transitional zone	Ganglion cell(+)/calretinin(+)	N/A	Non-HD
7	Ganglion cell(−)/calretinin(−)	N/A	Pull through	HD
8	Ganglion cell(−)/calretinin(−)	N/A	Pull through	HD
9	Ganglion cell(−)/calretinin(−)	N/A	Pull through	HD
10	Ganglion cell(−)/calretinin(−)	N/A	Pull through	HD
11	Ganglion cell(−)/calretinin(−)	N/A	Pull through	HD
12	Ganglion cell(−)/calretinin(+) Transitional zone	Ganglion cell(−)/calretinin(+)	Pull through Ganglion cell(+)	Non-HD
13	Ganglion cell(−)/calretinin(−)	N/A	Pull through	HD
14	Ganglion cell(−)/calretinin(+)	N/A	Pull through Ganglion cell(+)	Non-HD

HD: Hirschsprung disease.

used in rectal biopsies [8]. Five years later, in studies performed with rectal biopsies, one by Kapur and another by Guinard-Samuel, it was found that calretinin was a superior, and much simpler method for detection of HD than ACE [17,18]. In the guideline prepared by the Gastro 2009 International Working Group, of which Kapur was a member, the phrases "aganglionic calretinin immunohistochemical pattern" and "normal calretinin immunohistochemical pattern" were added to the report format for rectal biopsies, however they mentioned that re-biopsy is required in the absence of ganglion cells, in spite of calretinin positivity, as there was, as yet, insufficient data [2]. There are a few other articles about the use of calretinin staining in HD in the English literature [8,17–24].

Calretinin is a calcium binding protein and it plays an important role in the organization and function of the CNS. Calretinin immunoreactivity has been detected in the submucosal and myenteric ganglions and in the nerve cell bodies of the human gastrointestinal tract [25,26]. As already understood from previous studies, it seems that the evaluation of calretinin immunoreactivity is very simple [17–20,23]. Namely: any positivity in the ganglion cells or nerve fibers in the lamina propria, submucosa and muscularis mucosa would exclude HD, while negativity would provide a diagnosis of HD. In this study we aimed to evaluate the usability of calretinin in the diagnosis and exclusion of HD in rectal biopsies, which would normally have been evaluated in our clinic only through H&E stained serial sections.

Materials and method

A total of seventy-seven pathology reports, containing the key words "ganglion cell, rectal biopsy and HD", from the period 2009–2012, were identified from the pathology records of the computerized system of our hospital. A total of 42 patients had been subjected to 55 small colon biopsies, 36 full thickness rectal biopsies, 12 instances of colon pull through operation (Duhamel-Martin and transanal endorectal), 2 instances of partial intestinal resection, 10 colostomy closures, 5 appendectomies, 1 consultation and 29 frozen examinations. All slides of rectal biopsies (1–4 formalin-fixed biopsies and two slides including 15 sections, per patient) and materials of pull-through operations were taken out of the glass archive and reevaluated under the light microscope. Only blocks of rectal biopsies were chosen and the primary antibody of calretinin (clone CAL6, Bond ready-to-use) was used for immunohistochemical staining in a Leica Bond max automatic staining device. In the immunohistochemical evaluation any staining of nerve fibers and/or ganglion cells found in the lamina propria, muscularis mucosa or submucosa was accepted as positive immunostaining.

Results

Fifteen of the 33 patients in whom the full thickness rectal biopsy was done were females, and 18 were males. Seven patients (5 of whom were HD patients) were in the neonatal period, and the mean age of all patients was 4.06 years, but it was 2 years for the HD patients. In all of the pathology reports, an initial diagnosis or suspicion of HD was reported; and in some of them dilatation of the sigmoid colon and perforation of the appendix were described; while, in patients in whom the abdomen had been investigated due to their not producing stools, constipation, vomiting, ileus, acute abdomen, functional constipation, abdominal distension, anal atresia, neuronal intestinal dysplasia, an enterocolitis attack, and annular pancreas had all been suggested in the clinical notes.

In the first biopsies, ganglion cells were seen in 19 out of the 33 patients, and HD was excluded. In 11 patients a ganglion cell was not seen. In three patients a re-biopsy was needed because two of the biopsies were taken in the transitional zone, and one biopsy consisted only of lamina propria. Ganglion cells were seen in two of these three re-biopsies and HD was excluded. Twelve patients underwent a colon pull-through operation, 10 of these were diagnosed as HD, however ganglion cells were seen in all areas of the surgical specimens of two of the patients (Table 1).

In the immunohistochemical examination, nuclear and cytoplasmic calretinin staining revealed ganglion cells in a total of 21 cases in which ganglion cells were detected either in the first rectal biopsies or in the re-biopsies. In the nerve fibers of the lamina propria and superficial submucosa, staining was detected in granular patterns of different intensities (strong, intermediate, weak). In particular, the staining in the lamina propria was found to be typical and diagnostic (Fig. 1). Nerve fibers were stained with calretinin in a total of three biopsies, including the re-biopsies where the ganglions could not be seen (Table 1). On the other hand, in the 10 patients diagnosed as HD, calretinin staining was not detected in any area except in the mast cells. The staining in the mast cells was pale and cytoplasmic (Fig. 2).

Discussion

The standard approach in the diagnosis of HD is a rectal biopsy [4]. However at what distance from the dentate line should the biopsy be taken? Although there is no definite consensus on this, according to many surgeons, the position of the biopsy should be at least 2–3 cm above the dentate line. Ganglion cells are found in 3 different plexus in the rectum: Superficial, deep submucosal and myenteric [27]. In the anal canal ganglion cells are absent, or are