

Can We Stop Looking?

Immunohistochemistry and the Diagnosis of Hirschsprung Disease

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The Malformation

The enteric nervous system (ENS) is essential for normal motility and many other physiologic properties of the gastrointestinal tract.¹ It is composed of intrinsic neurons, the cell bodies and most processes of which are located inside the bowel wall, and extrinsic nerve fibers that project into the gut from autonomic and sensory ganglia. Although extrinsic innervation modulates the activity of intrinsic neurons, the latter are necessary and sufficient for the complex reflexes pathways that ensure peristaltic activity. Intrinsic neurons of the ENS are located in the ganglia of two interconnected myenteric and submucosal nerve plexuses. Nerves in both plexuses are a mixture of intrinsic and extrinsic fibers and specialized enteric glial cells. Enteric nerves are ultrastructurally distinct from the central and nonenteric peripheral nervous systems. They project to many sites in all layers of the bowel wall, including the muscularis propria, muscularis mucosa, and lamina propria.

Congenital intestinal aganglionosis, or Hirschsprung disease, is a malformation of the ENS, in which the obligate diagnostic feature is absence of intrinsic ganglion cells from the distal rectum and a variable length of contiguous bowel.² It affects an estimated 1:5,000 liveborns and enters into the clinical differential diagnosis for infants, children, and adults with severe and/or chronic constipation. Dysmotility is due to constriction of the aganglionic segment, which requires intrinsic innervation to relax.

In addition to absent intrinsic ganglion cells, other malformations of the ENS are usually present in the aganglionic rectum of the patient with Hirschsprung disease. Perhaps the most striking and diagnostically useful finding is the presence of hypertrophic nerve fibers in the myenteric and submucosal plexuses. The diameters of normal submucosal nerves are less than 40 μm . In contrast, 90% of suction rectal biopsies from patients with Hirschsprung disease contain nerves with diameters of 40 μm or greater.³ These hypertrophic nerves represent extrinsic fibers,

primarily from pelvic autonomic ganglia. They are ultrastructurally and immunohistochemically similar to extrinsic nerves that exist normally in the mesentery and serosa, and which adopt an “enteric” phenotype when they merge with the intrinsic innervation. In aganglionic bowel, small branches from hypertrophic nerves distribute to the muscularis propria and mucosa⁴ but do not express synaptic proteins or other antigens that characterize the normal intrinsic innervation of these targets.⁵⁻⁷

The Problem

Despite the seeming anatomic simplicity of this condition, diagnosis of Hirschsprung disease can be a stressful experience, particularly for pathologists who encounter the condition infrequently. Establishing a diagnosis of Hirschsprung disease commits the patient to a surgical procedure, often with no confirmation of the diagnosis until the rectum with or without the adjoining colon has been resected.

In most centers, the diagnosis is based on histopathologic analysis of suction rectal biopsies that sample mucosa and underlying submucosa.⁸ The cornerstone of diagnosis is failure to identify submucosal ganglion cell soma, despite adequate sampling. Two fundamental anatomic facts complicate the task.⁴ First, submucosal ganglia are relatively sparse and are most abundant along the internal layer of the muscularis propria, in the deep portion of the submucosa that is not sampled well by the suction biopsy technique. Second, the density of submucosal ganglion cells declines significantly in the terminal rectum, such that a physiologic hypoganglionic zone exists in a 1 to 3 cm long segment proximal to the pectinate line. Even in normal individuals a generous biopsy from this region may be aganglionic. Clinicians are taught to take biopsies just proximal to this region but caudal enough to detect very short segment aganglionosis. However, the occasional presence of anal squamous mucosa in suction rectal biopsies serves as a reminder that, despite an overt effort to

biopsy proximal to the terminal 1 to 3 cm of rectum, the targeting skills of those who procure the biopsy are sometimes imprecise.

In general, 2 different approaches have evolved to identify ganglion cells. The first, which is used in most pediatric pathology laboratories, is to evaluate numerous H&E-stained levels from each paraffin-embedded biopsy.⁸ The reliability of this method depends on the observer's ability to accurately distinguish a ganglion cell based on its H&E appearance. The relatively undifferentiated and nonneuronal appearance of "immature" ganglion cells that exist in the submucosa of neonates, particularly premature infants, is frequently cited as a difficulty associated with H&E-based diagnosis of Hirschsprung disease. A second approach relies on only frozen sections that are stained histochemically to resolve ganglion cell bodies based on their high levels of lactate dehydrogenase (mature) and/or nitric oxide synthase (immature) activity.⁹ The latter technique is primarily used to diagnose Hirschsprung disease in parts of Europe.

The primary challenge associated with the diagnosis of Hirschsprung disease is to confidently exclude the presence of even a single ganglion cell from a biopsy in which only a handful of ganglion cells may exist; with either the H&E- or histochemistry-based approach, adequate sampling is critical. Because submucosal ganglia are widely distributed, a generous amount of submucosa must be obtained, and 100 or more histologic sections may need to be analyzed.⁸ Ultimately, a pathologist must rely on his/her ability to recognize ganglion cells, usually, but not always, in the context of hypertrophic submucosal nerves and abnormal innervation of the mucosa.

For several decades, researchers have sought simple, reliable specific methods to facilitate Hirschsprung disease diagnosis from suction biopsies. The most widely utilized technique of this type is acetylcholinesterase histochemistry (AChE staining), which was introduced by Meier-Ruge et al.¹⁰ They observed that AChE staining resolves abnormally thick and numerous nerve fibers in the muscularis mucosa and lamina propria of suction rectal biopsies from patients with Hirschsprung disease, in contrast to relatively sparse, thin AChE-positive nerve fibers that are limited largely to the deep muscularis mucosa in normal rectal mucosa. Even though AChE staining requires frozen sections from a separate biopsy, many laboratories use this method as a complement to H&E-stained paraffin sections. The AChE stain does not obviate the need to thoroughly exclude ganglion cells, because a Hirschsprung disease–like pattern can occasionally be observed in other contexts.⁹

For decades many laboratories have used an H&E-based approach with or without ancillary AChE staining to diagnose Hirschsprung disease. Some experienced investigators report 99% sensitivity and no false-positive results using these methods.^{11,12} However, others have reported more variable results, as discussed by Tam and Owen.¹³ As a consequence, a search has continued for new simple and reliable diagnostic methods.

The Holy Grail?

The limitations associated with an H&E-based approach to the diagnosis of Hirschsprung disease, with or without AChE histochemistry, have fueled efforts to identify immunohistochemical markers that can simplify diagnosis. In addition to high sensitivity and specificity, an ideal immunohistochemical test would be easy to employ and interpret, work with paraffin sections of suction biopsies, and circumvent the need to analyze large numbers of histologic levels. However, despite a huge number of studies devoted to immunostaining patterns of various neural and nonneural antigens in Hirschsprung disease, no single antibody or panel of antibodies has achieved these properties, nor has immunohistochemistry received general acceptance in the routine diagnosis of Hirschsprung disease. These shortcomings stem in part from the properties of the antigens that have been studied, but also from deficiencies in study design.

Most of the antigens with altered distributions in the aganglionic rectum of patients with Hirschsprung disease can be placed in one of the five groups listed in **Table 1**.^{5-7,13-21} In principle, those that primarily highlight ganglion cell bodies can be used to accurately identify ganglion cells, which may be difficult to confidently recognize in H&E-stained sections. Papers that tout the value of these immunoreagents generally emphasize the potentially ambiguous H&E appearance of immature ganglion cells, a problem that many pathologists overcome with experience. While antibodies may facilitate identification of ganglion cells, they do not eliminate the need to examine numerous levels and therefore may require immunostaining several slides from each case. Concurrent immunoreactivity with nerve fibers labels hypertrophic submucosal nerves but can obscure ganglion cell bodies, whereas antigens expressed only in nerve fibers (eg, S-100) are claimed to improve the resolution of ganglion cell bodies by virtue of their negative staining.¹⁴ In my opinion, none of these "generic" neural markers offer a significant advantage over a well-stained, nicely cut H&E-stained section viewed under the microscope of an experienced observer.

In theory, an immunoreagent that selectively recognizes extrinsic or intrinsic nerve fibers might provide a sensitive and specific method to diagnose aganglionosis from a single histologic section. A selective marker of extrinsic nerves should stain hypertrophic fibers in the submucosa, as well as their thinner branches in the mucosa, thereby mimicking the results of AChE staining. Two antigens, NGFR and GLUT1, appear to discriminate between intrinsic and extrinsic nerves by labeling the perineurium of the latter.^{5,15} However, in published studies, antibodies directed against each of these proteins do not label the smaller mucosal branches of hypertrophic nerves. As such, they merely highlight hypertrophic nerves, which, if present, can generally be recognized in H&E-stained sections without immunostaining.

Several antibodies that preferentially label intrinsic neurons have been studied, most of which show absent or markedly

Table 1
Selected Studies That Suggest Immunohistochemistry as a Diagnostic Aid for Hirschsprung Disease (HSCR)

Predominant Immunoreactivity	Study	Antigen	HSCR Cases (N)	Controls	Purported Diagnostic Value
Ganglion cell bodies (may help resolve immature ganglion cells to exclude HSCR)	Karim et al ¹⁶	RET	SB and FT-R (38)*	cSB and HSCR prox colon	"...a combination of routine H&E stained sections with intervening RET IHC preparations is an optimal method for evaluating bowel specimens for [HSCR]."
	Brewer et al ¹⁷	BMPRIA	FT-R (3)	HSCR prox colon; surgical resections	"The antibody does not appear to identify any ganglion cells not seen on H&E staining but does greatly facilitate their identification."
	Wester et al ¹⁸	bcl-2 [†]	FT-R (11)	HSCR prox colon; ostomy closures and adult resections	"In Hirschsprung disease, we demonstrated that bcl-2 immunohistochemistry may serve as an additional marker for this diagnosis...."
Intrinsic and extrinsic neuronal cell bodies and nerves (may help resolve immature ganglion cells to exclude HSCR or hypertrophic nerves in the aganglionic segment)	Robey et al ¹⁴	NSE [‡]	SB (8)	cSB	"For definitive demonstration of mucosal nerve proliferations that were diagnostic for [HSCR]...acetylcholinesterase stain remained the method of choice... [but]...Immunoperoxidase staining for NSE and S-100 protein is helpful...particularly when fresh tissue is not available...."
Intrinsic and extrinsic nerves (may help resolve hypertrophic nerves in aganglionic segment)	Robey et al ¹⁴	S-100	SB (8)	cSB	See NSE (above).
	Tam and Owen ¹³	MAP-5	FT-R (5)	Ostomy closures	"...we have demonstrated that [MAP 5] immunostaining is similarly useful for the diagnosis of [HSCR]."
Extrinsic nerves (may help resolve hypertrophic nerve fibers in aganglionic segment)	Kakita et al ¹⁵	Glucose transporter-1 (perineurium)	FT-R (12)	cSB, FT-R, and autopsy	"Glucose transporter-1...may be a useful positive indicator in the diagnosis of [HSCR]."
	Kawana et al ¹⁹	GFAP	FT-R (25)	Ostomy closures	"Immunohistochemistry for GFA protein can be of excellent diagnostic value for the aganglionic colon."
Intrinsic nerves (reduced or absent in aganglionic segment)	Nirasawa et al ⁶	HPC-1/ Syntaxin 1A	FT-R (7)	HSCR prox colon	No explicit statement of potential diagnostic value, despite the observation that "HPC-1/syntaxin-1A was not recognized in the proliferated nerve fibers of the submucosal layer or the hypertrophied nerves of the aganglionic segment."
	O'Kelly et al ²⁰	nNOS	FT-R (7)	HSCR prox colon	"...[NOS histochemistry] may have a role as an adjunct to cholinesterase staining in equivocal cases."
	Barshack et al ²¹	Calretinin	FT-R (10)	HSCR prox colon	"The absence of calretinin expression may serve as a diagnostic aid in identifying aganglionic segments in HD."
	Kobayashi et al ⁷	NGFR [‡]	SB (10)	cSB	"...NGFR immunohistochemical staining of suction rectal biopsies provided 100% diagnostic accuracy in our patients, including newborns."
	Yamataka et al ⁵	SVP-38 kD	FT-R (8)	Non-HSCR resections	"...immunohistochemistry on the lamina propria alone can differentiate between normal and aganglionic bowel."

BMPRIA, bone morphogenic protein 1A; cSB, control suction rectal biopsies performed to exclude HSCR, but contained ganglion cells; FT-R, full-thickness sections from resection for HSCR; GFAP, glial fibrillary acidic protein; GLUT1, glucose transporter-1; HSCR prox colon, ganglionic portion of HSCR resection; IHC, immunohistochemistry; MAP-5, microtubule-associated protein-5; NGFR, nerve growth factor receptor; nNOS, neuronal nitric oxide synthase; NSE, neuron-specific enolase; SB, suction rectal biopsies without ganglion cells; SVP-38, synaptic vesicle protein-38 identified by MAb 171B5.

* A total of 55 blocks from the 38 patients (37 from biopsies and 18 from resections).

[†] Also stains B lymphocytes, which could be confused with immature neurons.

[‡] Refers to nerve fiber staining of intrinsic mucosal nerves, as opposed to perineural staining of extrinsic nerve fibers.

reduced immunoreactivity in the inter- and intramuscular plexuses of aganglionic bowel. Unfortunately, most of these do not appear to label mucosal nerve fibers in normal gut with sufficient intensity and/or reliability to rely on loss of staining in suction biopsies for diagnosis of Hirschsprung disease. A possible exception may be antibodies that recognize one or more synaptic proteins, as it appears that these are expressed in the normal intrinsic innervation of the mucosa but are lacking in the aberrant extrinsic mucosal innervation of aganglionic gut.⁵

The Sad State of Affairs

Many papers have been published that document differences in the distribution of various antigens in aganglionic vs "normal" bowel wall. Several suggest the usefulness of particular antibodies to diagnose Hirschsprung disease (Table 1). With rare exception (eg, Robey et al¹⁴), such claims seem unjustified and premature, as appropriately designed studies to evaluate diagnostic utility of these reagents, in the context of suction biopsies, are almost entirely lacking. Shortcomings common to

almost all of the examples cited in Table 1 include relatively small study groups, analysis of only full-thickness sections from surgical resections as opposed to suction biopsies, use of proximal ganglionic colon from Hirschsprung disease resections as a "normal" control, and unblinded slide review.

Some of these limitations are exemplified in the paper by Karim et al.¹⁶ In this study, ganglion cell identification was compared in RET (REarranged during Transfection, not "retinoblastoma oncoprotein" as stated in the abstract)-immunostained sections vs adjacent H&E-stained sections, as well as "original" nonadjacent H&E-stained sections from the same paraffin blocks. To their credit, the authors reported results from suction rectal biopsies. In 2 of 47 biopsy samples, ganglion cells were identified in RET-stained sections but not an adjacent H&E section. Despite their conclusions to the contrary, insufficient data exist to determine whether this difference reflects better sensitivity of the immunohistochemical approach, as opposed to the sampling issues inherent to histologic analysis of suction biopsies. A better study design might have been to collect 4 adjacent sections and stain them as follows: H&E, H&E, anti-RET, H&E, such that the discrepancy rate between H&E-stained sections could be ascertained simultaneously and levels superficial and deep to the RET-stained section would be sampled.

Other weaknesses of this study include the unblinded nature of the analysis, lack of relevant data from the 2 discrepant cases (whether the missed ganglion cells had immature cytology, whether these were missed diagnoses or other biopsies from the same patients excluded Hirschsprung disease), and the fact that the number of original H&E levels obtained in this case series (mean = 7.59; range = 2-25) is well below the standard of care.⁸ Some might argue that a perceived need for immunohistochemistry would not exist if an adequate number of levels were examined routinely.

While immunostaining for RET antigen (like other markers of ganglion cell somas) may facilitate diagnosis of Hirschsprung disease, the largely descriptive studies that have been published to date fail to provide compelling evidence that any immunocytochemistry-based method is easier, cheaper, more accurate, or in any other way more advantageous than existing approaches to Hirschsprung disease diagnosis.

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