

**Practical Pathology and Genetics of Hirschsprung Disease**  
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The only obligate diagnostic feature of the malformation, known as Hirschsprung disease (HSCR), is absence of ganglion cells in the myenteric and submucosal plexuses of the terminal rectum. Hypoganglionosis (paucity, but not complete loss of ganglion cells), segmental aganglionosis that does not involve the terminal rectum, and various forms of neuronal dysmorphogenesis in which ganglion cells are present, but dysplastic, should not be regarded as HSCR, even though the clinical presentation may be similar. It is very likely that the pathogenesis of these conditions is distinct from HSCR. The pathology of some of these other conditions is reviewed elsewhere.<sup>1-3</sup>

*Submucosal Biopsies*

Aganglionosis can be diagnosed by evaluation of either submucosal or myenteric ganglia. Since, in either instance, the pathologist must establish absence of ganglion cells, adequate sampling is essential and the recognition of ancillary histopathological findings that correlate with aganglionosis can be helpful. In most centers, initial diagnosis of HSCR is based on histopathological study of suction rectal biopsies. Diagnostic biopsies must be taken at least 1-1.5 cm proximal to the anorectal squamo-columnar junction, measure >2 mm in diameter, and include sufficient submucosa. When properly oriented and sectioned adequately (75-100 levels), H&E-stained, paraffin-embedded sections are generally sufficient to exclude the presence of submucosal ganglion cells and suggest the diagnosis of HSCR. The presence of hypertrophic nerve fibers (>40  $\mu$ m diameter) is observed in many, but not all cases, and helps establish the diagnosis. Similarly, the frequent, but not invariable, presence of abnormally thick and numerous AChE-stained nerve fibers in the muscularis mucosa +/- lamina propria can be used to confirm the diagnosis, particularly when neither ganglion cells nor hypertrophic nerves are observed.

The hypertrophic nerves that exist in most patients with HSCR arise from extrinsic autonomic and sensory fibers, which enter along with vessels from perirectal region and project for a finite distance rostrally. It is the number and diameter of these fibers that increases in HSCR, giving rise to the "hypertrophic" nerves that are frequently, but not always, observed in the myenteric plexus, submucosa, and mucosa of HSCR patients. Because these fibers only project for a finite distance proximal to the rectum, hypertrophic innervation may not be observed in biopsies taken rostrally in long-segment disease. Furthermore, extrinsic nerve hypertrophy of the rectum may not be observed in patients with combined deficiency of intrinsic ganglion cells and other peripheral ganglia (more common with long-segment HSCR) or very premature infants with delayed extrinsic innervation.

The average submucosal biopsy may be 3 mm across and the average distance between submucosal ganglia varies with age, but is probably about 500 microns and contains 2-7 ganglion cells, each of which measures 20-30 microns in diameter. With 3-5  $\mu$ m-thick paraffin sections, ganglia are missed in many random sections. Therefore, it is important to evaluate a lot of sections (>75). With an adequate, well-oriented, not crushed, H&E-stained biopsy one can be fairly confident about the diagnosis of HSCR simply by careful examination of each section. In this situation, the presence of certain ancillary features of HSCR can be quite helpful, but is not required. The problem is that very often the biopsies are suboptimal due to paucity of submucosa, crush, or both. It is in these cases, where AChE-staining can be particularly valuable.

### *AChE staining and interpretation*

Histochemical staining for AChE activity is a useful adjunct for the diagnosis of HSCR. The procedure is only performed with frozen sections and therefore requires a second suction biopsy, if paraffin sections are also going to be evaluated. The traditional protocol for AChE staining requires approximately 90 min,<sup>3</sup> but rapid procedures have been developed that require 5-10 minutes.<sup>4,5</sup> AChE staining in the rectum of normal children includes staining of nerves in the submucosa and small fibers in the muscularis mucosa. Usually the latter are confined to the inner half of the muscularis mucosa.<sup>6</sup> If fibers are positively stained in the lamina propria, they are extremely thin and few. Most, but not all, of the rectal submucosal biopsies from HSCR patients contain more densely packed, large, AChE-positive fibers through the full thickness of the muscularis mucosa. In addition, prominent AChE-positive fibers are often present in the lamina propria. The latter finding should not be relied upon too heavily because hypertrophic nerves are confined to the muscularis mucosa in biopsies from a significant subset of HSCR patients, particularly young infants.

### *Histochemical diagnostic approach to HSCR*

Considerable variation exists in the approaches taken by clinicians and pathologists to diagnose HSCR. In particular, the number of biopsies, number and section thickness, and use of AChE-staining differ between groups. In some parts of the world, diagnosis of HSCR is based solely on histochemically stained frozen sections of suction rectal biopsies; paraffin sections are not required.<sup>3</sup> The latter protocols generally stain separate sections from each biopsy for AChE and one or two markers of ganglion cell bodies, such as lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH). A potential added value of this histochemical battery is that it may disclose subtle forms of enteric dysganglionosis (e.g., ganglion cell dysmaturity), apart from HSCR, which may explain the HSCR-like symptoms in a patient with ganglion cells.<sup>3</sup>

With regard to diagnosis of HSCR, either the H&E/paraffin-based or histochemistry/frozen-based technique is valid, but experience with a given method is undoubtedly the most important variable that influences diagnostic accuracy. The pathologist must be secure with what constitutes adequate specimen, slides, orientation, etc. Above all, he/she should have the confidence to distinguish diagnostic from equivocal findings and clearly communicate the results to the clinician. In some instances, repeat biopsy is indicated, particularly if the specimen is inadequate or equivocal results are obtained.

### *Intraoperative Seromuscular Biopsies*

Many surgical approaches to HSCR are currently employed with a trend toward one-step procedures that are often transanal. In other instances, diagnosis based on suction rectal biopsy is followed by a two-stage procedure that begins with placement of an ostomy proximal to the aganglionic segment. Intraoperative seromuscular biopsies are important to determine that ganglion cells are present at the level where the ostomy or anastomosis will be placed. During ostomy placement, aganglionic gut is frequently not biopsied intraoperatively. However, whether the definitive surgery is done in one or two stages, it is helpful if the transitional zone between aganglionic and ganglionic gut is mapped intraoperatively prior to resection of any bowel. Generally this is accomplished by sequential seromuscular biopsies, from distal-to-proximal.

Seromuscular biopsies should be a minimum of 1 cm in length and extend for a depth of 3-5 mm, so as to include the longitudinal and most of the circular layers of the muscularis propria. Proper orientation of the biopsy for frozen sections greatly facilitates sampling and identification of ganglion cells. The goal is to cut perpendicular to the serosal surface, thereby visualizing the both muscle layers and their interface in the histological sections. With a well-oriented biopsy,

two-to-five sections are generally sufficient to confirm/exclude aganglionosis. Recognition of ganglion cells is usually not difficult, although inflammation sometimes obscures their cytological features.

#### *Analysis of HSCR Resections*

The pathologist's goals in analyzing resected aganglionic gut from an HSCR patient are to confirm the diagnosis of HSCR, map the transitional zone, and assess the integrity of the nervous system at the proximal end of the resection. Confirmation that the distal gut is aganglionic is straightforward. A map of the transition zone can be completed by sampling multiple areas along the length of the resected segment to document the presence/absence of submucosal and myenteric ganglia. Some pathologists prepare rolls from longitudinal strips of the entire length of the resection.<sup>3</sup> I prefer to use transverse sections because the circumferential interface between aganglionic and ganglionic gut is often irregular, which will not be apparent in any single longitudinal strip.<sup>7</sup> Full-circumference sections are needed, particularly if the aganglionic zone appears extend within two cm of the proximal resection margin. Construction of a decent map may require repeated sampling sessions.

Evaluation of the integrity of the proximal gut is the most challenging aspect of HSCR pathology. In principle, the genetic etiologies that produce distal aganglionosis may have more subtle effects on the number, distribution, circuitry, and/or differentiation of proximal neurons. Certainly, most HSCR patients harbor a transitional zone of variable length, in which the density of myenteric ganglia is obviously less than in normal gut. However, mild-to-moderate changes in neuronal density are extremely difficult to diagnose by routine analysis and yet may correlate with continued HSCR-like symptoms postoperatively. Incorporation of hypoganglionic transitional zone reportedly increases the likelihood of persistent post-surgical constipation that may necessitate a repeat operation. However, the pathological criteria used to define the transitional zone were not provided in these studies.

In addition to hypoganglionosis, submucosal hyperganglionosis (intestinal neuronal dysplasia, type B; IND) has been reported in the proximal gut of HSCR patients. IND is a controversial form of submucosal hyperganglionosis that has been defined entirely based on histochemically stained biopsies.<sup>8</sup> Similar changes have been observed as isolated "neuropathy" in some children with HSCR-like symptoms, as well as in contexts of other primary disorders of dysmotility, including the transitional zone of HSCR. The significance of Hirschsprung-associated IND is hotly debated in the literature; some authors have advocated screening for IND with frozen sections at the time of surgery so as to extend the resection proximal to the affected area. However, no compelling data exists to suggest that IND-like changes in HSCR either predict a poor outcome or should be managed any differently from isolated HSCR.

A variety of other poorly understood histopathological findings are observed in aganglionic bowel and or the transitional zone. Some of these are summarize in Table 1. While most of these findings have no established clinical and/or genetic significance, their potential correlations with specific genetic defects and / or post-operative complications have not been adequately studied.

<u>Histopathological Finding</u>	<u>Location</u>	<u>References</u>
Eosinophilic neural infiltrates	Aganglionic and transitional zones; transmural including nerve plexuses	<sup>9</sup>
Submucosal arterial fibromuscular dysplasia	Transitional zone	<sup>10</sup>
Loss of c-kit immunoreactive interstitial cells of Cajal	Conflicting data	<sup>11-13</sup>
Peripheral nerve pattern of laminin expression	Aganglionic and transitional zones	<sup>14</sup>
Loss of nNOS innervation in muscularis propria	Aganglionic zone	<sup>15-17</sup>

#### *Skip Lesions and Zonal Aganglionosis*

It is important to be aware of two rare forms of congenital aganglionosis that deviate from the classic pattern, in which the distal rectum and uninterrupted contiguous bowel are devoid of nerve cell bodies. In the intestinal tracts of persons with “zonal” (“segmental”) aganglionosis, ganglion cells are present in the distal rectum, but are absent from a proximal segment of gut. In contrast, “skip areas” are ganglion cell-containing segments of large intestine, flanked proximally and distally by aganglionic gut.

Zonal aganglionosis is considered to be an acquired lesion (disruption) that results when ganglion cells (or their precursors) in a fully colonized segment of gut die due to ischemic, viral, immunologic, or other types of injury. The aganglionic segment can occur in small or large intestine. In some cases, a specific etiology is suggested by history (e.g., necrotizing enterocolitis) or other pathological findings (e.g., viral cytopathy). Alternatively, it has been suggested that zonal aganglionosis might result from failure of vagal and sacral crest cells to converge in the gut wall. At the time this hypothesis was introduced, it was less certain that sacral crest cells naturally adopt an enteric neural fate. Given recent evidence that a subset of colonic neurons derive normally from the sacral crest, the proposal is more tenable. However, evidence for neuron formation in the distal colon does not occur in experimental models in which hindgut colonization by vagal crest cells is prevented (discussed above).

Some colleagues and I reviewed the subject of skip areas in 1995 and found that only eleven cases had been reported. Since then, I have been informed of several other cases that were encountered by colleagues, and I suspect the entity may be more common than the literature might suggest. With one exception, skip areas are located in the large intestine and bracketed by aganglionic areas that invariably include both the distal rectum and appendix plus variable lengths of contiguous large and small intestine. Pathologists and surgeons must be cognizant of skip areas, and not use biopsies of the appendix as a means to diagnose total colonic aganglionosis since relatively large skip areas can be present in which ganglion cells exist. In at least one patient, the skip area was recognized, preserved, and used to establish a functional anastomosis between small intestine and anus.

#### *Ultrashort-segment Hirschsprung Disease*

Ultrashort-segment HSCR is a controversial entity that has been defined differently by various investigators. Initially, the term “ultrashort-segment HSCR” was reserved to describe patients with clinical and radiological findings similar to those of HSCR, but with ganglion cells in their rectal biopsies. Others reserved the diagnosis for only those biopsies that contain a normal density of ganglion cells and demonstrate an HSCR-like AChE-staining pattern. In either case, the underlying assumption is that an extremely short aganglionic zone in the distal rectum, inferior to

the submucosal biopsy site, is responsible for obstructive symptoms. Another possibility is that such patients have distal hypoganglionosis, which is extremely difficult to document because ganglion cells are normally sparse or absent in the terminal 2-3 cm of rectum.

Patients who fulfill revised criteria for “ultrashort-segment HSCR” probably constitute a heterogeneous set of disorders that may include aganglionosis, hypoganglionosis and anal achalasia (internal sphincter achalasia).<sup>18</sup> In those patients with aganglionosis, the HSCR cannot be established by suction rectal biopsy because the aganglionic myenteric plexus does not extend proximal to the zone of physiological submucosal hypoganglionosis (2-3 cm from the dentate line). In patients that do not have aganglionosis, anatomic changes may not be evident and the diagnosis is based on physiological or histochemical criteria. Regulation of anal sphincter tone is a complex process, which is influenced by both the central and peripheral nervous systems. Heightened sphincter tone could be caused by psychogenic, myogenic, or neurogenic etiologies. A major stimulus for sphincter relaxation is nitric oxide (NO) release by efferent nerve fibers in the smooth muscle. Reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-diaphorase activity (nitric oxide synthase), a marker for NO-producing nerve fibers, has been observed in the internal sphincter of patients with anal achalasia. In some cases, the symptoms resolve after sphincter myotomy.

#### *Immunohistochemistry and the diagnosis of HSCR*

An unbelievable number of papers have been published which essentially describe some neuron-specific marker that the authors suggest could be used to facilitate the diagnosis of HSCR. Although most of these antibodies are fairly specific, none is used widely in practice because pathologists who regularly search for ganglion cells in H&E-stained sections or LDH/SDH histochemically stained sections are quite good at discriminating neuron cell bodies without the need for special stains. False positive diagnoses result either from inadequate sampling or observer inexperience.

As opposed to most of the published immunohistochemical targets which are expressed in ganglionic gut, a marker specifically expressed in aganglionic gut would be much more valuable. The fundamental problem in the diagnosis of HSCR is sampling and the fact that the primary feature is absence of ganglion cells, which are sparsely distributed. A reliable marker is needed that detects changes related to the absence of ganglion cells. AChE histochemistry does this to some degree, but the hypertrophic nerve fibers revealed by this technique represent a quantitative change that is subject to individual interpretation. Also, as noted above, abnormal AChE staining is not present in all cases and is age-dependent. Recently, laminin immunohistochemistry was shown to highlight changes in submucosal nerves that may distinguish aganglionic bowel from ganglionic bowel.<sup>14</sup> However, the sensitivity and specificity of this method have not been established.

#### *Molecular genetics and HSCR*

Hirschsprung disease (HSCR, intestinal aganglionosis) affects an estimated 1:5000 liveborn infants. The actual incidence of this malformation may be much higher since intestinal aganglionosis is frequently associated with other anomalies, and many embryos with multiple defects might die *in utero*. It is now clear that most, if not all, cases of HSCR have a genetic basis and that HSCR is a complex multigene disorder characterized by incomplete penetrance and variable associated anomalies.<sup>19</sup> Mutations in more than 11 different genes have been implicated in the pathogenesis of HSCR (Table 2); many were first recognized in murine models for this condition. However, according to most estimates, mutations in one or more of these genes only can be detected in about half of all HSCR cases. Therefore, other genetic and/or environmental factors are likely to be involved. RET is the gene in which mutations are most frequently

detected in patients with non-syndromic HSCR. In addition, recent studies indicate that non-coding polymorphisms (base-pair differences that do not affect protein structure and which are present in >1% of the normal population) in the proximal portions of the *RET* gene pose a significant risk for HSCR, possibly by reducing RET expression.<sup>20-22</sup>

<b>TABLE 2: SUSCEPTIBILITY GENES IN ISOLATED AND SYNDROMIC HSCR</b>			
<b>SYNDROME</b>	<b>GENETIC DEFECT</b>	<b>% WITH HSCR</b>	<b>COMMON PHENOTYPIC FEATURES</b>
<b>Isolated HSCR<sup>a</sup></b>	<i>RET</i> <i>EDNRB<sup>b</sup></i> <i>EDN3<sup>b</sup></i>	100%	
	<i>TTF-1</i>	NE	<i>TTF-1</i> mutations have been associated with hypothyroidism and cleft palate, but the only reported HSCR patient with a <i>TTF-1</i> mutation had isolated HSCR.
<b>Syndromic HSCR</b>			
MEN2A	<i>RET<sup>c</sup></i>	<1% <sup>23</sup>	Medullary thyroid carcinoma, pheochromocytoma, parathyroid hyperplasia
Smith-Lemli-Opitz	<i>DHCR7</i>	16% <sup>24</sup>	Growth retardation, pedal syndactyly, mental retardation, hypospadias, dysmorphic facies
Down	<i>Trisomy 21</i>	2-9% <sup>25</sup>	Prominent epicanthal folds, upslanting palpebrae, hypotonia, mental retardation, flat midface, single transverse palmar crease
Waardenburg-Shah	<i>SOX10</i> <i>EDN3<sup>b</sup></i> <i>EDNRB<sup>b</sup></i>	100% <sup>d</sup>	Deafness, piebaldism, other neurological deficits
Mowat-Wilson	<i>ZHFX1B</i>	62% <sup>26</sup>	Abnormal facies, cardiac malformations, mental retardation, genitourinary anomalies
Haddad	<i>PHOX2B<sup>e</sup></i>	100% <sup>f</sup>	Congenital central hypoventilation, neuroblastoma
Goldberg-Shprintzen	<i>KIAA1279</i>	100%	Microcephaly, various brain malformations, cleft palate
X-linked hydrocephalus	<i>LICAM</i>	NE	Cerebral aqueductal stenosis, hydrocephalus, absent corpus callosum
Cartilage-Hair Hypoplasia <sup>g</sup>	<i>RMRP</i>	NE	Skeletal dysplasia, sparse blond hair, immunodeficiency, anemia
Bardet-Biedl	<i>BBS1</i> , <i>BBS2</i> , <i>BBS4</i> , <i>BBS6<sup>h</sup></i> , <i>BBS7</i> , <i>BBS8</i>	2% <sup>27</sup>	Obesity, retinal degeneration, polydactyly, gonadal and renal malformation
Kauffman-McKusick	<i>MKKS<sup>h</sup></i>	NE	Polydactyly, congenital heart defect, hydrometrocolpos

<sup>a</sup>Since HSCR most often presents shortly after birth features of some syndromes (e.g., MEN2A) may not be obvious at the time of diagnosis.

<sup>b</sup>Isolated HSCR is more commonly associated with heterozygous mutations; Waardenburg-Shah syndrome is more commonly associated with mutations in both alleles.

<sup>c</sup>Missense mutations affecting one of three cysteine codons (Cys609, Cys 618, or Cys620) <sup>32</sup>.

<sup>d</sup>HSCR is a diagnostic feature that differentiates Waardenburg-Shah syndrome from other variants of Waardenburg syndrome.

<sup>e</sup>Congenital central hypoventilation syndrome has also been associated with *RET*, *GDNF*, and *EDN3* mutations in rare patients, but concurrent HSCR has only been associated with *PHOX2B* mutations <sup>49</sup>.

<sup>f</sup>HSCR is a diagnostic feature of Haddad syndrome, but *PHOX2B* mutations also occur in patients with isolated congenital central hypoventilation <sup>49</sup>.

<sup>g</sup>Many patients have been presented with a HSCR and a variety of limb defects different from cartilage-hair hypoplasia <sup>37</sup>.

<sup>h</sup>*BBS6* and *MKKS* are the same gene <sup>50,51</sup>.

NE, not established.

The gene products encoded by all established “HSCR genes” are either expressed by neural crest-derived enteric neural progenitors or by adjacent cells in the colonization pathway that progenitors follow. Many of these gene products are intercellular signaling components that influence proliferation, survival, migration, and/or differentiation of enteric neural crest cells and, in some cases, other cell types as well. Recent research using avian, zebrafish, and murine models is providing insight into when, where, and how these gene products influence enteric neurodevelopment. Although much is unknown about the molecular and cell biology of each HSCR gene product in normal enteric neurodevelopment, it appears that defects in these genes lead to intestinal aganglionosis by interfering with events at very different points in the colonization route that neural crest cells negotiate en route to colonization of the entire gut. At the same time, studies murine and human studies are beginning to elucidate complex interactions that exist between polymorphic alleles of these genes.

It is important to realize that intestinal aganglionosis is a malformation, analogous to other birth defects like absent radii, cleft lip, congenital heart malformations, etc. HSCR can occur as an isolated anomaly or in the context of multiple malformations, which in many instances constitute recognizable syndromes. Genotype-phenotype correlation in patients with HSCR is often not sufficient to suggest mutation in a particular candidate gene because considerable phenotypic overlap exists among HSCR patients with mutations in different genes. However, complete clinical evaluation and family history should be obtained as these details sometimes provide clues to the nature of the genetic defect.

A particularly challenging problem in the field of Hirschsprung genetics is the phenomenon of “incomplete penetrance”. Incomplete penetrance refers to the fact that only a subset of individuals who carry a particular mutation in one of the genes listed in Table 2 actually exhibit the HSCR phenotype (intestinal aganglionosis). Other persons, even immediate family members, with the same mutation are unaffected. Incomplete penetrance is observed in many other human genetic disorders and is usually attributed to one of three variables - genetic modifiers, environmental modifiers, or stochastic events. Substantial evidence has been gathered to suggest that genetic modifiers are responsible for some of the variable penetrance of HSCR mutations. In some cases, interactions between alleles of two different genes listed in Table 2 determine whether enteric neurodevelopment is perturbed. For example, subtle polymorphisms that alter the nucleotide sequence of the *RET* gene, but not the amino acid sequence of the RET protein, have been shown to influence penetrance of a particular missense mutation in the *EDNRB* gene.<sup>28</sup> In addition, genetic loci that influence penetrance of RET mutations, but do not correlate with any of the genes listed in the table, have been mapped. Thus, HSCR is a complex genetic disorder that challenges the comprehension of scientists and clinicians, let alone the families with an affected child.

At this time, the genetic data listed in Table 2 has limited practical value. Mutational analysis for many of the genes listed is not easily obtained and does not affect clinical management or counseling, except in rare contexts. One possible exception is testing for MEN2A mutations of the *RET* gene in patients with sporadic HSCR. Despite the fact that most patients with MEN2A do not have HSCR, some studies have reported relatively high rates (2.5-5%) of MEN2A-associated mutations in patients with what initially appears to be sporadic HSCR.<sup>29</sup> Early diagnosis of MEN2A allows better surveillance for neoplasia in the affected individual and screening of relatives who may also carry the same mutation.

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