The Identification of Ganglion Cells in Hirschsprung Disease by the Immunohistochemical Detection of ret Oncoprotein

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Abstract

The absence of ganglion cells (GCs) is the primary anatomic abnormality in Hirschsprung disease. Light microscopy is the mainstay in establishing this diagnosis. However, establishing a condition of aganglionosis may be challenging on routine H&E-stained sections of colonic biopsies and resections. We studied the identification of GCs by retinoblastoma oncoprotein (ret) immunoreactivity and routine H&E light microscopy by evaluating 53 blocks from 34 patients demonstrating GCs on original H&E-stained sections and 55 blocks from 38 patients lacking GCs on original H&E-stained sections. All blocks demonstrating GCs on H&E-stained sections also were positive for GCs on ret staining (100%). In 3 blocks that were negative for GCs by H&E staining (5%), GCs were shown on ret-stained sections. Immunoreactivity for ret has comparable specificity but slightly higher sensitivity to routine light microscopic evaluation in identifying GCs. GCs are identified more readily by ret immunoreactivity than by routine morphologic examination.

Hirschsprung disease occurs in approximately 1 in 5,000 live births. The classic description of Hirschsprung disease was credited to Harald Hirschsprung in 1887.1 This disease entity is characterized by absence of parasympathetic ganglion cells in the myenteric (Auerbach) and submucosal (Meissner) plexi of the affected colon, causing a sustained contraction of that segment. In approximately 80% of cases, the aganglionic segment involves the rectum and the sigmoid colon only, whereas in 20% of cases, the aganglionic segment involves the more proximal bowel, sometimes extending to variable lengths of the ileum.

The ganglion cells migrate to the bowel from the neural crest. Hirschsprung disease is thought to be a neurocristopathy, related to the premature arrest of the craniocaudal migration of these cells during the 5th to 12th week of gestation. Mutations in several genes have been associated with aberrant population of the fetal distal gut, including ret.2 In human fetal models of Hirschsprung disease, the ganglion cells had not been observed in the distal gut before their appearance in the proximal gut. This has significant implications for the surgical treatment of the disease because most of the time, there are no skip lesions. Once the transition zone has been identified, the surgeon can be confident that the exact extent of the disease is known.3

The diagnosis and extent of resection for the management of Hirschsprung disease depend on the sensitive and specific identification of ganglion cells. Despite the availability of special techniques such as acetylcholinesterase histochemical analysis and S-100 protein and neuron-specific enolase (NSE) immunohistochemical analysis, examination of serial H&E-stained sections remains the standard approach for establishing this diagnosis. However, documenting aganglionosis often is difficult on routine H&E-stained sections, acetylcholinesterase
histochemical analysis is technically challenging, and S-100
and NSE staining lack sensitivity and specificity.

In Hirschsprung disease, the ret signaling pathway has a
critical role. To briefly summarize, ret is a proto-oncogene with a
114 amino acid transmembrane receptor with a cadherin-like
extracellular domain, a cysteine-rich region, and an intracellular
tyrosine kinase domain. It is expressed in the developing central
tissue in the block or biopsy specimens that were too superficial
and peripheral nervous systems. Unlike in the cancer susceptibil-
ty syndromes, mutations in Hirschsprung disease are inactivat-
ing and lead to misfolding or failure to transport the protein to
the cell surface, effectively resulting in half the usual dose of the
functioning protein, a situation known as haploinsufficiency. Half
of the wild-type ret is not sufficient for normal enteric
development.4 This loss of ret expression resulting in agan-
glionosis can be exploited to establish the diagnosis of
Hirschsprung disease. We compared ret immunoreactivity with
H&E light microscopic examination in an effort to find a sensi-
tive and dependable marker for identifying ganglion cells.

Materials and Methods

A computer-based search was performed for cases of
Hirschsprung disease in the Department of Pathology, Baystate
Medical Center, Springfield, MA, from June 1990 to April 2004.
We retrieved 61 formalin-fixed, paraffin-embedded tissue blocks
from colonic and rectal biopsies relating to 46 patients with a
clinical suspicion of Hirschsprung disease. Another 47 blocks
from resection specimens from 14 patients with Hirschsprung
disease also were retrieved. Other possible cases were not
included owing to technical factors, including lack of sufficient
tissue in the block or biopsy specimens that were too superficial
or too distal. In 53 of these blocks from 34 patients (24 from
biopsies and 29 from resection specimens), ganglion cells were
evident on the original H&E-stained sections, whereas 55 blocks
from 38 patients (37 from biopsies and 18 from resection speci-
mens) were lacking ganglion cells on the original H&E-stained
sections. The number of original H&E-stained levels on the 61
biopsy blocks ranged from 2 to 25, with a mean of 7.59.

Results

The ret oncoprotein was detected in the cytoplasm of gan-
glion cells, where intense paranuclear distribution of immunore-
activity was present. Schwann cells and nerve trunks were weak-
ly immunoreactive, and, therefore, the hypertrophied nerve
fibers in aganglionic intestinal segments did not show a conspic-
uous reaction. Endothelial cells and fibroblasts were nonimmu-
noreactive for ret.

A significant observation was the ease with which ganglion
cells were identified by ret immunohistochemical analysis

Comparison of Original H&E and ret Immunohistochemical
Staining (With Adjacent H&E)

All 42 blocks with ganglion cells identified on the adjacent
H&E-stained sections also were positive for ganglion cells by ret
immunohistochemical analysis (Table 3, rows 1 and 5). There
were no cases in which ret immunohistochemical analysis failed
to demonstrate ganglion cells that were identified on the adjacent
H&E-stained sections (Table 3, row 2).

Of 65 blocks negative for ganglion cells on the adjacent
H&E-stained sections, 63 (97%) did not demonstrate ganglion
cells on ret immunohistochemically stained sections (Table 3, rows 3 and 4). However, 2 blocks that were negative for ganglion
cells on the adjacent H&E-stained sections (3%) showed gan-
glion cells on ret immunohistochemically stained sections (Table
3, row 6), and 1 block showed a ganglion cell that was stained
with ret but was not positively identified in the adjacent H&E-
stained section (Table 3, row 7).

Comparison of Original H&E and ret
Immunohistochemical Staining (With Adjacent H&E)

Original sections of 13 blocks with ganglion cells were nega-
tive for ganglion cells by ret immunohistochemical analysis and
on the adjacent H&E-stained sections (Table 3, row 3). This
highlights the variation in ganglion cell populations within the
paraffin blocks and is the reasoning for including an adjacent
section for true comparison between results of H&E and ret oncoprotein staining in identifying ganglion cells. Two blocks demonstrated ganglion cells on ret and adjacent H&E-stained sections, although the original H&E levels were negative for ganglion cells (Table 3, row 5), again indicating the ganglion cell population variability within the paraffin block.

Comparison of ret Immunohistochemical Stains and Other Immunohistochemical Stains Applied in the Original Diagnosis

The 6 blocks that originally were stained with NSE, S-100, and synaptophysin to aid the routine H&E examination in the identification of ganglion cells demonstrated the same results on ret staining. Of these 6 blocks, 1 was positive for ganglion cells on all stains, including the original immunohistochemical stains and the ret stain, whereas the remaining 5 blocks did not demonstrate any ganglion cells with these stains, including ret.

Discussion

Histologic examination of colorectal specimens for the presence of ganglion cells remains the standard method of evaluating patients with Hirschsprung disease and forms the basis for surgical treatment (Image 1A, Image 2A, and Image 3).
The difficulty in identifying neonatal ganglion cells by morphologic examination is well known. Fibroblasts and endothelial cells may be confused with ganglion cells. This difficulty in interpretation may result in inadequate resection of bowel segments or resection of unnecessarily long segments of bowel. Therefore, various techniques have been assessed to facilitate the determination of ganglionic or aganglionic bowel.5-8

The acetylcholinesterase histochemical stain has diagnostic value in Hirschsprung disease because it demonstrates ganglion cells and abnormal mucosal cholinergic nerve fibers.4,9-13 However, it requires frozen tissue with its associated compromise in morphologic features. Moreover, the preparations may be difficult to interpret because the histochemical procedure may produce variable results. Most laboratories perform the stain by hand. Several series also have documented false-negative14-16 and false-positive13,17 staining in biopsy specimens examined for Hirschsprung disease.18

Immunohistochemical evaluations have led to less labor-intensive procedures with the widespread application of semi-automated instrumentation. S-100 antibodies have been applied to the diagnosis of Hirschsprung disease. However, S-100 does not stain ganglion cells; rather, it is immunoreactive to nerve fibers—normal and hypertrophied—and the cytoplasm and nuclei of Schwann cells. This produces a contrast picture of the unmarked ganglion cells surrounded by axons and interstitial cells with intense immunoreactivity.19 Because a negatively stained cell would be difficult to identify, some researchers have advised using S-100 protein with NSE in the same histologic specimen to enhance the visualization of ganglion cells,18 which will stain positively with NSE along with all other intestinal neuronal elements, including Schwann cells and nerve fibers, but will not stain with S-100 protein.

The ret oncoprotein is expressed in the ganglia of normal colon and in the ganglionic colonic segments of patients with Hirschsprung disease.20 A partial synthesis of this receptor tyrosine kinase can be shown in the ganglionic and hypoganglionic segments of patients with total disruption of the ret gene structure (frameshift mutation with stop codon in the extracellular domain)20 or complete deletion of the ret gene.20,21 In these conditions, a dose effect could account for the migration arrest of the neuroblasts causing Hirschsprung...
disease. Therefore, antibodies against the ret oncoprotein can be used to detect ganglion cells.

Immunohistochemical detection of ganglion cells by using ret is technically routine, less labor-intensive than histochemical procedures, and reproducible, yielding slides that are easily interpreted (Images 1B and 2B). The ganglion cells are intensely immunoreactive in a clean background, resulting in a desirable high signal/noise ratio. Suture material stained nonspecifically in a biopsy specimen. However, the morphologic features were easily identified as a foreign body rather than a ganglion cell. Significantly, the immature ganglion cells of neonates, which may be confused with endothelial cells, macrophages, fibroblasts, or Schwann cells by routine H&E staining, were intensely immunoreactive with ret oncoprotein (Image 2B).

This study demonstrated that ret immunoreactivity has comparable specificity but slightly higher sensitivity than routine H&E staining in the identification of ganglion cells in biopsy and resection specimens from patients in whom there is a clinical suspicion of Hirschsprung disease. Ganglion cells are more readily identified by ret immunoreactivity than by routine morphologic examination. The immunohistochemical procedure is routine and may be performed on an automated immunohistochemical platform.

This study did not address the intraoperative assessment of biopsy or resection specimens for ganglion cells by frozen section. However, surgical resection of affected bowel commonly is preceded by an endoscopic biopsy specimen consisting of at least the submucosa. These may be multiple biopsy specimens, which subsequently are evaluated by routine formalin-fixed, paraffin-embedded processing.

Immunohistochemical analysis using ret should be considered as the standard adjunctive procedure in the evaluation of biopsy or resection specimens for ganglion cells in patients suspected of having or diagnosed with Hirschsprung disease. Although an exact protocol for the evaluation of such biopsy specimens has not been established based on sensitivity and specificity, our results suggest that a combination of routine H&E-stained sections with intervening ret immunohistochemical preparations is an optimal method for evaluating bowel specimens for Hirschsprung disease. Perhaps an alternative, more cost-effective method is to approach the tissue submitted for evaluation in 2 steps. The first step would include evaluation of H&E-stained sections while saving unstained intervening levels for future ret immunohistochemical analysis. If the initial examination identifies ganglion cells or deems a biopsy specimen insufficient, the unstained slides are not subjected to immunohistochemical evaluation. However, if the specimen is adequate and no ganglion cells are found by H&E examination, the intervening unstained slides are evaluated for ret oncoprotein by immunohistochemical analysis.

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