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REVIEW ARTICLE

Diagnostic histopathological tools in Hirschsprung disease and related disorders in childhood

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Summary

Diagnosing Hirschsprung disease (HD) and related disorders can be complex and demands a deep understanding of the mechanisms governing intestinal motility, which involves the enteric nervous system (ENS), interstitial cells of Cajal (ICCs), and the muscle layers of the intestine. The London classification identifies three groups of gastrointestinal neuromuscular disorders: neuropathies, myopathies, and ICC abnormalities. Hirschsprung disease, characterized by the absence of ganglion cells, is the most common intestinal neuropathy and it results from the impaired migration of neural crest cells during development. It affects about 1 in 5,000 live births and involves several genetic factors, notably the RET gene. HD typically affects the rectum and a part of the colon, with varying extents of aganglionosis. The diagnosis is based on the histopathological analysis of suction biopsies, the absence of ganglion cells, and the presence of thick submucosal nerves on a standard hematoxylin and eosin stain, supplemented by enzyme histochemistry (acetylcholinesterase method) or immunohistochemical methods (calretinin and other antibodies) staining. The treatment for HD involves surgical resection of affected bowel segments. Accurate intraoperative assessment of tissue margins is critical to preventing postoperative complications related to pseudoobstruction. Communication between surgeons and pathologists is essential to ensure successful treatment outcomes.

Other intestinal neuropathies include intestinal hypoganglionosis, hyperganglionosis, delayed maturation of ganglion cells, and gliopathies. Enteric myopathies are exceptionally rare conditions, with typical morphological changes such as atrophy of the muscularis propria, intracellular vacuolization of smooth muscle cells, and interstitial fibrosis. Disruption in ICC network and arrangement forms the morphological basis of slow transit constipation. Each of aforementioned disorders has unique characteristics and diagnostic challenges. Understanding and diagnosing these conditions often require a combination of histological, histochemical, immunohistochemical, and sometimes genetic analyses. The integration of these techniques is vital for accurate diagnosis and effective treatment planning.

In summary, the complexity of intestinal dysmotility disorders necessitates a thorough understanding of intestinal motility mechanisms and the utilization of advanced diagnostic methods to provide accurate diagnoses and effective treatments.

Key words: Hirschsprung disease, intestinal dysmotility, biopsy, immunohistochemical staining

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INTESTINAL DYSMOTILITY - INTRODUCTION

Diagnosis of Hirschsprung disease (HD) and allied disorders is often challenging. Accurate diagnosis requires comprehensive knowledge of all components involved in regulating intestinal motility, such as the enteric nervous system (ENS), network of interstitial cells of Cajal (ICCs), and the integrity and functionality of the muscular layers.

ENS is a part of autonomic nervous system, built from distinct types of cells which form a complex network (1). Its cells are derived from precursor cells of the vagal and sacral part of the neural crest. During embryonal development, these cells migrate along the primitive gut in the opposite directions, proliferate and differentiate into various cell types such as neurons, glial cells, and Schwann cells (2). Although precursor multipotent cells originate from two different segments of the neural crest, these two groups of cells give identical types of neurons and glial cells in the ENS. There are many different regulatory signaling pathways involved in ENS development where the RET (Rearranged during Transfection)/GDNF (Glial Derived Neurotrophic Factor) signaling pathway is detected as the most important and most studied. Recognition of a large number of signaling pathways involved in the ENS development and linked genetic and epigenetic factors has been important for better understanding of ENS developmental disorders, such as Hirschsprung disease and related disorders (3).

Two main parts of ENS are myenteric nervous plexus (MP) and submucosal nervous plexus (SP). MP is related to intestinal motility, while SP is important for regulation of blood supply and transepithelial ion transfer and has a minor role in the intestinal motility.

ICCs produce slow waves responsible for intestinal contraction (4). These cells are gracile, and immunohistochemistry is necessary for their detection and evaluation in daily practice. The most commonly used antibodies for this propose purpose are c-kit (CD117) and DOG1. A reduced number of ICCs is the main characteristic of some cases of chronic constipation and chronic intestinal pseudoobstruction (4, 5). Damage to ICCs or the disruption of c-kit immunohistochemical expression can be associated with other conditions. It has been observed in some cases of transient hypomotility following adequate HD surgery or in certain inflammatory conditions (6,7).

GASTROINTESTINAL NEUROMUSCULAR DISORDERS OF CHILDHOOD

According to the London classification of gastrointestinal neuromuscular disorders, three main groups of disorders are recognized: neuropathies, myopathies, and ICCs abnormalities (8). However, Kapur classified gastrointestinal neuromuscular disorders of childhood into five categories: enteric neuropathies, enteric myopathies, combined neuromuscular disorders, colonic desmosis and idiopathic disorders. In the group of enteric neuropathies five distinct disorders were recognized such as HD, hypoganglionosis, hyperganglionosis, delayed maturation of ganglion cells (DMGC) and gliopathies (9).

HIRSCHSPRUNG DISEASE

HD is bowel aganglionosis, which is a consequence of disrupted migration of pluripotent neural crest cells during the embryonal period, as previously mentioned. It is the most frequently diagnosed intestinal neuropathy (9,10). HD occurs in 1:5000 live births, with significant ethnic deviation (11). More than 11 genes can be involved in HD pathogenesis (12). The vast majority of HD patients have mutation of proto-oncogene RET (in about one-third of sporadic cases and a half of familial cases) (13). However, more than 100 different mutations of RET gene have been identified. HD can be sporadic, syndromic HD (within various syndromes such as Down syndrome, Waardenburg syndrome and others) and also, can be associated with other non-syndromic anomalies of heart, gastrointestinal tract, central nervous system, or genitourinary tract (11,12, 14).

Aganglionosis mainly affects the rectum and varying lengths of the large intestine, continuously. The most common HD type is short segment disease with rectum and sigmoid colon affection (80% HD). Less frequent HD forms are ultrashort HD (length of aganglionosis up to 3 cm), long segment disease (aganglionosis affects segment proximal to sigmoid colon or lienal flexure), total colonic aganglionosis (the absence of ganglion cells throughout the entire colon) and total intestinal aganglionosis (affected colon and terminal ileum). Male predominance (4:1) is characteristic of short HD segment, while long segment disease is equally present in both genders (11). For HD diagnosis, morphological findings are particularly important. Apart from the absence of ganglion cells, an important finding in affected bowel segment is overgrowth of extrinsic parasympathetic nerves. These nerves continually release acetylcholine, and this is associated with consequential smooth muscle contraction and pseudoobstruction. Elevated expression of actin alpha 2 of intestinal smooth muscle in the aganglionic segment leads to hyperactive contraction that also worsen pseudoobstruction (15). The number of more than two thick nerve bundles per high microscopic magnification is significant for HD diagnosis. Submucosal nerve bundles are typically thicker than 40 μm in diameter (16,17) **(Figure 1A)**. The funnel shaped segment (common lengths 1 to 3 cm) interposed between aganglionic and normoganglionic zone (NZ) is called transitional zone (TZ). Hypoganglionosis of MP and submucosal hypertrophic nerves are essential of TZ, while SP in TZ varies from agangli-

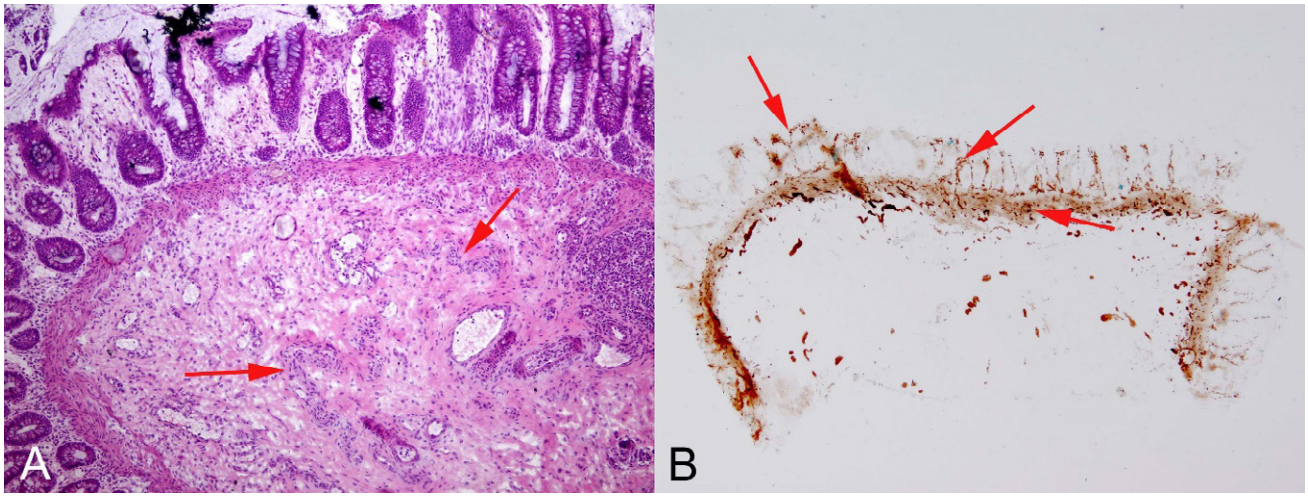

Figure 1. Typical morphology of HD: the absence of ganglion cells and thick nerves (arrows) in the rectal submucosa (A; H&E staining, 100x) and acetylcholine positive nerve fibers within mucosa and lamina muscularis mucosae (arrows) (B; AchE, 40x).

onic to hyperganglionic (12,18). Ectopic ganglia and abnormal "hybrid ganglia" with characteristics of extrinsic nerves could also be found within the transitional zone (19). On the other hand, in the long segment HD or total colonic/intestinal aganglionosis, nerve fibers are not prominent, and interstitial zone between two muscular layers may be inconspicuous, with close contact between the circular and longitudinal muscle layers (8,11,16). Multiple biopsies from different levels are necessary for the differentiation of long segment HD and total colonic/intestinal aganglionosis (21).

The gold standard in HD diagnosis involves histological analysis of suction biopsy samples containing mucosa and submucosa, or full-thickness biopsies of the rectum in children over 1 year old (8,12,20,22). Accurate diagnosis requires biopsy samples taken 2 to 3 cm above the dentate line to avoid the zone of physiological SP hypo- or aganglionosis (8,20). Suction biopsies should be taken from at least two sites in the rectum (23). HD diagnosis relies on detecting the absence of ganglion cells in serial hematoxylin and eosin stained (H&E) sections and/or visualizing hypertrophic and hyperplastic nerve fibers in serial frozen sections stained with acetylcholinesterase (AChE) technique **(Figure 1B)**. The AChE method is highly sensitive and requires meticulous section preparation (fresh tissue, proper orientation, serial cryostat sections, and always fresh substrate), as well as skilled pathologist interpretation (12). To mitigate these challenges, immunohistochemical staining methods are increasingly used for ganglion cell and nerve fibers detection in practice. Commonly applied antibodies in HD diagnostics include calretinin, S100, Glut-1, MAP-2, peripherin, synaptophysin, and PGP 9.5 (23,24) **(Figure 2A,B)**. Calretinin is most frequently utilized for its ability to detect ganglion cells and intrinsic nerve fibers in the mucosal lamina propria, enabling diagnostics even in superficial suction biopsies and when analysis is performed by unexperienced pathologist (25-28). The absence of calretinin immunostaining is a crucial feature for HD diagnosis (25,29).

Definitive therapy for HD involves surgical resection. There are several surgical techniques with different outcomes (30). The choice of surgical treatment is

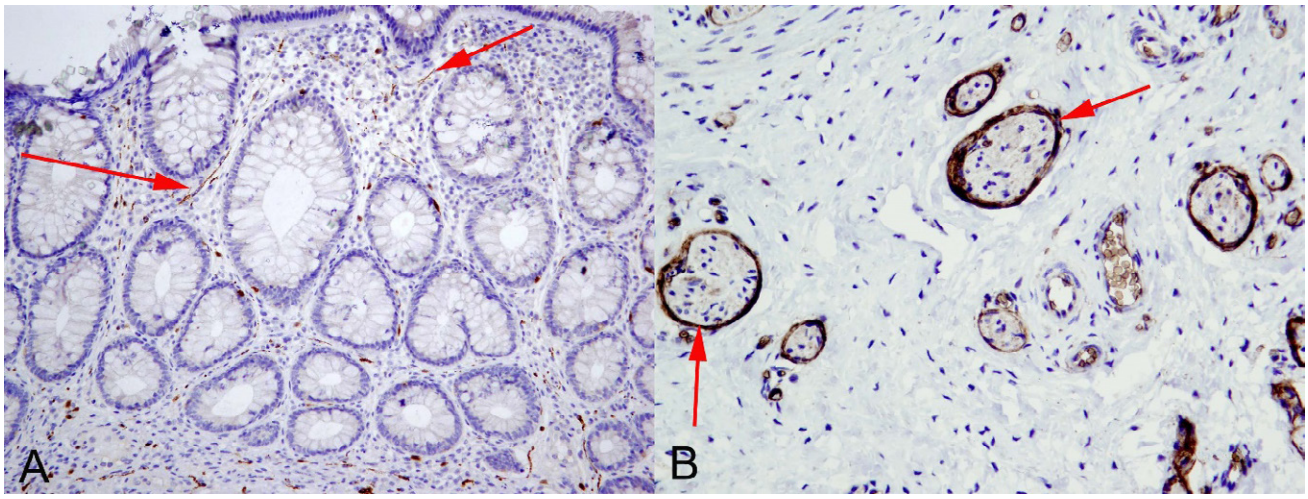

Figure 2. Immunohistochemical staining in HD diagnostics: calretinin positive intrinsic nerve fibers (arrows) in normoganglionic rectal mucosa (A; 200x) and Glut-1 positive perineurium of extrinsic nerves in HD (B; 200x)

conditioned by the length of affected bowel and general condition of the patient. The decision on the extent of bowel resection is based on intraoperative analysis of seromuscular or full-thickness biopsies. The presence of a TZ at the proximal surgical margin often leads to persistent pseudoobstruction, necessitating reoperation (18,23,31,32) or sometimes could be solved by botulinum toxin injections or some kinds of physiotherapy (33). To prevent retention of the TZ, it is recommended to resect at least 5 cm of ganglionic bowel and conduct frozen section examination of the entire proximal resection margin (23). Adopting a standardized and algorithmic approach can reduce anxiety and minimize diagnostic errors (29,31,32). Also, effective communication between the surgeon and the pathologist is crucial. When faced with diagnostic uncertainty, requesting additional tissue is a reasonable next step to ensure accurate diagnosis (31). Transplantation of human enteric nervous progenitor cells is a new approach in HD treatment that will probably play a significant role in the future (34). Pan et al. found that even Schwann cells from aganglionic bowel segment could be a potential autologous source of progenitor cells for regenerative therapy (35).

INTESTINAL HYPOGANGLIONOSIS

Isolated intestinal hypoganglionosis (IIH) is a rare condition (36). The conventional cutoff value for IIH in adults is <1 ganglion per 10 mm of bowel length, with an average of two ganglion cells or less per ganglion (37). Official criteria for pediatric cases are not established due to significant variability in the number of ganglion cells based on the age (37-39). According to Gastro International Working Group 2009, quantitative studies should be conducted by reference laboratories using their own control ranges, collected by the same observer using a standardized method (20,37). Typically, a full-thickness intestinal biopsy is necessary for diagnosis since the disorder predominantly affects the myenteric plexus in many cases. Immunohistochemistry with pan-neuronal markers can confirm hypoganglionosis. Reduced staining for calretinin and NeuN indicates a specific deficiency of intrinsic primary afferent neurons in this disorder (40).

HYPERGANGLIONOSIS

Hyperganglionosis includes diffuse intestinal ganglioneuromatosis and intestinal neuronal dysplasia (IND). Diffuse intestinal ganglioneuromatosis is a hamartomatous lesion of the ENS found in syndromes like multiple endocrine neoplasia type 2B or neurofibromatosis (8,9). In IND type A, there is an absence or significant hypoplasia of intestinal adrenergic nerves from extrinsic ganglia. In contrast, IND type B is characterized by an increased density of "giant" ganglia in the submucosal plexus, which contain at least eight ganglion cells (9,11,41).

DELAYED MATURATION OF GANGLION CELLS (DMGC)

DMGC is identified as the primary cause of constipation in infants during their first year of life. It is crucial that the maturation process of ganglion cells completes by the end of the fourth year of life to ensure proper gastrointestinal function and alleviate potential complications associated with delayed development during this critical period (36). The presence of immature ganglion cells in the ENS after the age of 4 is always a pathological finding, often associated with other ENS abnormalities (9).

GLIOPATHIES

Glial cells are crucial components of ENS ganglia. Traditionally, they were considered to have a supportive role within ganglia. Recent studies have highlighted their significant roles in maintaining ganglion cell homeostasis and neurotransmission. Moreover, they play an important role in intestinal inflammation, particularly in inflammatory bowel disease (IBD), where glial cells act as antigen-presenting cells (42). The Glial Cell Index (GCI) represents the ratio of glial cells to ganglion cells (37,43). Hoff et al. have identified GCI as a robust quantitative measure of the submucosal plexus (SP) and myenteric plexus (MP) within a species (43). Significant alterations in glial cell numbers and GCI have been observed in conditions like diverticular disease and IBD (44). Morphological abnormalities were noticed in aganglionic segment, TZ in HD and even in dilated proximal NZ segment of the bowel (45). Additionally, GCI could serve as a useful marker for TZ in HD (46). S100 and GFAP antibodies are commonly used for the identification of glial cells (43,44).

ENTERIC MYOPATHIES

Enteric myopathies are exceptionally rare conditions. For thorough histopathological evaluation, Masson-trichrome staining, Picrosirius red staining, and periodic acid-Schiff staining (PAS) are recommended. Typical morphological changes include atrophy of the muscularis propria, primarily affecting the longitudinal muscular layer, intracellular vacuolization of smooth muscle cells, and interstitial fibrosis. However, additional specific analyses are advised. Immunohistochemical staining for α-smooth muscle actin (α-SMA) is highly recommended. The loss of α-SMA expression in the circular muscular layer is observed in some cases of intestinal myopathies

(47). Aberrant α-SMA expression may also occur due to abnormalities of intracellular filaments (47). The use of an electron microscope can facilitate establishing the diagnosis **(Figure 3)** (48). Ultrastructural analysis is crucial and typically reveals myofilament degeneration, intracellular vacuolization, irregularity of membranes, variable cytoplasmic density, and increased interstitial collagen deposition (49-51). Morphological evaluation alone is insufficient for a definitive diagnosis, and genetic analyses are often recommended (49).

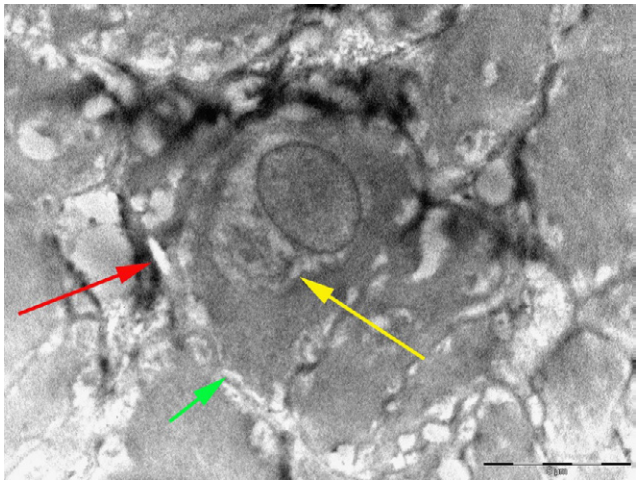

Figure 3. Ultrastructure of smooth muscle cell in visceral myopathy: perinuclear vacuoles (yellow arrow), subsarcolemmal vacuola (green arrow), collagen deposition (red arrow) (4000x).

DISORDERS OF INTERSTITIAL CELLS OF CAJAL (ICC)

ICC, identified by CD117 and DOG-1 immunopositivity, are located around MP ganglia where their density is highest, within the circular and longitudinal muscular layers, deep submucosa, and around submucosal ganglia. Disruption in ICC number and arrangement forms the morphological basis of slow transit constipation. Studies on ICC involvement in HD show conflicting results: while most authors indicate reduced ICC numbers exclusively in affected segments (52), some authors propose that lower ICC counts in the NZ of HD are linked to postsurgical complications, notably constipation (7,53,54). Decresed number of ICC is also described in cases of hypoganglionosis (55).

CONCLUSION

The diagnosis of intestinal dysmotility, particularly conditions like HD and related disorders, require a comprehensive approach integrating knowledge of all components involved in intestinal motility. Key diagnostic methods such as suction and full-thickness intestinal biopsy, along with immunohistochemical analyses, play pivotal roles in modern diagnostics of these conditions. The complexity of these disorders also requires standardization of diagnostic protocols and well-trained pathologists to ensure diagnostic precision, reduce errors, and facilitate accurate treatment for patients. Advances in understanding genetic and molecular mechanisms further enhance precise diagnosis and management of these challenging disorders.

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HISTOPATOLOŠKI ALATI U DIJAGNOZI HIRŠPRUNGOVE BOLESTI I SRODNIH OBOLJENJA

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Sažetak

Dijagnostika Hiršprungove bolesti (HB) i srodnih poremećaja može biti složena i zahteva dobro poznavanje mehanizama koji regulišu crevni motilitet, kao što su enterički nervni sistem (ENS), intersticijske Kahalove ćelije (IKĆ) i mišićni omotač creva. Londonska klasifikacija identifikuje tri grupe gastrointestinalnih neuromuskularnih poremećaja: neuropatije, miopatije i abnormalnosti IKĆ. Hiršprungova bolest koju odlikuje odsustvo ganglijskih ćelija, je najčešća crevna neuropatija i rezultat je poremećene migracije ćelija nervnog grebena tokom razvoja. Pogađa oko 1 od 5 000 živorođene dece i uključuje više gena, među kojima je najznačajniji *RET* gen. HB obično pogađa rektum i deo kolona, sa različitim stepenima aganglionoze. Dijagnoza se zasniva na histopatološkoj analizi sukcionih biopsija - odsustvu ganglijskih ćelija i prisustvu debelih submukoznih nerava na standardnom hematoksilin i eozin bojenom preparatu, dopunjenom enzimohistohemijskim bojenjem (metoda acetilholinesteraze) ili imunohistohemijskim (kalretinin i druga antitela) bojenjem. Lečenje HB podrazumeva hirurško resekciju aganglionarnog segmenta creva. Tačna intraoperativna procena tkivnih margina je ključna za prevenciju postoperativnih komplikacija uslovljenih pseudoobstrukcijom. Komunikacija između hirurga i patologa je od suštinskog značaja za postizanje uspešnog terapijskog ishoda.

Ostale crevne neuropatije uključuju intestinalnu hipoganglionozu, hiperganglionozu, odloženo sazrevanje ganglijskih ćelija i gliopatije. Intestinalne miopatije su izuzetno retka stanja, sa tipičnim morfološkim promenama kao što su atrofija mišićnog omotača creva, intracelularna vakuolizacija glatkih mišićnih ćelija i intersticijalna fibroza. Poremećaj u mreži i rasporedu IKĆ čini morfološku osnovu za konstipaciju sporog prolaza. Svaki od pomenutih poremećaja ima jedinstvene karakteristike i dijagnostičke izazove. Dijagnostikovanje ovih stanja često zahteva kombinaciju histoloških, histohemijskih, imunohistohemijskih i ponekad genetskih analiza. Integracija ovih metoda je od vitalnog značaja za tačnu dijagnozu i efikasno planiranje lečenja.

Ukratko, složenost poremećaja crevnog dismotiliteta zahteva detaljno razumevanje crevnog motiliteta i korišćenje naprednih dijagnostičkih metoda kako bi se pružile tačne dijagnoze i efikasni tretmani.

Ključne reči: Hiršprungova bolest, crevni dismotilitet, biopsija, imunohistohemijsko bojenje

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