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RESEARCH

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Immunohistochemical evaluation of CD34, CD117, and calretinin for diagnosis of hirschsprung's disease

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Abstract

Introduction Hirschsprung's disease (HD) is a neurogenic intestinal disorder attributed to incomplete neural crest cell migration during fetal intestinal development, leading to an aganglionic segment of the colon and functional obstruction. Associated malformations like intestinal atresia, hydronephrosis, and imperforate anus can accompany Hirschsprung's disease. this study aims to evaluate the efficacy of Calretinin and Cajal cells (CD34 and CD117) immunohistochemical staining in improving HD diagnosis.

Methods The study involved 70 pediatric patients suspected of Hirschsprung's disease. Clinical, histopathological, and immunohistochemical analyses were conducted, focusing on calretinin, CD34, and CD117 markers to identify ganglion cells and Cajal cells. Data were statistically analyzed using SPSS software.

Results In the examination of the samples, the calretinin marker exhibited a consistent accuracy of 100% in diagnosing Hirschsprung's disease (with sensitivity and specificity both at 100%). Regarding the markers for Cajal cells in cases of Hirschsprung's disease, an irregularity in the arrangement of Cajal cells was observed, which was absent in normal cases. These markers also demonstrated a specificity and sensitivity of 100% in diagnosing the disease.

Conclusion Hirschsprung's disease remains a complex condition with multifaceted pathophysiological mechanisms. Calretinin immunohistochemical staining offers enhanced diagnostic accuracy, while the debate surrounding ICC distribution underscores the need for advanced diagnostic techniques. Further research is warranted to unravel the intricacies of Hirschsprung's disease and its associated complications.

Keywords Hirschsprung's disease, Calretinin, CD34, CD117, Interstitial cells of Cajal

Introduction

Hirschsprung Disease (HD) is a complex motor disorder of the gastrointestinal tract resulting from the incomplete migration of neural crest cells, which are the precursors of enteric ganglion cells, during fetal intestinal development (Klein and Varga 2020). This leads to the formation of an aganglionic segment of the colon that fails to relax, causing functional obstruction and impairing peristalsis. In addition to its primary manifestations, HD can be associated with various coexisting malformations such as intestinal atresia (Ladan et al. 2023; Ohno 2021), hydronephrosis (Eelen et al. 2020), congenital heart defects

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(Fallahi et al. 2022), imperforate anus (Rougraff et al. 2022), and musculoskeletal or neurological abnormalities (Amiel et al. 2008). Recognizing these concurrent anomalies is essential for comprehensive patient care, even though they may not always be present.

HD occurs in approximately 1 in 5000 live births, with gender ratios varying depending on the extent of colonic involvement (Klein and Varga 2020; Ieiri et al. 2008; Suita et al. 2005). Mutations in several genes, particularly the RET proto-oncogene and the endothelin receptor B (EDNRB) gene, have been identified in patients with HD, leading to disrupted neural crest cell migration and aganglionosis (Amiel et al. 2008).

The underlying cause of HD is multifaceted, involving intricate molecular pathways and genetic factors. The widely accepted theory suggests a defect in the migration of neuroblasts originating from the neural crest, a process that occurs between the fourth and seventh weeks of gestation (Fu et al. 2004). This migration failure leads to an aganglionic segment, rendering it nonfunctional due to the absence of ganglion cells. Furthermore, defects in the differentiation of neuroblasts into ganglion cells and the potential destruction of these cells within the intestine may contribute to the disorder's pathogenesis (Klein and Varga 2020).

The enteric nervous system plays an essential role in coordinating gastrointestinal motility, but the interstitial cells of Cajal (ICC) are equally vital for generating the coordinated peristaltic waves necessary for normal bowel movement (Klein and Varga 2020). ICCs act as specialized pacemaker cells, generating slow waves that initiate contractions in various gastrointestinal tissues. Given their significance, disturbances or impairments in ICC function have been proposed to contribute to motility disorders in the gastrointestinal tract (Gfroerer and Rolle 2013).

Clinical presentation of HD often manifests during the neonatal period, with infants displaying symptoms of distal intestinal obstruction, including bilious emesis, abdominal distension, and a failure to pass meconium or stool (Khan et al. 2003; Gfroerer and Rolle 2015). While the absence of meconium passage within the first 48 h of life is a significant diagnostic indicator (Klein et al. 1984), there can also be an explosive expulsion of gas and stool after a digital rectal examination, known as the "squirt" or "blast" sign (Baaleman et al. 2022). Additionally, enterocolitis, characterized by sepsis-like symptoms such as fever, vomiting, diarrhea, and abdominal distension, can develop, requiring prompt intervention to prevent life-threatening complications (Hagens et al. 2022).

Diagnosing HD relies on rectal biopsies through suction or full-thickness techniques (Muise et al. 2016). These biopsies are considered the gold standard for confirming the absence of ganglion cells in the affected segments. Despite the utility of conventional hematoxylin-eosin (H&E) staining (Hwang and Kapur 2020), immunohistochemistry (IHC) has emerged as an invaluable tool to enhance diagnostic accuracy (Tran et al. 2016). Calretinin IHC, in particular, has proven to be highly specific and sensitive for identifying ganglion cells, making it a reliable alternative to traditional methods (Serafini et al. 2023). In this context, this study aims to evaluate the efficacy of Calretinin and Cajal cell (CD34 and CD117) immunohistochemical staining in improving HD diagnosis, especially in instances where conventional techniques may have limitations.

Materials and methods

Ethical approval

Approval for this study was secured from the Research Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran. Informed consent was obtained from every patient participating in the study.

Study population

Seventy patients, aged 0 to 16 years, referred to Tabriz Children's Educational and Medical Center within one year were included in this study. The patients were suspected of having Hirschsprung disease based on clinical signs. They underwent a full-thickness trans-anal biopsy following the established standard protocol.

Sample examination

Biopsy samples were subjected to standard pathological examination. Immunohistochemical staining was performed using KIT (CD117) (1:100 dilution of the rabbit monoclonal antibody R-0184) (Shanghai Long Island antibody), CD34 (1:100; QBEnd-10, Dako, Glostrup, Denmark) and Calretinin (monoclonal mouse antihuman antibody [DAKO]), (CLONE: DAK-Calret 1, Code: IR627). Immunohistochemical (IHC) analysis was performed to assess the biochemical markers of Cajal cells and Calretinin. The collected data were compared with the surgeon's clinical diagnosis and definitive pathological outcomes.

Data collection

Demographic information and the frequency of symptoms such as gas passage, defecation, vomiting, enterocolitis, and gas and stool expulsion after digital rectal examination (squirt sign or blast sign) were recorded for each patient.

Pathological evaluation

Paraffin blocks and slides were assessed by a pathologist to determine the presence or absence of ganglion cells and the transitional zone anatomical region. The transitional zone was defined as the segment between the contracted aganglionic segment and the normal or dilated ganglionated bowel. Diagnostic criteria for Hirschsprung disease (HD) were the presence of hypertrophic nerve bundles in the submucosa and the absence of ganglion cells. The diagnostic criteria for non-HD were identifying at least one ganglion cell in one or more tissue sections. Hematoxylin and eosin(H&E) staining was used to confirm the initial diagnosis.

Immunohistochemistry (IHC)

Sections of 4 μm were obtained from the slides and fixed with polylysine. The slides were dewaxed, rehydrated, and incubated with primary monoclonal antibodies against calretinin and Cajal cells (CD117, CD34). The immunoreactivity and staining pattern for ganglion cells were evaluated in the IHC-stained slides.

Statistical analyses

The data gathered underwent analysis using SPSS version 18 software. Qualitative data were assessed using the chi-square test, with a significance level of $P<0.05$, indicating statistical significance for this investigation.

Results

Patient demographics

The study comprised 37 boys and 29 girls, with 20 boys and 17 girls in the normal group and 21 boys and 12 girls in the Hirschsprung Disease group ($P=0.4$). The normal group had a mean age of 370.3 days (3 months), while the HD group had a significantly lower mean age of 99.6 days (12 months) ($P=0.002$). Similarly, the patient group exhibited a significantly lower mean weight of 4909.009 g compared to the normal group's mean weight of 7570.2 g ($P=0.007$).

Clinical characteristics

Among the initial complaints, 3 cases (4.2%) reported vomiting, 8 cases (11.4%) presented with abdominal swelling, and 59 cases (84.2%) complained of non-defecation or difficult defecation. Only two HD patients had a history of enterocolitis, compared to none in the normal group ($P=0.4$). Twenty patients in the HD group exhibited explosive expulsion of gas and stool after the digital rectal examination (squirt sign or blast sign), yielding a symptom sensitivity of 60.6% and specificity of 100%. This symptom was not observed in patients over one year old, with only 5 cases older than one month and the remaining 15 cases under one month old.

A summary of the patient's characteristics is provided in Table 1.

Diagnostic markers

The rectosigmoid index, calculated by dividing the widest diameter of the rectum by that of the sigmoid loop under full distension, yielded a rectosigmoid index<1 in 26 cases (78.7%) of HD and 2 cases (5.4%) of normal individuals. The rectosigmoid index<1 demonstrated a sensitivity of 77.77% and specificity of 94.59% in disease diagnosis. Additionally, 21 patients with HD and two normal individuals exhibited no stool passage within the first one to two days of life, resulting in a sensitivity of 63.63% and specificity of 94.59%. A summary of the sensitivity and specificity of diagnostic markers in diagnosing HD is shown in Fig. 1.

Histological findings

Microscopic examination of 70 specimens using H&E staining revealed the absence of ganglion cells in all samples of the HD group, with two false-positive cases in the normal group. IHC staining with Calretinin was negative in all HD cases and positive in all normal cases, with a sensitivity and specificity of 100% for disease diagnosis. HD specimens showed hypertrophied nerve fibers at submucosal layers. Also, the mentioned hypertrophied nerve fibers were noted between circular and longitudinal muscular layers in HD specimens.

On IHC staining, ICCs present as thin and bipolar cells around nerve trunks. For the scoring of the number of ICCs, we used qualitative scoring such as some articles () as follows: (0: absent, +: few ICCs, ++: moderate number of ICCs, +++:many ICCs). According to CD117 staining in HD specimens, ICCs stained sparely

Table 1 Prevalence of symptoms and gender distribution among analyzed patients in the Hirschsprung and control groups

	HD	Control
Male	21(63.63%)	20(54.05%)
Abdominal Distention and lack of gas or stool passing	32(96.96%)	35(95.59%)
Vomiting	1(3.03%)	2(5.40%)
Enterocolitis	2(6.06%)	0
Failure to defecate in the first 48 hours after birth	21(63.63%)	2(5.40%)
Absence of rectal inhibitory reflex and forceful defecation with pressure upon stimulation	20(60.60%)	0
Total	33	37

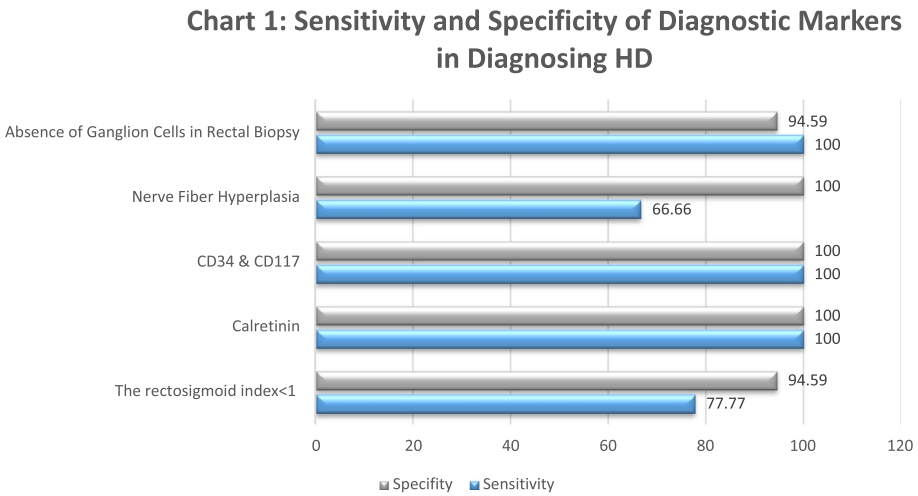

Fig. 1 Sensitivity and specificity of diagnostic markers in diagnosing HD

and as single cells around hypertrophied nerve plexuses. (0 and + of scoring) On the other hand, CD117 staining in normal specimens showed clusters of ICCs around nerve plexuses. (++ and +++ of scoring).

CD117 and CD34 markers indicated positive staining for Cajal cells in all samples(100% sensitivity and specificity in marking Cajal cells), with a marked lower number of Cajal cells in HD samples and irregular, widespread cell placement distinguishing ganglionic from aganglionic

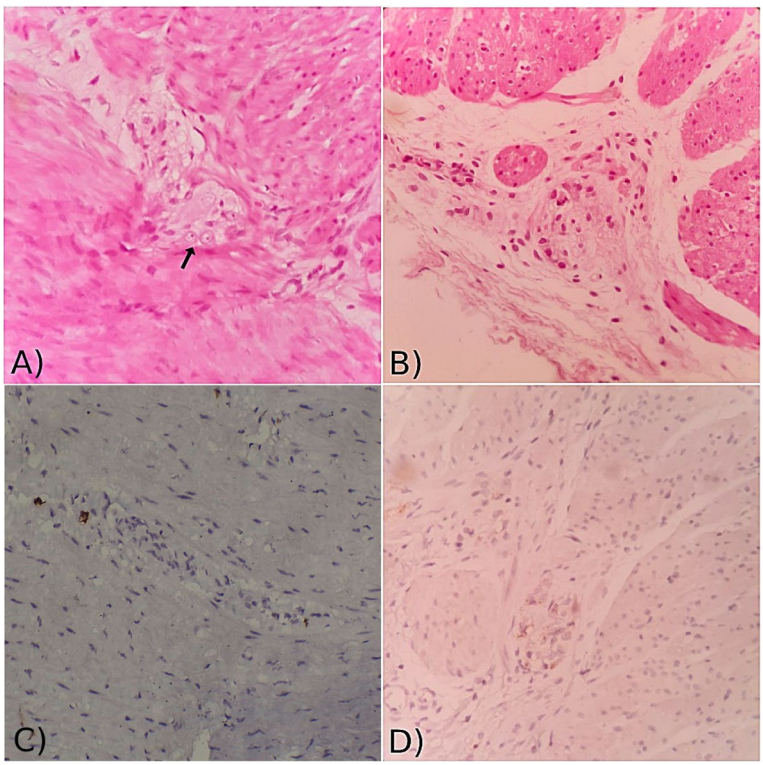

Fig. 2 An example of the obtained samples. **A** A normal sample with hematoxylin-eosin (H&E) staining showing Ganglion Cells(arrow). **B** An H&E-stained sample of Hirschsprung Disease (HD) demonstrating hypertrophic nerve fibers. **C** An ICC CD117 IHC sample showing the irregular distribution of ICCs in a HD smaple. **D** A normal sample of Calretinin IHC demonstrating weak staining of Ganglion Cells

tissue samples. A few of the samples are demonstrated in Fig. 2.

Discussion

HD is a relatively common disease that occurs in almost all races and can significantly negatively impact patients' quality of life with symptoms of chronic constipation and obstructive periods. In severe cases, HD is a potentially fatal disease that can cause death due to enterocolitis or functional obstruction and perforation of the intestines. Proper diagnosis and early treatment have particular importance.

Despite the various diagnostic methods invented so far since Harold Hirschsprung described the disease, rectal biopsy plays a leading diagnostic role in pathology centers worldwide. It is a well-known method for diagnosing intestinal motility disorders such as Hirschsprung. At the same time, the patient's clinical symptoms, manometry, and barium enema can also be used to diagnose the disease.

Interstitial Cells of Cajal (ICCs) play a crucial role in coordinating intestinal motility. These specialized cells are found within the human bowel wall, specifically at the myenteric plexus between the longitudinal and circular muscle layers, as well as within the deep muscular plexus. Previous research has identified three primary functions of ICCs (Friedmacher and Rolle 2023):

1. Pacemaker Cells: ICCs serve as pacemakers for smooth muscle contractions.
2. Electrical Propagation: They facilitate the active spread of electrical events.
3. Neurotransmission: ICCs are involved in mediating communication between nerve cells.

Some studies also suggest that ICCs may produce nitric oxide, which enhances inhibitory neurotransmission (Rolle et al. 2002).

Altered distributions of ICCs have been observed in various human intestinal motility disorders, including hypertrophic pyloric stenosis, HD, intestinal pseudo-obstruction, slow-transit constipation, and ulcerative colitis (Friedmacher and Rolle 2023). Reduced myenteric ICC counts and disordered myenteric ICC networks, which are only sporadically dispersed along hypertrophic nerve trunks, are two of the major pathogenic characteristics of HD (Friedmacher and Rolle 2023). Moreover, a significant reduction in muscle ICCs has been seen in the HD bowel. It's interesting to note that, as compared to healthy controls, a complete decrease in ICCs has also been seen in the proximal ganglionic colon of HD patients (Gfroerer and Rolle 2013). In the aganglionic HD bowel (where ganglion cells are absent), ICCs are usually scarce and exhibit a disrupted network, while in the ganglionic bowel of HD (with normal ganglion cells), the distribution of ICCs resembles that observed in healthy controls. However, these ICCs do not form a network and show no clear relation to hypertrophic nerve trunks (Rolle et al. 2002; Chen et al. 2014).

This study examined the immunohistochemical staining of Calretinin and Cajal cells in biopsy specimens of the rectum's full thickness in patients with suspected Hirschsprung's diagnosis One of the results obtained in this study was the ease of performing and interpreting IHC staining compared to H&E In examining the existence of ganglia.

In this study, calretinin IHC staining was 100% sensitive and specific for the diagnosis of HD In the case of CD117 and CD34 markers for Cajal cells' presence, results showed us 100% sensitivity and specificity. However, the critical point is that these markers were observed in all samples in two groups. Still, In the samples of patients with HD, these cells were scattered and irregular, and the interpretation of the results required the observation of an experienced pathologist in this field; thus Cajal cells IHC have less value compared to calretinin staining.

Also, if the first defecation is after the first 48 h, it has a very high specificity in HD's diagnosis in our study. Another significant finding in our study was the high-pressure and explosive excretion of feces after digital rectal examination in patients with HD. This symptom was observed only in patients under one year of age. Most of them were under one month old.

In a study by Chen et al., surgical resection samples of both proximal and narrow segments, from HSCR patients and normal controls were compared (Chen et al. 2014). The study found that both the presumed early progenitor and committed progenitor cells of interstitial cells of Cajal (ICC) exist in the adult normal colon as well as in the narrow and proximal parts of the Hirschsprung's disease (HSCR) colon. However, the proportions of mature, early, and committed progenitors of ICC were dramatically reduced in the narrow segment of the HSCR colon. Similarly, the proportions of mature and committed progenitors of ICC in the proximal segment of the HSCR colon were lower than in the adult normal colon. These results were consistent with our findings.

Coyle et al. investigated the utility of ANO1 in evaluating the colonic ICC network in HD in 2016 (Coyle et al. 2016). They evaluated the presence of ICCs using ANO1 and c-kit expression and found that the distribution of ANO1-positive ICCs was sparse in the aganglionic colon, with a modest reduction in ICCs seen in the ganglionic colon in HD samples compared to controls. Although they used different markers to evaluate ICCs, their results were consistent with ours.

In a study by Morris and colleagues in 2013, in some cases where acetylcholinesterase staining was not diagnostic for HD, Calretinin staining was completely diagnostic. In our study, we achieved comparable findings regarding Calretinin as well (Morris et al. 2013).

In the study of Bjorn et al. in 2018, 555 patients were evaluated for adequacy and suitable location for biopsy. 5.9% of biopsies were inappropriate and non-diagnostic, and 6.6% of patients had complications caused by biopsy. In our study, all samples were appropriate, and none of the patients had complications (Bjørn et al. 2018).

In the study of Muise and colleagues on 47 patients using suction biopsy and full-thickness biopsy, the number of insufficient biopsies was not statistically significant, and no patient had complications as a result of the biopsy. Both methods in the comparisons were completely diagnostic. Of course, in our study, biopsy suction was not performed, but full-thickness samples were perfectly suitable (Muise et al. 2016).

In a study by Lorencao et al. in 2014, acetylcholinesterase and Calretinin staining to diagnose HD were compared with H&E staining. This study showed that calretinin staining has higher accuracy than H&E. The biggest problem with acetylcholinesterase staining was the need to see hypertrophic nerve fibers in tissue samples (Arruda Lourenção et al. 2014). Similar results were obtained in our study. In a 2016 study conducted by Dr. Hatami and colleagues, an assessment was conducted to compare permanent and frozen sections for evaluating HD. The frozen section yielded a negative predictive value of 97% and a positive predictive value of 80%, underscoring the limitations of the freezing incision method in achieving a conclusive diagnosis for HD cases (Hatami et al. 2016). But in the study of Dr. Aslanabadi et al. In 2010, very high accuracy for the frozen section samples and their high compatibility with permanent incisions were obtained (Aslanabadi et al. 2010).

Conclusion

Immunohistochemical staining utilizing Calretinin has demonstrated exceptional diagnostic accuracy in identifying HD, surpassing the conventional Hematoxylin and Eosin (H&E) staining method. This immunohistochemistry method is especially effective in eliminating false positive results, improving the diagnosis's accuracy. The use of CD34 and CD117 markers, on the other hand, does not demonstrate adequate specificity in confirming the presence of Cajal cells, and these markers do not demonstrate superior performance when compared to the established conventional staining methods. It is crucial to clarify that CD34 expression is predominantly observed in a small subset of immature or progenitor ICCs, rather than in mature ICCs. This distinction is important because using CD34 as a general marker for ICCs could lead to misconceptions about its applicability across different maturation stages of these cells. Our findings align with the observations reported in the study by Chen et al., which also highlight CD34's role in identifying progenitor cells. In light of this clarification, we investigated the diagnostic effectiveness of this marker along with calretinin and we suggest that these markers would make an adequate means of diagnosing HD in the absence of more accurate markers.

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Authors' contributions
Ah. L., P. MY. And S. A. Contributed to study design and preparation of the manuscript. Ah. L., D. B., M. J., E. F., N. H. contributed to data gathering. Ah. L. And S. A. contriduted to data quality assessment and data analysis. Ah. L., P. MY. And S. A. contributed to Finalizing the study results and manuscript. The final form of the manuscript was reviewed and approved by all authors.

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Declarations

Ethics approval and consent to participate
This work has been ethically approved by Tabriz University of Medical Sciences with Ethical Code IR.TBZMED.REC.1398.1026. Human Ethics and Consent to Participate was waived by the ethical committee. The study was carried out in accordance with the Declaration of Helsinki.

Availability of data and materials
The data that support the findings of this study are available on request from the corresponding author.

Competing interests
The authors report no conflict of interest.

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