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Hirschsprung's Disease Diagnosis: A Comparison of Calretinin and CD56 Immunohistochemistry

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Abstract

Background & objectives: Hirschsprung's disease is attributable to the congenital lack of ganglion cells in the far intestine. Rectal biopsy is deemed critical for its testing. In some instances, regular techniques fail to detect it. This study aims to evaluate the testing role of calretinin and CD56 immunohistochemistry and correlate the results to routine hematoxylin & eosin stained samples.

Methods: This retrospective study was conducted in Rizgary Teaching Hospital, Erbil, Kurdistan region, Iraq. Rectal biopsies and colonic resection specimens of the clinically suspected Hirschsprung's disease patients were collected and stained with calretinin and CD56 then their findings were compared to the hematoxylin and eosin stained sections during the period between February 2016 to October 2021.

Results: Fifty patients aged from 3 days to 8 years with a male-to-female ratio of 3.6:1 were examined for rectal and colonic biopsies. Forty out of 50 cases were detected as HD by H&E staining. The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy rate of calretinin were coherent with that of H&E-stained samples (100%), the likelihood ratio was 50 and the kappa test was 1, were superior to CD56 results with sensitivity (100%), specificity (90%), positive predictive value (97.5%), negative predictive value (100%), accuracy rate (98%), likelihood ratio was 27.77 and kappa test was 0.805.

Conclusions: Immunohistochemical expression of calretinin is more specific and accurate than CD56 in HD diagnosis. Calretinin is a trustworthy, additional diagnostic tool for better morphological evaluation of ganglion cells and thereby assists in making a reliable diagnosis of HD.

Keywords: Hirschsprung's disease, Ganglion cells, Immunohistochemistry, Calretinin, and CD56.

Introduction

A developmental condition of the enteric nervous system known as Hirschsprung's disease (HD) is consisting of ganglion cells loss in the myenteric (Auerbach) and

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submucosal (Meissner) nerve plexuses, the rectosigmoid area is where HD usually manifest, but in few cases, the whole colon can be impacted. Functional intestinal blockage at the level of aganglionosis is typical in HD patients.[1] Hirschsprung's disease affects 1 in 5000 live births,[2] with a 4:1 male-to-female ratio.[3]This ratio is the same in Iraq.[4] While a study in the Kurdistan area found that there were 1.1 times as many males as females there.[5] The etiologic idea that is most widely recognized is based on a malfunction in the neuroblasts' early embryonic migration along their axis.[6] Most patients are diagnosed in the early newborn time due to failure to initiate bowel motion, others manifest later, with intestinal blockage, and persistent constipation[7]. The patient's presenting symptoms, rectal manometry, radiographic findings, and the histological characteristics of rectal biopsy are frequently combined to make the diagnosis of HD.[8] The presence of hypertrophied nerve fibers in the submucosa of the aganglionic segment and the absence of ganglion cells in the submucosal and myenteric plexuses are considered the gold standard for the diagnosis in histopathological examination of rectal biopsy utilizing routine standard hematoxylin and eosin (H&E) staining.[9] Several supplementary approaches, such as acetylcholinesterase (AChE) histochemistry and different immunohistochemistry (IHC) markers, have been developed to help with the diagnosis of HD because locating the ganglion cells can require several serials cut sections and have many drawbacks.[10] The AChE histochemistry approach has limitations, such as the need to treat frozen sections, interpretive issues, ambiguous or false-positive results, and technological obstacles.[11] Numerous immunohistochemical stains have been researched recently with the advancement of immunohistochemistry.[10] The shape and function of the growing central nervous system depend on the calcium-binding protein calretinin, which is vitamin D dependent and implicated in calcium signaling.[12] Calretinin immunohistochemical stain, a helpful supplementary diagnostic technique for HD, accurately identifies ganglions by staining their nuclei, cytoplasm, and specific nerve fibers.[13] The majority of tissues produced from neuroectoderm exhibit the calcium-binding protein CD56, also called neural cell adhesion molecule (NCAM), which is essential for neural cells to adhere to one another as they mature. Anti-CD56 marks Schwann cells, lymphocyte subsets, neurons, glia, and natural killer cells in healthy tissue.[14-16] A definitive histological diagnosis of isolated hypoganglionosis is shown to benefit from CD56.[16] This study intended to compare the diagnostic significance of calretinin immunostaining to CD56 in clinically suspected HD patients and to correlate the results with H&E-stained materials.

Material and methods

The records of the Histopathology Department at Rizgary Teaching Hospital and private laboratories in Erbil city were collected for a retrospective cross-sectional study (survey). The researchers collected 50 formalin-fixed paraffin-embedded specimens of full-thickness rectal biopsies and colonic resections of suspected cases of Hirschsprung's disease from February 2016 to October 2021. Kurdistan Higher Council of Medical Specialties granted approval for this study.

From each block, three sections were removed, two of which were used for immunohistochemical analysis for calretinin and CD56 expression to further confirm the

recognition of ganglion cells and nerve fibers. One section from each block was dyed with H&E for histological analysis regarding the presence or absence of ganglion cells.

Hirschsprung's disease diagnostic criteria: Patients had to have clinically suspected HD symptoms to be included (constipation, belated passage of stool, abdominal expansion, etc.) and no ganglion cells by H&E stained rectal samples but enlarged nerve bundles. Non-disease Hirschsprung's diagnostic standards (NHD): Clinical characteristics unrelated to HD and the identification of minimally one ganglion cell in one or more tissue pieces serve as exclusion criteria[17].

Calretinin from Dako (FLEX monoclonal mouse Anti-Human calretinin Clone DAK-Calret, ready to use (Dako Autostainer/Autostainer Plus)) was used for immunohistochemical staining. Both CD56 from Dako (FLEX monoclonal mouse Anti-Human CD56 Clone 123C3, ready to use (Dako Autostainer/Autostainer Plus)) and CD56 from the latter. EnVision FLEX+, Mouse, High PH (Dako Autostainer/Autostainer Plus) is the suggested visualization system (Code K8018). Positive controls: for CD56, the nerve fibers serve as an internal positive control, whereas for calretinin, a known mesothelioma case. Negative controls involve leaving out the principal antibodies for both markers in each run.

Immunohistochemical evaluation: Cases were classified as positive or negative for either marker. Calretinin: Positive categorization was given to unmistakably positive staining of the nerve fibers in the muscularis propria, mucosa, and lamina propria, along with significant cytoplasmic and nuclear staining of the ganglion cells; otherwise, they would be classified as negative[18]. CD56: Positive categorization was given to stained nerve fibers and ganglion cells (membranous and granular cytoplasmic), whereas negative categorization was given if only hypertrophied nerve fibers and bundles were stained but no ganglion cells were found[16].

Data were analyzed using the Chi-square test of association to compare proportions. A *p*-value of ≤ 0.05 was considered statistically significant. All the validity tests including sensitivity, specificity, positive predictive value, negative predictive value, and accuracy rate of both calretinin and CD56 were calculated in addition to the likelihood ratio and Kappa tests.

Results

A total of 50 patients were enrolled in this study investigating the diagnosis of Hirschsprung's disease. The mean age ± std. deviation of participants was 23.46 ± 29.18 months. The demographic and clinical features are presented in **Table (1).**

Results revealed that the majority (80%) of cases were positive for HD by hematoxylin and eosin stain and an equal percentage (80%) for the immune stain with calretinin. The same specimens stained with CD56 revealed a little higher percentage (82%) tested positive.

For Calretinin, in non-HD cases, there was intense nuclear and granular cytoplasmic staining of ganglion cells and nerve fibers, while completely absent in all neural components in HD cases. Regarding CD56, in non-HD, there was membranous and granular cytoplasmic staining of the ganglion cells as well as nerve fibers, while only the hypertrophied nerve bundles were stained in the aganglionic tissue (HD cases) Figure (1).

Table (1): Demographic, clinical presentation and type of specimens.

Variables	Categories	No.	%
Age groups	<1 year	25	50
	1 – 2 years	14	28
	> 2 years	11	22
Gender	male	39	78
	female	11	22
Clinical presentation	delayed passage of meconium	7	14
	chronic constipation	37	74
	intestinal obstruction	6	12
Type of specimens	Full thickness rectal biopsy	23	46
	colon resection	11	22
	recto-sigmoid resection	16	32

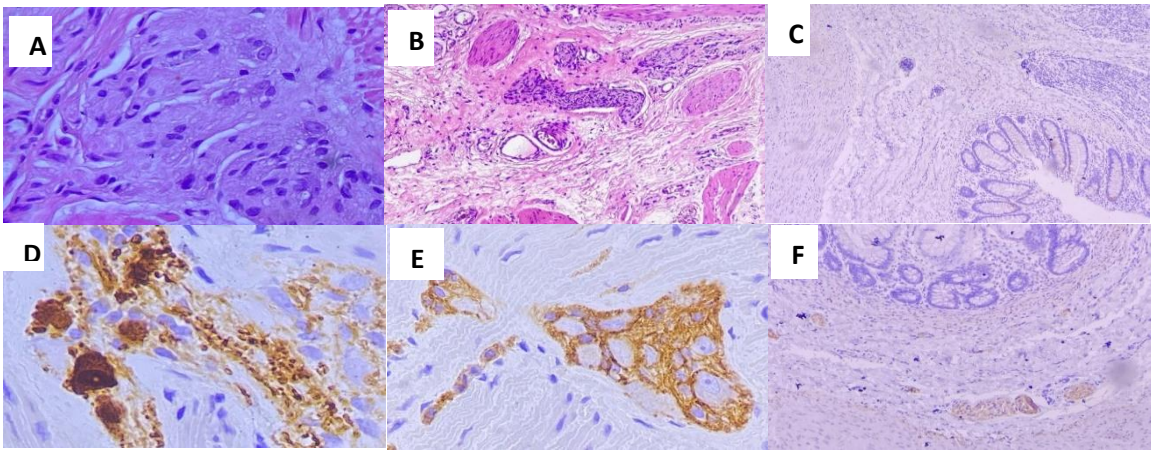

Figure (1): (A) ganglion cells in non-HD case (H&E-stain 400X), (B) Lack of ganglion cell with nerve hypertrophy in the submucosa of the rectum in HD patient (H&E-stain 200X), (C) Total Calretinin negativity in HD (IHC 200X), (D) Calretinin staining in non-HD (IHC 400X). (E) CD56 staining in non-HD (IHC 400X), and (F) CD56 stains only the nerve fibers in the HD case (IHC 200X).

All the validity tests namely: sensitivity, specificity, positive predictive value, negative predictive value, and accuracy rate of calretinin were 100%, the likelihood ratio was 50, and the Kappa test was 1, meaning a perfect and total agreement between screening and gold standard test Table (2).

Table (2): Sensitivity, specificity, PPV and NPV of calretinin as a screening test for Hirschsprung's disease.

Screening tests		Hematoxylin and eosin positive	Hematoxylin and eosin negative	Total	p-value
Calretinin	positive	40	0	40	< 0.001
	negative	0	10	10	
Total		40	10	50	

The validity tests of CD56 as a utilized screening tool for HD, were as follows: sensitivity was 100%, specificity was 90%, positive predictive value was 97.5%, negative predictive value was 100%, the accuracy rate was 98%, likelihood ratio was 27.77 and Kappa test was 0.805 meaning a substantial agreement between screening and gold standard test Table (3).

Table (3): Sensitivity, specificity, PPV and NPV of CD56 as a screening test for Hirschsprung's. disease.

Screening test		Hematoxylin and eosin positive	Hematoxylin and eosin Negative	Total	p-value
CD56	positive	40	1	41	<0.001
	negative	0	9	9	
Total		40	10	50	

Discussion

Hirschsprung's disease is one of the most prevalent causes of newborn intestinal obstruction, a particular gene in the development of the enteric nervous system and the migration of ganglion cells has significantly advanced its molecular diagnostics cause.[18-21] The patient's presenting symptoms, radiographic findings, and the histological characteristics of a rectal sample are frequently used to make the diagnosis of HD.[22] In this research, most of the patients presented in the newborn period. This is consistent with how patients present in industrialized countries, where most do so early, primarily in the newborn stage.[23,24] With a male-to-female ratio of 3.6:1, which is analogous to the universal ratio of 2.9: 1 to 4.5: 1, the current study demonstrated that males were more afflicted than females,[25] 75% of our HD-positive patients were suffering from chronic constipation which is compatible with other published studies.[23,26] The second most frequent symptom was delayed meconium passage, while 10% of children reported intestinal obstruction, and our clinical assessment was consistent with the clinical findings in the literature.[24] Rectal biopsy continues to be the gold standard for definitively diagnosing HD. Histopathological analysis using standard H&E staining demonstrates that the Meissner's and myenteric plexuses are devoid of ganglion cells and that the submucosa of the aganglionic segment contains hypertrophied nerve fibers.[9] The intensity of ganglion cells declines at the end of the rectum, making it difficult to locate the ganglion cells. To overcome possible sampling issues, taking the biopsy at least 2 cm on top of the dentate line is advisable. Another issue might arise if there is insufficient submucosa in the biopsy. Therefore, the biopsy specimen should have a sufficient amount of submucosa.[12] Additionally, during the neonatal period, GC is immature and challenging to distinguish from non-neuronal cells.[27]

Several studies have indicated that calretinin immunohistochemical stain is very helpful for resolving these problems and creating a conclusive diagnosis with H&E stain.[27] Calretinin, which has a crucial role in regulating neuronal activity, shows positive immunoreactivity in the nerve structures, and ganglion cells. In aganglionic segments, any positive calretinin immunoreactivity was not seen in hypertrophic nerve fibers[12]. After analyzing H&E-stained slides for the current study, we determined that 40 patients were HD and 10 instances were NHD. All cases that tested positive for ganglion cells by H&E staining displayed strong calretinin positivity, whereas all aganglionic segments tested negative for immunoreactivity by H&E staining. Calretinin staining is positive in both ganglia and nerve fibers in normal colons and negative in all aganglionic areas in HD cases, according to Barshack et al[28] and Maldyk et al.[29] The Kappa test result was 1, the likelihood ratio was 50, and the accuracy rate, sensitivity, specificity, positive predictive value, and negative predictive value were all 100%. The same details as those provided by Fakhry et al.[30] According to Hiradfar et al., rates of sensitivity, specificity, positive, and negative predictive values were, respectively, 93.3%, 100%, 100%, and 93.8%.[31] Our data were in accord with earlier published research that asserted calretinin staining was a viable auxiliary test for the detection of HD, particularly in difficult cases, and were very significant (p 0.0001). Calretinin IHC is easier to interpret than AChE, according to Zuikova et al[32].

The other marker, CD56, was well known for being helpful in making a conclusive

histological diagnosis of isolated hypoganglionosis.[16] The study revealed that CD56 stains both the ganglion cells and the nerve fibers in the ganglionic segment, while in the aganglionic segment, only the hypertrophied nerve bundles will be stained. Our results were related to Yoshimaru et al.[16] findings, in which the CD56 stained only the nerve fibers and bundles in the ganglion-deficient parts of HD patients. According to Park et al, CD56 showed significant expression of each of the enteric nerve plexus's constituents.[33] Because all of the nerve bundles were stained with CD56 when GC was absent, interpreting negative instances for GC was more challenging than for Calretinin. While Calretinin is negative. There is significant agreement between CD56 and H&E when compared. The sensitivity was 100%, specificity was 90%, positive predictive value was 97.5%, negative predictive value was 100%, the accuracy rate was 98%, and likelihood ratio was 27.77. The correlation was completely accurate statistically ($k = 0.805$, $P < 0.001$).

Conclusions

To rule out HD, our work showed that both calretinin and CD56 IHC were sensitive and specific in identifying ganglion cells and nerve fibers. Calretinin, however, demonstrated greater precision than CD56. Interpreting calretinin was also simpler. When coupled with H&E microscopic results in the testing of HD, we concluded that calretinin immunostain is a trustworthy supplementary diagnostic tool.

Conflicts of interest: There were no conflicts of interest.

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