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Original Article

Evaluation of the Diagnostic Value of Calretinin Immunohistochemistry Assay in the Superficial Rectal Biopsy of Children Suspected of Hirschsprung's Disease

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ABSTRACT

Background: Hirschsprung's disease (HD) is the most common cause of colon obstruction in neonates. Lack of calretinin immunohistochemistry (IHC) in colon lamina propria could be associated with aganglionosis.

Methods: This cross-sectional retrospective study examined 64 patients suspicious of HD and conducted on collected-demographic data and Hematoxylin/Eosin (H & E) results of the surgical rectal biopsy from the archive of Imam Hossein Hospital, Isfahan, Iran. Calretinin IHC was achieved for all the specimens, and the results of the two methods were compared. The receiver operating characteristic (ROC) chart shows sensitivity changes according to (1-specificity). Moreover, sensitivity, specificity, as well as the positive and negative predictive values (PPV and NPV) of calretinin staining in lamina propria and muscularis mucosa, compared to H & E staining, was calculated in this study.

Results: The number of males and females was the same in this study (n=32; 50%). The age of the patients ranged from 1 month to 156 months (13 years). Out of 64 patients, 8 cases were diagnosed with HD, and calretinin staining was negative in 7 patients; moreover, only one case showed weak positive staining. In 56 patients, who were not HD, two patients with negative calretinin staining had a positive H&E staining report. The current study aimed to check the diagnostic value of calretinin IHC in lamina propria of patients suspicious of HD. Compared to H&E staining, which is the routine method in thick wall biopsies, the statistics show that calretinin IHC has a high diagnostic value ($P<0.001$), sensitivity of 87%, specificity of 96%, PPV of 77%, and NPV of 98%.

Conclusion: In conclusion, calretinin IHC utilized in specimens seems superficial and inadequate; moreover, the association with the high specificity percentage (96%) could help physicians with less invasive biopsy methods, such as endoscopic rectal biopsy.

Keywords: Calretinine, Immunohistochemistry, Hirschsprung, Rectal biopsy

Introduction

Hirschsprung's disease (HD) is a functional disorder of the distal gastrointestinal tract and the most common cause of colon obstruction in neonates (1-3), which is resulted from the absence of ganglion cells in the submucosal plexus or myenteric plexus due to impaired migration of neural crest cells during fetus development (4, 5). Diagnosis of HD is based on clinical presentations, screening methods, such as rectum manometry, and radiologic contrast enema, followed by a confirmatory histopathology study (6-8). Based on clinical presentation, contrast enema is the first modality (9) that is a crucial guide to evaluate the next step and surgery plan (10). Colonoscopy or a rectal suction biopsy should be performed to obtain the pathology of the samples of Hematoxylin/Eosin (H&E) staining that is the most common method to evaluate biopsies (11). Although this method is used worldwide, it leads to some interpretation challenges, including the lack of cytoplasm in immature ganglion cells and biopsy with some problems (i.e., inadequate

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muscular bundles or submucosa in the sample depending on the abilities of an observer) (3, 12, 13). Therefore, new approaches have been developed to improve the accuracy of the diagnosis and various techniques, such as Acetylcholine esterase histochemistry (AChE), S-100 protein, neuron-specific enolase, and glial fibrillary acid protein immunohistochemistry (IHC) have been investigated in this regard (14-16). Among the mentioned methods, AChE is more proper with high specificity; however, fresh tissue for AChE staining causes this technique to make many problems in HD patients. AChE staining could also be challenging to perform and interpret in several cases, even for experienced pathologists (3, 17). During recent years, researchers have assessed different IHC stains (18, 19), and calretinin IHC seems more advantageous than the others (20). Calretinin is a vitamin D-dependent calcium-binding protein associated with calcium signaling, presenting in nerve fibrils of muscularis mucosa and lamina propria. The important diagnostic feature of aganglionosis is the absence of immunoreactive small nerves or individual neurites in the lamina propria and muscularis mucosa, and the lack of calretinin in tissue could be associated with aganglionosis (2, 21, 22). Furthermore, calretinin can be easily applied to even small and superficial specimens obtained during colonoscopy and interpreted even by inexperienced pathologists (21, 23, 24).

According to the beneficence of calretinin IHC in superficial specimens and limitations in using thick wall biopsies, the current study aimed to evaluate the diagnostic value of the calretinin IHC method in colon mucosa (lamina propria) and compare the results with the classic ganglion cell assay in deep surgical biopsies as a primary diagnostic test.

Methods

This cross-sectional retrospective study was conducted on 73 patients at the Pathology Department of Imam Hossein Paediatric Hospital, Isfahan, Iran, from October 2018 to November 2020. After explaining the aim of the study, written informed consent was taken from the parents; moreover, demographic characteristics and general information of the patients were obtained using a questionnaire. Considering the clinical presentations suspicious of HD in all patients, they were referred to the paediatric surgery department of the same health centre after contrast studies. Afterward, three samples from three positions were provided from 3-4 centimetres above the anal verge, fixed using formalin 10%, and embedded in paraffin blocks. Subsequently, paraffin-embedded samples were cut into 4µm thick sections. H&E staining was applied, and ganglion cells were assayed in the neural plexus, resulting in reporting the results registered in our questionnaire. During these years, patients with positive HD had been referred to the surgery department to perform a colon resection surgery based on H&E staining results. The size of the resection depends on the length of the aganglionic colon. The exclusion criteria were the patients 1) who were reluctant to participate in the study, 2) who had inappropriate biopsy samples (lack of colon mucosa or lamina propria, ruined samples), and 3) whose biopsies had not been stained with the aforementioned methods.

Calretinin staining detects small nerve, individual neurites, and ganglions in lamina propria, muscularis mucosa, and submucosa in rectal biopsies, resulting in ruling out the HD. In addition, the same rectal biopsy blocks were obtained from the sample bank, and calretinin IHC staining was conducted using monoclonal antibodies of the mouse (Dako Company; Clone-DAK Calret 1) with a dilution of 1:100. Samples were provided to a PH=9 buffer at 120°C for 30 min, and the purification was then performed for 30 min using the Dako technique. In each run of the staining, appendix tissue served both as negative and positive controls. Demonstration of any specific light gray to light brown in muscularis mucosa or lamina propria showed nerve fibre (Figures 1, 2). An experienced pathologist

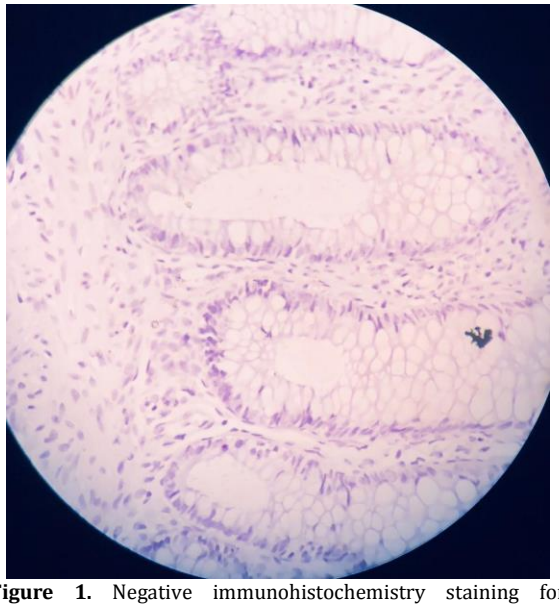

Figure 1. Negative immunohistochemistry staining for calretinin staining in rectal biopsy (×40)

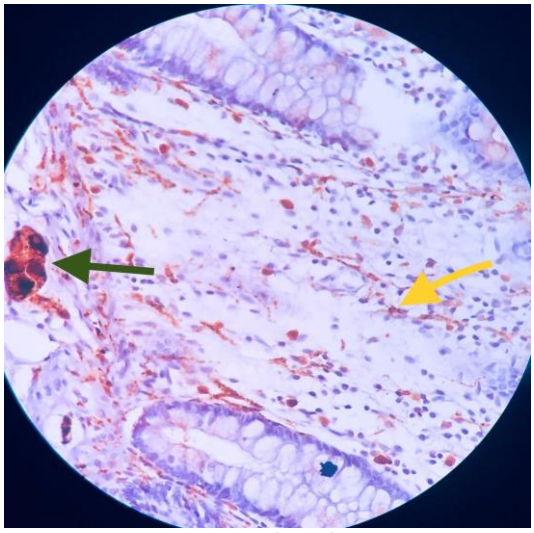

Figure 2. Positive immunohistochemistry staining for calretinin staining in rectal biopsy (×40); yellow arrow: nerve fibers; green arrow: ganglion cells

interpreted the calretinin only in lamina propria and muscularis mucosa without any H&E staining reports to evaluate superficial specimens. The calretinin staining results were compared to the H&E staining and final diagnosis.

Data recorded on these worksheets were analyzed using SPSS software (version 25). Firstly, descriptive data, such as age and gender distribution were evaluated using descriptive analysis, and the receiver operator characteristic curve of IHC, compared to H&E, was used for the diagnostic value of the tests in HD. The receiver operating characteristic (ROC) chart shows sensitivity changes according to (1-specificity). Furthermore, sensitivity, specificity, PPV, and NPV of the calretinin staining in lamina propria and muscularis mucosa, compared to H&E staining, and the final diagnosis were then calculated (Table 1).

Table 1. Diagnostic value of immunohistochemistry

		Disease +	Disease -	Predictive value	
Test	+	7	2	Positive predictive value: 77%	Total positive results: 9
	–	1	54	Negative predictive value: 98%	Total negative results: 55
Sensitivity and Specificity		Sensitivity: 87% All diseased patients: 8	Specificity: 96% All non-diseased patients: 56		

Results

From 73 suspected cases, five and four patients were without the pathology report and with destroyed samples, respectively. Accordingly, a total of 64 eligible samples (32 males and 32 females) were included in the study with the age ranging from 1 month to 156 months (13 years). Furthermore, the mean ages of the boys and girls were obtained at 41.56±7.54 and 31.5±5.86, respectively, and the mean age of female patients was significantly less than that of male cases (P<0.001). Out of 64 patients, 8 cases were diagnosed with HD, calretinin staining was negative in 7 cases, and only one patient showed weak positive staining. Additionally, in 56 patients who were not HD, 2 patients with negative calretinin staining had a positive H&E staining report. Figure 3 shows the high diagnostic value of the immunohistochemical assay to evaluate calretinin marker in paediatric rectal biopsies suspected of the HD, and the P-value (P<0.001) also indicates that calretinin IHC has a positive and significant diagnostic value for diagnosing HD in rectal biopsies of children. Based on the confidence interval (CI), the area under the curve (AUC) is between 78.2% and 100% with

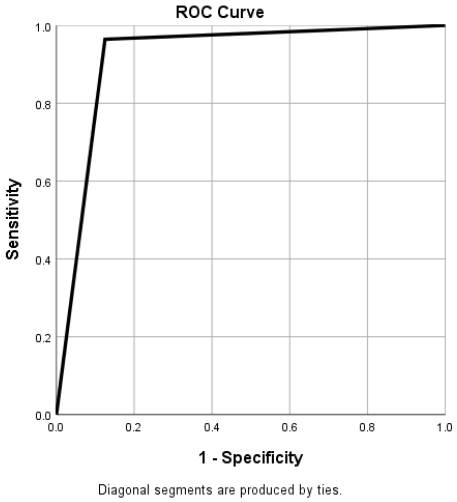

Figure 3. Area under the curve (P<0.05)

Area Under the Curve

Test Result Variable(s): Calretinin test

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval Lower Bound	Upper Bound
0.92	.070	.000	.782	1.000

The test result variable (s): Calretinin test has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

95% CI (i.e., the predictive power of the immunohistochemical method for the diagnosis of calretinin marker is at least 78.2% and at most 100% for 95% CI) (Figure 3). The sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of the variables were evaluated in this study (Table 1).

Discussion

The current study aimed to check the diagnostic value of calretinin IHC in lamina propria of patients suspicious of HD. Considering H&E staining, which is the routine method in thick wall biopsies, the statistics show that calretinin IHC has a great diagnostic value (P<0.001), sensitivity of 87%, specificity of 96%, PPV of 77%, and NPV of 98%. Firstly, the specificity of 96% shows a reliable way to diagnose the disease, providing the positive test (negative staining) that results in releasing the patient from surgery in colonoscopy biopsy. However, the results show that this method can be used on the missing patients (sensitivity: 87%). Some studies checked the calretinin marker in HD patients. In a study in 2015 on adolescents suffering from HD, the IHC results showed 96.2% specificity and 93.6% sensitivity, which were consistent with those of the current study. Out of 94 subjects, the result of IHC and H&E were not equal only in 3 cases (5). In another study conducted by Musa et al. on 40 HD patients, sensitivity and specificity were 100% using calretinin IHC (12). The results of other studies are also consistent with these findings (i.e., the high diagnostic values of calretinin IHC that the sensitivity and specificity were more than 90% in most of them) (25, 26). In a study conducted by Mukhopadhyay et al., 89 patients diagnosed with H&E and S-100 marker were investigated. The PPV, NPV, sensitivity, and specificity were 84.6%, 100%, 100%, and 97%, respectively, which are in line with those of our study; however, the sensitivity rate is markedly higher, compared to the present study (1). These criteria in other diagnostic methods, such as AchE histochemistry are also near 90% in different studies (13, 14). In France, a study conducted on 131 HD suspicious patients compared calretinin staining with a method combined with H&E and acetylcholinesterase IHC on the frozen section, called the standard method. Only one false-negative diagnosis was using calretinin, and 12 cases were also considered suspicious. Moreover, all these cases were diagnosed accurately by calretinin IHC (3), and in another study conducted in Vietnam by Tran et al., 92 patients with possible HD diagnoses were investigated the sensitivity and specificity of whom were 99.1% and 100%, respectively (2). In the current study, a full-thickness study did occur as well.

As mentioned, calretinin IHC can be applied to specimens considered superficial and inadequate. This feature and the high specificity percentage (96%) could also help physicians with less invasive biopsy methods, such as endoscopic rectal biopsy. In this method, inhalation anesthesia is enough and prevents some patients from an unnecessary procedure. The limitation of this study was the only use of H&E staining to compare with calretinin; however, the current study was a census study and all available cases were involved. More patients would lead to more generalized results. Therefore, further multicentre studies are recommended with a more significant patient population.

Conclusion

According to the obtained high sensitivity and specificity, calretinin IHC has a significant diagnostic value to diagnose HD in rectal biopsies of children (AUC=0.92, 95% CI=78.2%-100%, sensitivity 87%, and specificity 96%).

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Conflicts of interest

The authors declare that they have no conflict of interest.

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