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Diagnostic Value of Calretinin and S100 Immunohistochemistry in Hirschsprung's Disease

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Abstract

Background

Rectal biopsy and its histopathological study with hematoxylin and eosin (HE) is the gold standard for Hirschsprung's disease (HD) diagnosis. However, there are some limitations in the diagnosis of ganglion cells in HE approach. Recently, it was reported that the utility of Calretinin is a reliable ancillary immunohistochemistry (IHC) test for HD diagnosis. We aimed to investigated Calretinin and S100 IHC staining as ancillary methods to diagnose HD.

Materials and Methods: In this cross sectional study, 36 rectal biopsies taken from suspected HD patients were evaluated in pathology department of Mofid children's Hospital. Patients ranged from 1 day to 60 months. Data were collected in a 2-year period from 2014 to 2016 in Mofid Children Hospital, Tehran, Iran. The histological study was done observing HE stained tissue sections by two pathologists and diagnoses were: twenty-four HD (aganglionic), and twelve non-Hirschsprung's (NHD) (normoganglionic) patients. Then Calretinin and S100 IHC were performed on the slides. The IHC slides were evaluated by two pathologists and the diagnostic value of Calretinin and S100 was determined in comparison with gold standard which is the presence or absence of ganglion cells in serial HE stained sections of rectal biopsies.

Results

The results in this study demonstrated that sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) for S100 were 61.9%, 93%, 93%, and 62%, respectively. Also, sensitivity, specificity, NPV, and PPV for Calretinin were all 100%.

Conclusion

Based on the findings it may be concluded that Calretinin Immunohistochemistry had good diagnostic value and S100 Immunohistochemistry had intermediate level diagnostic value for Hirschsprung's disease.

Key Words: Calretinin, Immunohistochemistry, Hirschprung's disease, Sensitivity, S100.

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1- INTRODUCTION

Hirschsprung's disease (HIRSH-sproongz) is a congenital intestinal motility disorder (and a congenital rectal malformation which is a genetic disease in enteric related nervous system) characterized by the absence of parasympathetic ganglion cells in the submucosal (Meissner), and intramural (Auerbach) plexuses of distal colonic wall, which can lead to sustained contraction of aganglionic bowel segment, functional colonic obstruction and colonic dilatation proximal to affected segment (1-8).

The incidence rate of Hirschsprung's disease (HD) was reported 1 of 4,417 live births (9), and it is more prevalent in male (80%), 10% of the patients have Down syndrome, and 5% have other serious neurologic abnormalities (10). In some studies, it was reported that HD internationally affects 1 case per 5,000 live births (11, 12), with 4:1 male-female ratio (13, 14), and also in some other studies it was reported that HD affects about 1 per 4,500 to 1 per 5,500 live births and in males, it is about three times more common than in females (15, 16). Its prevalence in the UK and Ireland was 1.8 per 10,000 live births with male/female ratio 3.3 (16), and 1.63 per 10,000 live births in the north of England (17).

The prevalence rate of this disease in a register-based study in the European countries reported 1.09 per 10,000 live births with a trend that has significantly increased over time (18). The etiology of HD is not obviously described, but can be caused by either failure in migration of ganglion cells from neural crest during the development of intestine or by immune-mediated neuronal necrosis (10). HD is detected throughout the first year of life in the most patients, but it can present later, rarely, even in adulthood (10). Approximately 90% of patients present in infancy with constipation, abdominal distention, vomiting and delay of meconium passage. Toxic megacolon and enterocolitis can affect some cases (19, 20), but presentation later in life, with ongoing constipation is more common (21). HD diagnosis is based on histopathological study, and lack of ganglion cells in submucosal and myenteric plexuses is an indicator of the disease (19). The traditional approach to establish a pre-operative diagnosis has been the rectal full wall biopsy which tested in terms of presence of ganglion cells in myenteric plexus. Consistent with standard guidelines, the biopsy must be taken at a point 2 cm and 3 cm above the anal valve in infants and older children, respectively (10).

There are some limitations in routine hematoxylin and eosin (HE) stained histologic method which result in over or under-diagnosis of HD including: i) immature phenotype of ganglion cells could be confused with lymphocytes, endothelial cells and other elements that have similar morphology, in neonates and infants, ii) if the location of the biopsy is too distal, analysis can be problematic because paucity of ganglion cells in this site, iii) it is a time consuming process because analyzing many serial histologic sections is necessary before reporting a biopsy as "Negative for Ganglion cells", and finally, in some patients the samples are too superficial and there is not enough submucosal layer (19, 22, 23); therefore, immunohistochemistry (IHC) stains were introduced to simplify the HD diagnosis as an ancillary method to discover ganglion cells and nerve hypertrophy (24).

Calretinin is a vitamin D-dependent calcium-binding protein which plays an important role in the organization and functioning of the central nervous system (2, 22). In addition to Calretinin, S100 marker can be used to check the thickness and number of nerve fibers (25). Rakhshani et al. (8) revealed that IHC for calretinin is an appropriate technique in

HD diagnosis. In another study by Kacar et al. (20) it was concluded that Calretinin IHC is highly sensitive and specific in diagnosing aganglionic segments. In a previous similar study, the diagnostic value of calretinin and synaptophysin immunostaining in diagnosis of HD was compared (14), and in the current study, we compared the diagnostic value of Calretinin and S100 IHC staining. Given the controversy and the lack of consensus on the diagnostic tests of this disease, and also, according to the mentioned points, this study was designed to investigate the diagnostic value of Calretinin and S100 IHC staining as an ancillary method to detect aganglionosis (HD).

2- MATERIALS AND METHODS

2-1. Case selection

This cross sectional study was carried out in the Pathology Department of Mofid Children's Hospital in Tehran, Iran (one of the national referral centers in Iran) in a 2-year period from 2014 to 2016 in Mofid Children's Hospital, Tehran city, Iran. In this study, thirty-six rectal biopsy tissue samples in the form of formalin-fixed and paraffin-embedded (FFPE) blocks from suspected HD patients (age range:1 day-60 months) were collected. All cases were selected from clinically suspected HD patients. As the standard method in our laboratory for all suspected cases of HD, rectal biopsy was evaluated on paraffin-embedded tissue sections by HE staining to screen for the presence or absence of ganglion cells. All slides were analyzed by two pathologists (pathologist 1: an experienced pathologist in HD diagnosis, and pathologist 2: inexperienced in HD diagnosis), independently. The definitive diagnosis of HD suspected patients was done by HE stained tissue samples (the gold standard method), subsequently, twenty two Hirschsprung's disease (aganglionic), and fourteen non-Hirschsprung's disease were recognized. Additional sections from FFPE blocks were prepared for immunohistochemical staining including Calretinin and S100 markers. Also, patient's medical records were extracted from patient's files by a resident of pathology and the required data was recorded in a checklist.

2-2. Immunohistochemical staining

IHC process was performed using FLEX Monoclonal Mouse Anti-Human Calretinin reagent, Clone DAK-Calret 1 and FLEX Polyclonal Rabbit Anti-S100 Ready-to-use reagent (Dako, Glostrup, Denmark). Paraffin wax embedded, 4 μm thick tissue sections were cut on a microtome and mounted on positively charged slides and then air-dried by leaving them at room temperature overnight. The xylene and alcohol solutions were used for deparaffinization and rehydration of the tissue sections. Heat-induced epitope retrieval was performed at 95 ˚C for 30 min. Endogenous peroxidase activity was eliminated by treating the slides with H_2O_2 diluted in methanol for ten min in darkness at room temperature, and washed with distilled water.

The slides were incubated with the diluted primary antibody at room temperature in a humidified chamber for 60 min, and rinsed 3×5 min in Tris-Buffered Saline (TBS). Then the sections were incubated with EnVision solution for one hour in room temperature and rinsed 3×5 min in TBS buffer. The sections were then incubated with DAB CHROMOGEN for 5 min for visualization of the peroxidase reaction (23). After being washed in running cold tap water for a few minutes sections were counterstained with hematoxylin for 1 min, washed with tap water, dehydrated in alcohol, cleared in xylene and mounted (23). An ovarian granulosa cell tumor and schwannoma were selected as positive control (PC) for Calretinin and S100, respectively. The omission of primary

antibodies from staining process was considered as negative controls (22). Calretinin was considered as positive for ganglion cells (moderate to strong nuclear and cytoplasmic pattern), and also weak to moderate intensity, granular staining pattern for nerve fibers in all four layers of rectal wall (lamina propria, muscularis mucosa, submucosa and muscularis propria). Peripheral macrophages staining were excluded. And also S100 was considered as positive for peripheral nerve fibers and Schwann cells with nuclear and cytoplasmic staining reaction. Calretinin and S100 immunoreactivity and pattern of staining were evaluated on IHC slides without knowing the definitive diagnosis by two pathologists independently and finally, results were compared with gold standard method (HE stained sections).

2-3. Ethical Consideration

This study was approved by Ethical Committee of Shahid Beheshti University of Medical Sciences (Ethical code: IR.SBMU.RAM.REC.1394.461, IR.SBMU.RAM.REC.1394.462). Then the study was conducted with the permission and coordination of the research deputy of Shahid Beheshti University of Medical Sciences and Mofid Children's Hospital officials.

2-4. Statistical Analysis

Histopathologic diagnosis based on HE stained tissue sections were taken as 'gold standard'. The diagnostic values (sensitivity, specificity, positive predictive value and negative predictive value, etc.) of both Calretinin and S100 IHC markers and the Kappa statistic and agreement percent between two pathologists were calculated. Data analysis was done by R software (version 3.4.4) by running **epiR** package (version 0.9-97), and Stata 14.0 (Stata Corp LLC, College Station, Texas, USA). A p-value of less than 0.05 indicated statistical significance.

3- RESULTS

3-1. Clinical profile

A total number of 36 cases were included in this study, of which 24 patients were male (66.7%), and rest, 12 patients (33.3%), were female. The age ranged from 1 day to 60 months. The average age of HD patients was 6.5 months, and NHD patients was 22 months. Delayed passage of meconium (58.3%) was the main diagnostic symptom in most cases, followed by abdominal distension (44.4%). Other presenting symptoms were bilious vomiting (36.1%), and chronic constipation (30.6%). Three patients (8.3) had family history of Hirschsprung's disease in their first-degree relatives.

3-2. Histopathological examination

The diagnosis based on our 'gold standard' HE stained tissue sections evaluation showed 24 cases with absence of ganglion cells of submucosal and muscularis propria nerve plexuses (HD), and 12 non-HD patients showed presence of ganglion cells in the submucosal and muscularis propria layers.

3-3. Calretinin expression

In this study, Calretinin immunoexpression highlighted the ganglion cells with intense nuclear and cytoplasmic pattern in the myenteric, and meissner plexuses of normoganglionic sections (**Figure.1**). Calretinin IHC also showed granular staining of nerve fibers in lamina propria, muscularis mucosa and submucosa in normoganglionic biopsies (**Figure.2**). Also, Calretinin immunoreaction (nerve fibers or ganglion cells) was negative in all layers (lamina propria, muscularis mucosa, submucosa and muscularis propria) in aganglionic biopsies (**Figure.3**), and according to mentioned patterns Calretinin immunostaining was recorded as either positive or negative. During tissue processing of Calretinin immunostaining, two specimens lost some colonic wall

layers, so we had one biopsy without muscularis propria and the other one with absence of lamina propria and muscularis mucosal layers. Based on Calretinin IHC, experienced pathologist (P-1) made 22 HD diagnoses, and reported negative Calretinin immunoreaction in all layers (lamina propria, muscularis mucosa, submucosa and muscularis propria) of 22 sections (100%) diagnosed as HD by gold standard technique; and according to pathologist 2 (P-2), Calretinin immunoexpression was negative in all layers in 20 sections (90.9%). P-2 made HD diagnosis in 20 cases and failed to diagnosis two cases of HD, due to considering non-specific staining in lamina propria of cases 12 and 16 as positive reaction.

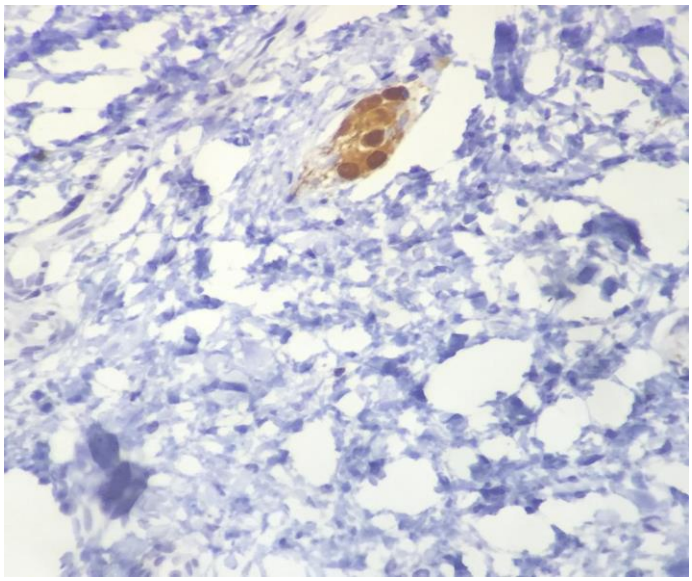

Fig.1: Intense nuclear and cytoplasmic Calretinin immunoreactivity in ganglion cells of submucosa (x100).

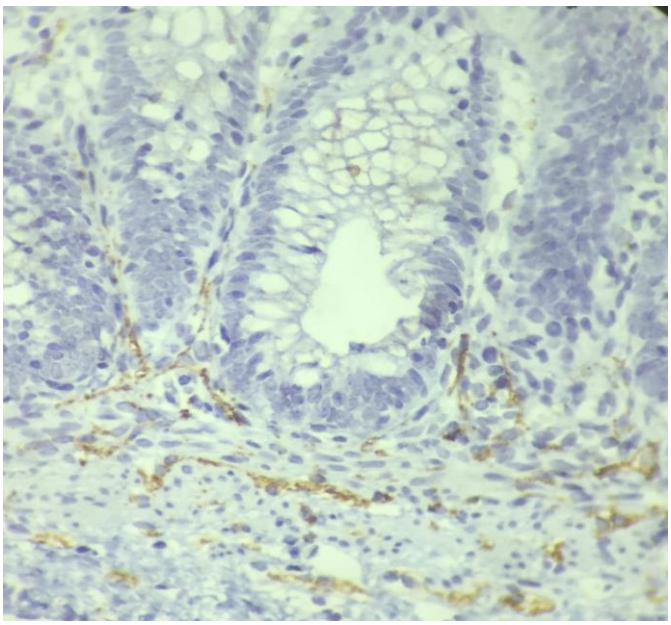

Fig.2: Lamina propria and muscularis mucosa show granular pattern of Calretinin immunoreactivity in nerve fibers (x100).

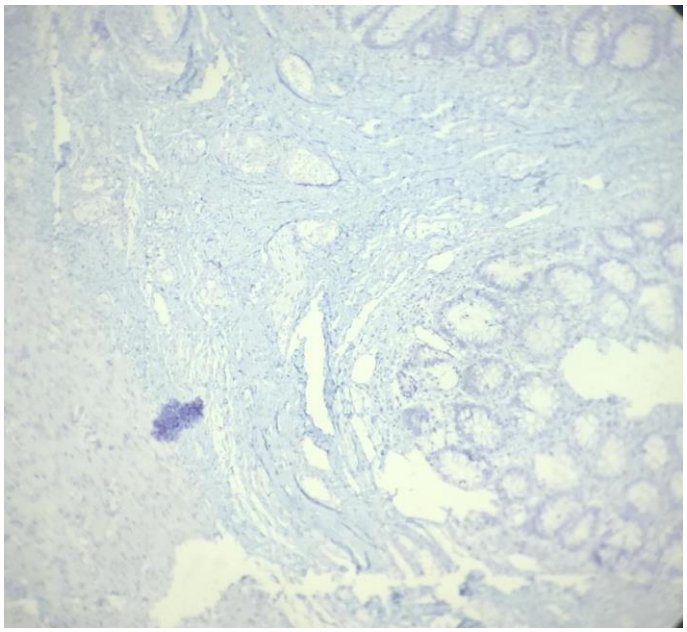

Fig.3: No Calretinin immunoreactivity in lamina propria, muscularis mucosa and submucosa (x40).

Both pathologists diagnosed 14 cases of non-HD accurately based on positive Calretinin immunoexpression in all four layers except case No 5 with loss of lamina propria and muscularis mucosa; and both pathologists reported no immunoreactivity in muscularis mucosa of case No. 7, moreover, P-2 has reported negative Calretinin result in lamina propria of case No. 2 and 9 and in muscularis mucosa of case No. 2 (**Table.1**). The diagnostic value of Calretinin IHC for experienced pathologist according to final diagnosis for each layer was calculated: sensitivity, specificity, PPV, and NPV of Calretinin for lamina propria, submucosa and muscularis propria were all 100%; and for muscularis mucosa were 100%, 92.3%, 95.6%, and 100%, respectively as mentioned above (**Table.2**). The agreement between two pathologists on Calretinin evaluation was statistically excellent: 100% for submucosa and muscularis propria, and good: 86.1% for lamina propria, and 94.4% for muscularis mucosal layer (**Table.3**).

Table-1: Calretinin Immunoreactivity in Patients with and without HD.

	Pathologist 1					**Pathologist 2**				
Case NO.	LP	MM	SM	MP	Final DX	LP	MM	SM	MP	Final DX
1	Neg	Neg	Neg	T	HD	Neg	Neg	Neg	T	HD
2	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
3	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
4	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
5	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
6	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
7	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
8	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
9	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
10	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
11	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
12	Neg	Neg	Neg	Neg	HD	Pos	Neg	Neg	Neg	NHD
13	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD

14	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
15	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
16	Neg	Neg	Neg	Neg	HD	Pos	Neg	Neg	Neg	NHD
17	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
18	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
19	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
20	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
21	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
22	Neg	Neg	Neg	Neg	HD	Neg	Neg	Neg	Neg	HD
Calretinin Immunohistochemistry Results in Non-HD patients										
1	Pos	Pos	Pos	Pos	NHD	Pos	Pos	Pos	Pos	NHD
2	Pos	Pos	Pos	Pos	NHD	Neg	Neg	Pos	Pos	NHD
3	Pos	Pos	Pos	Pos	NHD	Pos	Pos	Pos	Pos	NHD
4	Pos	Pos	Pos	Pos	NHD	Pos	Pos	Pos	Pos	NHD
5	T	T	Pos	Pos	NHD	T	T	Pos	Pos	NHD
6	Pos	Pos	Pos	Pos	NHD	Pos	Pos	Pos	Pos	NHD
7	Pos	Neg	Pos	Pos	NHD	Pos	Neg	Pos	Pos	NHD
8	Pos	Pos	Pos	Pos	NHD	Pos	Pos	Pos	Pos	NHD
9	Pos	Pos	Pos	Pos	NHD	Neg	Pos	Pos	Pos	NHD
10	Pos	Pos	Pos	Pos	NHD	Pos	Pos	Pos	Pos	NHD
11	Pos	Pos	Pos	Pos	NHD	Pos	Pos	Pos	Pos	NHD
12	Pos	Pos	Pos	Pos	NHD	Pos	Pos	Pos	Pos	NHD
13	Pos	Pos	Pos	Pos	NHD	Pos	Pos	Pos	Pos	NHD
14	Pos	Pos	Pos	Pos	NHD	Pos	Pos	Pos	Pos	NHD

LP: Lamina propria; SM: Submucosa; MM: Muscularis mucosa; MP: Muscularis propria; T: Tissue partially lost after IHC staining; NHD: Non-Hirschsprung disease; HD: Hirschprung disease; Neg: Negative staining; Pos: Positive staining.

Table-2: Diagnostic values of muscularis mucosae according to experienced pathologist results.

Diagnostic values		Histopathological examination	
		Positive	Negative
Muscularis mucosa	Negative	22	1
	Positive	0	12
Total		22	13
Sensitivity		1.00 (0.85, 1.00)	
Specificity		0.92 (0.64, 1.00)	
Positive predictive value		0.96 (0.78, 1.00)	
Negative predictive value		1.00 (0.74, 1.00)	
Diagnostic accuracy		0.97 (0.85, 0.99)	
Youden's index (J)		0.92 (0.48, 0.99)	
Likelihood ratio of a positive test		13.00 (1.98, 85.46)	
Likelihood ratio of a negative test		0	

Table-3: Agreement and kappa statistics of two raters (Pathologists) regarding submucosa, muscularis propria, lamina propria and muscularis mucosa.

Variables	Agreement	Kappa statistics	Z	P-value
Submucosa	100%	1	6.00	0.001
Muscularis propria	100%	1	5.92	0.001
Lamina propria	88.89%	0.77	5.05	0.001
Muscularis mucosal	97.14%	0.93	5.54	0.001

3-4 S100 Expression

In our study S100 was used as qualitative test to identify nerve trunk hypertrophy in submucosa which is a histological marker to define aganglionosis (**Figure.4**). Immunohistochemical stains for S100 were available for evaluation of a total number of 35 cases. One S100 prepared section had no sufficient submucosa for accurate evaluation of nerve trunks. Both pathologists reported no nerve hypertrophy in 13 out of 14 ganglionic sections, and only one case revealed nerve hypertrophy among non-HD patients. P-1 reported 13 cases of nerve hypertrophy in a total of 21 aganglionic sections and P-2 reported 12 cases. The agreement between two pathologists on S100 evaluation for submucosal nerve hypertrophy was good (97.14 %), and also the Kappa statistic of two pathologists on S100 was 93.98% (Z=5.57, P=0.001). PPV, NPV, sensitivity and specificity for S100 IHC according to experienced pathologist results for S100 were 92.8%, 61.9%, 61.9% and 93%, respectively (**Table.4**).

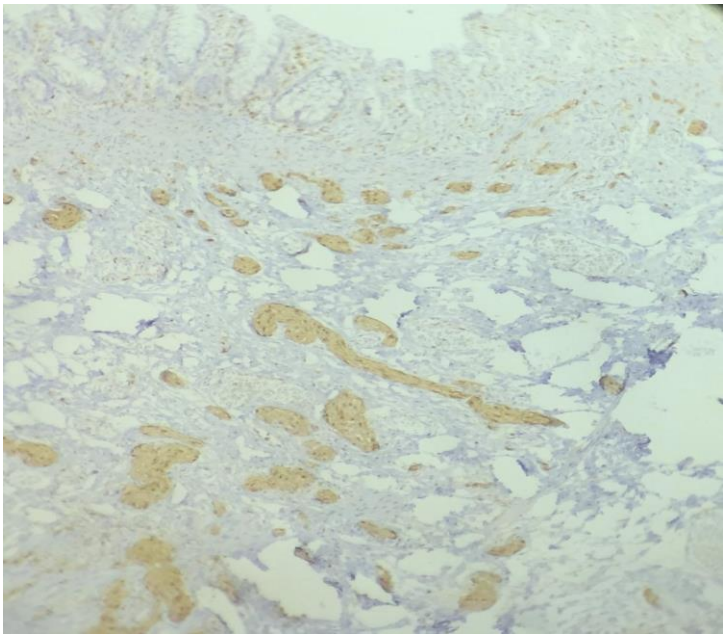

Fig.4: S100 immunoreactivity shows nerve hypertrophy in aganglionic case.

Table-4: Diagnostic values of S100 IHC according to experienced pathologist results.

Diagnostic values		Histopathological examination	
		Positive	Negative
S100 IHC	Positive	13	1
	Negative	8	13
Total		21	14
Sensitivity		0.62 (0.38, 0.82)	
Specificity		0.93 (0.66, 1.00)	
Positive predictive value		0.93 (0.66, 1.00)	
Negative predictive value		0.62 (0.38, 0.82)	
Diagnostic accuracy		0.74 (0.57, 0.87)	
Youden's index (J)		0.55 (0.04, 0.81)	
Likelihood ratio of a positive test		8.67 (1.27, 59.01)	
Likelihood ratio of a negative test		0.41 (0.23, 0.72)	

IHC: Immunohistochemistry.

4- DISCUSSION

In this study, we aimed to investigate the diagnostic values of Calretinin and S100 IHC staining as ancillary methods in diagnosis of HD. According to our findings it may be concluded that Calretinin Immunohistochemistry had good diagnostic value and S100 Immunohistochemistry had intermediate level diagnostic value for Hirschsprung's disease. Therefore, it seems that Calretinin is a very useful, valuable, sensitive and specific marker for detecting aganglionosis in patients who are suspected to have HD. There are several methods to diagnose HD, the most reliable of which is Swenson biopsy of rectum. This full wall thickness rectal biopsy should be taken 2 cm above dentate line in infants and 3 cm above it in older children, because there is a normal paucity of ganglion cells below this level.

Biopsies are taken for evaluation of ganglion cells in the Meissner and Myenteric plexuses. Absence of ganglion cells and the presence of unequivocally hypertrophic nerves (>40 µm) are diagnostic. The main approach involves visual counts of ganglion cells appropriately prepared 3-5µm-thick; Hematoxylin and eosin stained sections from paraffin blocks. In most centers, the diagnosis is based on histopathologic evaluation of rectal suction biopsies that include mucosa and underlying submucosa (26-29). However, there are limitations to this technique such as immature cytologic feature of ganglion cells in neonates and infants (23); also the need for evaluation of 75-100 properly oriented Hematoxylin and eosin (HE) stained sections to exclude the presence of ganglion cells and suggest the HD diagnosis (22, 23, 30, 31), which is a time consuming process (23). As a result, a number of ancillary methods such as acetyl cholinesterase (AChE), and Calretinin immunohistochemistry have been introduced to facilitate the diagnosis (23).

Kacar et al. in 2012 investigated Calretinin immunoreactivity in 10 HD patients and 23 non-HD patients as control group. Calretinin was expressed in all sections from control group and had negative immunoreactivity in HD patients, one case with positive nerve fibers staining in lamina propria of aganglionic zone. Calretinin was found to be highly sensitive and specific in detecting aganglionic segments (20). In another study in 2009, Guinard-Samuel et al. retrieved 131 suction rectal biopsies carried out for HD suspected patients from pathology repository and compared Calretinin immunohistochemistry with the standard method of their laboratory (AChE and HE staining). Their diagnosis based on Calretinin IHC was accurate for all HD patients according to standard technique, except one patient who revealed positive immunoreaction in some nerve fibers (false negative case). It is important to note that 12 additional cases considered as suspicious for HD using standard technique were accurately diagnosed by Calretinin immunohistochemistry. They concluded that Calretinin is more accurate than AChE to complete histology (22).

Another recent study by Hiradfar et al. in 2012 (23) showed positive immunostaining of nerve fibers in the lamina propria, muscularis mucosa, submucosa and muscularis propria in control group and ganglionic segments of HD patients group. There were also nuclear and cytoplasmic staining of ganglion cells in submucosa and muscularis propria in all specimens of both control group (100%), and ganglionic bowels (100%). Calretinin immunoexpression was negative in all except two cases (6.7%) of aganglionic segments (false positive). They found false positive immunostaining only in hypertrophic nerve fibers in muscularis propria in a ganglionic segment.

Their method had sensitivity of 93.3%, and specificity of 100% for diagnosis of HD in full thickness specimens of intestinal wall. Positive predictive value was 100% and negative predictive value was 93.8%. They suggested Calretinin immunostaining in submucosa can be used on suction rectal biopsies as a reliable and adjunctive method to diagnosis HD. In our study, sensitivity, specificity, PPV and NPV of Calretinin for lamina propria, submucosa and muscularis propria were all 100%, and for muscularis mucosa were 100%, 92.3%, 95.6% and 100%, respectively. Bachmann et al. in 2014 studied immunohistochemical panel of MAP2, Calretinin, S100 and GLUT1 for HD diagnosis on 69 specimens from 37 patients. Calretinin and MAP2 stained ganglia in both myenteric and submucosal plexuses reliably. In normal tissue, S100 staining revealed very few nerve fibers in the submucosa with weak staining.

In HD-affected tissue, the number of nerve fibers visible in the submucosa was great, and they were thicker with stronger staining. There was almost no GLUT1 staining in normal tissue; however, in HD-affected tissues, numerous thick nerve fibers were visible in submucosa. By combination of these markers they were able to recognize normally innervated tissue, as well as HD-affected tissues, reliably. It was concluded that this method was specific and showed good agreement with the gold standard method (25). In another similar study by Anbardar et al. which was published in 2015, Calretinin IHC in the detection of aganglionosis (HD) was investigated. They collected 27 HD patients and 28 non-HD patients during the period of study. The comparison between decision only on lamina propria and also only on submucosa and gold standard (HE serial sections) showed good agreement (1). Kacar et al. (20) also mentioned that nerve staining in lamina propria, muscularis mucosa and submucosa is useful for superficial biopsies as well as for suction biopsies. De la Torre et al. (24) studied Calretinin and S100 on rectal biopsies for HD diagnosis in 2012. S100 protein was used to identify nerve hypertrophy and showed low sensitivity (41.7%). In our study S100 marker evaluation showed higher sensitivity (61.9%). In another study by Holland et al. in 2010, S100 staining was used on 136 rectal biopsies from patients suspected of having HD. S100 staining was performed for 107 patients with ganglion cells and neural hypertrophy (nerve bundles >40µm) was identified in four (4%) of these cases. Ganglion cells were absent in 27 patients and of these Hirschsprung disease cases, neural hypertrophy was identified in 22 patients (81%, mean 54µm diameter).

They also performed Diff Quick staining on 26 frozen samples of colon resection and biopsies. Diff Quick protocol stained the cytoplasm of ganglion cells bright blue, expediting the intraoperative evaluation. Of a total of 57 frozen sections performed on patients, a 95% (54 of 57) similarity with permanent section was obtained. They proved that S100 and peripherin were a highly specific and sensitive modality for the identification of ganglion cells in suction rectal biopsies (32). According to our study, Calertinin is a good marker to detect ganglion cells in rectal biopsies. This method is easy to perform in pathology laboratory because unlike the AChE method it does not need fresh frozen tissue and is achievable on paraffin embedded blocks, and also the interpretation is easy for pathologists who are inexperienced in HD diagnosis.

4-1. Study Limitations

There were limitations to this study. Due to the rare nature of Hirschsprung's disease and low prevalence rate, the most important limitation of this study was the low sample size of patients which could reduce the power of the study. Therefore, it is recommended to consider this point in

the interpretation and generalization of the findings.

5- CONCLUSION

In conclusion, it seems that Calretinin is a very useful, valuable, sensitive and specific marker for detecting aganglionosis in patients who are suspected to have HD. Calretinin evaluation in lamina propria, submucosa and muscularis propria revealed reliable results to detect ganglion cells and nerve fibrils, and also showed great concordance with gold standard method. So it is a reliable ancillary method to diagnose HD which can be used by inexperienced pathologists when there is diagnostic doubt, even in suction rectal biopsies. This technique may eliminate the need for repeat biopsy and serial sectioning of blocks which are time consuming, also bothersome for the patient. Calretinin interpretation is simple and does not require any special experience. S100 marker showed moderate sensitivity and specificity to detect nerve hypertrophy, as a qualitative test in confirming patients with aganglionosis, although the use of an ocular micrometer gives more precise results of S100 evaluation. Given the limited sample size of our study, it is recommended that the interpretation and application of the results be done with more caution.

6- ABBREVIATIONS

HE: Hematoxylin and Eosin,
HD: Hirschsprung's Disease,
IHC: Immunohistochemistry,
NHD: Non-Hirschsprung's disease,
NPV: Negative Predictive Value,
PPV: Positive Predictive Value,
TBS: Tris-Buffered Saline,
PC: Positive Control,
AChE: Acetylcholinesterase,
FFPE: Formalin-Fixed and Paraffin-Embedded.

7- CONFLICT OF INTEREST: None.

8- ACKNOWLEDGMENTS

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9- REFERENCES

1. Anbardar MH, Geramizadeh B, Foroutan HR. Evaluation of Calretinin as a New Marker in the Diagnosis of Hirschsprung Disease. Iranian journal of pediatrics. 2015;25(2):e367.

2. Barshack I, Fridman E, Goldberg I, Chowers Y, Kopolovic J. The loss of calretinin expression indicates aganglionosis in Hirschsprung's disease. Journal of clinical pathology. 2004;57(7):712-6.

3. Anderson JE, Vanover MA, Saadai P, Stark RA, Stephenson JT, Hirose S. Epidemiology of Hirschsprung disease in California from 1995 to 2013. Pediatric surgery international. 2018;34(12):1299-303.

4. Chung PHY, Yu MON, Wong KKY, Tam PKH. Risk factors for the development of post-operative enterocolitis in short segment Hirschsprung's disease. Pediatric surgery international. 2019;35(2):187-91.

5. Nakamura H, O'Donnell AM, Marayati NF, Tomuschat C, Coyle D, Puri P. Altered expression of inflammasomes in Hirschsprung's disease. Pediatric surgery international. 2019;35(1):15-20.

6. Taghavi K, Goddard L, Evans SM, Hobson A, Beasley SW, Sankaran S, et al. Ethnic variations in the childhood prevalence of Hirschsprung disease in New Zealand. ANZ journal of surgery. 2018:[Epub ahead of print].

7. Zhu T, Sun X, Wei M, Yi B, Zhao X, Wang W, et al. Optimal time for single-stage pull-through colectomy in infants with short-segment Hirschsprung disease. International journal of colorectal disease. 2019;34(2):255-9.

8. Rakhshani N, Araste M, Imanzade F, Panahi M, Safarnezhad Tameshkel F, Sohrabi

MR, et al. Hirschsprung Disease Diagnosis: Calretinin Marker Role in Determining the Presence or Absence of Ganglion Cells. Iranian journal of pathology. 2016;11(4):409-15.

9. Spouge D, Baird PA. Hirschsprung disease in a large birth cohort. Teratology. 1985;32(2):171-7.

10. Rosai J. Rosai and Ackerman's surgical pathology. 9th ed. Edinburgh: Mosby: Elsevier Health Sciences; 2004.

11. Touré AM, Landry M, Souchkova O, Kembel SW, Pilon N. Gut microbiota-mediated Gene-Environment interaction in the TashT mouse model of Hirschsprung disease. Scientific reports. 2019;9(1):492.

12. Heuckeroth RO. Hirschsprung disease—integrating basic science and clinical medicine to improve outcomes. Nature Reviews Gastroenterology & Hepatology. 2018;15(3):152.

13. Chung PHY, Yu MON, Wong KKY, Tam PKH. Risk factors for the development of post-operative enterocolitis in short segment Hirschsprung's disease. Pediatric surgery international. 2019;35(2):187-91.

14. Sheir M, Samaka RM, Fakhry T, Albatanony AA. Comparative study between use of calretinin and synaptophysin immunostaining in diagnosis of Hirschsprung disease. International Surgery Journal. 2019;6(3):658-63.

15. Gustafson E, Larsson T, Danielson J. Controlled outcome of Hirschsprung's disease beyond adolescence: a single center experience. Pediatric surgery international. 2019;35(2):181-5.

16. Bradnock TJ, Knight M, Kenny S, Nair M, Walker GM. Hirschsprung's disease in the UK and Ireland: incidence and anomalies. Archives of disease in childhood. 2017;102(8):722-7.

17. Best KE, Glinianaia SV, Bythell M, Rankin J. Hirschsprung's disease in the North of England: prevalence, associated anomalies, and survival. Birth defects research Part A, Clinical and molecular teratology. 2012;94(6):477-80.

18. Best KE, Addor MC, Arriola L, Balku E, Barisic I, Bianchi F, et al. Hirschsprung's disease prevalence in Europe: a register based study. Birth defects research Part A, Clinical and molecular teratology. 2014;100(9):695-702.

19. Maldyk J, Rybczynska J, Piotrowski D, Kozielski R. Evaluation of calretinin immunohistochemistry as an additional tool in confirming the diagnosis of Hirschsprung disease. Polish journal of pathology: official journal of the Polish Society of Pathologists. 2014;65(1):34-9.

20. Kacar A, Arikok AT, Azili MN, Ekberli Agirbas G, Tiryaki T. Calretinin immunohistochemistry in Hirschsprung's disease: An adjunct to formalin-based diagnosis. The Turkish journal of gastroenterology: the official journal of Turkish Society of Gastroenterology. 2012;23(3):226-33.

21. Alexandrescu S, Rosenberg H, Tatevian N. Role of calretinin immunohistochemical stain in evaluation of Hirschsprung disease: an institutional experience. International journal of clinical and experimental pathology. 2013;6(12):2955-61.

22. Guinard-Samuel V, Bonnard A, De Lagausie P, Philippe-Chomette P, Alberti C, El Ghoneimi A, et al. Calretinin immunohistochemistry: a simple and efficient tool to diagnose Hirschsprung disease. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2009;22(10):1379-84.

23. Hiradfar M, Sharifi N, Khajedaluee M, Zabolinejad N, Taraz Jamshidi S. Calretinin Immunohistochemistery: An Aid in the Diagnosis of Hirschsprung's Disease. Iranian journal of basic medical sciences. 2012;15(5):1053-9.

24. De la Torre L, Santos K. Hirschsprung disease: Evaluation of calretinin and S-100 as ancillary methods for the diagnosis of aganglionosis in rectal biopsies. Acta Pediátrica de México. 2012;33(5):246-51.

25. Bachmann L, Besendorfer M, Carbon R, Lux P, Agaimy A, Hartmann A, et al. Immunohistochemical panel for the diagnosis

of Hirschsprung's disease using antibodies to MAP2, calretinin, GLUT1 and S100. Histopathology. 2015;66(6):824-35.

26. Rahman Z, Hannan J, Islam S. Hirschsprung's disease: Role of rectal suction biopsy - data on 216 specimens. Journal of Indian Association of Pediatric Surgeons. 2010;15(2):56-8.

27. Szylberg L, Marszałek A. Diagnosis of Hirschsprung's disease with particular emphasis on histopathology. A systematic review of current literature. Przeglad gastroenterologiczny. 2014;9(5):264-9.

28. Gonzalo DH, Plesec T. Hirschsprung Disease and Use of Calretinin in Inadequate Rectal Suction Biopsies. Archives of Pathology & Laboratory Medicine. 2013;137(8):1099-102.

29. Muise ED, Cowles RA. Rectal biopsy for Hirschsprung's disease: a review of techniques, pathology, and complications. World Journal of Pediatrics. 2016;12(2):135-41.

30. Kapur RP. Practical pathology and genetics of Hirschsprung's disease. Seminars in Pediatric Surgery. 2009;18(4):212-23.

31. Qualman SJ, Jaffe R, Bove KE, Monforte-Munoz H. Diagnosis of hirschsprung disease using the rectal biopsy: multi-institutional survey. Pediatric and developmental pathology : the official journal of the Society for Pediatric Pathology and the Paediatric Pathology Society. 1999;2(6):588-96.

32. Holland SK, Hessler RB, Reid-Nicholson MD, Ramalingam P, Lee JR. Utilization of peripherin and S-100 immunohistochemistry in the diagnosis of Hirschsprung disease. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2010;23(9):1173-9.