The Role of Calretinin Immunohistochemistry in Detection of Ganglion Cell in Hirschsprung Disease

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ABSTRACT:

BACKGROUND:

Hirschsprung's disease is a developmental abnormality of enteric nervous system characterized by lack of ganglion cell and neural hypertrophy in the Meissner and Auerbach's plexuses within variable length of the colon, the Calretinin Immunohistochemistry is one of the newer methods overcome difficulties in diagnosis of Hirschsprung's disease especially immature ganglia in neonates in submucosal plexus.

AIM OF STUDY:

This study was performed to evaluate the diagnostic value of Calretinin immunohistochemistry in the Hirschsprung's Disease diagnosis.

MATERIALS AND METHOD:

A retrospective and prospective case series study at the period from (Jan2018-Jan2020) included a total 50 formalin-fixed paraffin-embedded blocks of distal colonic and rectal incisional biopsies from infant and young children patient who suspected with Hirschsprung's disease, The study was undertaken in the department of pathology, collage of medicine/ University of Mosul, a clinicopathological data including (age, gender, site, type of surgical procedure) were obtained from patients' files This study was performed by using the primary antibody of Calretinin.

RESULTS:

The study done on 50 cases their age from 1 day to 14 years. The universal sensitivity in this study to diagnose presence or loss of enteric ganglia by calretinin immunostaining was 90% and the specificity is 100% with positive and negative predictive value of 100% and 97 % respectively.

CONCLUSION:

Calretinin marker considered as a reliable, valuable marker in Hirschsprung disease diagnosis and carry high sensitivity and specificity.

KEY WORDS: Hirschsprung disease, Calretinin.

INTRODUCTION:

Hirschsprung disease (HD) defined as enteric nervous system (ENS) disorder with complex pattern of inheritance, Herald Hirschsprung was first described this disease as aganglionic megacolon and a malformation of the distal gut in 1886 [1,2] it had been important cause of constipation in infants and children and commonest cause of neonatal bowel obstruction which characterized by complete absence of parasympathetic intrinsic ganglia within the variable length of colon that constituting the submucosal (Meissner's plexus) and myenteric (Auerbach's plexus [3], their Occurrence is predominant in males with a male to female ratio of 4:1.1 and 1/5,000 live births [4,5,6,7,8].

The clinical extent of HD divided into short segment aganglionosis (a ganglionic segment includes only the sigmoid colon and rectum) which considered commonest type 80% of cases, whereas in 20% of the aganglionic segment include the more proximal bowel (Long segment aganglionosis), and in rare condition may affect the entire colon (Total colonic aganglionosis) [9], overall the clinical presentation of HD disease mostly during infancy period as gastrointestinal symptoms predominantly constipation the best method for treatment of Hirschsprung disease is the surgical removal of aganglionic segment and an appropriate anastomosis between the innervated intestine and distal rectum [3,4].

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In spite of the importance of using Hematoxylin-eosin staining for rectal suction biopsy(RSB) in the diagnosis of Hirschsprung's disease that considered the gold standard test with sensitivity can reach 100%[8], but the diagnosis with these methods are not possible every time, because had many limitations, like the immaturity of ganglion cells particularly in neonates and premature infants and small, irregular distribution of ganglia in the sub-mucosal area are made their identification is difficult and requires high experience [3,4] Immunohistochemistry (IHC) is the important reliable and valuable method to detect ganglion cells and neural hypertrophy [5,6]. Calretinin is a vitamin D dependent calcium-binding protein which constituent in enteric neural tissue of the muscularis mucosae and lamina propria and play an important role in the diagnosis of HD where lack of calretinin immunoreactivity in the ganglionic cells or nerve fibers reported with aganglionosis, Calretinin IHC has other advantages it can be used in paraffin-embedded biopsies, is simple, easy to test and had a high specificity and sensitivity rate for HD diagnosis.[9]

This study was performed to assess the diagnostic value of Calretinin immunostaining in the irschsprung's Disease diagnosis.

MATERIALSANDMETHOD:

study design and setting

A retrospective and prospective case series study was done at period from October 2019- August 2020 which included a total 50 formalin –fixed paraffin embedded distal colonic and rectal incisional biopsies from infant and young children patient who clinically presented with symptoms suspicious for HD, the cases were received from Al-khansa Teaching hospital and private laboratories in Mosul city /Iraq through two consecutive years (Jan2018- Jan2020) , and immunohistochemical stain was conducted in Veen medical laboratory/ Dohuk city/ Iraq, Autostainer Link48 / Dako, calretinin rabbit monoclonal antibody, code 129954. The study was undertaken in the department of pathology, college of medicine/ The University of Mosul, a clinic- pathological data including (age, gender, site, type of surgical procedure) were obtained from patients' files. Calretinin was considered as positive if any of the specific findings below were present:

1) Intense, granular and linear staining of nerve fibers in the lamina propria, muscularis mucosa, submucosa and muscularis propria

2) Diffuse strong cytoplasmic and nuclear staining of ganglion cells in the submucosal and/or in the myenteric plexus, and the supporting Schwann cells and nerve cells were evaluated as follows (Fig.4)

0–No immunostaining or impossible to visualize ganglion cell,
1–Mildimmunostaining,
2–Moderateimmunostaining,
3–Strongimmunostaining.

Score (0-1) considered as negative immunoreactivity for calretinin while (2-3) considered as positive immunoreactivity for calretinin.(Fig.5)

calretinin	29KD-Calbidine
Clone	DAK-calret1
Code	129954
Isotype	IgG1-Kappa
Reactivity	Human
Localization	Nuclear and cytoplasm
Control	Mesothelial cells

Tumor marker

Statistical analysis was done on IBM Statistical Package for Society Study (SPSS) statistical software version 19.0. Qualitative data were presented using the frequency and related percentage, by using of T-test and Chi-square or Fisher exact test for qualitative comparison of variables between groups. The sensitivity and specificity of the marker and their combination in the diagnosis of PTC was done through equation (below). A P value of <0.05 was considered as significant statistically with confidence interval of 95%.

Sensitivity=True(+ve)
True(+ve)+False(-ve)

Specificity=True(-ve)
True(-ve)+False(+ve)

RESULTS:

Clinicopathological data

A total 50 cases from 2018 to 2020 in Nineveh province (governorate and private hospitals) underwent colorectal full-thickness or pull through biopsy and histopathology confirmed for presence or absence of ganglionic cell, the Patient's age ranged from 1 day to 14years the median age of presentation was 4.4 year, of these 50 cases 26 (50%) was less than 2 years old, 14 (28%) patients between(3-5)year of age and 6(12%) patients between (6-8) year of age,3(6%) from (9-11)year and 1(2%) between (12-14) year of age there is statistical significant P value <0.05 (Fig.1), descriptive analysis for gender demonstrated 19(38%) female ,31(62%) male and, Male to Female ratio was 1,6:1 P value >0.05 no association between gender type and immunohistochemical expression.(Fig2)

Fig.1DistributionofHirschsprungdiseaseaccordingtoagegroup Fig.2Gender distribution among Hirschsprung disease.

0-2Years3-5Years6-8Years9-11Years12-14Years

Female

Male

Calretinin Immunestain results:

Fig.3Correlation between H&E and Calretinin immunohistochemistry in expression of Ganglionic Cells in Hirschsprung disease patients.

N=50.*P*-value0.032<0.05statisticallysignificance

Table 1 :The sensitivity, specificity, positive productive value and negative productive value for calretinin in detection ganglion cell in bowel segment.

BOWEL SEGMENT	Final diagnosis by gold standard	Sensitivity	Specificity	PPV %	NPV %	*P-value*		
+	-							
Calretinin (Ganglion Cells)	+	11	0	90%	100%	100	97	<0.05
-	1	29						

PPV: Positive productive value

NPV: Negative productive value

No false-positive result in this study while only one case was false negative

21

19

29

31

50

10

15

20

25

30

35

H&ECalretinin

PositiveNegative

A- H&E section shows submucosal –Meissner's plexus with ganglion cell. B- Calretinin immunostaining shows positive ganglion cells. C- H&E section shows myenteric (Auerbach's plexus). D- Calretinin immunostaining shows positive ganglioncell.(400X)

A

D

B

C

A- Calretinin immunostaining shows no reactivity in HD bowel segment (0). (40X). B- faint calretinin immunoreactivity (+1). (100X) .C- (+2) granular nuclear and cytoplasmic calretinin immunostaining. (400X) (D- (+3) diffuse intense pattern calretinin immunostaining (.400X).

A**B**

CD

DISCUSSION:

Age and Gender

Hirschsprung disease is a common developmental anomaly that affects pediatric age group and it is mostly diagnosed during first year of life [10,11] to the current study which is done on 50 suspected cases of Hirschsprung's disease, the Patient' age ranged from 1 day to 14years most of the cases were < 24 month ,this result in agreement with several studies as Musa ZA et al in Iraq at 2017, Mukhopadhyay et al study at 2019 and L

Kannaiyan in at 2013 in India [6,10,12] and Alexandrescu S et al in California at 2013 [13]. The descriptive analysis for gender demonstrated Male to Female ratio was 1,6:1 which are non-significance statistically (P>0,05) this result less than other studies which reported the ratio of male to female 4:1 (10-16) and other study conducted by L-Kannaiyan et al who found male to female ratio 3:1 [12] the possible cause is the number of sample in this work is smaller than others.

Histopathological type

Histopathological examination of this study two features was evaluated the first is the absence of ganglion cells in submucosal plexus and muscularis propria plexus, second is presence of nerve bundle hypertrophy in these regions, which are good predictive feature for diagnosis HD. [15]

Calretinin immunohistochemical expression

According to this study, the calretinin immunohistochemical method is less challenging and can be interpreted more easily than other methods with lesser requirements for several serial sections of tiny rectal biopsy and assist in detection and recognition of small immature ganglion cell [10,14,17,18],

The examination of spastic colon/rectal segment by calretinin reveals great diagnostic value of calretinin IHC in the diagnosis of aganglionosis in HD patients which was 100% specificity and 90% sensitivity, so it is a useful tool in the diagnosis of HD and it was statically significance P value <0.05 . this agreement with several studies as Musa ZA et al in Iraq at 2017 record calretinin reliable and perfect diagnostic aid to histopathological examination of HD where reported sensitivity and specificity 100% [10] ,also other studies conducted by Kacar et al in turkey at 2012([11]), L-Kannyain et al in India at 2013, Hiradfar et al in Iran at 2012 [12,14].

Holland et al in Georgia at 2011 shows the benefit of using calretinin IHC as easy method it work on paraffin block section and less the need fora large number of H&E levels(17) Zuikova et al in Riga in 2015 found calretinin as the most valuable IHC marker of three evaluated Calretinin ,chromogranin and synaptophysine[18], , Kapur et al in Washington at 2009 and Barshak et al at 2004(19,20) and Maldyk et al in Poland at 2014 found the expression of calretinin IHC was positive in all ganglionic biopsies while negative in aganglionic segments [21] Guinard –Samuel et al in Paris at 2009 found with calretinin can pass the difficulties facing in histopathological examination by acetylcholinesterase and diagnosis be more accurate and easy without false-positive results [22] The study reveals one false-negative result this study are compatible with several previous studies that revealed the possibility of false-negative results of calretinin IHC were Hiradfar et al showed similar false negative staining of nerve fibers of HD patients also Kapur et al record false negative immunoreactivity in submucosal nerve fibers in the some patients of HD could be due to staining technical causes [19-23], in this work no false positive result recorded opposite to other several previous studies revealed the possibility of false positive results in calretinin IHC , due to immunostaining in some non-neuronal cells such as histiocytic and mast cells which considered as internal positive control shows cytoplasmic and nuclear immunoreactivity [16].

CONCLUSION:

The Calretinin immunohistochemical marker considered a very valuable, reliable and extremely useful sensitive and specific test for detection ganglionic cell and internal nerve fibers cells in doubtful cases of Hirschsprung disease can be used by inexperienced pathologist, calretinin IHC overcome the difficulties facing in H&E method and improve diagnostic approach of HD diagnosis.

REFERENCES:

1. Mukhopadhyay B, Mukhopadhyay M, Mondal KC, Sengupta M, Paul A.Hirschsprung's Disease in Neonates with Special Reference to CalretininImmunohistochemistry. J Clin Diagn Res. 2015;9:6-9.
2. Aghdam MK, Khoddami M, Mollasharifi T, Almasi-Hashiani A. Diagnostic Value of Calretinin and S100 Immunohistochemistry in Hirschsprung's Disease. Int J Pediatr. 2019;7:9577-89.
3. Rakhshani N, Araste M, Imanzade F, Panahi M, Tameshkel FS, Sohrabi MR, et al. Hirschsprung Disease Diagnosis: Calretinin Marker Role in Determining the Presence or Absence of Ganglion Cells. Iran J Pathol. 2016;11:409-15.
4. Bajaj A. Omitted or Deficient – Hirschsprung Disease. EC Gastroenterology and Digestive System. 2019:445-56 .
5. Sikandar M, Nagi AH, Sikandar K, Naseem N, Qureshi I. Calretinin Expression in Hirschsprung's Disease–A Potential Marker of Ganglion Cells. APMC. 2017;11:181-86.

6. Mukhopadhyay M, Das C, Kumari M, Mukhopadhyay B, Saha AK.Evaluation of calretinin expression in diagnosis of hirschsprung's disease: as an adunct to histopathological diagnosis. Int J Sci Res. 2019;8:6-8.
7. Morales-Miranda A. Congenital intestinal stenosis and Hirschsprung's disease: two extremely rare pathologies in a newborn puppy. BMC Vet Res.2019;15:92.
8. Roed-Petersen K. Harald Hirschsprung og den kongenitte aganglionose[Harald Hirschsprung and congenital aganglionosis]. Ugeskrift for laeger.2007;169:2866.
9. Elhalaby RE, Oreiby RM, Hasby EA, Sallam FA. Evaluation of the role of Immunohistochemical markers in the diagnosis of Hirschsprung's Disease.Journal of American Science. 2016;12:41-50.
10. Musa ZA, Qasim BJ, Ghazi HF, Al Shaikhly AW. Diagnostic roles of calretinin in hirschsprung disease: A comparison to neuron-specific enolase.Saudi J Gastroenterol. 2017;23:60-66.
11. Kaçar A, Arikök AT, Azili MN, Ekberli Ağirbaş G, Tiryaki T. Calretinin immunohistochemistry in Hirschsprung's disease: An adjunct to formalinbased diagnosis. Turk J Gastroenterol. 2012 ;23:226-33.
12. Kannaiyan L, Madabhushi S, Malleboyina R, Are NK, Reddy KR, Rao B.Calretinin immunohistochemistry: A new cost-effective and easy method for diagnosis of Hirschsprung's disease. J Indian Assoc Pediatr Surg. 2013;18:66-68.
13. Alexandrescu S, Rosenberg H, Tatevian N. Role of calretinin immunohistochemical stain in evaluation of Hirschsprung disease: an institutional experience. Int J Clin Exp Pathol. 2013 ;6:2955-61.
14. Hiradfar M, Sharifi N, Khajedaluee M, Zabolinejad N, Taraz Jamshidi S.Calretinin Immunohistochemistery: An Aid in the Diagnosis of Hirschsprung's Disease. Iran J Basic Med Sci. 2012 ;15:1053-59.
15. Anbardar MH, Geramizadeh B, Foroutan HR. Evaluation of Calretinin as a New Marker in the Diagnosis of Hirschsprung Disease. Iran J Pediatr. 2015; 25:367.
16. de Lorijn F, Kremer LC, Reitsma JB, Benninga MA. Diagnostic tests in Hirschsprung disease: a systematic review. J Pediatr Gastroenterol Nutr. 2006;42:496-505.
17. Holland SK, Ramalingam P, Podolsky RH, Reid-Nicholson MD, Lee JR. Calretinin immunostaining as an adjunct in the diagnosis of Hirschsprung disease. Ann Diagn Pathol. 2011;15:323-28.
18. Zuikova V, Franckevica I, Strumfa I, Melderis I. Immunohistochemical diagnosis of irschsprung's disease and allied disorders. Acta Chirurgica Latviensis. 2016;15:50-57.
19. Kapur RP, Reed RC, Finn LS, Patterson K, Johanson J, Rutledge JC. Calretinin immunohistochemistry versus acetylcholinesterase histochemistry in the evaluation of suction rectal biopsies for Hirschsprung Disease. Pediatr Dev Pathol. 2009;12:6-15.
20. Barshack I, Fridman E, Goldberg I, Chowers Y, Kopolovic J. The loss of calretinin expression indicates aganglionosis in Hirschsprung's disease. J Clin Pathol. 2004;57:712-16.
21. Małdyk J, Rybczyńska J, Piotrowski D, Kozielski R. Evaluation of calretinin immunohistochemistry as an additional tool in confirming the diagnosis of Hirschsprung disease. Pol J Pathol. 2014;65:34-39.
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. Mod Pathol. 2009;22:1379-84.
23. Lim KH, Wan WK, Lim TK, Loh AH, Nah SA, Chang KT. Primary diagnosis of Hirschsprung disease–Calretinin immunohistochemistry in rectal suction biopsies, with emphasis on diagnostic pitfalls. World Journal of Pathology.2014;9.