Hirschsprung disease. Evaluation of calretinin and S-100 as ancillary methods for the diagnosis of aganglionosis in rectal biopsies

Luis De la Torre MD [1], Karla Santos MD [1]

ABSTRACT

Introduction. Rectal biopsy and its histopathologic study with hematoxylin and eosin (HE) is the gold standard for Hirschsprung disease (HD) diagnosis. However, this procedure is a common challenge and wrong diagnoses arise when an inadequate rectal biopsy or inaccurate histological process and/or examination are made. Recently, to increase the diagnostic accuracy of HD, an ancillary immunohistochemistry (IHQ) test with calretinin has been used. The aim of this study was to establish the concordance, sensibility and specificity of calretinin in our laboratory to diagnose aganglionosis.

Methods. Twelve rectal biopsies of patients admitted because suspected HD were included. The histologic study was done observing fifty sections stained with HE and diagnoses were: eight HD (aganglionic) and four idiopathic constipated (normoganglionic) patients. These biopsies were re-evaluated blindly and independently by three pathologists. S-100 and calretinin IHQ stains were added for this study. The analysis of the results was performed with the SPSS 19.0. The concordance was established with kappa test.

Results. The global kappa showed that calretinin had a perfect concordance and was statistically significant (p=<0.0001) being better than HE (p=0.01) and PS100 (p=0.12).

Conclusion. HE is the stain that should be used in the study of rectal biopsies to observe ganglion cells. Calretinin is a good ancillary method that can be used for pathologists, it showed great sensibility and specificity and a perfect concordance between observers.

Keywords: Aganglionosis, calretinin, Hirschsprung disease, rectal biopsy.

RESUMEN

Introducción. El estándar de oro para el diagnóstico de la enfermedad de Hirschsprung (EH) es la interpretación histopatológica de las biopsias rectales teñidas con hematoxilina y eosina (HE). Sin embargo, este procedimiento tiene retos que ocasionan errores diagnósticos: por una mala toma de biopsia, por un proceso o un estudio histológico inadecuados. Recientemente para aumentar la certeza en el diagnóstico de EH se ha utilizado una prueba de inmunohistoquímica (IHQ) con calretinina. El objeto de este trabajo es demostrar la concordancia, la sensibilidad y la especificidad de este estudio con calretinina en nuestro laboratorio.

Material y métodos. Se incluyeron doce biopsias rectales de pacientes con sospecha de EH. El estudio histológico se hizo observando 50 muestras teñidas con HE y los resultados fueron: ocho pacientes con EH (aganglónico); cuatro con constipación idiopática (normoganglónico). Las biopsias fueron reevaluadas en estudio ciego por tres patólogos. Se incluyeron estudios de inmunohistoquímica de S-100 y calretinina. El análisis de los resultados se realizó con SPSS 19.0. La concordancia se estableció con la prueba de kappa.

Resultados. La prueba de kappa global mostró que la calretinina tuvo perfecta concordancia y fue estadísticamente significativa (p = <0.0001), mejor que la HE (p=0.01) y S-100 (p = 0.12).

Conclusión. La tinción con HE es la que se usa para observar células ganglionares en las biopsias rectales. El estudio con calretinina es un buen método auxiliar que puede ser usado por los patólogos, muestra gran sensibilidad y especificidad y una perfecta concordancia entre los observadores.

Palabras clave: Aganglionosis, calretinina, enfermedad de Hirschsprung, biopsia rectal.

[1]   Department of Surgery. Centro Colorrectal para Niños de México y Latinoamérica. Hospital para el Niño Poblano.

Hirschsprung disease (HD) is a congenital rectal malformation that can affect more proximal segments of the colon. The histological characteristics of HD are absence of ganglion cells and nerve hypertrophy in the submucosal and myenteric plexus [1]. Contrast enema and anorectal manometry have been used to attempt diagnosis of HD. However, the histological study remains the gold standard using paraffin embedded biopsies, observing many sections stained with HE. This protocol is adequate to confirm or discard aganglionosis [2-6]. This protocol required a good biopsy and an

experienced pathologist. Nonetheless, pathologists should be alert in preterm or newborns where the phenotype of an immature ganglion cell, which is smaller, apolar, ovoid with small or absent nucleoli and scarce cytoplasm could be present which are different from the classical phenotype of the mature ganglion cells. An immature ganglion cell could be confused with lymphocytes, endothelial cells, among others that have similar phenotype (Figure 1). Nerve hypertrophy also demands experience to be defined [5-8]. These morphological variables make the diagnosis of HD a real challenge for pathologists, moreover, when they do not see regularly rectal biopsies to diagnose HD. This situation results in over or under-diagnosis of HD with different consequences. IHQ stains have been used as a supplementary method to identify ganglion cells and nerve hypertrophy. An advantage of IHQ is that it uses paraffin embedded biopsies. S-100 is a nerve sheath marker used to identify the nerve hypertrophy [8] and has been suggested as indirect evidence of the presence of ganglion cells by staining the Schwann cells adjacent to them [9]. Barshack et al reported in 2004 that calretinin is absent in colonic nerve fibers of patients with HD while it is present in normal patients; additionally, calretinin is present in the cell body of ganglion cells and its prolongations [9]. Kapur et al compared calretinin versus acetylcholinesterase (AChE)

in normal and HD patients concluding that calretinin is superior to AChE as an adjunct diagnostic method [10].

The origin of the problems in the histological diagnosis in HD may have one or two possibilities: the biopsy (surgeon dependent) and/or, its process and examination (pathologist dependent). Wrong diagnoses in rectal biopsies to confirm aganglionosis are common and it is not informed in the literature. Two situations may be possible: over-diagnose and under-diagnose. The first situation, results in unnecessary colostomies, ileostomies, partial or total colonic resection and pulled-throughs. The second circumstance, causes a delayed diagnosis with the possibility of increased morbidity and mortality.

The aim of this study was to establish the utility of calretinin to aid in the histological diagnosis of aganglionosis in rectal biopsies.

METHODS

Twelve transanal, mucosa-submucosa rectal biopsies performed under general anesthesia and obtained with Iris scissors at least 3 cm above of the dentate line were included in this study. All biopsies were done in patients with suspected of Hirschsprung disease and their definitive diagnoses were: eight Hirschsprung diseases (aganglionic) and four idiopathic constipation (normoganglionic). Patient age at biopsy ranged from 1 to 116 months, with an average of 20 months. Criteria to include the cases for this study were: 1. Confirmed histological diagnosis of normoganglionic or aganglionic made by an experienced pediatric pathologist using 50 consecutive sections stained with HE, which was considered the "gold standard" for this study. 2. The original 50 sections stained with HE should be available. 3. The stored tissue embedded in the paraffin blocks from the biopsies was sufficient for at least ten new additional sections for IHQ stain with calretinin and, another ten for S-100 except in one case where the tissue was insufficient for this stain. IHQ process was performed using DAKO EnVision™ system. The monoclonal antibodies used were mouse anti-human calretinin (Dako Cytomation®) and rabbit anti-cow S100 (Dako®).

Three pediatric pathologists with different experience diagnosing Hirschsprung disease participated as observers. Two pathologists received at least 15 Hirschsprung cases per year; the other one received 0-1 case of HD per year.
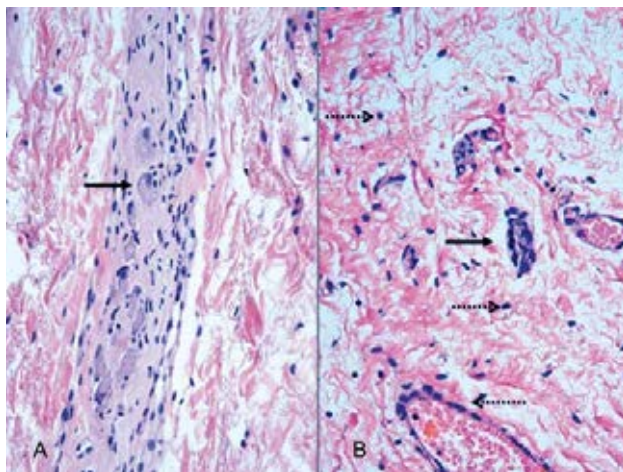

Figure 1. Different phenotypes of mature versus immature ganglion cells with HE at 40X in submucosal plexus of rectal biopsies. (A) Submucosal ganglia of normal rectum with HE at 40 x, the phenotype of mature ganglion cells is showed (arrow). (B) A group of immature ganglion cells are showed (arrow); this phenotype could be confused with other type of cells such as endothelial cell, lymphocytes or mesenchymal cells (3 dotted arrows).

The study was divided in three stages; each one consisted of a blind and independent evaluation of the biopsies. In the first stage, the pathologists did a re-evaluation of the 50 original stored HE stained sections, to establish if they were aganglionic or normoganglionic. During the second phase, pathologists defined if nerve hypertrophy was present in the new sections stained with S-100. Finally in the third stage, pathologists determined in the sections stained with calretinin positivity or negativity to this antigen. Diagnostic criteria are summarized in Table 1. The informed diagnoses from the three pathologists were registered and a statistical analysis was performed with SPSS 19.0®. The concordance was established with kappa test with a significance level of p<0.05. The global kappa was established with the Epidat 4.0® program; also sensibility and specificity were determined for each stain.

RESULTS

Hematoxylin and eosin allow the correct diagnosis in all cases for pathologist 2 (P-2). Pathologist 1 (P-1) diagnosed correctly eleven cases and only one normoganglionic case (case 12) was incorrectly diagnosed. Pathologist 3 (P-3) diagnosed correctly nine cases, eight aganglionic and one normoganglionic; and diagnosed incorrectly three normoganglionic cases where he could not identify ganglion cells (cases 5, 8 and 12).

When protein S-100 was used as an auxiliary method to evaluate nerve hypertrophy in aganglionic cases, this correlation was observed by P-2 in 5 cases (case 1, 7, 9, 10 and 11), by P1 in 4 (case 7, 9, 10 and 11) and P-3 only in one (case 11). Protein S-100 in the three normoganglionic cases where IHQ could be done (case 5, 6, and 8), the correlation to observe the nerves without hypertrophy was informed by the three pathologists.

Calretinin used as an ancillary IHQ stain to observe positivity in normoganglionic cases and negativity in aganglionic cases, allowed to observe this relationship in all cases by the three pathologists (Table 2).

Inter-observer evaluation showed a concordance for HE of 0.30 to 1, for S-100 of 0.07 to 0.47 and for calretinin was 1. Sensibility, specificity, positive and negative predictive values (PPV and NPV) are also showed in Table 3.

Global evaluation showed a concordance for HE of 0.99 (p 0.01), for S-100 of 0.14 (p 0.12) and for calretinin was 1 (p < 0.0001). Sensibility of the three stains was: HE 100%, S-100 41.7% and calretinin 100%. Specificity was: for HE 63.6%, S-100 and calretinin 100%. PPV was: HE 85.7%, S-100 and calretinin 100%. NPV was: HE 100%, S-100 39.1% and calretinin 100% (Table 4).

DISCUSSION

The problem of histopathologic diagnosis of aganglionosis is that the pathologists should "demonstrate something that does not exist", in other words: they have to confirm the absence of ganglion cells in the submucosal, in the myenteric plexus or in both. This dilemma is resolved for any pathologist when ganglion cells are definitely identified, it does not matter which histological technique was used. However, the pathologist needs: a good rectal biopsy and, an appropriate histological process of the sample following an adequate protocol for diagnosis of aganglionosis.

To reduce these problems, a good rectal biopsy is mandatory. Rectal biopsy should be obtained from the rectum at least 3 cm above the dentate line and, it should contain adequate amount of submucosa. One should keep in mind that, the first centimeters of the anal canal are normally aganglionic [4,11,12]. For this reason, we recommend a transanal biopsy using scissors obtaining mucosa and adequate amount of submucosa; also a suction biopsy instrument could be used; however, the amount of submucosa could be small. In our experience, more deep transanal biopsies like full-thickness rectal biopsy and posterior rectal myectomies, as the Lynn procedure, to diagnose aganglionosis may cause serious consequences: damage to the anal canal, and fibrosis of the rectal wall, making a difficult and laborious pull-through and, leaving the possibility of fecal

Table 1. Criteria used to define aganglionic and normoganglionic in rectal biopsies.

Diagnosis	HE	S-100	Calretinin
Aganglionic	Absence of ganglion cells and nerve hypertrophy	Nerve hypertrophy	Negative stain
Normoganglionic	Identify ganglion cells	None nerve hypertrophy	Positive stain

HE: hematoxylin and eosin

Table 2. Diagnoses of twelve rectal biopsies studied blindly and independently by three pathologists.

Case	Age (month)	Confirmed Diagnostic	HE			PS-100			Calretinin		
			P-1	P-2	P-3	P-1	P-2	P-3	P-1	P-2	P-3
1	2	A	A	A	A	WO	W	WO	-	-	-
2	1	A	A	A	A	WO	WO	WO	-	-	-
3	8	A	A	A	A	WO	WO	WO	-	-	-
4	1	A	A	A	A	WO	WO	WO	-	-	-
5	6	N	N	N	A	WO	WO	WO	+	+	+
6	64	N	N	N	N	WO	WO	WO	+	+	+
7	24	A	A	A	A	W	W	WO	-	-	-
8	20	N	N	N	A	WO	WO	WO	+	+	+
9	1	A	A	A	A	W	W	WO	-	-	-
10	116	A	A	A	A	W	W	WO	-	-	-
11	2	A	A	A	A	W	W	W	-	-	-
12	1	N	A	N	A	I	I	I	+	+	+

A= aganglionic
N=normoganglionic
W= with nerve hypertrophy
WO= without nerve hypertrophy
+ = positive stain
- = negative stain
I = Insufficient paraffin block for S-100 stain.
P-1= Pathologist 1, P-2= Pathologist 2 and P-3= Pathologist 3.

Table 3. Statistical evaluation of three histopathological techniques used for Hirschsprung disease according to each observer.

Stain	Pathologist	kappa	p	Sensibility	Specificity	PPV	NPV
HE	1	0.8	0.005	100%	75%	88.9%	100%
	2	1	0.001	100%	100%	100%	100%
	3	0.308	0.140	100%	25%	72.7%	100%
S-100	1	0.353	0.125	50%	100%	100%	42.9%
	2	0.476	0.064	62.5%	100%	100%	50%
	3	0.072	0.521	12.5%	100%	100%	30%
Calretinin	1	1	0.001	100%	100%	100%	100%
	2	1	0.001	100%	100%	100%	100%
	3	1	0.001	100%	100%	100%	100%

kappa=Concordance
p: significance
PPV: Positive predictive value
NPV: Negative predictive value.

Table 4. Global statistical evaluation of three histopathological techniques used for Hirschsprung disease.

Stain	Global kappa	p	Sensibility	Specificity	PPV	NPV
HE	0.999	0.01	100%	63.6%	85.7%	100%
S-100	0.147	0.120	41.7%	100%	100%	39.1%
Calretinin	1	<0.0001	100%	100%	100%	100%

HE: hematoxylin and eosin
Global kappa: concordance
p: significance
PPV: Positive predictive value
NPV: Negative predictive value

incontinence. On the contrary, rectal and colon biopsies made by endoscopy commonly only obtain mucosa and inadequate amount of submucosa, leaving the possibility that the biopsy could be wrongly interpreted as aganglionic. These kinds of biopsies are not recommended for diagnosis of aganglionosis.

HE remains the main stain used to identify ganglion cells in the study of the rectal biopsy of a child suspected with HD. However, if there is any doubt or when the pathologist does not have experience an ancillary method should be used. Rectal biopsies of newborns and premature patients may express a phenotype of immature ganglion cells that are difficult to recognize and ancillary histological methods have been used in these situations. Many IHQ stains have been used, among others, S-100, neuron specific enolase, catepsin-D, peripherin but their sensibility and specificity are low and do not resolve the diagnostic problem. Other popular adjuvant is acetylcholinesterase (AChE) but, it is a hystochemical method which requires frozen tissue and we strongly oppose its use for initial definitive diagnosis of HD [10,13]. It has been proven that calretinin has better specificity than other IHQ stains [6]. In the present study global kappa results for the three staining techniques showed that calretinin had a perfect concordance and was statistically highly significant ($p<0.0001$). Additionally, calretinin showed high specificity with better PPV compared with HE and S-100. (Table 4) In the present study case 12 was a difficult one. It was a newborn without HD. Rectal biopsy with HE showed immature cells, nerve hypertrophy was not present. This situation lead to a wrong diagnosis by two observers informing the case as aganglionic; however, the third observer, an experienced pathologist, diagnosed it as normoganglionic and pointed out the difficulty to define the ganglion cell due to its immature phenotype. This case was positive to calretinin and consequently the three observers correctly diagnosed the case as normoganglionic (Figure 2).

Nerve hypertrophy is a histological marker used to define aganglionosis. This finding becomes weak and less useful in preterm and some term newborns, long segment HD and total colonic aganglionosis. HE is also the stain to observe nerve hypertrophy. Nonetheless, this finding is difficult to define with HE for pathologists with poor experience. S-100 has been proposed as an IHQ stain used to identify this marker. S-100 is a qualitative test with a low sensibility, in our study it was 41.7%. S-100 stain could be
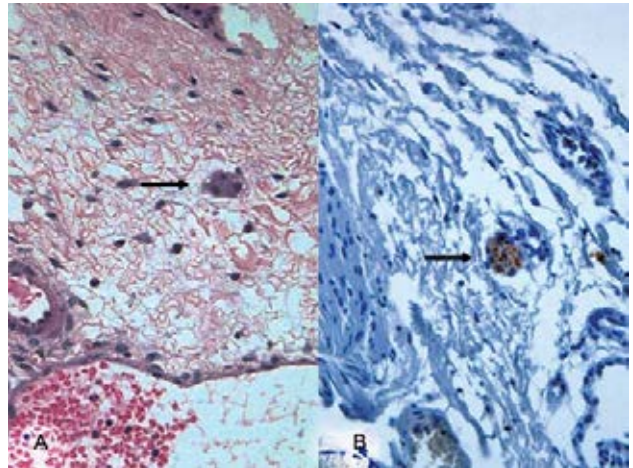

Figure 2. Normal rectal biopsy at 40X of a newborn patient. (A) Biopsy with HE showing the immature ganglion cells; nerve hypertrophy was not observed. (B) Biopsy positive to calretinin; the three observers correctly diagnosed the case as normoganglionic.

positive in different cells including glia and Schwann cells [8] causing inconsistent interpretation by the pathologists.

Over-diagnosis and under-diagnosis of HD are not unusual and, in some places they are common, thus prompting unfortunate consequences in children. These situations are not informed in the literature. HE is an inexpensive stain and this study corroborated that it continues to be the initial method and gold standard to study rectal biopsies for Hirschsprung disease in view of its high sensibility and specificity. Calretinin demonstrated a great sensibility, specificity and concordance, so it is an excellent option which can be used by pathologists in case of diagnostic doubt or when they are not familiar with the interpretation of biopsies for HD, even in small bowel intestine (Figure 3).
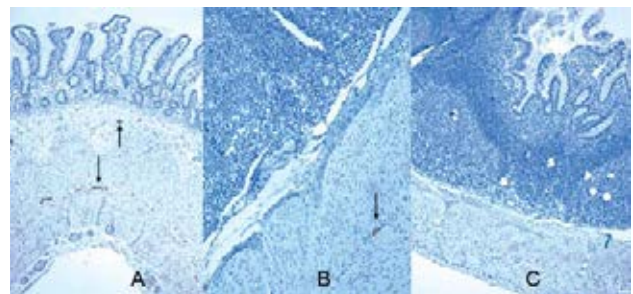

Figure 3. Photomicrograph at 40x with calretinin of 3 ileal biopsies of a total colonic aganglionosis. Photomicrographs at 40X with calretinin. (A) Proximal normoganglionic ileum positive to calretinin in both plexus. (B) Transitional zone positive only in the myenteric plexus. (C) Aganglionic ileum with negative calretinin in both plexus.

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