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Calretinin immunohistochemistry in Hirschsprung's disease: An adjunct to formalin-based diagnosis

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Aim: *This study was designed to assess the utility of calretinin immunohistochemistry in the diagnosis of Hirschsprung's disease.* ***Material and Methods:*** *Eleven definitive resection materials from 10 Hirschsprung's disease patients and 3 initial full-thickness rectal biopsies of these patients were retrieved from the pathology archives. Additionally, 15 distal colon and 13 proximal colon full-thickness samples from 23 non-Hirschsprung's disease patients were also evaluated as the control group. All material was reevaluated by light microscopy for the presence or absence of ganglion cells and immunostained with calretinin, including proximal surgical margins and aganglionic zone samples from each resection material.* ***Results:*** *Immunohistochemistry for calretinin provided highly compatible results with hematoxylin-eosin findings in Hirschsprung's disease and non-Hirschsprung's disease patients, except in one Hirschsprung's disease patient with very rare nerve stainings at the distal surgical margin.* ***Conclusions:*** *Calretinin immunohistochemistry was found to be highly sensitive and specific in detecting aganglionic segments. New research should be conducted in order to clarify calretinin staining patterns of the transitional zone, rare Hirschsprung's disease types, pure hypoganglionosis patients, and the anorectal junction, and for the mapping of fetal and neonatal colonic specimens. The technique seemed very effective for lowering the need for excessive sectioning and practical regarding the erratic nature of the acetylcholinesterase staining.*

Key words: Hirschsprung's disease, calretinin, acetylcholinesterase, intestinal pseudo-obstruction, dysmotility

Hirschsprung hastalığında kalretinin immünhistokimyası: Formalin bazlı tanıda yardımcı bir teknik

Amaç: *Bu çalışma Hirschsprung hastalığında kalretinin immünhistokimyasının yararlılığını araştırmak amacıyla planlanmıştır.* ***Yöntem ve Gereç:*** *On Hirschsprung hastasından 11 cerrahi rezeksiyon materyali ve bunlara ait 3 başlangıç tamkat rektal biyopsi çalışma için kullanılmıştır. Ek olarak, 23 Hirschsprung dışı hastadan, 15 distal ve 13 proksimal tam kat kolon örnekleri kontrol grubu olarak kullanılmıştır. Tüm örnekler ganglion hücrelerinin varlığı veya yokluğu açısından tekrar ışık mikroskobunda değerlendirilmiş ve bütün rezeksiyon materyalleri üzerinde proksimal cerrahi sınırlar ve agangliyonik zonu içerecek şekilde kalretinin immünhistokimyası uygulanmıştır.* ***Bulgular:*** *Kalretinin immünhistokimyası Hirschsprung ve Hirschsprung dışı örneklerde hemotoksilen-eozin bulgularla yüksek uyumluluk göstermiştir. Yalnızca 1 Hirschsprung örneğinde distal cerrahi sınırda nadir sinir boyanması gözlenmiştir.* ***Sonuç:*** *Kalretinin immünhistokimyası agangliyonik segmenti belirlemede yüksek sensitivite ve spesifisite göstermiştir. Transisyonel zonda, nadir Hirschsprung hastalığı tiplerinde, hipogangliyonoziste, ayrıca fetal neonatal kolon örneklerinin ve anorektal bileşkenin haritalaması için ek çalışmalar yapılmalıdır. Teknik asetilkolinesteraz boyanmasında pratikte yaşanan güçlükler gözönüne alındığında uygulaması kolay ve zaman kazandırıcı bulunmuştur.*

Anahtar kelimeler: Hirschsprung hastalığı, kalretinin, asetilkolinesteraz, intestinal psödo-obstruksüyon, dismotilite

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INTRODUCTION

Hirschsprung's disease (HD) is the most important type of intestinal pseudo-obstruction in neonatal pathology. It is a congenital malformation of the enteric nervous system characterized by the absence of ganglion cells from submucosal (Meissner) and myenteric (Auerbach) nerve plexus, and is diagnosed histopathologically on the basis of absence of these cells from the distal rectum and a variable length of contiguous bowel. HD is thought to be a neurocristopathy, related to the premature arrest of the craniocaudal migration of these cells during the 5th to 12th weeks of gestation. Alternatively, etiologies including microenvironment theory, breakdown in apoptosis-related systems, smooth muscle cell pathology, genetic factors, and decreased expression of Ca++ channel receptors were also implied in the pathogenesis (1-5). In approximately 70% of cases, the aganglionic segment involves the rectum and the sigmoid colon only (classical HD), whereas in 20% of cases, the aganglionic segment involves the more proximal bowel, with the total colon being aganglionic in 8-10%. It affects an estimated 1:5000 liveborns and enters into the clinical differential diagnosis for infants, children and adults with severe and/or chronic constipation.

The diagnosis and extent of resection for the management of HD depend on the sensitive and specific identification of ganglion cells. However, documenting aganglionosis is often difficult and tedious on routine hematoxylin-eosin (H&E)-stained sections; thus, either H&E evaluation of serial sections from full-thickness biopsies or acetylcholinesterase (AChE) staining from fresh superficial biopsies is preferred for histopathologic diagnosis. Since its introduction, AChE has evolved as the gold standard in diagnosing HD; however, this histochemical analysis is technically challenging, and to date, has not gained worldwide utilization and applicability. Diagnostic pathologists remain uncomfortable with the diagnosis of HD via rectal (mucosal/submucosal) biopsy and with performance and interpretation of the associated AChE assay.

Recently, calretinin, a calcium-binding protein that plays an important role in the organization and functioning of the central nervous system, has been introduced as a new simple and reliable tool for the diagnosis of HD (6). Moreover, studies in which the results were compared with those of AChE staining protocols showed that calretinin was potentially superior to AChE as an adjunctive diagnostic method for evaluating suction rectal biopsies for HD (7-9).

Owing to the limited number of relevant studies on this topic and the need for a reliable and simple diagnostic method in our routine practice with HD, we planned to review our material for calretinin immunohistochemistry and to compare results with our standard method used for diagnosing HD (histology from full-thickness samples of rectal biopsies and definitive operation materials).

MATERIALS AND METHODS

Eleven definitive resection materials from 10 HD patients (age range: 1 month-9 years; median: 28 months) seen from 2005-2011 were retrieved from the pathology archives. There were 8 male and 2 female patients (Table 1). All cases were selected from clinically well-followed HD patients. Cases with poor clinical information or follow-up were not included in the study. Only the blocks presenting unequivocal existence of mature ganglion cells that were full-thickness were selected as positive controls. No giant ganglia were observed except in one ostomy resection, which was thought to be a secondary finding. The resected specimens of the colon obtained at pull-through surgery were sampled at 1.5 cm intervals and sections were stained with H&E; samples were numbered sequentially from the dentate line proximally. The samples were taken on two sides (mesenteric and anti-mesenteric) in parallel, and the distal and proximal surgical margins were separately sampled totally according to a topographic scheme. In total, 28 formalin-fixed, paraffin-embedded tissue blocks were analyzed.

Additionally, 15 distal colon full-thickness samples from 13 patients (3 imperforate anus, 2 eosinophilic colitis, 1 enterogenous rectosigmoid cyst, 1 spontaneous cecum perforation, 4 intestinal pseudo-obstruction other than HD, 1 aplastic/atrophic desmosis, 1 neuronal intestinal dysplasia autopsy case), and 13 proximal colon full-thick-

Table 1. Demographic data

Variables	Control group	HD group
Number of cases	23	10
Age (months), *Mean±SD* *(Range)*	28.6±57.1 (1-192)	27.8±34.1 (1-108)
Gender		
Male, n (%)	12 (52.2)	8 (80.0)
Female, n (%)	11 (47.8)	2 (20.0)

HD: Hirschsprung's disease.

ness samples from 10 patients (4 necrotizing enterocolitis, 1 duodenal atresia, 1 spontaneous cecum perforation, 1 brid ileus, 1 invagination, 1 chronic constipation-related megacolon, 1 ostomy resection for imperforate anus) were also evaluated as the control group. This group included 12 male and 11 female patients, with an age range from 1 month to 16 years (median: 29 months). In total, 28 formalin-fixed, paraffin-embedded blocks were used from these cases as control.

All the material was reevaluated by light microscopy for the presence or absence of ganglion cells, and serial sections were performed when needed. Blocks lacking sufficient tissue and those that were superficial or too distal were not included.

In total, 56 paraffin blocks from 33 patients were processed for the study (Table 2). All the blocks were sectioned (4 µm), deparaffinized, cleared, and rehydrated in graded ethanol concentrations. Ready-to-use calretinin from cell marque was used (cat. no: 232A-78, Rocklin, CA). Immunohistochemical analysis was performed with a Ventana, GX Benchmark Autostainer using BMK iVIEW DAB Paraffin Protocol. Mast cells and nerve stainings served as internal control, and mesothelial tissue was used as external control.

Calretinin staining was analyzed without knowledge of the diagnosis. This analysis was performed independently by two pathologists - one experienced in diagnosing HD (AK) and one unaccustomed to handling HD cases (ATA). Calretinin was considered as positive if any specific staining (excluding mast cells) was present within the submucosal nerve plexus, muscularis mucosa or lamina propria mucosa. All three anatomic portions were scored separately for positivity for calretinin, and the absence or presence of ganglion cells was also noted for each case. Serial H&E sections from each block were performed when needed.

Data analysis was performed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, US). Data were shown as mean±standard deviation or number of cases and percentages. Kappa coefficient was applied for determining the agreement level between the 1st and 2nd observer. Diagnostic performances (e.g. sensitivity, specificity, positive and negative predictive values, and accuracy) of the clinical evaluation regarding biopsy (i.e. gold standard) were also calculated. The association between clinical evaluation and biopsy was analyzed by Pearson's chi-square test. A p value less than 0.05 was considered statistically significant.

Table 2. Documentation of Hirschsprung's disease patients (total number of samples: 28)

Patient	Age	Sex	Operation procedure	Samples analyzed
1		M	Rectosigmoid resection	1 DSM 2 PSM 1 DSM
			Duhamel	1 PSM
2		F	Duhamel	1 DSM 1 PSM
3		M	Swenson	1 DSM 1 PSM
4		M	Transanal pull-through	AZ 1PSM 1 IRB 1 OR
5		M	Transanal pull-through	AZ 1 PSM 1 IRB
6		M	Duhamel	1 DSM 1 PSM 1 IRB
7		M	Duhamel	1 DSM 1 PSM
8		F	Duhamel	1 DSM 1 PSM
9		M	Soave	AZ 1 PSM
10		M	Transanal pull-through	2 AZ 1 PSM

DSM: Distal surgical margin. PSM: Proximal surgical margin. AZ: Aganglionic zone. IRB: Initial rectal biopsy. OR: Ostomy resection.

RESULTS

Calretinin immunohistochemistry was evaluated as negative or positive in the lamina propria, muscularis mucosa and submucosa by two pathologists blinded to the corresponding H&E sections and histopathologic diagnoses. It was found that a high consensus existed between the two interpreters (κ=0.953 ve p<0.001) (Table 3).

All but one aganglionic zone sample and all initial rectal biopsies (3 cases) obtained from HD patients were interpreted as negative with calretinin. The only patient with positive staining at the distal

Table 3. Distribution of samples according to observers' evaluation

1st Observer	2nd Observer	Number of samples
Negative	Negative	14/56 (25.0%)
Negative	Positive	1/56 (1.8%)
Positive	Negative	0/56 (0.0%)
Positive	Positive	41/56 (73.2%)

surgical margin had very discrete nerve fibers stained positively for calretinin in the lamina propria in the aganglionic zone, despite the absolute absence of ganglion cells in deep sectioning in all layers (Figures lA, B). This case resulted in a discrepancy between the two observers. It was noted as negative by the first observer while the second observer regarded the sample as positive for calretinin. All other sections obtained from proximal surgical margins and ostomy resection material stained positively for the antibody in this group.

The control group on the whole showed a universal positivity for calretinin in all layers, which was straightforward in all cases, and no discrepancy in interpretation was noted between the two observers.

The concordance of the calretinin results with the histology was excellent. There was 100% agreement between the gold standard test (i.e. biopsy) and decisions of the 1st observer (κ=1.000 and $p<0.001$). All of the diagnostic indicators (i.e. sensitivity, specificity, positive and negative predictive values, and accuracy) of the first observer were determined as 100%. The results were highly satisfactory for the inexperienced observer as well, with the positive predictive value and the specificity being 97.6% and 93.3%, respectively (Table 4).

In general, the nerve stainings were more appreciable in the lamina propria and in the adjacent submucosa than in the muscularis mucosa in all patients.

The nerve stainings were peculiar with their granular non-homogeneous staining pattern and with their very fine structure, focally forming a fine fibrillary network in the lamina propria (Figure 2). Ganglion cells were also stained strongly with the antibody.

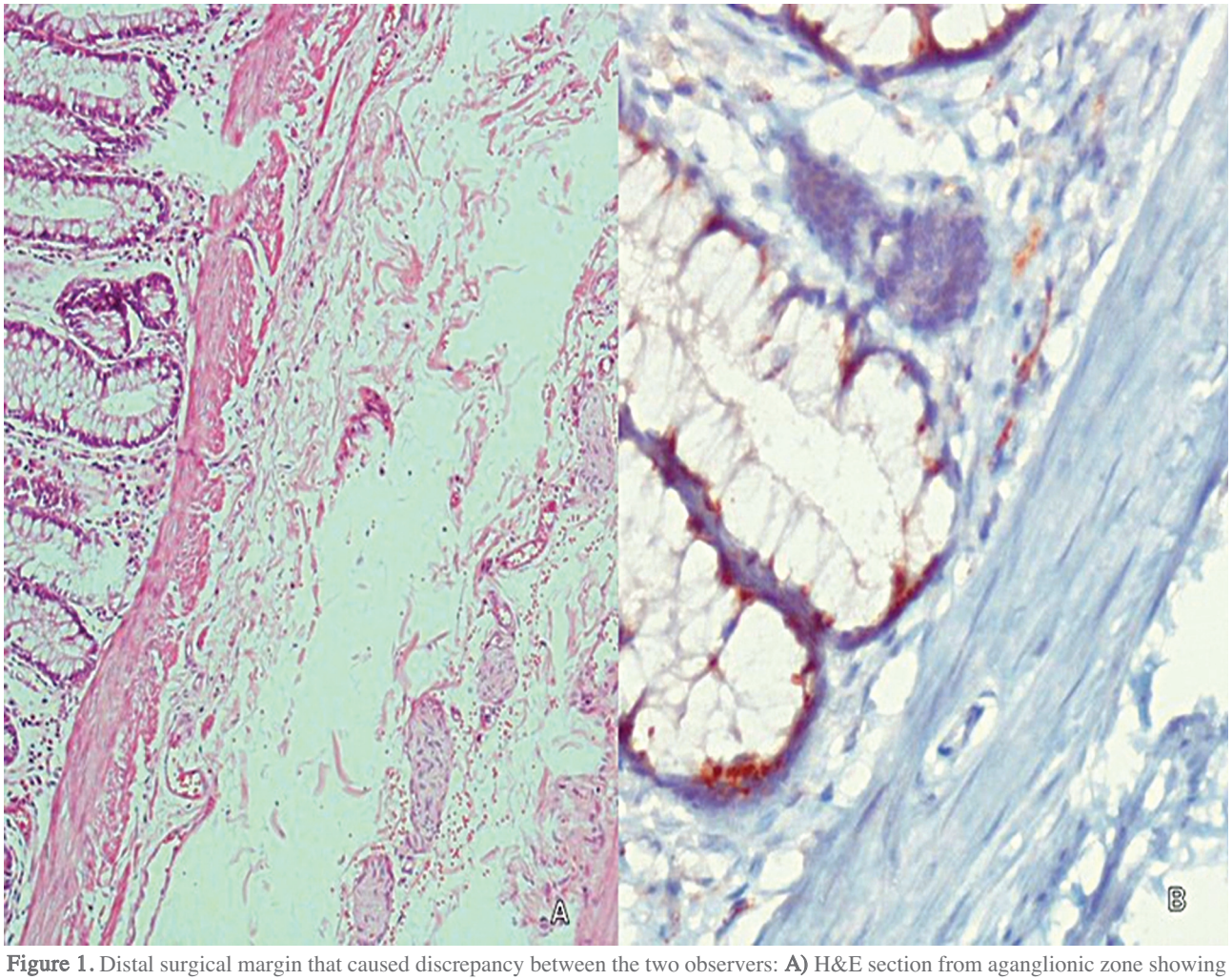

Figure 1. Distal surgical margin that caused discrepancy between the two observers: **A)** H&E section from aganglionic zone showing hypertrophic nerve fibers in the submucosa (x40). **B)** Discrete nerve fibers stained positively for calretinin in the lamina propria of this patient (x400).

Table 4. Predictive values and sensitivity/specificity of immunohistochemical evaluation

Indicators	Definitions	1st Observer	2nd Observer
Number of samples	N	56	56
Sensitivity	TP/(TP+FN)	41/41 (100.0%)	41/41 (100.0%)
Specificity	TN/(TN+FP)	15/15 (100.0%)	14/15 (93.3%)
PPV	TP/(TP+FP)	41/41 (100.0%)	41/42 (97.6%)
NPV	TN/(FN+TN)	15/15 (100.0%)	14/14 (100.0%)
Accuracy	(TP+TN)/(N)	56/56 (100.0%)	55/56 (98.2%)

TP: True positive. FN: False negative. TN: True negative. FP: False positive. PPV: Positive predictive value. NPV: Negative predictive value.

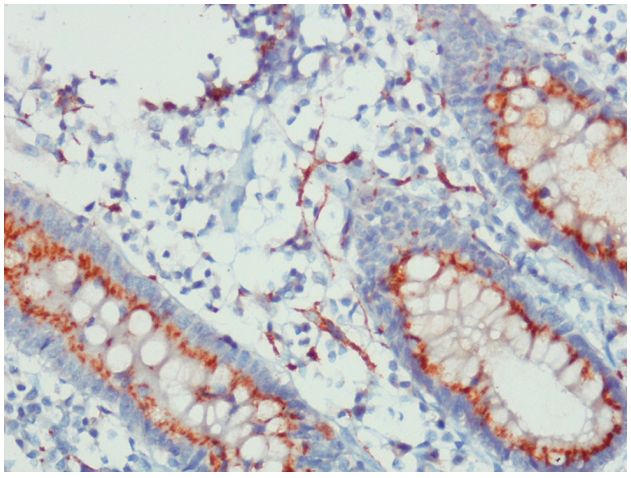

Figure 2. Granular non-homogeneous staining pattern of the nerve fibers (calretinin x400).

Owing to the overall architectural staining pattern, the nerve fibers were continuously present in the lamina propria, muscularis mucosa and adjacent submucosa without skip areas, except in the lymphoid follicle regions. In other words, the calretinin nerve staining was continuous in each layer and between all three layers, most markedly in the lamina propria; no focal staining was noted in any of the samples. In the low-power magnification, at first glance, the positive samples appeared more intense in color, while the negative samples appeared totally pale (Figure 3A, B).

The mast cells stained homogeneously without granular staining and showed distinct cytoplasmic borders.

DISCUSSION

The biopsy diagnosis of congenital colorectal aganglionosis, or HD, commonly poses difficulties for the pathologist. Detection of ganglion cells may be difficult for several reasons, but in particular because few may be represented in small mucosal biopsies and because the ganglion cells of young infants (the population most often investigated for HD) are morphologically immature and can be readily confused with endothelial cells and plasma cells among others. With either the H&E- or histochemistry-based approach, adequate sampling is critical. Because submucosal ganglia are widely distributed, a generous amount of submucosa must be obtained, and if not obtained from the appropriate level of the rectum, analysis of 100 or more histologic sections may be needed in H&E-based diagnosis (6,10).

AChE staining is still the most widely utilized technique for facilitating HD diagnosis in this context (11,12). The value of AChE staining depends on the positivity of the hyperplastic nerve fibers seen in the lamina propria and muscularis mucosa in aganglionosis; however, these features are not always present, particularly in the early postnatal period, when detection of ganglion cells is most difficult. Hence, the AChE stain does not obviate the need to thoroughly exclude ganglion cells, because an HD-like pattern can occasionally be observed in other contexts (6,13,14). The utility of the technique is compromised by the need for frozen sections of biopsies -sometimes oriented under a dissecting microscope-, positive and negative control frozen sections, and a pathologist experienced in interpreting the results of the stain. Moreover, the stain itself is somewhat erratic, and in laboratories where it is performed infrequently, several attempts may be required to obtain satisfactory preparations. In our country, despite the efforts over many years, this technique still cannot be used as a diagnostic aid in H&E-based diagnosis of this disease because of technical difficulties and poor standardization in employing the method. Almost all pathology laboratories in Turkey still use histopathology for diagnosis of HD in full-thickness biopsies with the evaluation of serial H&E sections.

Because AChE staining techniques are mostly

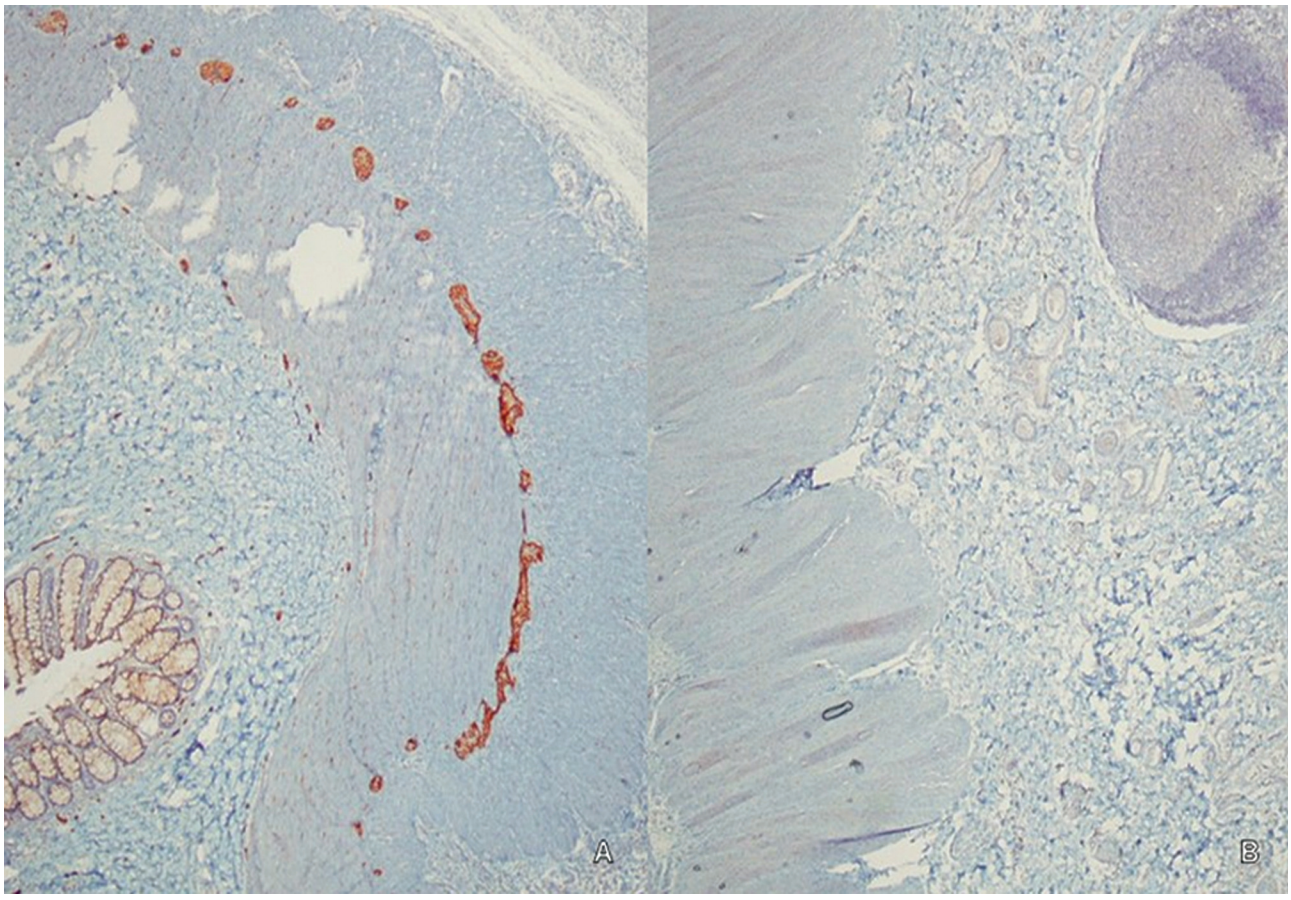

Figure 3. A) Sample positive for calretinin. Compare the tone of the slide with that of a negative sample **(B)** (x40).

confined to specialized centers, many pathology units in the rest of the world have a problem in obtaining access and rely on H&E staining, which may present difficulties especially in identifying immature ganglion cells. Therefore, inability in employing AChE causes limitations in the HD diagnosis, and hence influences the data accumulation negatively and hampers the performance of worldwide standardized methods in the histopathologic diagnosis of HD.

As a consequence, search has continued for new simple, less time-consuming and reliable methods. Researches have been concentrated on immunohistochemical analysis because of its worldwide availability (6,15-31). No immunohistochemical marker, either alone or in combination, has emerged from those researches that is as promising as calretinin. Calretinin immunohistochemistry holds several advantages, such as: it is carried out on a formalin- embedded superficial rectal biopsy, its staining pattern is simple and distinct, and it is either positive or negative (7-9).

The functional interpretation of calretinin immunohistochemistry in HD might be the subject of further studies, but the Ca++-deficient status of the aganglionic bowel might be related to deficient Ca++ receptor expression, which was shown by Piotrowska et al. in their study (32).

In our study, calretinin positivity in all ganglion cell bodies and peripheral nerve fibers was one of an intense granular cytoplasmic reactivity. The granules crowded the cytoplasm and showed a heterogeneous staining pattern with more intense and less intense cytoplasmic areas. The stainings had a fibrillar pattern in the majority, while dot-like granular or small stain aggregates were also seen (Figure 4A, B, C). In the submucosa, the only other immunoreactive cells were the mast cells, but they could be distinguished easily from ganglion cells and nerve endings by their characteristic nuclear features, distinct cytoplasmic borders, and their occurrence singly and unassociated with nerves, along with their less intense homogeneous staining pattern. Owing to the overall architectural staining pattern, the nerve staining was conti-

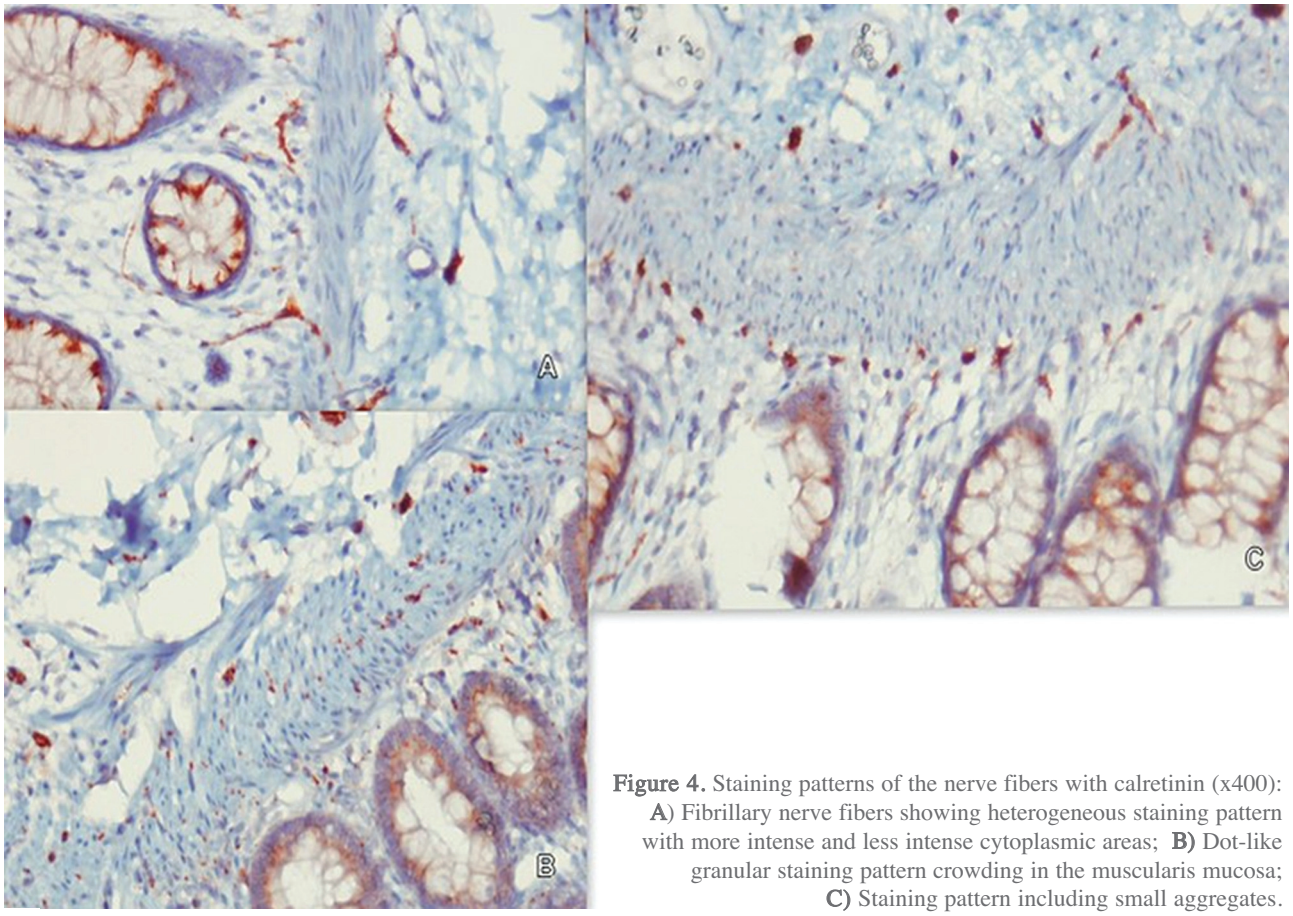

Figure 4. Staining patterns of the nerve fibers with calretinin (x400): **A**) Fibrillary nerve fibers showing heterogeneous staining pattern with more intense and less intense cytoplasmic areas; **B)** Dot-like granular staining pattern crowding in the muscularis mucosa; **C)** Staining pattern including small aggregates.

nuously present in the lamina propria, muscularis mucosa and adjacent submucosa without skip areas, except in the lymphoid follicle regions. We think that this observation of a contiguous staining pattern would help to solve the representation problems regarding small biopsies, which can cause major concern in interpretation.

Our results showed a near perfect match between calretinin immunohistochemistry and histopathology. The only equivocal result was in a sample obtained from the aganglionic zone of a patient with HD. The very discrete presence of the positively stained nerve fibers was considered as negative by one observer, while the other observer interpreted this finding as positive.

In conclusion, our study demonstrated that calretinin immunohistochemistry is a very sensitive and specific method for detecting aganglionic tissue, may eliminate the need for repeat biopsy and may lower the need for excessive sectioning. The utility is for suction biopsy or full-thickness biopsy material, as well as in this study, in the evaluation of the resection specimen. This study differs from the previous studies in the way that a large spectrum of negative control cases have also been checked, and it was shown for the first time that calretinin staining is continuous without skip areas between the lamina propria, muscularis mucosa and submucosa. This feature renders this technique also useful for superficial biopsies as well as for suction biopsies.

Worldwide histopathologic diagnosis of HD still relies largely on formalin-fixed and paraffin-embedded tissues, and we believe that H&E-based studies including immunohistochemical methods should be conducted and encouraged in every possible challenging area of the intestinal pseudo-obstruction pathology. We believe this would help to drive the process forward, and access of the practicing majority to the intestinal pseudo-obstruction pathology should be an achievable goal. Further study is warranted in the understanding of calretinin staining patterns in ultrashort and long HD, the transitional zone, pure hypoganglionosis patients, the anorectal junction, and for the mapping of fetal and neonatal colonic samples. It is advisable that calre-

tinin immunohistochemistry should always be used in addition to limited H&E sections because of the heterogeneous nature of the disease.

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