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Immunohistochemical study of enteric nervous system in hirschsprung's disease and intestinal neuronal dysplasia

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Summary. Background. Hirschsprung's disease (HD) is one of the most common motility disorders in pediatric age groups and it is very important that it be differentiated from other types of motility disorders, especially intestinal neuronal dysplasia B (IND B). Although many studies regarding the differences between the two disorders by immunohistochemical studies exist, there is as yet no consistent result. The purpose of this research was to study the immunohistochemical findings of enteric nervous system in these two motility disorders in comparison with colectomies without motility disorder. Methods. Full wall thickness specimens of three groups of patients (HD, IND B and non motility disorders) were included in the study to be evaluated by immunohistochemistry (IHC). Markers were specific for neuronal cells and pace maker cells composed of PGP 9.5, c-kit, synaptophysin, S100 and CD56. The number of cells was evaluated in the muscularis properia, and myenteric plexus. Results. The number of all the IHC markers i.e. PGP9.5, c-kit, synaptophysin, S100 and CD56 was completely different in HD from the two other groups, while IND B was quite similar to control group. Conclusion. Our finding suggests that there is a marked and significant difference between HD and IND B by IHC markers, which can be used as an additional test for the diagnosis of HD with more accuracy. Further multicenter studies with a greater number of cases would be necessary to find a cut-off point for every IHC marker to differentiate HD and IND B.

Key words: Hirschprung's disease, Intestinal neuronal dysplasia, Immunohistochemistry, Enteric nervous system

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Introduction

Pediatric motility disorders constitute a complex array of clinicopathologic disturbances which result from abnormalities in the neuronal, muscular, neuromuscular or interstitial cell of Cajal (Feichter et al., 2010).

The best characterized type of these disorders is Hirschsprung's disease (HD), but there are also other disorders in this category such as intestinal neuronal dysplasia, neuronal immaturity, disorders of Cajal cells etc (Karim et al., 2006).

The diagnosis of motility disorders remains a challenge for both the clinician and the pathologist, given the confusion in the diagnosis of pediatric motility disorders. The most important question is how best to make a differential diagnosis between HD and other less common diseases. For histologic diagnosis of HD, it is necessary to see the ganglion cells by serial sections in formalin-fixed tissue and use frozen section for acetylcholine esterase enzyme histochemistry (Moore and Johnson, 2005) however, difficulties often arise in situations, such as identifying ganglion cells with confidence in neonates (Guinard-Samuel et al., 2009).

Several methods were used in the past years to identify ganglion cells, but few of them used the difference of various neuronal markers between HD, neuronal intestinal dysplasia B (IND B) and normal intestinal motility, so the diagnosis of intestinal neuronal dysplasia remains a subject of controversy (Corsois et al., 2004; Holland et al., 2010; Petchaswan and Pintong, 2000).

Therefore, an effort was made to use several Immunohistochemical markers composed of S100, CD56, synaptophysin, PGP9.5 and c-kit in the full thickness intestinal wall i.e., mucosa, submucosa and muscularis properia to investigate the difference of the expression of the above-mentioned markers in three

groups of children.

Materials and methods

Patients

All of the children who were referred with chronic constipation and required surgery in the department of pediatric surgery in the biggest referral center of the country, between 2008 and 2011 were evaluated. Three categories were considered:

29 patients with the clinical impression of HD, who were confirmed by histology. There were 20 boys and 9 girls ranging from 4 days to 8 years of age.

29 patients with chronic constipation and clinical impression of HD in which histologically ganglion cells were found and who met the diagnostic criteria of IND B. (Meier-Ruge et al., 2004) This group consisted of 19 boys and 10 girls aged from 14 months to 14 years of age. In this group, only patients above 1 year of age were included in order to exclude ganglion cell immaturity and incomplete differentiation.

Twelve age and sex matched patients as a control group were also included in this study. There were 6 boys and 6 girls (12 months to 14 years of age). They have been operated for reasons such as abdominal mass, intussception, and etc., in which segments of the colon were resected. None of the patients showed any history or complaints regarding gastrointestinal motility disorder.

Methods

All of the hematoxylin and eosin (H&E) slides from the distal colon (left colon) were retrieved from the file and studied for the best full wall thickness section (in all 70 patients).

Immunohistochemistry (IHC) was performed for PGP9.5, c-kit, synaptophysin, S100, and CD56, and is shown in Table-1.

Immunohistochemistary (IHC) was performed on 5 μm sections obtained from formalin- fixed, paraffin embedded blocks using avidin-biotin peroxidase complex method. Unstained tissue sections were collected onto poly L- Lysinized slides for IHC staining. Tissue sections were deparaffinized in xylene (3 times, 10 minutes each) and gradually rehydrated with ethanol (100%, 96%, 70%) and distilled water (2 minutes). Washing was then performed by phosphate buffer saline (PBS) for 5 minutes. After blocking endogenous peroxidase by treatment of hydrogen peroxide (H_2O_2 3%, for 20 minutes), the sections were incubated at 100°C for 20 minutes in citrate buffer (pH=6) as an antigen retrieval step for all the markers except CD 56, for which tris EDTA was used. After washing with PBS (5 minutes) the sections were marked with Dakopen and subsequently incubated for 20 minutes at room temperature with horse serum (diluted to 1:10 in PBS). The primary antibody of each antibody was applied with a certain dilution (Table 1) and incubated overnight at 4°C. The sections were then washed with PBS for 20 minutes and a secondary antibody (Envision, Dako, Denmark) was added, incubated at room temperature for 30 minutes and washed with PBS for 30 minutes.

The final reaction product was revealed by incubation with diaminobenzidine (DAB) (DAKO, Denmark) for 10 minutes. After 10 minutes washing with PBS, nuclei were counterstained with Hematoxylin.

-PGP9.5: The number of enteric nervous cells which were stained by this marker was counted in 5 high power field (HPF x 400) in the inner circular and outer longitudinal muscle layer separately. The positive cells in the myenteric plexus were not counted because they were too crowded to be accurately counted (Geramizadeh et al. 2009) (Fig. 1a,b).

-c-kit: The number of interstitial cells of Cajal (ICC) cells stained by c-kit (CD117) was counted in 10 HPF (x400) in both the longitudinal and circular muscle as well as myenteric plexus, separately. As mast cells are also c-kit positive, but are round and contain cytoplasmic granules, only cells with cytoplasmic process were included (Fig. 2a,b).

- Synaptophysin: The number of cells in all three locations (inner circular, outer longitudinal and myenteric plexus) was counted in 5 HPF (x400), separately.

-S100: This marker is positive in the nuclei and cytoplasm of the enteric nervous system. The number of S100 positive cells was counted in the three locations mentioned above separately in 5 HPF (x400).

-CD56: Neuronal cell adhesion molecule (NCAM) or CD56 is a surface glycoprotein which is important for the interaction of neurons with muscle cells during synaptogenesis (Corsois et al., 2004). CD56 positive cells were counted in the inner circular and outer longitudinal muscle layers in 5 HPF (x400), separately.

Table 1. Characteristics of the antibodies which have been used in this study.

Antibody	Source	Clone	Titer	Antigen retrieval	Company
PGP9.5	Rabbit	Polyclonal	1/200	Citrate	DAKO
c-Kit	Rabbit	polyclonal	1/1000	Citrate	DAKO
Synaptophysin	Mouse	SY38	1/50	Citrate	DAKO
S100	Rabbit	Polyclonal	predilute	Citrate	DAKO
CD56	Mouse	123C3	1/200	Tris EDTA	DAKO

In the myenteric plexus, they were too crowded to be accurately counted (Geramizadeh et al. 2009).

Statistical Methods. The ANOVAs test was applied for quantitative evaluation of IHC markers. P value <0.05 was considered statistically significant.

Results

The results of the IHC markers were as shown below:

-PGP9.5: The number of positive cells in HD, IND B and the control groups in the inner circular muscle layer was 39-350 (mean=169.44), 130-421 (mean=258.10) and 159-290 (mean=220.66) respectively. This number was statistically different between HD and IND (P value <0.05), but no differences were seen between IND and control group (Graph 1a).

The number of PGP9.5 positive cells in the outer longitudinal muscle layer in HD, IND B and the control groups was 25-190 (mean=80.96), 60-210 (mean=113.20), and 53-160 (mean=88.25) respectively (Graph 1b). This number was lower in HD in comparison with the control group, but was not statistically significant (P value=0.596).

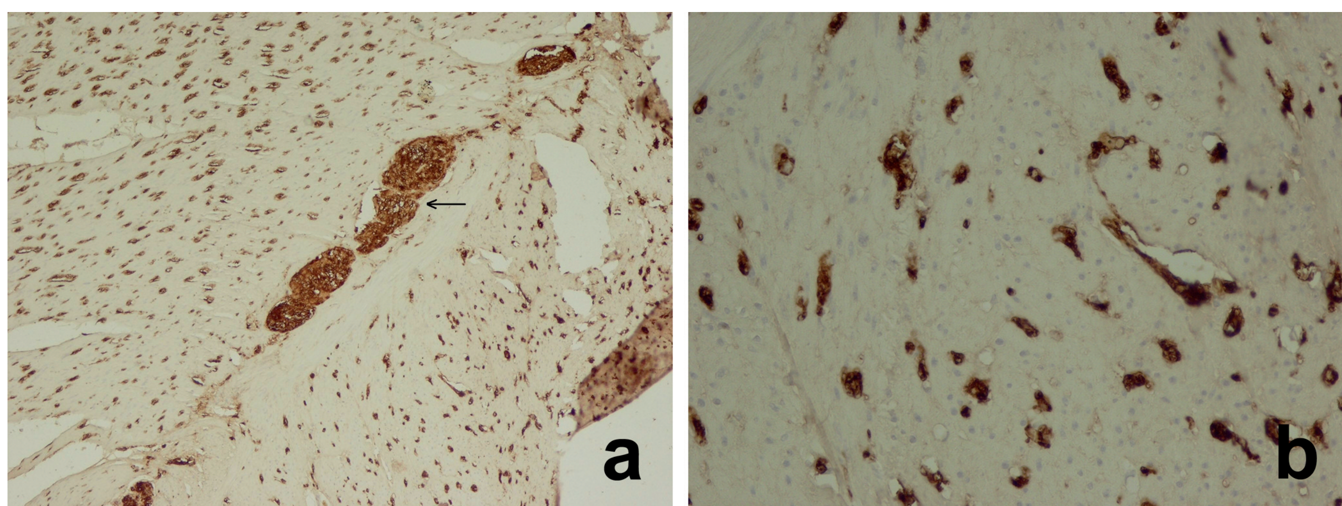


Fig. 1. a. Low power of the section from the intestinal wall muscle stained with PGP 9.5.The arrow shows the thick and crowded nerve plexus which cannot be counted. **b.** High power shows the staining pattern in the longitudinal muscle which was counted and compared in the three groups of this study. a, x 250; b, x 400.

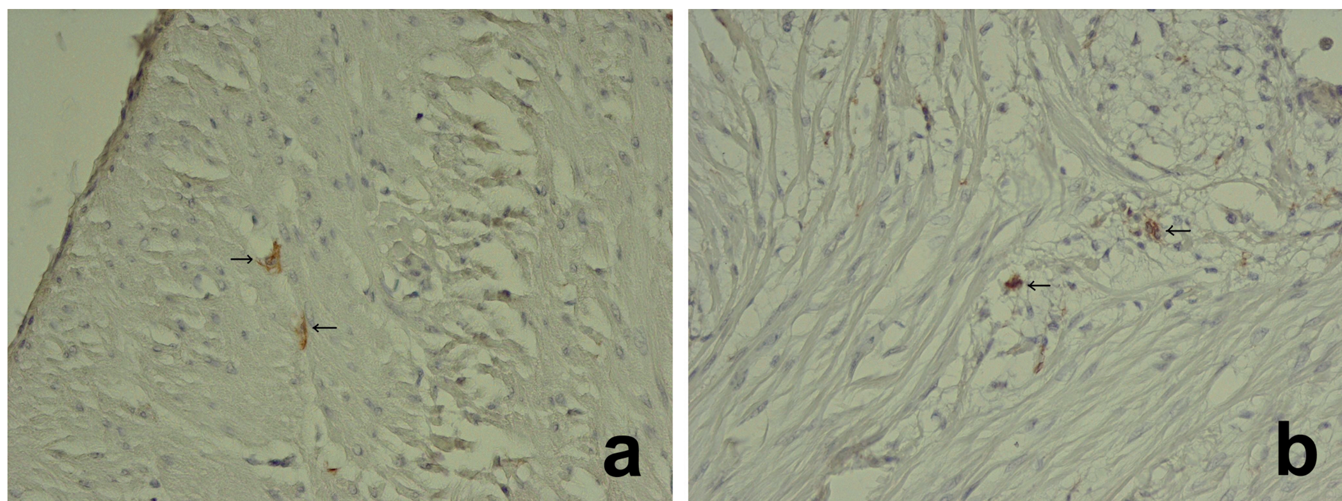
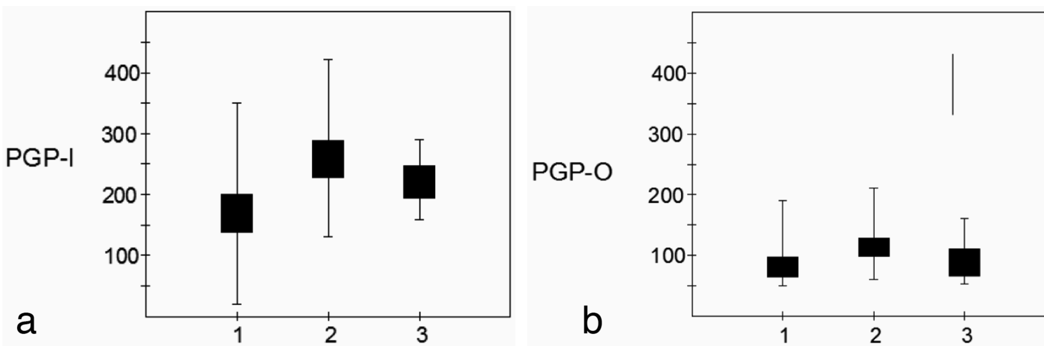


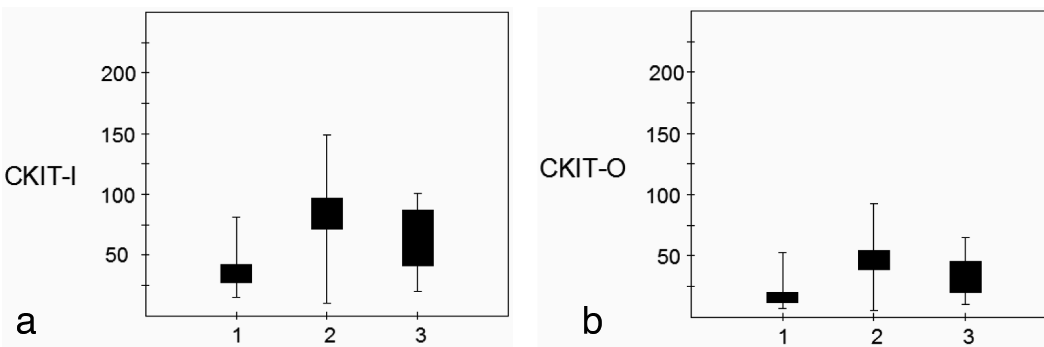
Fig. 2. Sections stained with c-kit. **a.** Two c-kit positive Cajal cells in the muscular layer are shown by the arrows. **b.** Nerve Plexus shows two c-kit positive Cajal cells which are marked b y arrows. x 400

-c-kit: The number of ICCs, stained by c-kit in HD, IND B and control group in the inner circular layer was 15-81 (mean=34.81), 10-49 (mean=84.02) and 20-101 (mean= 64.41) respectively (Graph 2a). The difference between HD and the two other groups was statistically significant (P value=0.003, 0.004) respectively, but the difference between IND B and control group was not significant (P value=0.054). The number of ICCs in the outer longitudinal layer of HD, IND B and control group was 7-53 (mean=16.24), 5-93(mean=46.27), and 10-65 (mean=32.58) respectively (Graph 2b). A statistically significant reduction in the number of ICCs was present in the HD compared with the two other groups (P value= 0.005). The difference between the average numbers of ICCs in the myenteric plexus of the three groups was not statistically significant.

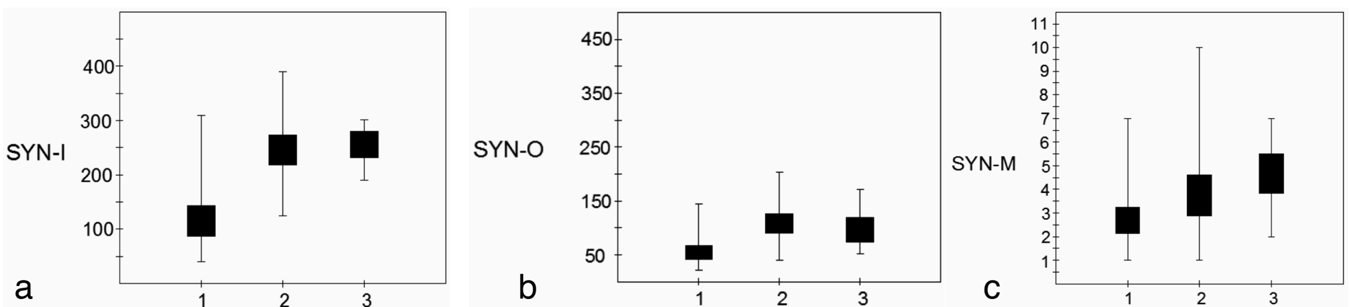
-Synaptophysin: The number of synaptophysin positive cells of the enteric nervous system in the inner circular layer in HD, IND B and control group was 41-310 (mean=115.27), 125-390 (mean=246.58), and 19-302 (mean=256.58). A statistically significant reduction in the number of these cells was present in HD in



Graph 1. a. Distribution of PGP 9.5 positive cells in inner circular layer in three groups (1: HD, 2: IND B, 3: Normal). **b.** Distribution of PGP 9.5 positive cells in outer longitudinal layer (1: HD, 2: IND B, 3: Normal).



Graph 2. a. Distribution of ICC positive cells in inner circular layer (1: HD, 2: IND B, 3: Normal). **b.** Distribution of ICC positive cells in outer longitudinal layer (1: HD, 2: IND B, 3: Normal).



Graph 3. a. Distribution of Synaptophysin positive cells in inner circular layer in three groups (1: HD, 2: IND B, 3: Normal). **b.** Distribution of Synaptophysin positive cells in outer longitudinal layer in three groups (1: HD, 2: IND B, 3: Normal). **c.** Distribution of Synaptophysin positive cells in myenteric plexus in three groups (1: HD, 2: IND B, 3: Normal).

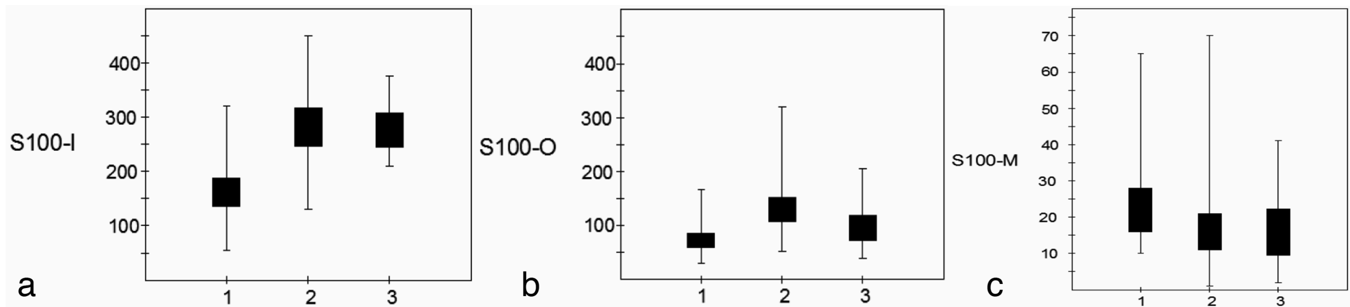
comparison with IND B (P value=0.000), but there was no significant difference between IND B and control group (P value= 0.671) (Graph 3a).

The number of synaptophysin positive cells in the outer longitudinal layer in HD, IND B and control group was 21-145 (mean=53.75), 40-203 (mean=106.68), and 51-171(mean=96) respectively (Graph-3b). A statistically significant reduction was present in HD compared with IND B (P value=0.000) and control group (P value= 0.002).This result showed no significant difference between IND B and control group (P value=0.378).

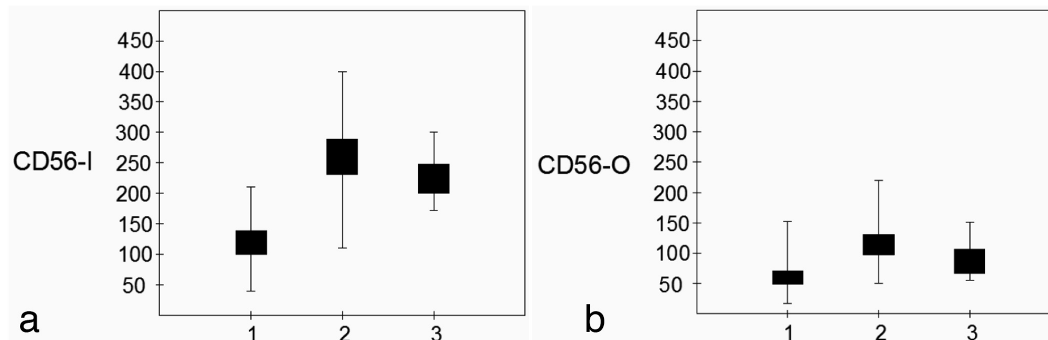
The number of the above- mentioned cells in the myenteric plexus was 1-7 (mean=2.68), 1-10 (mean=3.75) and 2-7 (mean=4.66) in HD, IND B and control group respectively (Graph 3c). This number showed a significant reduction in HD compared with the two other groups (P value= 0.030 for IND B and 0.003 for control group). The results showed no difference between control group and IND B (P value=0.155).

-S100: The number of S100 positive enteric nervous cells in the inner circular layer in HD, IND B and the control groups was 55-320 (mean=160.86), 131-450 (mean=281.13), and 210-375 (mean=275.58) respectively (Graph 4a). The number of the above-mentioned cells in the outer longitudinal layer in HD, IND and the controls was 30-167 (mean 72.51), 52-320 (mean=129.34) and 40-205 (mean=117.58) respectively (Graph 4b). There was a significant difference between HD and the other two groups (P value= 0.006 and 0.000 respectively), but no difference between IND B and normal cases (P value= 0.465). S100 positive cells in the myenteric plexus in the three groups of HD, IND B and the control groups were 10-65 (mean=22.10), 1-70 (mean=16.37), and 2-41 (mean=15.91). There was no significant difference between these three groups (Graph 4c).

-CD 56: The number of CD 56 positive cells of the enteric nervous system in the inner circular layer in HD, IND B and control group was 40-210 (mean=119.75), 110-400 (mean=260-37), and 172-300 (mean=228.83) respectively (Graph-5a). The CD56 positive cells were significantly lower in the HD in comparison with the other two groups (P value= 0.000 for both groups). This number was not different in the IND B and control groups (P value= 0.086). The above-mentioned cells in the outer longitudinal layer in HD, IND B and the control groups were 17-152 (mean= 60.37), 50-220 (mean= 118.89), and 55-151 (mean= 86.50) respectively (Graph 5b). This showed a significantly lower number in



Graph 4. a. Distribution of S100 positive cells in inner circular layer in three groups (1: HD, 2: IND B, 3: Normal). **b.** Distribution of S100 positive cells in outer longitudinal layer in three groups (1: HD, 2: IND B, 3: Normal). **c.** Distribution of S100 positive cells in myenteric plexus in three groups (1: HD, 2: IND B, 3: Normal).



Graph 5. a. Distribution of CD56 positive cells in inner circular layer in three groups (1: HD, 2: IND B, 3: Normal). **b.** Distribution of CD56 positive cells in outer longitudinal layer in three groups (1: HD, 2: IND B, 3: Normal).

HD in comparison with the two other groups (P value= 0.000 and 0.039 respectively).

Discussion

The diagnosis of HD is based on a combination of clinical features, radiologic appearances and histologic findings (Guinard-samuel et al., 2009). Histologic examination of colorectal specimens for the presence or absence of ganglion cells remains the standard method for the diagnosis of HD and forms the basis for the decision of surgical treatment. In children with chronic constipation, the major task of the pathologist is to differentiate between HD and other motility disorders, especially IND B (Park et al., 2005).

The difficulty in identifying neonatal ganglion cells by morphological examination is well known, which can result in inadequate resection of intestinal segments or resection of unnecessary long segments of intestine (Martuciello et al., 2007).

In previous studies, IHC has been used for different markers, such as synaptophysin, BCl2, calretinin, Pten, and etc. (Guinard-samuel 2009; O'Donnell and Puri, 2011). However, there is no consistent finding in previous studies and controversial issues in the diagnosis of HD versus other motility disorders using IHC markers still remain.

One of the most important IHC markers is c-kit for ICCs (pace maker cells), which mediate input from enteric motor nerves to the muscle cells and generate slow waves (Wester et al., 1999; Newman et al., 2003). It has been claimed that c-kit can be a good marker for the differential diagnosis of HD and IND B, even intraoperatively by frozen section (Piotrowska et al., 2003).

In our study c-kit positive ICCs were significantly reduced in both circular and longitudinal muscle layers of patients with HD, but in IND B the number of these cells was very similar to colectomies from nonmotility disorders.

Also, 4 IHC markers were used to stain different kinds of neurons, such as PGP 9.5, S100, CD56 and synaptophysin. The former is a good marker for enteric nervous system, and was significantly lower in HD in comparison to both normal and IND B. On the other hand, this marker was quite similar in the cases with IND B and normal colectomies with no motility disorder. This marker has rarely been used in previous studies (Geramizadeh et al., 2009).

Synaptophysin can mark the neuronal synaptic vesicle membrane, responsible for neurotransmission, which is absolutely essential for normal bowel motility. (Bettolli et al., 2006).

In our study there was a change in the distribution of synaptophysin positive synapses in two layers of muscularis properia i.e., there was marked reduction of synaptophysin positive cells in the affected colon of HD compared to IND B and normal colectomies of patients with no motility disorder. This finding was also true for S100, which is a specific marker for evaluation of neuromuscular innervations (Park et al., 2005).

Some studies have shown that the distribution and numbers of c-kit positive Cajal cells are related to the presence of neurons, so the lack of enteric cells can have an influential effect on the differentiation and development of interstitial cells of Cajal (Taguchi et al., 2003; Wang et al., 2009).

CD 56 was also used as a marker of neuromuscular junction (NCAM), which is considered to be important for the interaction of neurons with muscle cells during synaptogenesis (Kobayashi et al., 2003; Dzienis-Koronkiewicz et al., 2005).

In our patients the number of CD 56 positive cells in both layers of the muscularis properia was significantly decreased in HD in comparison with the two other groups. This shows that the disturbances in HD may be basically abnormal intestinal innervation.

In conclusion, our results suggest that HD is a true neuropathy and completely different from IND B and normal colon. The immunohistochemical study of IND B showed that the disease is neurologically quite similar to normal colon. Further multicenter studies can identify a valuable cut-off number for differential diagnosis of HD and IND by immunohistochemistry.

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