

# Histopathological Evaluation of Pediatric Intestinal Pseudo-Obstruction: Quantitative Morphometric Analysis of Pathological Changes in the Enteric Nervous System

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**Background :** This study was done to obtain comprehensive data on changes in the structural components of the enteric nervous system in pediatric patients with intestinal pseudo-obstruction (IPO). We evaluated routinely processed, in formalin-fixed tissues by quantitative morphometric analysis. In addition, we used formalin-fixed tissue to explore the possibility of using previously proposed diagnostic criteria to evaluate frozen serial sections for intestinal neuronal dysplasia (IND) type B and hypoganglionosis. **Methods :** We analyzed data for 19 IPO cases. Morphometric analysis for quantification of ganglia and ganglion cells (GCs) was done for the myenteric and the submucous plexus. In addition, we determined the presence of immature GCs and the distribution of nerve fibers and interstitial cells of Cajal (ICC). **Results :** Nine patients showed combined hypoganglionosis, IND, and decreased ICC; others showed various combinations of these. Several morphometric factors were significantly different between patient groups as well as being different than the control group. **Conclusions :** Our pediatric IPO cases showed extensive overlapping of pathological findings. And the findings suggest the utility of using previously proposed morphometrically measured factors in multiple frozen sections as diagnostic criteria for IND type B and hypoganglionosis in formalin-fixed tissue.

**Key Words :** Intestinal pseudo-obstruction; Morphometric analysis; Quantitative

Intestinal pseudo-obstruction (IPO) is characterized by loss of contractility or normal slow waves of the intestinal wall, and has been classified into neuropathic, myopathic, and idiopathic types according to the causative pathological changes. The neuropathic type is associated with various abnormalities of the enteric nervous system (ENS) and has been subclassified as aganglionosis (Hirschsprung's disease, HD), intestinal neuronal dysplasia (IND) types A and B, hypoganglionosis, immaturity of ganglion cells (GCs, nerve cells) and internal anal sphincter neurogenic achalasia.<sup>1-12</sup> In addition to abnormal findings for GCs, previous studies have documented an association between IPOs and defective innervations of the neuromuscular junction (NMJ) or abnormal distribution of c-kit-positive intestinal pacemaker cells (interstitial cells of Cajal, ICC).<sup>13-16</sup>

The proper interpretation of pathological changes in the ENS

(intestinal dysganglionosis) is very important for diagnosis and treatment. Compared with a diagnosis of HD based on the absence of GCs, the diagnosis of other neuropathic diseases including IND and hypoganglionosis has often been controversial due to the lack of definitive diagnostic criteria or the lack of exact reference values based on clearly defined evaluation techniques. In addition, although many pathologists still effectively use hematoxylin and eosin (H&E) staining for the diagnosis of these diseases, most of the well established diagnostic criteria used currently are based on multiple serial sections (more than 30 sections) of fresh frozen tissue with enzymatic histochemistry and immunohistochemical studies, which in practice are hard to perform as routine procedures in most surgical pathology laboratories.<sup>17-27</sup>

Ever since the first description of IND by Meier-Ruge,<sup>8</sup> there

have been continuous controversies on the existence of this entity as a distinct histopathologic entity. The characteristic histologic features of IND type B include hyperganglionosis of the submucous and myenteric plexuses, giant ganglia, hypogenetic GCs, heterotopic GCs, and increased acetylcholinesterase (AChE) activity in the lamina propria and around submucosal blood vessels, as well as lactate dehydrogenase and succinate dehydrogenase positive reactions in the submucous plexus.<sup>1-4,7,9</sup> In addition to many slightly different diagnostic criteria, previous morphometric studies have defined the diagnostic criterion of more than four giant ganglia (more than seven or eight GCs per ganglion) in whole submucous plexus if the specimen was not large (less than several centimeter length) or more than 20% of submucosal ganglia showing giant ones per 30 serial sections counted.<sup>1,4-7,10,17</sup> Hypoganglionosis is an entity with a diminished number of ganglia and GCs in the myenteric plexus, but its existence as a separate clinical disease has been questioned.<sup>2,5,12-14</sup> Since knowledge of GC density in the normal human ENS is scanty, and since previous reports have shown huge variations in normal density of GCs in myenteric plexus, the diagnosis of hypoganglionosis or hyperganglionosis in myenteric plexus is very difficult. The previously reported density variation of more than 200 fold among different studies could be due to the difference in technical procedures, because the whole-mount preparation technique produces a 3-dimensional picture showing the complex neuronal networks much better than conventional thin sections. In addition, the number of GCs is variable depending on the thickness of the sections as well as on the stain used.<sup>2,13,17,21-25</sup>

Our study was done to identify underlying pathology in the ENS (number and size of myenteric and submucosal ganglia, number and maturation of GCs, nerve fibers in the intestinal wall, and distribution of ICC) as well as to identify a possible association of myopathy in pediatric patients with IPO. In addition, we evaluated the effectiveness of presently known diagnostic criteria and non-obligatory, possibly diagnostic histopathological findings for identifying IND and hypoganglionosis (which

were proposed to be used in serial frozen sections) in routinely processed formalin-fixed surgical specimens by quantitative morphometric analysis using an image analyzer.

## MATERIALS AND METHODS

### Patients

We included in this study 19 pediatric patients with clinical symptoms of IPO who underwent surgical treatment. The patient group consisted of nine male and ten female cases. Seventeen cases with known gestational age (GA) consisted of nine cases of preterm (less than 37 weeks GA; range, 26.5 to 36.2 weeks; mean,  $33.06 \pm 3.43$ ) and eight cases of term (more than 37 weeks GA; range, 37.2 to 42 weeks; mean,  $39.10 \pm 1.77$ ). As age-matched controls, seven sections of normoganglionic segments of colon and one section of small intestine resected from patients with HD were examined. The diagnosis of HD was confirmed pathologically by the presence of an aganglionic segment. The eight cases in the control group consisted of seven male cases and one female case. Clinical features of patients and age-matched control cases are summarized in Table 1.

The patients presented with symptoms and signs of IPO such as distended abdomen, decreased bowel sound, and vomiting. In addition to gastrointestinal problems, five preterm patients (GA, 26.5 to 34.3 weeks) were associated with hyaline membrane disease and bronchopulmonary dysplasia. Two of the five had persistent ductus arteriosus. Ten of the 19 patients underwent segmental resection such as small bowel segmental resection, colon segmental resection, and right hemicolectomy. Four underwent Duhamel's or a modified Duhamel's operation accompanied by colostomy, ileostomy, or segmental resection. Transient stoma formation such as ileostomy, colostomy, and double barrel ileostomy-colostomy were selectively done in the remaining six cases. Two preterm babies expired with disseminated

**Table 1.** Clinical characteristics of patient groups and the age-matched control group

	n (M : F)	Gestational age (wk)	Birth body weight (kg)	Age at operation (mo)
Preterm (< 37 wk)	9 (3 : 6)	$33.06 \pm 3.43$ (26.50-36.20)	$2.2 \pm 0.68$ (0.87-2.90)	$4.18 \pm 5.07$ (0.06-14.17)
Term ( $\geq$ 37 wk)	8 (5 : 3)	$39.10 \pm 1.77$ (37.2-42.0)	$3.68 \pm 0.53$ (3.10-4.70)	$5.36 \pm 2.50$ (0.13-9.00)
Unknown	2 (1 : 1)		4.7 <sup>a</sup>	$6.00 \pm 0.00$
Patient (n = 19)	19 (9 : 10)		$2.71 \pm 1.01$ (0.87-4.03)	$5.12 \pm 4.06$ (0.07-14.17)
Control (n = 8)	8 (7 : 1)	$39.48 \pm 1.28$ (37.50-42.00)	$3.27 \pm 0.39$ (2.44-3.63)	$4.63 \pm 2.97$ (1.00-10.00)

Unknown, two cases with unknown gestational age.

<sup>a</sup>One patient with unknown birth weight.

M, male; F, female.

intravascular coagulation, acute respiratory distress syndrome, and shock-one after three days, the other after two months. During follow-up periods of variable duration (one month to seven years), three patients complained of frequent loose stool (4 to 5/day) or needed an enema. The others have showed good bowel habits after treatment.

### Immunohistochemical study

Twenty two sections of surgically resected small and large intestine from the 19 patients were evaluated. These included 12 cases of small intestine and 10 cases of large intestine. All specimens were fixed with 10% buffered formalin and processed by routine techniques with 2 to 3  $\mu\text{m}$  thick sectioning and H&E staining. H&E stained slides were used to select one or two representative sections of the lesion. Immunohistochemical staining was done on 3 to 8  $\mu\text{m}$  thick sections of formalin-fixed paraffin-embedded tissue by Bond™ Automated Immunohistochemistry (Vision Biosystems, Inc., Mount Waverley, Australia) and bond polymer detection system with counterstaining (Vision Biosystems, Inc.). Heat-induced epitope retrieval was carried out to facilitate staining by immersing the slides in citrate buffer (pH 6.0) and microwaving at 90°C to 100°C for 10 minutes. Sections were incubated with the primary antibodies for 60 minutes at 25°C. Commercial antibodies were obtained and used against neuronal cell adhesion molecule (NCAM, CD56; 1 : 100, monoclonal, NCL-L-CD56-1B6, Novocastra, Newcastle, UK), neuron specific enolase (NSE; 1 : 200, monoclonal, NCL-L-NSE2, Novocastra), synaptophysin (1 : 200, monoclonal, NCL-SYNAP-299, Novocastra), cathepsin D (1 : 400, monoclonal, NCL-CDm, Novocastra), c-kit (CD117; 1 : 400, monoclonal, Dako, Carpinteria, CA, USA), S-100 protein (S-100; 1 : 400, polyclonal, NCL-L-S100p, Novocastra), alpha smooth muscle actin (SMA; 1 : 400, monoclonal, NCL-SMA, Novocastra) and bcl-2 oncoprotein (bcl-2; 1 : 200, monoclonal, NCL-L-bcl-2, Novocastra). A negative antibody control was obtained by omitting the primary antibodies. Two pathologists independently reviewed the H&E and immunohistochemical stained slides and agreed on diagnosis by consensus.

### Evaluation of pathological changes

The mature GC was identified by a large cell body with dispersed chromatin and a prominent nucleolus. If only part of a cell body with the typical cytoplasm was clearly identified, it was also counted. Since the characteristic features of mature GCs

(prominent cytoplasm and nucleoli) were absent in immature GCs, they were confirmed by immunostaining with various neuronal markers (cathepsin D and bcl-2) as well as a marker for myenteric glial cells or satellite cells of the submucous plexus (S-100). A ganglion (or nerve plexus) was defined as a group of continuous nerve cell bodies that were not separated by connective tissue and appeared in aggregates of more than three GCs. The ganglia were subclassified as submucosal or myenteric according to their location. Submucosal giant ganglia were defined as aggregates of more than seven GCs.

### Evaluation of myenteric plexus

The myenteric ganglia were evaluated as follows. Two photographs of the myenteric ganglia area were taken at 100  $\times$  magnification by an Olympus BX51 microscope (Tokyo, Japan) and a ProgRes® Capture Pro 2.5 digital camera system (JENOPTIK Laser, Optik, Systeme GmbH, Jena, Germany). The area included in one photograph was 1.23 mm in length. Total numbers of ganglia and GCs were counted in two photographs and numbers of ganglia and GCs per mm were calculated (as ganglia/mm and GCs/mm, respectively). In addition, the total plexus area, ganglion length (a virtual line that connects one point of a ganglion to another point), and ganglion distance (the closest interval between two ganglia) were measured by image analyzer (JENOPTIK Laser, Optik, Systeme GmbH). The plexus area/mm ( $10^3 \times \mu\text{m}^2/\text{mm}$ ), mean ganglion area ( $10^3 \times \mu\text{m}^2$ ), mean ganglion length ( $\mu\text{m}$ ), and mean ganglion distance ( $\mu\text{m}$ ) were calculated.

### Evaluation of submucous plexus

Submucosal ganglia were evaluated as follows. In formalin-fixed paraffin embedded tissue with H&E staining and in various immunostains with neuronal markers, ten photographs of the submucosal layer were taken at 200  $\times$  magnification by an Olympus BX51 microscope and a digital camera. The area included in the ten photographs was 2.82 mm<sup>2</sup>. The number of ganglia and giant ganglia as well as the total number of GCs in ganglia were counted in all fields of the ten photographs and a mean GC number (n/ganglion), maximum GC number (n/all ganglia), and the percentage of giant ganglia in the total number of counted ganglia (%) were calculated. The presence of heterotopic GCs in the proper muscle layer, muscularis mucosa, and lamina propria were evaluated as was the presence of immature GCs, bud-like GCs and anisomorphic GCs.



### Evaluation of nerve fiber distribution and ICC

We evaluated nerve fiber distribution in each layer (outer muscle layer, inner muscle layer, muscularis mucosa, and lamina propria) for NCAM, NSE, and S-100 immunostained sections. The degree of nerve fiber distribution was recorded as follows: 0, no visible nerve fibers; 1, a few positive fibers; 2, moderate numbers of positive fibers; 3, many positive fibers.

The c-kit positive ICC was evaluated as follows. Compared with normal age-matched controls, c-kit positive reactions were evaluated in three areas: ICC-MY (myenteric, at the interphase between myenteric ganglia and adjacent muscle), ICC-IM (intramuscular, scattered in proper muscle layer), and ICC-SM (submucosal, superficial plexus on the submucosal surface of the circular muscle layer). The grading of positive reactions was recorded as follows: 0, no visible positive reaction; 1, markedly reduced numbers of positive fibers; 2, mildly reduced positive fibers; 3, normal.

### Evaluation of the muscle layer

The muscle layers (outer longitudinal layer, outer circular and inner circular layer, muscularis mucosa) were evaluated by immunostaining with SMA antibody. Compared with the positive reaction in the submucosal vascular wall, the grading of positive reactions was recorded as follows: 1, markedly reduced positive reaction; 2, mildly reduced positive reaction; 3, normal.

### Statistical analysis

Determination of statistical significance was done using SPSS ver. 12 (SPSS Inc., Chicago, IL, USA). A nonparametric two-independent samples test (Mann-Whitney U test) was used. A p-value of less than 0.05 was regarded as statistically significant.

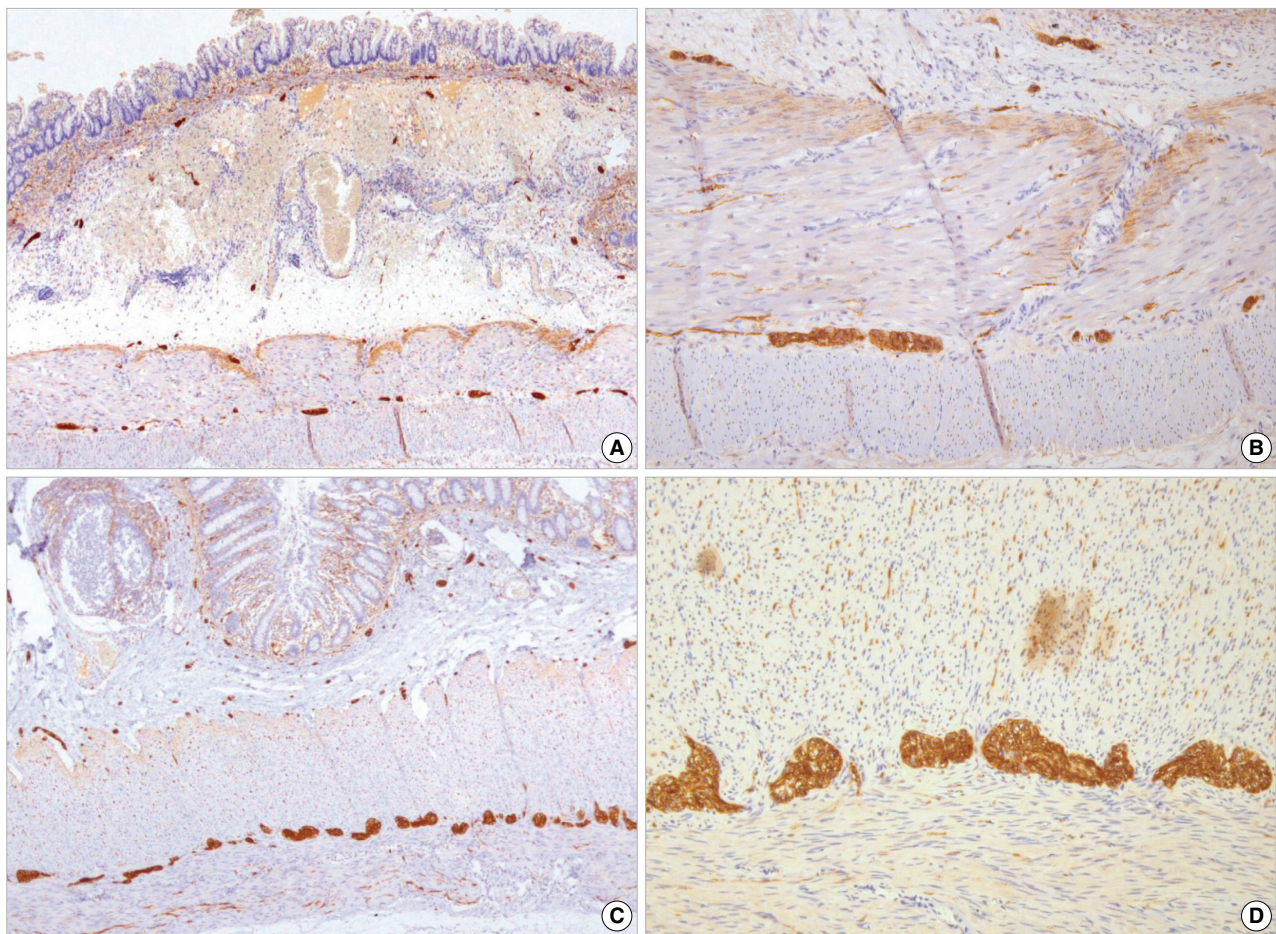


Fig. 1. Hypoganglionosis of myenteric plexus. Lower and high magnification (A, B) of a hypoganglionosis case (3.25 nerve cells/mm) shows a markedly decreased number and size of myenteric ganglia (at nine days after birth, gestational age [GA] 31.4 wk) compared with a control case (at five months after birth, GA 39 wk) (C, D) (immunostaining with neuronal cell adhesion molecule antibody).

## RESULTS

### Analysis of myenteric plexus and evaluation of diagnostic criteria for hypoganglionosis

The number, size (area), and distribution of myenteric ganglia as well as the size, maturation, and numbers of GCs in myenteric ganglia were clearly identified by H&E staining and immunostaining with various antibodies (Fig. 1). With previously used diagnostic criterion of hypoganglionosis (less than 10 GCs/mm in myenteric plexus), 12 of 22 sections (54.6%) were compatible with hypoganglionosis; findings were compared with the results from other analysis factors (Table 2). The difference in GCs/mm between the two patient groups (hypoganglionosis group and non-hypoganglionosis group) was statistically significant. The evaluation of various other diagnostic criteria for hypoganglionosis showed significant differences in plexus area/mm and mean inter-ganglionic distance of myenteric plexus between the two patient groups as well as between the hypoganglionosis group and the control group (all eight sections of control cases) (Table 2). Mean ganglion area showed a remarkable but not a significant difference between the two patient groups ( $p = 0.056$ ) while there was a significant difference between the hypoganglionosis group and the control group ( $p = 0.039$ ).

When 10 sections of colon from patients were compared with controls (seven sections of colon), plexus area/mm showed a significant difference between the two patient groups ( $15.37 \pm 2.49$  vs  $20.35 \pm 27.35$ ,  $p = 0.038$ ) as well as between the hypoganglionosis group and the control group ( $15.37 \pm 2.49$  vs  $25.43 \pm 7.53$ ,  $p = 0.008$ ). Mean inter-ganglionic distance in the hypoganglionosis group was significantly greater than that in the control group

( $153.01 \pm 46.96$  vs  $90.77 \pm 36.07$ ,  $p = 0.035$ ), although the two patient groups were not significantly different ( $153.01 \pm 46.96$  vs  $136.74 \pm 29.54$ ,  $p = 0.914$ ). When 12 sections of small intestine from patients were compared with controls (all eight sections of control cases), plexus area/mm and mean inter-ganglionic distance of myenteric plexus were significantly different between the two patient groups ( $10.76 \pm 3.58$  vs  $19.26 \pm 6.70$ ,  $p = 0.004$  and  $207.72 \pm 65.76$  vs  $126.06 \pm 72.56$ ,  $p = 0.015$ ,

**Table 3.** Summary of pathology findings

	No. of patients (n = 19) (%)
Hypoganglionosis	10 (52.6)
+ IND + Decreased ICC	8 (80) <sup>a</sup>
+ Decreased ICC	1 (10) <sup>b</sup>
Isolated	1 (10)
IND	12 (63.2)
+ Hypo + Decreased ICC	8 (66.7) <sup>a</sup>
+ Decreased ICC	3 (25)
Isolated	1 (8.3) <sup>c</sup>
Decreased ICC	15 (78.9)
+ Hypo + IND	8 (53.3) <sup>a</sup>
+ Hypo	1 (6.7) <sup>b</sup>
+ IND	3 (20)
Isolated	3 (20)
Immature GCs	13 (68.4)
+ Hypo + IND + Decreased ICC	4 (30.7)
+ IND + Decreased ICC	2 (15.4)
+ Hypo	1 (7.7)
+ IND	1 (7.7) <sup>c</sup>
+ Decreased ICC	3 (23.1)
Isolated	2 (15.4)

<sup>a</sup>Including one case with two sections showing same findings; <sup>b</sup>Including one case with two sections showing partly different findings; <sup>c</sup>One patient with two consecutive biopsies showing immature GCs and IND on first biopsy and nonspecific findings on second biopsy. IND, intestinal neuronal dysplasia; ICC, interstitial cells of Cajal; Hypo, hypoganglionosis.

**Table 2.** Quantitative morphometric analysis of myenteric plexus in patients with intestinal pseudo-obstruction according to the number of ganglion cells in myenteric ganglia

No. of myenteric plexus GCs	< 10/mm (n = 12)	≥ 10/mm (n = 10)	p-value <sup>a</sup>	Control (n = 8)	p-value <sup>b</sup>
Age at operation (mo)	5.03 ± 3.82 (0.06-11.00)	5.24 ± 4.53 (0.10-14.16)	0.872	5.24 ± 4.54 (0.10-14.17)	0.851
Ganglia/mm (n)	3.15 ± 0.65 (2.03-4.47)	3.86 ± 0.99 (1.62-8.13)	0.722	3.86 ± 1.21 (1.63-5.69)	0.070
GCs/mm (n)	6.57 ± 1.79 (3.25-8.95)	14.31 ± 3.05 (10.98-20.74)	0.000	14.08 ± 2.40 (10.17-17.89)	0.000
Plexus area (10 <sup>3</sup> × μm <sup>2</sup> )/mm	13.08 ± 3.78 (4.74-18.37)	19.69 ± 2.71 (13.98-23.80)	0.000	26.81 ± 8.00 (18.02-36.21)	0.001
Mean ganglion area (10 <sup>3</sup> × μm <sup>2</sup> )	4.16 ± 1.09 (2.33-5.66)	6.13 ± 2.65 (2.33-11.67)	0.056	8.33 ± 5.44 (3.17-19.68)	0.039
Mean ganglion length (μm)	148.42 ± 37.46 (92.17-205.24)	154.77 ± 88.43 (57.38-333.06)	0.742	183.95 ± 116.14 (84.19-435.59)	0.851
Mean ganglion distance (μm)	180.37 ± 65.52 (107.18-312.80)	130.33 ± 24.44 (103.16-168.03)	0.041	93.72 ± 34.42 (51.12-151.81)	0.001

<sup>a</sup>< 10 vs ≥ 10 nerve cells/mm group; <sup>b</sup>< 10 nerve cells/mm group vs control group. GC, ganglion cell.



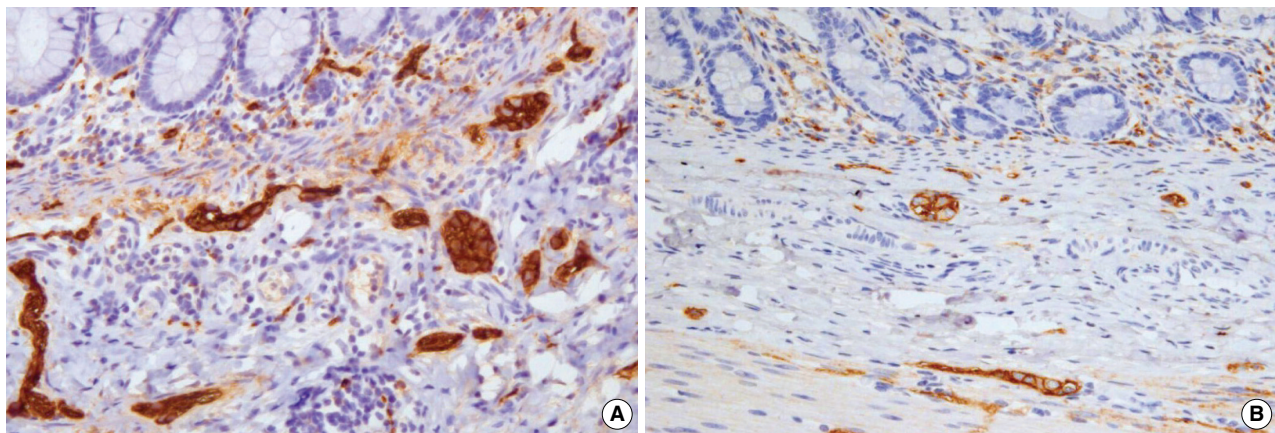
respectively) as well as between the hypoganglionosis group and the control group ( $10.76 \pm 3.58$  vs  $26.81 \pm 8.00$ ,  $p = 0.001$  and  $207.72 \pm 65.76$  vs  $93.72 \pm 34.42$ ,  $p = 0.005$ , respectively). Mean ganglion area in the hypoganglionosis group was significantly different from the control group ( $3.36 \pm 0.72$  vs  $8.33 \pm 5.44$ ,  $p = 0.013$ ), although the two patient groups were not significantly different ( $3.36 \pm 0.72$  vs  $5.13 \pm 2.13$ ,  $p = 0.310$ ).

Nine of 12 sections with hypoganglionosis (75%) also showed obligatory criteria of IND (more than 20% of submucosal ganglia were giant ones) as well as a decreased number of ICC compared with age-matched control sections (Table 3). An additional two sections (16.7%) also showed decreased ICC and only one section (8.3%) of 12 showed isolated hypoganglionosis.

#### Analysis of the submucous plexus and evaluation of diagnostic criteria for IND

The number of submucosal ganglia as well as number, size,

and maturation of GCs in submucosal ganglia were clearly identified by H&E staining and immunostaining with various antibodies (Fig. 2). When previously known obligatory diagnostic criterion of IND was used (more than 20% of ganglia in the submucous plexus are giant ones because all the sections were larger than several centimeter in length), 13 of 22 sections (59.1%) were compatible with IND. These findings were compared with results from evaluation of various diagnostic criteria for IND (Table 4). Statistical analysis showed a significant difference between the two patient groups (IND and non-IND groups). In addition, number of giant ganglia, mean GC number, and maximum GC number also showed significant differences between the two patient groups as well as between the IND group and the control group (six sections of colon and one section of small intestine). Total GC number showed a remarkable but not a significant difference between the two patient groups ( $p = 0.06$ ), compared with a significant difference between the IND group and the control group ( $p = 0.030$ ).



**Fig. 2.** Giant ganglia in submucous plexus. An intestinal neuronal dysplasia case with markedly increased numbers of giant ganglia (36.4%) (at six months three weeks after birth, gestational age [GA] 37.5 wk) (A) compared with a control case with 14.3% of giant ganglia (at three months after birth, GA 35.2 wk) (B) (Immunostaining with neuronal cell adhesion molecule antibody).

**Table 4.** Quantitative morphometric analysis of submucous plexus in patients with intestinal pseudo-obstruction according to the percentage of giant ganglia in relation to total number of counted ganglia

Giant ganglia/total numbers of counted ganglia (%)	≥ 20% (n = 13)	< 20% (n = 9)	p-value <sup>a</sup>	Control (n = 7)	p-value <sup>b</sup>
Age at operation (mo)	$1.69 \pm 0.49$ (1.10-2.35)	$2.04 \pm 0.59$ (1.10-3.00)	0.292	$3.86 \pm 2.19$ (1.00-7.00)	0.877
Submucosa ganglia	$24.23 \pm 9.19$ (4-14)	$21.78 \pm 5.17$ (17-29)	0.601	$20.00 \pm 5.03$ (15-29)	0.275
Number of giant ganglia	$7.77 \pm 3.00$ (4-14)	$2.89 \pm 1.27$ (1-5)	0.000	$2.57 \pm 1.13$ (1-4)	0.000
Giant ganglia/ganglia (%)	$32.51 \pm 7.05$ (24.00-47.37)	$13.03 \pm 4.12$ (5.88-17.65)	0.000	$12.95 \pm 5.20$ (5.88-20.00)	0.000
Total GC number	$141.69 \pm 48.77$ (70-254)	$107.78 \pm 37.44$ (70-190)	0.060	$96.71 \pm 23.86$ (72-143)	0.030
Mean GC number	$5.94 \pm 0.73$ (4.80-7.74)	$4.88 \pm 0.67$ (4.12-6.55)	0.002	$4.85 \pm 0.25$ (4.33-5.06)	0.002
Maximum GC number	$16.15 \pm 6.09$ (9-27)	$10.67 \pm 4.42$ (7-22)	0.004	$10.71 \pm 4.15$ (7-17)	0.037

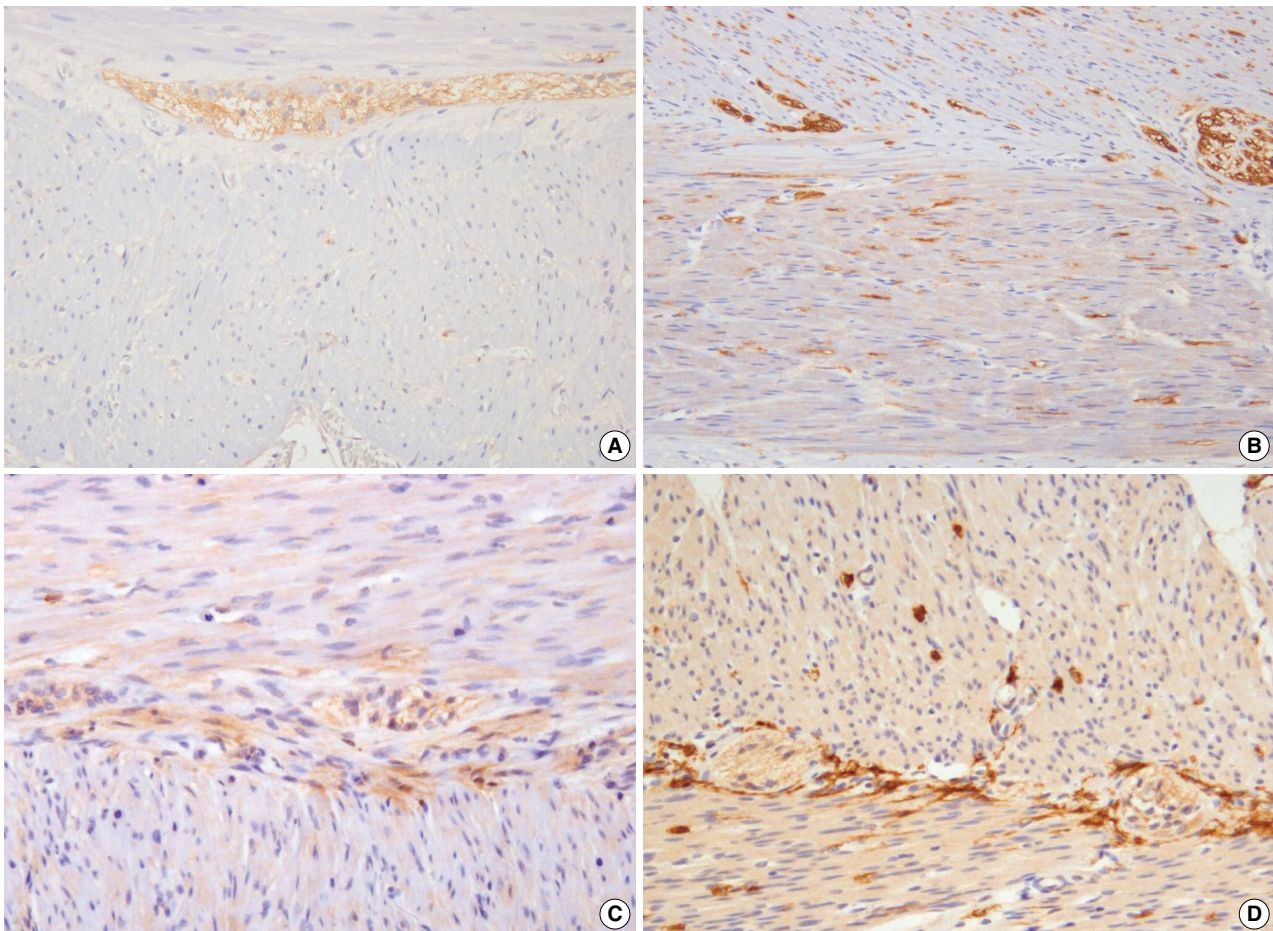
<sup>a</sup>Giant ganglia/ganglia < 20% vs ≥ 20%; <sup>b</sup>Giant ganglia/ganglia ≥ 20% vs control group. GC, ganglion cell.

When ten sections of colon from patients were compared with control (six sections of colon), number and percentage of giant ganglia, and mean GC number showed significant differences between the two patient groups as well as between the IND group and the control group ( $p < 0.01$ ). When 12 sections of small intestine from patients were compared with control (six sections of colon and one section of small intestine), the two patient groups showed a significant difference in the number and percentage of giant ganglia and maximum GC number ( $p = 0.003$ ,  $p = 0.048$ , respectively). The IND group showed a significant difference from control group in number and percentage of giant ganglia, total GC number, and mean GC number ( $p = 0.001$ ,  $p = 0.002$ , and  $p = 0.011$ , respectively).

Nine of 13 sections (69.2%) from IND cases were combined with hypoganglionosis and decreased ICC (Table 3). An additional three sections (23.1%) were associated with decreased ICC and only one section (7.7%) showed isolated IND findings.

### Analysis of other histopathological findings

Statistical analysis of the nerve fiber distribution in the outer muscle layer, inner muscle layer, muscularis mucosa, and lamina propria by NCAM immunostaining according to the numbers of myenteric GCs (hypoganglionosis vs non-hypoganglionosis groups) showed significantly decreased positive nerve fibers in the hypoganglionosis group compared with the control group only in the outer longitudinal muscle layer ( $1.2 \pm 0.42$  vs  $1.9 \pm 0.74$ ,  $p = 0.022$ ) (Fig. 3A, B). The outer longitudinal muscle layer also showed a significantly lower positive NCAM reaction in the IND group compared with the control group ( $1.23 \pm 0.44$  vs  $1.9 \pm 0.74$ ,  $p = 0.026$ ). In addition, NSE immunostaining showed a significantly lower positive reaction in the hypoganglionosis group compared with the non-hypoganglionosis group only in the lamina propria ( $1.5 \pm 0.85$  vs  $2.23 \pm 0.79$ ,  $p = 0.022$ ). Compared with NCAM and NSE, S-100 immunos-



**Fig. 3.** Expression of neuronal cell adhesion molecule (NCAM) positive nerve fibers and c-kit positive interstitial cells of Cajal-myenteric (ICC-MY). A few NCAM positive fibers (1+) of the outer muscle layer (A) compared with many positive fibers (3+) (B); Markedly decreased c-kit positive ICC-MY in the same case as Fig. 1A (C) compared with a case with a normal positive reaction (at eight months after birth, gestational age 34.3 wk) (D).

**Table 5.** Expression of NCAM and c-kit positive ICC in patient groups

	Myenteric plexus GCs (n)			Giant ganglia/Ganglia (%)		
	< 10/mm (n = 12)	≥ 10/mm (n = 10)	p-value	≥ 20% (n = 13)	< 20% (n = 9)	p-value
NCAM-OL	1.2 ± 0.42	1.63 ± 0.67	0.06	1.23 ± 0.44	1.75 ± 0.71	0.08
NCAM-IC	2.3 ± 0.48	2.73 ± 0.47	0.08	2.46 ± 0.52	2.63 ± 0.52	0.37
NCAM-MM	1.9 ± 0.74	1.64 ± 0.67	0.53	1.92 ± 0.76	1.5 ± 0.53	0.13
NCAM-LP	2.0 ± 0.67	2.36 ± 0.81	0.19	2.31 ± 0.63	2.00 ± 0.93	0.41
ICC-MY	1.33 ± 0.89	1.70 ± 1.16	0.54	1.15 ± 0.69	2.00 ± 1.22	0.14
ICC-IM (L)	1.50 ± 0.80	1.92 ± 0.99	0.31	1.54 ± 0.88	1.89 ± 0.93	0.32
ICC-IM (C)	1.50 ± 0.80	1.60 ± 0.97	0.87	1.54 ± 0.88	1.56 ± 0.88	0.90
ICC-SM	1.42 ± 0.09	1.60 ± 0.84	0.67	1.31 ± 0.75	1.78 ± 0.97	0.26

NCAM, neuron cell adhesion molecule (CD56); ICC, interstitial cells of Cajal; GC, ganglion cell; OL, outer longitudinal; IC, inner circular; MM, muscularis mucosa; LP, lamina propria; MY, myenteric; IM (L), intramuscular (longitudinal); IM (C), intramuscular (circular); SM, superficial plexus on submucosal surface of circular muscle layer.

taining did not show a significant difference in positive fibers between hypoganglionosis vs non-hypoganglionosis groups as well as between IND vs non-IND groups.

The c-kit positive ICC distribution in ICC-MY, ICC-IM, and ICC-SM showed decreased ICC in 17 of 22 sections (77.3%) (Fig. 3C, D). In addition to nine of 17 (52.9%) with hypoganglionosis and IND, three of 17 (17.6%) and two of 17 (11.8%) showed findings of IND and hypoganglionosis, respectively (Table 3). The c-kit positive ICC distributions were compared between hypoganglionosis and non-hypoganglionosis groups as well as between IND and non-IND groups (Table 5). There were no significant differences between patient groups (hypoganglionosis vs non-hypoganglionosis groups or IND vs non-IND groups).

In comparison with colon sections showing a similar positive reaction to SMA antibody in outer and inner circular muscle layers, the small intestine sections showed a weak positive reaction in the outer circular muscle layer compared with the inner circular muscle layer. The positive reactions of the muscle layers after SMA immunostaining are summarized according to hypoganglionosis vs non-hypoganglionosis groups as well as IND vs non-IND groups (Table 5). The distribution of SMA positive reactions was not different between patient groups.

The previously designated, non-obligatory criteria for IND type B such as the presence of heterotopic GCs in the proper muscle layer, muscularis mucosa, and lamina propria, bud-like GCs and anisomorphic GCs did not show a significant difference between hypoganglionosis vs non-hypoganglionosis groups or between IND vs non-IND groups. Immature GCs were noted in 13 of 22 sections (59.1%) and most of them (11 cases, 84.6%) were combined with other abnormalities (Table 3).

## DISCUSSION

Since the identification of immature GCs is very important for diagnosis of intestinal dysganglionosis in pediatric patients with IPO, the characteristic features of immature GCs should be recognized in H&E stained sections, especially to achieve an adequate intraoperative pathological evaluation of the extent of disease and avoid postoperative complications. Many previous studies identified useful antibodies for immature GCs such as antibodies against PGP 9.5, peripherin, NADPH, bcl-2, cathepsin D, glial cell line-derived neurotrophic factor, or type 1 bone morphogenetic protein receptor in coordination with antibodies specific for satellite cells of the submucosal ganglia and enteric glia of the myenteric ganglia.<sup>2,7,10,11,17,19</sup> The present study also showed well-documented immature GCs by cathepsin D and bcl-2 antibodies. For GC counting, NSE and PGP9.5 are less suitable because they stain not only the cell bodies but also the axonal processes. Recently, cuprolinic blue stain has been proposed as the method that stains the largest number of GCs compared with other antibodies.<sup>24</sup> Furthermore, it is easy to distinguish the individual cells because it stains only neuronal cell bodies and not axons.

For the correct diagnosis of intestinal dysganglionosis (IND type B, hypoganglionosis, or immaturity of GCs), many studies have been done to find the best method for evaluation of ganglia and GCs in myenteric and submucous plexus as well as for changes in GCs and for reference control data according to patient age and lesion location.<sup>20,25,26</sup> Since there are considerable differences in the number of ganglia and GCs in the ENS according to location and other factors, the location of the biopsy site as well as cutting direction and thickness of the section are important for quantitative analysis of nerve plexuses.

Two obligatory criteria (hyperplasia of the submucous plexus



and an increase in AChE-positive nerve fibers around submucosal blood vessels) and two additional criteria (neuronal heterotopias and increased AChE activity in the lamina propria) were proposed for the diagnosis of IND type B. Even though the giant ganglia and other findings have been known to be characteristic histopathological features and important diagnostic criteria for IND type B for a long period, there have been continuous suggestions and reports suggesting that those pathologic changes might be parts of normal developmental changes or secondary phenomena associated with congenital obstruction, inflammatory disease or other causes.<sup>2,3,6</sup> Recently, hyperganglionosis and giant ganglia have been accepted as the most important features for the diagnosis of IND in suction rectal biopsies, except in newborns, where hyperganglionosis is a normal finding.<sup>24,25</sup>

There have been controversies on the definition of hyperplasia of submucosal ganglia or the number of giant ganglia required for the diagnosis of IND. A recently proposed requirement was that more than 15% to 20% of all ganglia should be giant ones on 30 serial sections to fulfill the criteria for diagnosis of IND.<sup>1,4,5,7,10,17</sup> In our study, the diagnosis of IND was made using the same diagnostic criteria (more than 20% of submucosal ganglia are giant ones), which was modified in formalin-fixed thin sections. Thirteen of 22 sections (59.1%) were compatible with IND. In addition to the percentage of giant ganglia, the mean number and variability range of giant ganglia showed a distinction with no or minimal overlapping between the IND group and the control group ( $7.77 \pm 3.00$  vs  $2.57 \pm 1.13$  and 4 to 14 vs 1 to 4, respectively,  $p = 0.000$ ). In contrast, the mean and maximum GC numbers also showed a significant difference between the two patient groups as well as between IND and control groups, but there was a large range and overlapping between groups, which reduced their diagnostic significance (Table 4). These findings were similar to findings of previous studies, although the analytic procedures as well as the sites of the biopsies were different.<sup>17</sup> The difference in results of statistical analysis depending on location (colon or small intestine) of the lesion and control sections noted in this study suggests the importance of proper age- and location-matched reference values. Although the characteristic histopathological features of IND include hyperganglionosis and giant ganglia in the myenteric plexus, our study showed combined hypoganglionosis of the myenteric plexus in nine sections (69.2%). Similar findings were reported in previous studies.<sup>5,9</sup>

Hypoganglionosis has been considered to be characterized by a diminished number of ganglia and GCs in myenteric plexus. It has been reported as an isolated colon disease or as a HD-asso-

ciated malformation.<sup>7,12,15</sup> The examination of large amounts of full thickness biopsies or segmentally resected surgical specimens are mandatory for the diagnosis of hypoganglionosis, because the density and size of ganglia in myenteric plexus are variable according to intestinal luminal distension and patient age.<sup>3,5,12</sup> In addition, the proper control data for diagnosis are not available in most cases. Meier-Ruge *et al.*<sup>13</sup> proposed less than two ganglia/mm of myenteric plexus and/or less than ten GCs/mm of myenteric plexus as diagnostic criteria for hypoganglionosis.<sup>14</sup> A previous study on caudocranial coiled and cryocut specimens with HD and on proximal areas of hypoganglionosis and dysganglionic hypoganglionosis by Meier-Ruge and Brunner<sup>14</sup> showed that the most characteristic parameters of a hypoganglionosis were a decrease in mean ganglion cross-sectional area and the number of GCs per mm colon in myenteric plexus. The mean number and variability range of GCs per mm colon in dysganglionic hypoganglionosis part were significantly lower than in the normally innervated colon segment ( $7.4 \pm 2.1$  vs  $14.5 \pm 3.3$  and 5 to 9 vs 11 to 18 GCs, respectively). In addition, HD-associated hypoganglionosis of myenteric plexus was characterized by a significant decrease in mean ganglion cross-sectional area (-56.2%) and plexus area per mm colon (-53.5%) with an increase in inter-ganglionic distance (+20%).

Hypoganglionosis was diagnosed depending on reduced numbers of myenteric GCs (less than 10 GCs/mm) in this study and 12 of 22 sections (54.5%) were compatible with hypoganglionosis. The mean number and range of GCs per mm in the hypoganglionosis group did not overlap with the control group ( $6.57 \pm 1.79$  vs  $14.08 \pm 2.40$  and 3.25 to 8.95 vs 10.17 to 17.89 GCs, respectively). Although plexus area/mm and mean ganglionic distance also showed significant differences between the groups, the large range and the overlapping between groups reduced their diagnostic significance (Table 2). The hypoganglionosis of myenteric plexus could not be properly diagnosed by the numbers of ganglia in this study, which was similar to previous studies.<sup>13,14</sup> And the difference in results of statistical analysis of findings in myenteric plexus depending on location of lesion and control sections noted in this study also suggests the importance of proper age- and location-matched reference values.

Although c-kit positive ICC fibers were decreased in 17 of 22 sections (77.3%) in this study, the findings did not show a significant relationship associated with the presence or absence of hypoganglionosis or IND. Previous studies showed a loss of c-kit positive ICC in association with motility disorders of the bowel such as secondary IPO (IND, scleroderma, meconium

ileus, eosinophilic enteritis) as well as mechanical obstruction (carcinoma, Crohn's disease with stricture).<sup>3,10,16,23</sup> A case with congenital ICC hyperplasia with IND was also reported.<sup>24</sup>

Immaturity of GCs was noted in 13 of 22 sections (59.1%) and most of them (11 sections) also had other histopathological findings. Six of them were premature (GA, 27.4 to 35.6 weeks) and biopsies were done during between 3 days and 4 months after birth. Seven of them were born at 37.2 to 42 weeks GA and biopsies were done during between day 4 and month 9. Immature GCs belong to a variant of HD and are usually seen in biopsy results from premature infants presenting with IPO.<sup>3,5,6</sup> The extensive overlapping of histopathological findings of various diseases in cases with pediatric IPO suggests the possibility of a common pathogenesis of these diseases.<sup>5-7,10,22,27</sup>

The decreased NCAM positive nerve fibers of the outer muscle layer in association with hypoganglionosis as well as the IND noted in this study suggest an abnormality in the NMJ in those diseases, which was also reported in previous studies.<sup>3,22,27</sup> The significance of the localization of a decreased reaction in the outer muscle layer is uncertain in this study and further studies with more cases should be helpful. Although the significance of a decreased SMA positive reaction is uncertain, the decreased SMA positive reaction in patients with IPO has been associated with various muscle diseases.<sup>3,10,27</sup> A myopathic type of IPO showed a fatal clinical course.

In conclusion, pediatric IPO cases show extensive overlapping of pathological findings. The present study suggests the possibility of using the same analytic method for the diagnosis of IND type B and hypoganglionosis in formalin-fixed specimens. For the practical use of these methods for the diagnosis of IPO cases in formalin-fixed tissue, proper age, sex, and location-matched reference values based on clearly defined analytic techniques are mandatory.

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