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Diagnostic utility of Bcl-2 immunohistochemical expression in pediatric functional bowel obstruction cases with ganglionated specimens

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Abstract

Background: Functional disturbances of the gastrointestinal tract are caused by a number of neurodysplastic conditions, including diseases that are rarer than Hirschsprung's disease (HSCR), such as ganglion cell immaturity and intestinal neuronal dysplasia (IND). Bcl-2 shows positive immunoreactivity in degenerative and immature ganglion cells (IGCs). This work evaluates the implication of the immunohistochemical expression of Bcl-2 in cases of pediatric intestinal pseudo-obstruction (IPO) with ganglionated specimens.

This 2-year prospective observational study was conducted from January 2017 to December 2019 on all intestinal specimens for cases referred from a pediatric surgery department with clinical data suggesting IPO (abdominal distension > 3 months, intermittent bilious vomiting, no radiological evidence of the transitional zone of HSCR or evidence of mechanical obstruction). The exclusion criteria were patients with IPO related to myopathic disorders, aganglionic biopsies, and specimens with inadequate tissue blocks. The same number of intestinal specimens of cases without IPO was used as a control group. All specimens underwent a histologic examination of ganglion cells and nerve bundles and were also evaluated for Bcl-2 immunohistochemical expression.

Results: Twenty-one specimens were analyzed, including six colonic resection specimens and 15 intestinal biopsies taken by full-thickness transanal biopsy ($n = 12$) or incisional biopsy from inadequately functioning stomas ($n = 3$). The mean ± standard deviation age of the patient cohort was 22 ± 7.4 months (range, 19–153 months), and there were 13 (61.9%) male patients. Bcl-2 protein was strongly positive in 57.1% of the cases, weakly positive in 19%, and negative in 23.8% as well as in all 21 control specimens. A highly significant correlation was observed between Bcl-2 expression and ganglion cell number ($P < 0.001$), where all hyperganglionic specimens showed strong positivity compared with the hypoganglionic and adequately ganglionic specimens. A positive association was also found between Bcl-2 expression and IND compared with other disorders of dysganglionosis ($P = 0.04$) and post-HSCR cases ($P = 0.002$).

Conclusion: Bcl-2 immunohistochemistry is a valuable tool to diagnose allied disorders of HSCR through its expression in IGCs, which are difficult to identify by conventional hematoxylin and eosin staining.

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Background

Pediatric motility disorders constitute a complex array of clinicopathologic disturbances [1]. Intestinal pseudo-obstruction (IPO) is a disorder characterized by the inability of the gastrointestinal tract to propel its contents, mimicking mechanical obstruction in the absence of a lesion occluding the gut [2]. IPO may be caused by a disturbance in any of the components of the neuromuscular apparatus in the bowel [3]. It is a rare disease, with scant epidemiological data and poorly defined incidence and prevalence in both adult and pediatric populations [2].

A number of congenital neurodysplastic conditions exist that cause varying degrees of functional disturbances of the gastrointestinal tract. Some patients have symptoms similar to those of Hirschsprung's disease (HSCR) (HSCR-like conditions) despite the presence of ganglion cells in the rectum [4]. Detailed data on patients with allied disorders of Hirschsprung's disease (ADHD) is limited. In 2014, the Japanese Study Group of ADHD attempted the collection of data on all cases of ADHD from 2001 to 2010 in Japan, and the mean number of cases per institution was only 3.7 [5, 6].

Many pathological findings of the enteric nervous system have been attributed to IPO. Of them, hyperganglionosis is the most discussed and open to controversy, primarily because of its association with the diagnosis of intestinal neuronal dysplasia (IND) type B [7]. IND-B is regarded as a phenotype of relative enteric neural immaturity that can only be recognized with confidence after the age of 1 year and often spontaneously disappears by the age of 4 years [8]. It is rarely reported in adult patients [9] and is characterized by hyperplasia of the myenteric nerves accompanied by giant ganglia [10]. IND remains surrounded by controversies related to its definition, etiopathogenesis, diagnostic criteria, and therapeutic possibilities [11].

Immature ganglion cells (IGCs) are known for their relationship with intestinal motility and their impact on postoperative functional outcomes of HSCR [12]. The recognition of IGCs can be challenging, and they have a smaller, darker nucleus without a recognizable nucleolus. Special staining methods may be necessary to clarify the ganglion cell morphology and identify immature cells [13].

The B cell lymphoma/leukemia-2 gene (*BCL2*) was first identified in B cell follicular lymphomas [14] and is an important regulator of cell death [15]. It is also a much more efficacious technique to identify dysplastic (immature) ganglion cells since it was reported as specifically expressed in IGCs, with a positive immunoreactivity in degenerative ganglion cells but not in mature ganglion cells [16].

This study evaluates the implication of the immunohistochemical expression of Bcl-2 in various pediatric IPOs, including ADHD and cases of post-HSCR surgeries, to aid the diagnosis of these challenging conditions and facilitate more appropriate management.

Methods

Patient and tissue data

After obtaining the institutional ethics committee approval in November 2016, this comparative study was conducted over 2 years (from January 2017 to December 2019) on all intestinal specimens referred from the Pediatric Surgery Department for cases with clinical data suggesting IPO (abdominal distension > 3 months, intermittent bilious vomiting, and no radiological evidence of the transitional zone of Hirschsprung's disease or evidence of mechanical obstruction). The routine representative paraffin-embedded tissue blocks of the specimens of these cases received at the Pathology Department underwent sectioning and hematoxylin and eosin (H&E) staining, and Sects. 3–4-μm thick were prepared and examined. Cases of patients with IPO related to myopathic disorders (those cases were histologically identified by the presence of muscle degeneration resulting in fibrosis, cytoplasmic vacuolation, and variation in muscle fiber size [3]), as well as aganglionic biopsies and specimens with inadequate tissue blocks, were excluded from the study.

At the end of the study period (December 2019), since the number of referred IPO specimens was known, we obtained the same number of intestinal specimens from patients without IPO referred from the Pediatric Surgery Department in the same period. These specimens underwent Bcl-2 IHC staining and were used as a control group.

Ethics statement

Written informed consent was obtained from the patients' parents or guardians before colonic resection or intestinal biopsies.

Histopathological and immunohistochemical evaluation

Demographic data were collected for all cases, and all specimens underwent histopathological and immunohistochemical evaluation.

Evaluation of ganglion cell status and nerve bundle hypertrophy

Mature ganglion cells were identified by the presence of a large cell body with abundant cytoplasm and a nucleus with a prominent nucleolus, while IGCs were characterized by a smaller, darker nucleus. The giant ganglia in IND cases were defined as aggregates of > 6 ganglion cells. Hypoganglionosis was defined as ≤ 2 neurons per ganglion. Nerve bundle hypertrophy was evaluated morphometrically by assessment of nerve trunk caliber, according to Subramanian et al. [17], who considered a nerve trunk thickness > 37.85 μm as hypertrophic.

Bcl-2 immunohistochemistry (IHC) staining

For all specimens, 4–5-μm thick sections of paraffin-embedded tissue were cut on positively charged slides and subjected to Bcl-2 immunohistochemical staining. Briefly, the paraffin sections were deparaffinized in xylene and rehydrated through a graded series of alcohol and ending with dH_2O. Antigen retrieval was performed using a high-temperature treatment in citrate buffer for 5 min in a microwave oven, and the sections were then left to cool for 20 min. Next, a peroxidase-blocking solution was applied for 10 min at room temperature. Thereafter, the slides were incubated at room temperature for 2 h with the primary antibody with a prediluted ready-to-use anti-human rabbit monoclonal antibody (Roche) to detect Bcl-2 protein, followed by rinsing in phosphate-buffered saline for 2 min. This was followed by a secondary biotin-conjugated antibody for 45 min and peroxidase-conjugated streptavidin for another 45 min, both at room temperature. Finally, a substrate/chromogen (3,3′-diaminobenzidine) mixture was added for 10 min. Then, the section was counterstained in Harris hematoxylin and mounted. Negative controls were obtained by substituting the primary antibody with a nonimmune antibody. A positive Bcl-2 reaction in normal colonic mucosa and the mantle zone of lymphoid follicles served as a positive control, while negative staining of germinal centers of lymphoid follicles served as a negative control [18].

Bcl-2-positive staining was defined as cytoplasmic staining of ganglion cells. The stained tissue preparations were scored for Bcl-2 according to Wang et al. [16] as follows: 0: negative, no staining; 1+: minor staining only (< 2 ganglion cells less and/or faint yellow); 2+: moderate staining (3–5 ganglion cells and/or deep brown); and 3+: heavy staining (> 6 ganglion cells and/or dark brown). Scores of 2+ and 3+ were considered strongly positive.

Statistical analysis

SPSS v23 software (IBM Corp.) was used for all statistical analyses. A P value < 0.05 was considered significant. Quantitative data were expressed as means ± standard deviation (SD) and qualitative variables as a number and percentage. Chi-square and Fisher's exact tests were used to compare qualitative data between groups.

Results

During the study period, 38 intestinal specimens for cases with clinical data suggestive of IPO were referred to the Pathology Department. We excluded 17 cases, of which 14 were aganglionic specimens, and three cases showed pathological evidence of a myopathic disorder. Of the remaining 21 specimens, six were from colonic resections, and 15 were intestinal biopsies taken by full-thickness transanal biopsy ($n = 12$) or incisional biopsy from inadequately functioning stomas ($n = 3$). The mean ± SD age of the patient cohort was 22 ± 7.4 months (range, 19–153 months), and there were 13/21 (61.9%) male patients.

Clinically, 13/21 (61.9%) patients were well-known cases of HSCR who underwent pull-through procedures with good ganglionated proximal resection margins, 5/21 (23.8%) presented as the delayed passage of meconium and IPO manifestations, 2/21 (9.5%) presented as perforation, and 1/21 (4.8%) showed IPO manifestations following staged repair of associated high anorectal malformation.

Surgical procedures were performed on 8/21 (38.1%) patients, with 5/21 (23.8%) undergoing permanent ileostomy or colostomy, 2/21 (9.5%) undergoing partial colectomy and pull-through surgeries, and 1/21 (4.8%) undergoing total colectomy and pull-through surgeries. By the end of the study period, 2/21 (9.5%) patients had died from severe sepsis and electrolyte imbalance.

The control group of 21 intestinal specimens which were resected from 7 cases with neglected intussusception, 5 cases with a strangulated hernia, 5 cases with adhesive bowel obstruction, and 4 from intestinal biopsies resected for being related to lymphatic malformations or mesenteric cysts. The mean ± SD age for the children with specimens collected for the control group was 18 ± 4.4 months (range, 14–65 months), and 11/21 (52.4%) were males.

Pathological assessment of the specimens

Evaluation of ganglion cell status and nerve bundle hypertrophy

There were seven (33.3%) hyperganglionic, six (28.6%) hypoganglionic, and eight (38.1%) adequately ganglionic specimens (Table 1 and Fig. 1). Nerve bundle hypertrophy was identified in 12/21 (57.1%) and absent in 9/21 (42.9%) cases. Associated colitis was observed in 9/21 (42.9%) cases. IND was reported as the final diagnosis in 6/21 (28.6%) cases (Table 1 and Fig. 2). IGCs were detected in 15/21 (71.4%) cases by H&E staining (Fig. 3) and were not detected in the remainder (6/21, 28.6%) (Table 1).

Bcl-2 IHC staining

Examination of the immunohistochemically stained slides by Bcl-2 revealed that 12/21 (57.1%) cases were strongly positive for Bcl-2 protein, with six (28.6%) cases scoring 2+ and the other six cases (28.6%) scoring 3+. Meanwhile, four (19.0%) cases were weakly positive and scored 1+, and 5 (23.8%) cases were negative, scoring 0 (Table 1 and Fig. 4).

A significant correlation was detected between Bcl-2 expression and ganglion cell number, with all hyperganglionic specimens showing strong positivity compared with the hypoganglionic and adequately ganglionic specimens ($P<0.001$) (Table 2).

Table 1 Pathological data and Bcl-2 status of cases ($N=21$)

Characteristic		*N*	%
Ganglion cell number	Hypoganglionic	6	28.6%
	Adequate	8	38.1%
	Hyperganglionic	7	33.3%
Immature ganglion cells stained by H&E	Detected	15	71.4%
	Cannot be detected	6	28.6%
Nerve bundle hypertrophy	Present	12	57.1%
	Absent	9	42.9%
Colitis	Present	9	42.9%
	Absent	12	57.1%
Bcl-2 status	Strongly positive	12	57.1%
	Weakly positive	4	19.0%
	Negative	5	23.8%
Bcl-2 score	0	5	23.8%
	1+	4	19.0%
	2+	6	28.6%
	3+	6	28.6%
Final diagnosis	IND	6	28.6%
	Another type of dysganglionosis	15	71.4%

H&E, hematoxylin and eosin; *IND*, intestinal neuronal dysplasia

A positive and statistically significant association was also found between Bcl-2 expression and IND cases compared with other disorders of dysganglionosis ($P=0.04$) and between IND cases and post-HSCR cases ($P=0.002$) (Tables 3 and 4 and Fig. 5).

A significant correlation was demonstrated between ganglion cell number and nerve bundle hypertrophy, with 85.7% of the hyperganglionic specimens showing nerve bundle hypertrophy compared with 62.5% of the adequately ganglionic and 16.7% of the hypoganglionic specimens ($P=0.02$) (Table 5).

All control group specimens showed normal ganglion cells and nerve bundle patterns. Bcl-2 expression was negative in all the control specimens, with a highly statistically significant relationship between the cases and control specimens regarding Bcl-2 expression ($P<0.001$) (Table 6).

Discussion

The correct interpretation of pathological changes in the enteric nervous system (intestinal dysganglionosis) is crucial for IPO diagnosis and treatment. Compared with a diagnosis of HSCR based on the absence of ganglion cells, the diagnosis of other neuropathic diseases, including IND and hypoganglionosis, has often been controversial due to the lack of definitive diagnostic criteria [19]. The differential diagnosis of other dysganglionosis conditions that are rarer than HSCR, such as ganglion cell immaturity, whether IND or not, should be considered in neonates with signs of functional obstruction of the digestive tract.

Ganglion cell immaturity was first described by Spencer in 1966 [20]. IGCs are related to intestinal motility and impact the postoperative functional outcomes of HSCR [12]. Compared with routine H&E staining, Bcl-2 provides a much more efficacious method to identify dysplastic ganglion cells [16]. In HSCR and its allied disorders, Bcl-2 immunostaining detects the IGCs, and it is considered the most valuable biomarker for discriminating immature small neurons [10, 21, 22].

The age range of children with IPO in our study was 19–153 months. This was in agreement with Singh et al. [23], who reported an age range of 2 days to 11 years but was lower than that of Thapar et al. [2], whose study population consisted of children aged 0–18 years. In our current study, 5/8 (62.5%) patients presented with delayed passage of meconium. This was slightly similar to the study done by Mallick et al. [24], who reported 3/8 patients (37.5%) with delayed meconium passage. Meanwhile, Markiewicz-Kijewska et al. [25] reported 12/15 (80%) cases with this presentation. Our study included 2/8 (25%) cases who underwent pull-through surgery and 5/8 (62.5%) who underwent stoma formation. This was

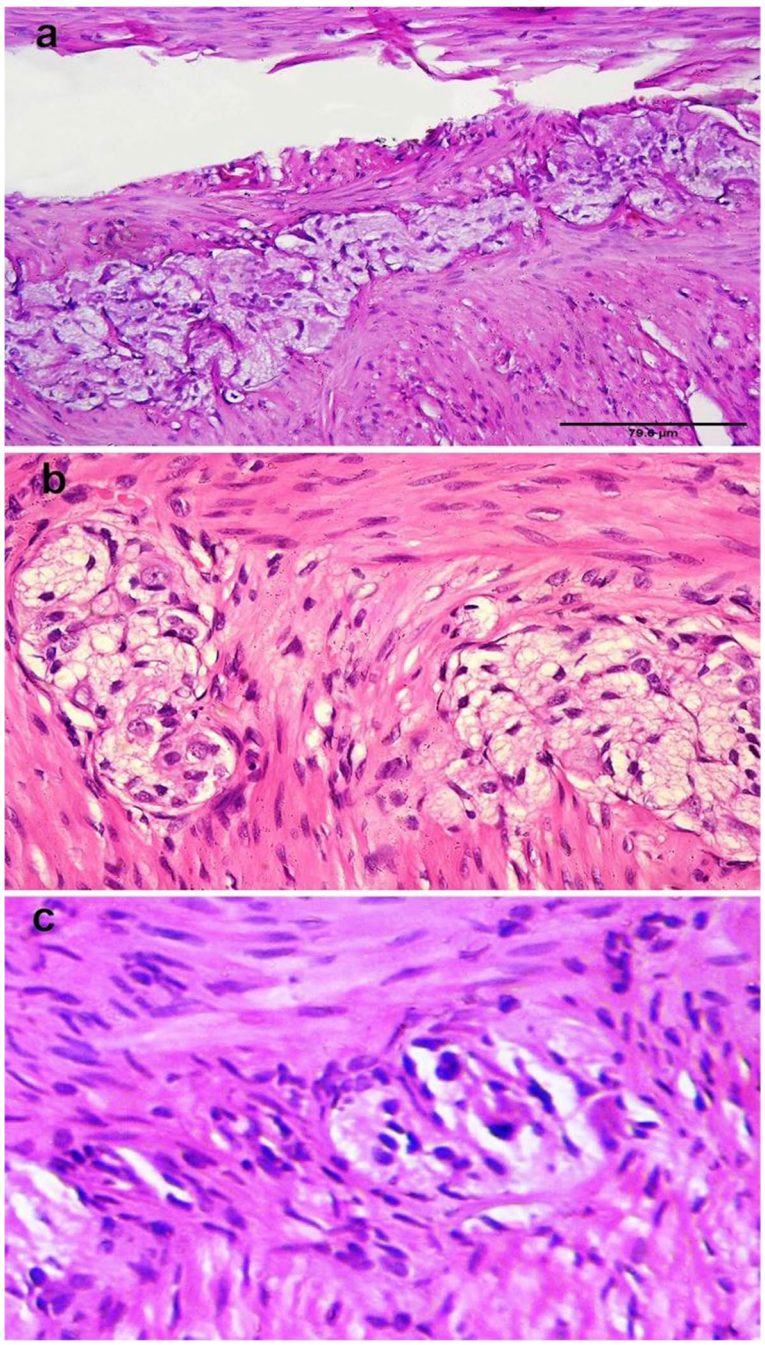

Fig. 1 Intestinal segments with different numbers of ganglion cells: hyperganglionic segment with giant ganglia displaying > 10 ganglion cells (H&E, × 200) (**a**). An adequately ganglionic specimen in a case of clinically queried Hirschsprung's Disease (H&E, × 400) (**b**). A case of hypoganglionosis with degenerated ganglion cells (H&E, × 400) (**c**)

in agreement with Kim et al. [19], whose study included 4/19 (21.1%) cases who underwent pull-through surgery, while stoma formations, such as ileostomy, colostomy, and double-barrel ileostomy-colostomy, were performed in 6/19 (31.6%) cases. Ieiri et al. [26] reported ileostomy in 69.2% of their cases.

Our studied cases were classified according to the number of ganglion cells as hyperganglionic (7/21, 33.3%), adequately ganglionic (8/21,38.1%), or hypoganglionic specimens (6/21,28.6%), one of which was associated with HSCR, another one with colitis, and a third one had combined colitis and HSCR (i.e., there were

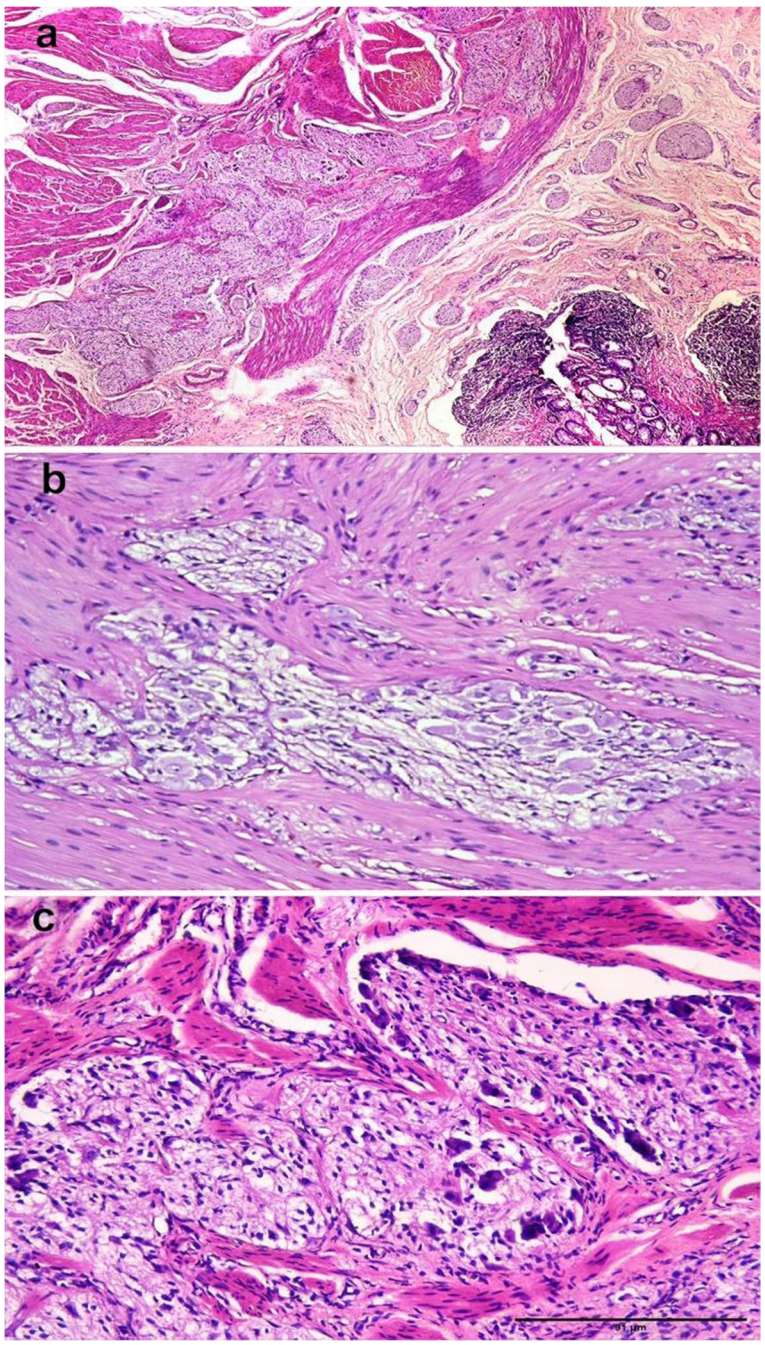

Fig. 2 Intestinal neuronal dysplasia by H&E: Giant ganglia and hypertrophied submucosal and myenteric nerve plexuses (H&E, × 40) (**a**). Higher power view showing giant ganglia with large and small neurons as well as hypertrophied myenteric nerve plexus (H&E, × 200) (**b**). Giant ganglia comprising ~ 15 ganglion cells (H&E, × 200) (**c**)

3/21 isolated cases of hypoganglionosis, constituting 14.3%). This was in near agreement with the classification of ADHD in a Japanese survey performed by Ieiri et al. [26] and the study of Wang et al. [16], which included 37 patients with hyperganglionosis and eight with hypoganglionosis. This can be explained by the fact that isolated hypoganglionosis is one of the rarest subtypes of intestinal innervation disorders, and there is only a limited number of cases in the published literature [27]. In contrast, the study done by Matsufuji [28] included 54 cases of hypoganglionosis and 15 cases of hyperganglionosis.

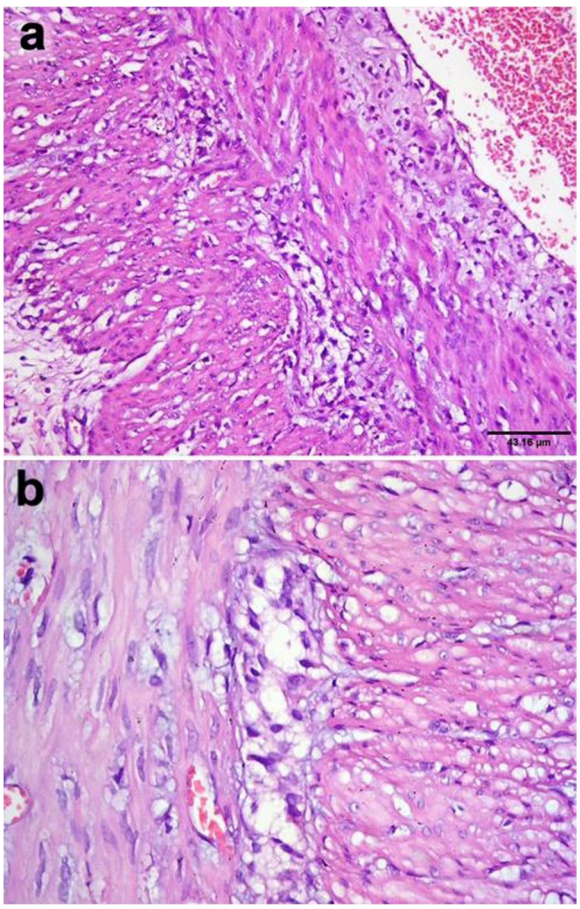

Fig. 3 A case of clinically queried Hirschsprung's disease: small immature ganglion cells and hypertrophied myenteric nerve plexus (H&E, × 200) (**a**). Higher magnification showing the myenteric plexus, including immature ganglion cells with a high nuclear-cytoplasmic ratio and inconspicuous nucleoli (H&E, × 400) (**b**)

In our study, the eight (38.1%) cases with adequately ganglionic specimens showed IGCs. This was higher than the study by Taguchi et al. [5], in which immature ganglia constituted 7.8% of cases, and lower than the study by Kim et al. [19], who reported IGCs in 13/22 cases (59.1%), with most of them also showing other histopathological findings. The variation in the reported frequency of these cases may be due to variations in the number of cases in different studies and the different approaches in diagnosing IGCs. Subramanian et al. [17] stated that the diagnosis was straightforward in biopsies showing a definitive absence of ganglion cells combined with the presence of hypertrophic nerve trunks. Similarly, the unmistakable presence of adequate ganglion cells without nerve trunk hypertrophy categorically rules out the pathological segment of HSCR. However, concerns arise when there is ambiguity in the identification of either of these parameters. The overdiagnosis of HSCR results in unwarranted surgical procedures leading to the loss of a significant portion of the colon in neonates in two conditions that mimic HSCR clinically but do not benefit from surgical resection, namely, IND-B and immaturity of ganglion cells [17]. This was in agreement with our study, which showed a statistically significant relationship ($P=0.02$) between the hyperganglionic specimens showing nerve bundle hypertrophy (85.7%) compared with the adequately ganglionic specimens (62.5%) and the hypoganglionic specimens (16.7%).

The IND incidence is estimated at approximately one in every 7500 newborns [29]. However, the frequency of isolated IND cases seems to be highly variable, with reported rates of 0.3–40% of all rectal biopsies. Some authors have found IND in up to 44% of their patients with HSCR, while others have rarely observed this combination [30]. IND was reported in six patients in our study, representing 28.6% of cases, and two of them were combined with HSCR. This was more than the cases reported by Taguchi et al. [5], whose sample included 5.1% of IND cases. Furthermore, the study done by Matsufuji et al. [28] included 15/77 (19.5%) cases of IND. In contrast, a higher frequency of cases was reported by Wang et al. [16], Park et al. [21], and Zani et al. [31], where IND cases constituted 82%, 48.8%, and 55% of their samples, respectively. The difference in the frequency of cases diagnosed as IND can be explained by the high variability of the patient ages, specimen types, and applied staining methods, which resulted in considerable confusion in the published literature regarding accurate diagnostic criteria [30].

In our study, Bcl-2 staining assisted in the identification of IGCs and helped to diagnose ADHD, where 57.1% of cases showed strong positivity, 19% were weakly positive, and 23.8% were negative. This was in agreement with Park et al. [21] and Yang et al. [12], who reported Bcl-2 positivity in 48.8% and 96.5% of cases, respectively. Also, Wang et al. [16] stated that all 45 ADHD cases in their study were positive for Bcl-2 expression. Meanwhile, Taguchi et al. [5] only reported a 7.9% rate of Bcl-2 positivity.

Our study showed a positive association between Bcl-2 protein expression and hyperganglionic specimens (100% strong positivity) in contrast with the hypoganglionic specimens (0% strong positivity and 16.7% weak positivity) and the adequately ganglionated specimens (62.5% strong positivity and 37.5% weak positivity), which was highly significantly significant ($P<0.001$).

A comparison of the cases finally diagnosed as IND with the other types of dysganglionosis (such as hypoganglionosis and immaturity of ganglia) found a positive association between Bcl-2 expression and IND, since all cases (100%) were strongly positive compared

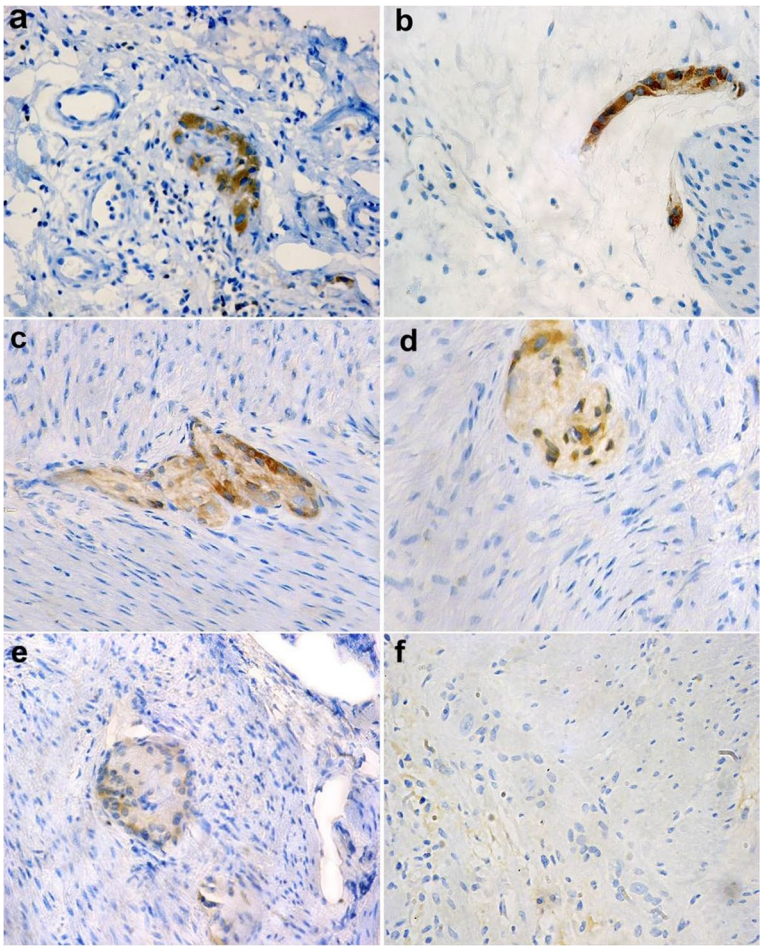

Fig. 4 Immunohistochemical expression of Bcl-2: Strong Bcl-2 positivity of > 6 ganglion cells (IHC score 3 +, × 400) (**a**, **b**). Moderate Bcl-2-positive staining of five ganglion cells with negative staining of mature ganglion cells (IHC score 2 +, × 200) (**c**). Two ganglion cells with weak staining for Bcl-2 (IHC score 1 +, × 400) (**d**). Negative Bcl-2 staining in ganglion cells (IHC score 0, × 400) (**e**). A case of intestinal adhesive loops (control) showing negative Bcl-2 expression (IHC score 0, × 400) (**f**)

Table 2 Relationship between Bcl-2 status and ganglion cell number (*N* = 21)

Ganglion cell number	Bcl-2 status						X^{2*}	*P* value
	Strongly positive		Weakly positive		Negative			
	N	%	*N*	%	*N*	%		
Hypoganglionic	0	0.0%	1	16.7%	5	83.3%	17.63	< 0.001**
Adequate	5	62.5%	3	37.5%	0	0.0%		
Hyperganglionic	7	100%	0	0.0%	0	0.0%		

* Fisher's exact test

** *P* values < 0.05 were considered statistically significant

with the other types of dysganglionosis, in which 40% showed strong positivity, 26.7% showed weak positivity, and 33.3% were negative, which was statistically significant ($P = 0.04$).

In contrast, Wester et al. [32] and Wang et al. [16] reported Bcl-2 immunostaining as valuable for the diagnosis of disorders characterized by both hypoganglionosis and hyperganglionosis. Kim et al. [19] stated that 7/12

Table 3 Relationship between Bcl-2 status and a final diagnosis of IND ($N=21$)

Final diagnosis	Bcl-2 status						X^{2*}	*P* value
	Strongly positive		Weakly positive		Negative			
	N	%	*N*	%	*N*	%		
IND	6	100%	0	0.0%	0	0.0%	5.21	0.04**
Another type of dysganglionosis	6	40.0%	4	26.7%	5	33.3%		

IND intestinal neuronal dysplasia

* Fisher exact test

** *P* values < 0.05 were considered statistically significant

Table 4 Relationship between Bcl-2 status and diagnosis ($N=11$)

Diagnosis	Bcl-2 status						X^{2*}	*P* value
	Strongly positive		Weakly positive		Negative			
	N	%	*N*	%	*N*	%		
IND	6	100%	0	0.0%	0	0.0%	10.20	**0.002****
Post-Hirschsprung's disease	0	0.0%	3	60.0%	2	40.0%		

IND intestinal neuronal dysplasia

* Fisher's Exact test

** *P* values < 0.05 were considered statistically significant

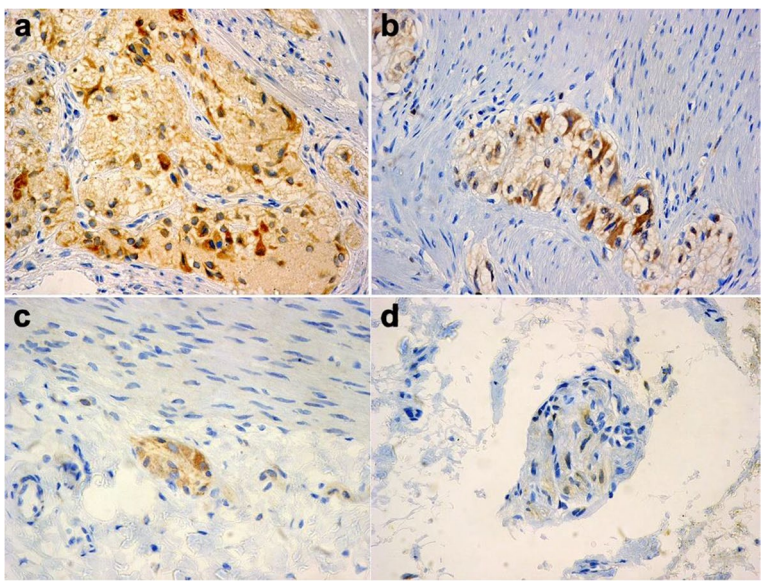

Fig. 5 Bcl-2 immunohistochemical expression in some of the studied cases: a case of IND showing strong Bcl-2 positivity in > 6 immature ganglion cells (IHC score 3 +, × 400) (**a**, **b**). A case of post-Hirschsprung's disease showing weak positivity in one ganglion cell (IHC score 1 +, × 200) (**c**). A case of post-Hirschsprung's disease showing a hypoganglionic with negative staining for Bcl-2 (IHC score 0, × 400) (**d**)

Table 5 Relationship between ganglion cell number and nerve bundle hypertrophy ($N=21$)

Nerve bundle hypertrophy	Ganglion cell number						X^{2*}	*P* value
	Hypoganglionic		Adequate		Hyperganglionic			
	N	%	*N*	%	*N*	%		
Present	1	16.7%	5	62.5%	6	85.7%	6.0	0.02**
Absent	5	83.3%	3	37.5%	1	14.3%		

* Fisher's exact test

** *P* values < 0.05 were considered statistically significant

Table 6 Comparison of Bcl-2 status between cases and controls

Group	Bcl-2 immunohistochemical expression						X^{2*}	*P* value
	Strongly positive		Weakly positive		Negative			
	N	%	*N*	%	*N*	%		
Cases	12	57.1%	4	19.0%	5	23.8%	27.54	< 0.001**
Controls	0	0.0%	0	0.0%	21	100.0%		

* Fisher's exact test

** *P* values < 0.05 were considered statistically significant

(58.3%) IND cases showed Bcl-2 positivity compared with 5/10 (50%) of hypoganglionic cases and 2/3 (66.6%) of the adequately ganglionated specimens.

In agreement with our study, Park et al. [21] reported Bcl-2 positivity in 48.8% of patients with IND and 6.3% of those with hypoganglionosis. Also, the study by Mallick et al. [24] included four IND cases, all of which showed Bcl-2 positivity. The variability in the reported percentages could be attributed to the different sample sizes of the studies as well as different ratios of the diagnoses. Furthermore, discrepancies in IHC staining methods and protocols could play a role in this variation as well as the different types of specimens and subjectivity of the interpretation of cases.

IGCs are also known for their impact on the postoperative functional outcomes of HSCR [12]. Our study included 5/21 (23.8%) post-HSCR cases, of which 60% showed weak positivity for Bcl-2, and 40% were negative for Bcl-2. In contrast, Yang et al. [12] reported that 96.5% of their patients were positive for Bcl-2 and that intestinal dysmotility was not observed in the Bcl-2-negative group. However, because there was no statistical significance between the two groups, they concluded that considering that HSCR is diagnosed at a young age and the rate of IGC is very high, and it might be inappropriate to relate IGC to a functional outcome in young patients.

Our study also compared the usefulness of Bcl-2 immunostaining in both IND and post-HSCR entities and demonstrated that IND cases showed strongly positive Bcl-2 expression in contrast to post-HSCR cases, which showed weak positivity or negative expression that was highly statistically significant ($P=0.002$).

The current study was limited by relatively small number of cases, performing acetyl cholinesterase staining which was not applicable in this study since it should be done on frozen sections, and the inclusion of different entities of intestinal pseudoobstruction; using a full panel of neuromuscular and mesenchymal labeling techniques to facilitate the diagnosis of Hirschsprung's disease and its allied disorders.

Conclusion

Bcl-2 IHC is a valuable tool to diagnose ADHD through its expression in the IGCs, which may be challenging to identify by conventional H&E staining.

Abbreviations

HSCR: Hirschsprung's disease; IND: Intestinal neuronal dysplasia; IGCS: Immature ganglion cells; IPO: Intestinal pseudo-obstruction; ADHD: Allied disorders of Hirschsprung's disease; BCL2: B cell lymphoma/leukemia-2 gene; H&E: Hematoxylin and eosin; IHC: Immunohistochemistry; SD: Standard deviation.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by LAM, ABR, and SAH. The

first draft of the manuscript was written by LAM, and all authors commented on previous versions of the manuscript. The authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
Obtained in November 2016, Ain-Shams University, Faculty of Medicine ethical committee (No. 1/11/2016). Written informed consent was obtained from the patients' parents or guardians before colonic resection or intestinal biopsies.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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