

Original Article

Utilization of RET, Bcl-2 and CR immunohistochemistry in the diagnosis of Hirschsprung disease and its allied disorders

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Received April 4, 2016; Accepted August 1, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Diagnosis of Hirschsprung disease (HSCR) is quite entirely based on the histopathological analysis of suction rectal biopsies. Identification of ganglion cells can be difficult sometimes, especially in Hirschsprung's allied disease (HAD) and in newborns. In this study, we developed a protocol using Bcl-2, RET and calretinin (CR) immunostaining to diagnosis and differential diagnosis of HSCR and HAD. Sample from a total of 168 HSCR patients, 45 HAD patients and 20 age-matched normal samples from patients with colorectal trauma or undergoing anorectal colostomy were collected from 2011 to 2015 and full-thickness tissues were obtained for evaluation. Our results showed that only Bcl-2 has the positive immunoreactivity in the degenerative and immature ganglion cells, CR showed negative immunoreactivity in the degenerative ganglion cells of myenteric plexus in the intestinal. Besides, RET showed positive immunoreactivity in both immature and mature ganglion cells. Combined immunohistochemical detection of CR and RET facilitates can be used for diagnosis and differential diagnoses of HSCR and HAD. And the specific expression of Bcl-2 and CR play an essential role in diagnosis of HAD and newborn HSCR.

Keywords: Hirschsprung disease, hirschsprung allied disorders, immunohistochemistry, diagnosis

Introduction

Hirschsprung disease (HSCR), or congenital intestinal aganglionosis, is a malformation of the enteric nervous system (ENS), with an incidence of 1 in 5000 live births [1, 2]. A properly developed mature enteric ganglia require for motility of the gastrointestinal tract. The absence of enteric ganglion cells in the myenteric and submucosal plexus leads to HSCR [3]. HSCR is a complex genetic disorder [4]. During the development of embryo, migration failure of neural crest cells (NCCs) to their correct distal intestinal position results in the loss or decrease of ENS, which leads to the function defects of gut. Furthermore, the proliferation and differentiation of NCCs are also failed result in the abnormalities in the microenvironment [5-7]. Spasmodic contraction of the abnormal intestine occurred, due to the lack of

enteric ganglion cells [8]. HSCR is considered as a kind of neurocristopathy: a disorder that presents failure to pass meconium within the first 48 hours of life, follow with constipation, abdominal distention, emesis, and occasionally diarrhea. Some patients with HSCR are at risk for enterocolitis and/or potentially lethal intestinal perforation. Generally, diagnosis involves clinical history, X-ray, barium enema, anorectal manometry and suction rectal biopsy. However, X-ray, barium enema and anorectal manometry are inaccurate in judging the properties and position of HSCR [9]. Hematoxylin and eosin (H&E) staining and acetyl cholinesterase (AChE) histochemistry in suction rectal biopsy were remained as the gold standard for diagnosis in most cases [10]. However, finding ganglion cells and proving the absence of ganglion cells is time consuming, moreover, identification of ganglion cells in patients with Hirschsprung-

associated enterocolitis or newborn can be difficult. Furthermore, AchE needs fresh frozen tissue, and there are high rates of false positive and false negative results in AchE staining [11]. In addition, Hirschsprung's allied disease (HAD) including Intestinal Neuronal Dysplasia (IND), Hypoganglionosis and Ganglioneuromatosis [12] has the similar clinical features with HSCR which is difficult to be distinguished from HSCR through AchE staining, due to the immature ganglion cells can be mixed together with inflammatory cells, mesenchymal cells, or endothelial cells [13].

For this reason, many institutions have used a plenty of new immunohistochemical markers to help identify ganglion cells, such as S-100 protein [14], peripherin [14], neurone-specific enolase [15], cathepsin D [16], ret oncoprotein [17], bcl-2 [18], and Calretinin (CR). CR now has been proved to be successful in highlighting ganglion cells which can be used as a useful and valuable method in demonstrating aganglionosis in HSCR patients [19, 20].

In this study, we evaluated the value of Bcl-2, RET and CR immunostaining as a diagnosis tool for HSCR and HAD. We retrieved 168 sporadic HSCR, 45 HAD and 20 normal samples to investigate the expression of these markers in different lesion intestinal segment of HSCR and HAD, and to explore its possible role in diagnosis and differential diagnosis of HSCR and HAD. Our results showed that only Bcl-2 has the positive immunoreactivity in the degenerative and immature ganglion cells, CR showed negative immunoreactivity in the degenerative ganglion cells of myenteric plexus in the intestinal. RET showed positive immunoreactivity in both immature and mature ganglion cells. Combining with Bcl-2, RET and CR immunostaining are useful strategy for differential diagnosis of HSCR and HAD.

Materials and methods

Ethics statement

The study was approved by the Ethical Committee Psychology (ECP) affiliated to the Chongqing Medical University, China. All guardians of participants signed an informed consent prior to the study and were debriefed after the assessment. All guardians of participants were informed that participation was voluntary

and that they had the right to refuse or stop participating in the study at any time. All of the experiments in the research were in accordance with the government policies and the approved guidelines.

Clinical cases for HSCR and HAD

This case series consisted with 168 sporadic HSCR, 45 HAD and 20 normal samples. All HSCR cases attained from 136 males and 32 females were affected with common-segment aganglionosis. Patients' age ranged from 14 days to 8 years. Nine of the patents were less than 1 month, 31.5% (53/168) of the patients were one month to three months, 51.2% (86/168) were three months to one year old and 11.9% (20/168) were more than 1 year old at presentation. HAD samples were obtained from 30 males and 15 females. Patients' age ranged from 1 year to 12 years. Thirty-seven samples were obtained from patients with Intestinal Neuronal Dysplasia (IND) and eight samples were obtained from patients with Hypoganglionosis (HG). Another 20 cases are age-match control (children died of non-digestive diseases) intestinal samples, mature ganglion cells found in the myenteric and submucosal plexus with H&E staining. Both experimental group and control group were Chinese Han in Chongqing.

Immunohistochemistry

Paraffin sections were first rehydrated, and then rehydrated sections were incubated with a 1:400 dilution of rabbit anti-human primary antibody against RET (Abcom, USA), Bcl-2 (Abcom, USA) and CR (Abcom, USA) or control IgG (1:1000) overnight at 4°C. The tissue sections were washed in PBS and then incubated with a 1:100 dilution of biotinylated secondary sheep anti-rabbit or goat anti-rabbit IgG (Jingmei BioTech, Shenzhen, China). After washing with PBS, tissue sections were incubated with an avidin-biotin complex and developed in 0.075% (w:v) 3,3 diaminobenzidine (DAB). After lightly counterstaining with haematoxylin, the sections were dehydrated.

The intensity of immunostaining around the stent struts was scored as follows: -, no staining; +, minor staining only (ganglion cells less than 2 and/or faint yellow); ++, moderate staining (ganglion cells were from 3 to 5 and/or deep

Table 1. Expression of RET, CR and bcl-2 in the myenteric plexus of HSCR and HAD

Group	RET	CR	Bcl-2
HSCR spasm segment	-	-	-
HSCR distension segment	++	+++	-
HG spasm segment	+	+/-	+
HG distension segment	++	+++	+
IND spasm segment	+++	+/-	+
IND distension segment	++	++	+

brown); and +++, heavy staining (ganglion cells more than 6 and/or dark brown). Intensities of ++ and +++ were considered strongly positive. Scoring was performed on every third strut in each vessel beginning with the strut closest to the top of the slide by an investigator blinded to the treatment allocation.

The color of positive cells were showed brown or deep brown, five views were randomly selected to count the number of ganglion cells and myenteric plexus, diameter of the ganglion cells (μm) were measured. The average area of plexus were calculated by a pathological image analysis system (YC.YX-2050, Zeiss, German).

Statistical analysis

Two-tailed Student's t-test was employed for analyzing in all data and all values were expressed as mean \pm standard deviation. Two-way ANOVA was applied to analyze comparison between groups. All analysis were performed by SPSS version 11.0 software (SPSS Inc., Chicago, USA). A threshold of $P < 0.05$ was defined as statistically significant.

Results

In the spasm segment of HSCR, the expression of CR, RET and bcl-2 were missing. In HSCR and normal intestinal tissue, CR was mainly expressed in the mature ganglion cells and nerve fibers, there was no or low expression in the immature ganglion cells. Both mature and immature ganglion cells have the expression of RET. Bcl-2 was mainly expressed in the immature ganglion cells (**Table 1**).

RET

In the control group, the expression of RET were observed mainly in mature ganglion cells (**Figure 1A**). However, in the spasm segment of

HSCR, there was a number of nerve fibers, showing strong expression of RET, but no ganglion cells were observed (**Figure 1B**). Immature ganglion cells can be found in the HG groups (**Figure 1C**). In the IND group, there was ectopic myenteric plexus with positive expression (**Figure 1D**).

Bcl-2

There were no Bcl-2 expression found in the normal control group and HSCR. In the nine newborn cases, low expression of Bcl-2 were found in the distension segment. The stained ganglion cells were small, and no staining found in the submucosal nerve fibers (**Figure 2A**) and there was no expression in the spasm segment. In all HAD 45 cases showed positive expression in ganglion cells in the spasm and distension segment. In the IND, the number and size of myenteric plexus were significantly increased, but the size of ganglion cells were small (**Figure 2B**). In the HG, the number and size of myenteric plexus were significantly decreased, and the size of ganglion cells were also small (**Figure 2C**).

CR

There were CR positive expression were found in the ganglion cells and nerve fibers in the normal control group and distension segment of HSCR (**Figure 3A**), but showed negative expression in the spasm segment of HSCR. In the 45 HAD cases of HAD, there were positive expression of CR were found in ganglion cells and nerve fibers in the distension segment (**Figure 3B**), but showed negative expression of CR in ganglion cells and no/low expression of nerve fibers in the spasm segment. The other nine newborn cases with HSCR, there were no positive expression of CR in ganglion cells and no/low expression of nerve fibers in the distension and spasm segment.

Quantitative analysis of immunohistochemical staining

Number, diameter and plexus size of RET-positive ganglion cells: Loss of neurons and glial cells was observed in the nerve plexus of HSCR lesions, and marked hyperplasia of the nerve plexus was observed, which was 3-4 times size of that of the normal group. The area of the myenteric plexus in the normal segment of HSCR was $4952.29 \mu\text{m}^2$, while the area of

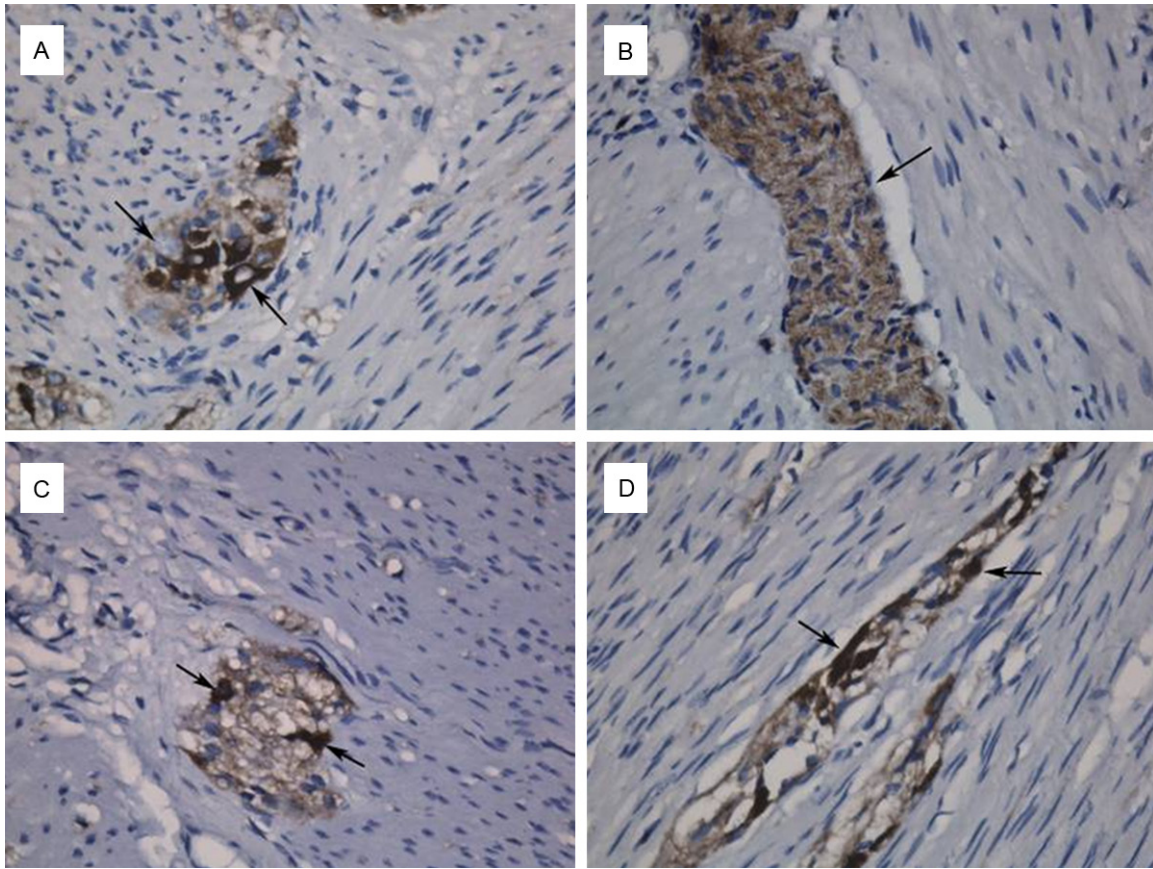


Figure 1. A. Specimen from distension segment of HSCR patient showing RET immunoreactivity ganglion cells (arrow) and nerve fibres in myenteric plexus ($\times 400$). B. Specimen from spasm segment of HSCR patient showing RET immunoreactivity nerve fibres (arrow) and absence of ganglion cells in myenteric plexus ($\times 400$). C. Specimen from spasm segment of HG patient showing RET immunoreactivity immature ganglion cells (arrow) in myenteric plexus ($\times 400$). D. Specimen from spasm segment of IND patient showing RET immunoreactivity ectopic plexus and ganglion cells (arrow, $\times 400$).

the myenteric plexus of HG was $2996.79 \mu\text{m}^2$ ($P < 0.01$), which was significantly smaller than that of the normal myenteric plexus. Additionally, the area of the myenteric plexus of IND was $7991.3 \mu\text{m}^2$ ($P < 0.01$), which was significantly greater than that of the normal myenteric plexus. Integrated ganglion cells containing a nucleus were identified and counted in histological sections. In the normal intestine, 4.25 integrated ganglion cells were observed within each myenteric plexus. Only 1.53 cells were found in HG, and in some cases there was no integrated ganglion cell at all. Moreover, 7.54 ganglion cells were observed in the myenteric plexus of IND-B lesion; this number was significantly higher than that of the normal group ($P < 0.01$) (Table 2).

Number, diameter and plexus size of Bcl2 -positive ganglion cells: There was a obvious hyper-

plasia in the spasm segment of HSCR, and the myenteric plexus area can up to 4 times larger than normal plexus, but no ganglion cell. The plexus area ($6571.33 \pm 873.98 \mu\text{m}^2$) in the normal segment of HSCR was larger than HG ($2425.87 \pm 789.45 \mu\text{m}^2$), but smaller than IND ($9241.43 \pm 1127.15 \mu\text{m}^2$). The number of ganglion cells were counted. In the normal group, there were 4.22 ganglion cells in every myenteric plexus. There were only 1.24 ganglion cells or even absence in HG group. Compared with the normal group, ganglion cells were up to 8.27 in the IND group (Table 3).

Discussion

Previous studies shown that both HSCR and HAD can be characterized by the absence of ganglion cells or ganglion cells shown abnormal morphology or the insufficient number of gan-

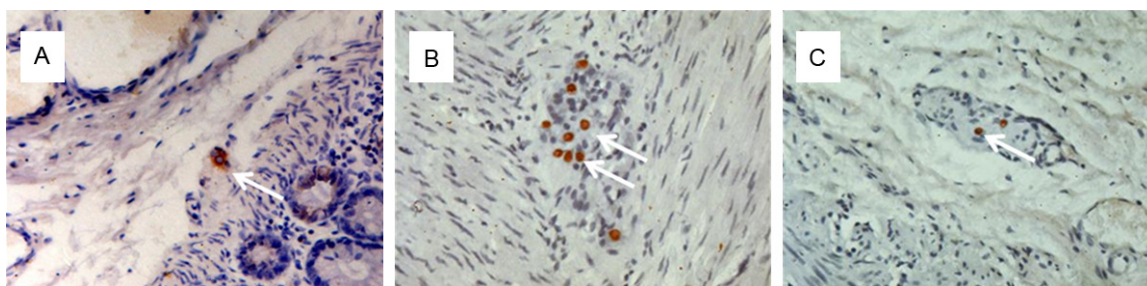


Figure 2. A. Specimen from distension segment of HSCR newborn patient showing Bcl-2 immunoreactivity small ganglion cells (arrow) in sub mucosal plexus ($\times 400$). B. Specimen from spasm segment of IND patient showing Bcl-2 immunoreactivity small ganglion cells (arrow), number and size of plexus were significantly increased in myenteric plexus ($\times 400$). C. Specimen from spasm segment of HG patient showing Bcl-2 immunoreactivity small ganglion cells (arrow), number and size of plexus were significantly decreased in myenteric plexus ($\times 400$).

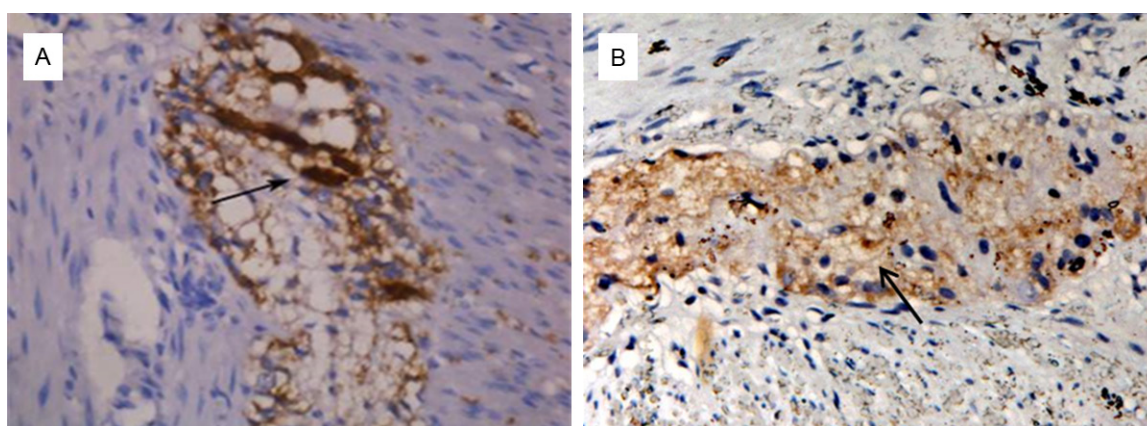


Figure 3. A. Normal expression of CR-immunoreactive ganglion cells (arrow) and nerve fibres in myenteric plexus ($\times 400$). B. Specimen from distension segment of HAD patient also showing CR-immunoreactive ganglion cells (arrow) and nerve fibres in myenteric plexus ($\times 400$).

glion cells, as well as reduced function of intestinal tract [21]. However, the morphology changes are different in HSCR and subtypes of HAD. HAD is a group of enteric nervous system dysganglionosis including Intestinal Neuronal Dysplasia (IND), Hypoganglionosis and Ganglioneuromatosis [12]. IND is characterized by a hyperplasia ganglion cells or huge ganglion [22], including increased immature ganglion cells in per unit length intestinal wall or number of ganglion cells more than 7 (normal is 2-5) in submucosal plexus and the size of plexus was three times larger than normal. Both plexus and ganglion cells were decreased in HG, and there were only 1-2 ganglion cells in myenteric plexus. In this study, we measured plexus size, diameter and number of Bcl-2 stained ganglion cells. Our data shown that, compared with normal group, in IND group, the size of Bcl-2⁺/CR⁺ cells were smaller, but the number of ganglion cells were significant increased (up to 7-12),

and the size of plexus was larger. In HG, the number of Bcl-2⁺/CR⁺ cells were significant decreased, there was only 1-2 ganglion cells and smaller plexus size. In distention segment of HSCR, the size and number of Bcl-2⁺/CR⁺ cells were slightly decreased, but the size of plexus 2-3 times larger than the control group. However, compared with Bcl-2 and CR staining, HE stained dysplastic ganglion cells have the similar morphology with Swann cells, microglial cells and inflammatory cells. Distinguishing of dysplastic ganglion requires the pathologists are familiar with normal morphology and number of ganglion cells. Bcl-2 and CR can provide us a much more efficacious way in finding dysplastic ganglion cells.

Previous study showed that Bcl-2 was specifically expressed in the dysplasia ganglion cells [18]. In this study, we proved that Bcl-2 showed positive staining in mature ganglion cells, mi-

Table 2. RET-immunoreactive ganglion cells, ganglion cell diameter, ganglion cell number and myenteric plexus size in different intestinal tissue expression ($\bar{x} \pm s$)

Group	n	Diameter (μm)	Number	Plexus size (μm^2)
IND spasm segment	26	21.02 \pm 1.85*	7.54 \pm 2.49*	7991.30 \pm 1188.19*
HG spasm segment	15	16.62 \pm 1.04#	1.53 \pm 0.64#	2996.79 \pm 1162.14#
HSCR distension segment	26	18.29 \pm 0.91	3.81 \pm 0.98	4952.29 \pm 1227.3
Normal control	20	18.87 \pm 0.99	4.25 \pm 1.16	4677.11 \pm 2533.19

The values were calculated from three independent technicians. t-test, compared with HSCR distension segment and normal control group, *P < 0.01, #P < 0.01.

Table 3. Bcl2-immunoreactive ganglion cells, ganglion cell diameter, cell number and myenteric plexus size in different intestinal tissue expression ($\bar{x} \pm s$)

Group	n	Diameter (μm)	Number	Plexus size (μm^2)
IND spasm segment	15	17.69 \pm 0.78*	8.27 \pm 0.74*	9241.43 \pm 1127.15*
HG spasm segment	4	18.89 \pm 1.25	1.24 \pm 0.67*	2425.87 \pm 789.45*
HSCR normal segment	24	18.12 \pm 0.53	3.11 \pm 0.98	6571.33 \pm 873.98
Normal control	17	21.11 \pm 1.29	4.22 \pm 1.12	4681.56 \pm 1125.98

The values were calculated from three independent technicians. t-test, compared with HSCR distension segment and normal control group, *P < 0.01.

croglial cells and inflammatory cells. However, Bcl-2 showed positive staining in immature ganglion cells in the distension segment of HSCR in the 9 newborn patients, might because in the intestinal lesion of HSCR development of ganglion cells were affected by the micro-circumstance. The other reason for the diameter decrease in dysplasia ganglion cells might be that ganglion cells mature in 2-4 weeks after birth but the age of those new born cases were less than 2 weeks. Parts of non-newborn patients also have Bcl-2 positive expression in the distension segment, which suggest not only absence of ganglion cells of spasm segment but also ganglion cells dysplasia in relatively normal intestinal. This is part of the reason of why it is hard from recovery of defecation function in the postoperation of HSCR. In the 45 HAD patients, there were Bcl-2 positive staining in dysplasia ganglion cells in the lesion intestinal. However, there were no Bcl-2 positive stained cells in part of distension segment of HAD patients, ganglion cells number and morphology were similar to the normal intestinal, due to milder patients did not affect the ganglion cells development of distention segment.

RET, a receptor tyrosine kinase, which encodes a transmembrane tyrosine kinase receptor, refers to cell growth and differentiation, is the

major susceptibility gene for HSCR [23]. Mutations in RET gene constitute the most frequently observed genetic alteration in patients with HSCR disease, including 15-20% of sporadic cases and 50% of familial cases [24]. Gath et al investigated Single strand conformational polymorphism (SSCP) of RET, showed that three RET mutations were detected in patients with HSCR, but no mutations were observed in IND [25]. In this study, we measured the ganglion cells diameter and number of ganglion cells and the size of plexus. The data shown an absence

of ganglion cells within spasm segment and/or small immature ganglion cells could be found in spasm segment of HSCR samples. However, in IND, the plexus size was 1.71 times larger than normal plexus, and cell number were increased with larger size. The incidence of HG is 3%~5% in intestinal dysganglionosis. The size of myenteric plexus is smaller and only 1-2 small ganglion cells could be found with normal morphology. Our results shown the similar results with the previous studies [12, 26]. Compared with CR, the expression of RET were much stronger in the immature and mature ganglion cells which can be used for differential diagnosis between HAD and HSCR.

Calretinin (CR) is a vitamin D dependent calcium-binding protein involved in calcium signaling [27]. CR has an important role in the functioning and organization of the central nervous system, which lack of its immunoreactivity in the ganglion cells and nerve fibers of myenteric plexus has been claimed to be characteristic of HSCR. Recently CR has been introduced as a useful marker for the diagnosis of HSCR [19, 20, 28]. In this study, there were no CR positive stained ganglion cells and nerve fibers in submucosal and myenteric plexus of 9 newborn patients. However, in the distension segment of full-term birth patients, which with Bcl-2 posi-

tive stained ganglion cells, there were no or low CR positive expression of plexus. There were no CR positive staining in spasm segment of 45 HAD patients. There were no or low CR immunoreactivity in HAD with Bcl-2 positive expression patients. CR positive staining can be observed in all of normal control patients' intestinal ganglion cells. Our data shown that CR has strong immunoreactivity in mature ganglion cells and nerve fibers but no or low immunoreactivity in immature ganglion cells.

Our results also showed that the absence of ganglion cells or dysplasia not only in the spasm segment but also in distension segment of certain HSCR and HAD, both results in a disordered motor function of the alimentary tract. We also found no Bcl-2 positive expression staining in neurogliaocyte, satellite cell and inflammatory cell. Combined with the characteristics of CR immunoreactivity in ganglion cells and nerve fibers, CR and Bcl-2 were useful to confirm the dysplasia of ganglion cells.

In conclusion, our study demonstrated for the first time in a large series of histopathological analysis to show that CR, Bcl-2 and RET can be used as a valuable tool in HSCR and HAD diagnosis and differential diagnosis. Extensive expression of neural markers CR and RET were observed in the affected segments in HSCR. The area of the nerve plexus, the diameter of the ganglion cells, and the number of ganglion cells were increased in the affected intestinal segments of IND, but decreased in the intestinal wall of HG. In normal intestinal segments and intestinal tissue in the control group, CR expression was observed mainly in mature ganglion cells and nerve fibers; however, CR was weakly expressed or not expressed in immature ganglion cells. Immature ganglion cells and mature neurons had strong RET expression. Bcl-2 was specially expressed in the dysplasia ganglion cells, not mature ganglion cells. Combined immunohistochemical detection of neural markers CR and RET facilitates the diagnosis and differential diagnoses of HSCR and HAD. And the specific expression of Bcl-2 and CR took an essential role in the diagnosis of HAD and newborn HSCR.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No.81200258)

and Foundation for Science & Technology Research Project of Chongqing (CSTC2015-jcyjA10080).

Disclosure of conflict of interest

None.

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