Pathology International

Pathology International 2014; **64**: 432–442 doi:10.1111/pin.12191

Original Article

Developmental markers of ganglion cells in the enteric nervous system and their application for evaluation of Hirschsprung disease

Hitomi Kawai,[1]* Kaishi Satomi,[2]* Yukio Morishita,[3] Yoshihiko Murata,[4] Masato Sugano,[2] Noriyuki Nakano[4] and Masayuki Noguchi[2]

[1]*Department of Pathology, Tsukuba University Hospital,* [2]*Department of Diagnostic Pathology, Faculty of Medicine and* [4]*Department of Diagnostic Pathology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba and* [3]*Department of Diagnostic Pathology, Tokyo Medical University Ibaraki Medical Center, Ami, Japan*

Hirschsprung disease (HSCR) is a congenital disease resulting from failure of neural crest-derived ganglion cells to colonize the colon. Conventional diagnostic methods are insufficient for evaluating the 'functional' prognosis of HSCR. In order to elucidate the maturation of ganglion cells, 17 immunohistochemical markers were examined. We examined the digestive tracts of 2 human early delivery patients, 2 miniature swine fetuses, 4 little infants, 3 infants, 3 children, 6 adults, and 3 aged individuals. With increasing age, the labeling index (LI) for both calretinin and tyrosine hydroxylase (TH) increased, whereas that for SOX10 decreased. We then examined the 'transitional zone' of HSCR in 21 affected patients and 18 controls for these three markers. The LI of calretinin and TH were significantly lower than in the controls (median: 3.7 in HSCR and 8.2 in controls, $P < 0.001$, median: 27.9 in HSCR and 44.4 in controls, $P < 0.001$, respectively). In contrast, the LI for SOX10 showed no significant difference (median: 33.7 in HSCR and 29.2 in controls, $P = 0.666$) however, hierarchical cluster analysis was able to divide HSCR patients into two groups. These results suggest that immature ganglion cells are present in the transitional zone of HSCR, and that HSCR may have two different pathophysiological processes.

Key words: calretinin, ganglion cells, Hirschsprung disease, immunohistochemistry, maturity, SOX10, tyrosine hydroxylase

Correspondence: Kaishi Satomi, MD, PhD, Department of Diagnostic Pathology, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-shi, Ibaraki 305-8575, Japan. Email: kaishis@md.tsukuba.ac.jp

Disclosure: The authors have no conflicts of interest to declare.

*Authors HK and KS contributed equally to this paper.

Received 9 January 2014. Accepted for publication 8 July 2014.

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd

The enteric nervous system (ENS) is a ganglionic network of neurons and glial cells within the intestinal wall. Approximately 400–600 million enteric ganglion cells arise from the neural crest during human embryonic development,[1,2] and the neurons migrate from the oral to the anal side of the intestinal wall in a continuous manner, maturing into ganglion cells at the same time.

Hirschsprung disease (HSCR) is a congenital disease that results from failure of neural crest-derived ganglion cells to colonize the colon.[3] This aganglionosis results in spastic constriction or 'megacolon' associated with specific positions or lengths of the aganglionic segment. Many genetic mutations associated with HSCR have been reported, and these affect molecules produced by the gut mesenchyme or epithelium, cell-surface molecules, transcription factors, and intracellular molecules in neural crest-derived cells.[4] Although *RET* is the most frequently mutated gene (in over 80% of cases),[5] none of the genes affected directly influences neuronal migration alone. Thus, coordinated function of the genes is needed for ENS development, and the pathogenesis of HSCR is therefore complex.

The pathological and clinical features of HSCR differ among patients, and it is very difficult to predict the functional outcome, even if extensive pathological examinations are performed on surgical specimens. Surgical resection of the aganglionic segment of the intestine is usually performed, especially in short-segment cases.[6] Sometimes, however, surgical treatment cannot be performed for HSCR patients with long-term complications. More than half of the patients with short-segment HSCR have been reported to show chronic complications during childhood, including fecal incontinence, constipation and enterocolitis.[7] Long-segment HSCR

is associated with less favorable functional outcomes than short-segment HSCR.[8] Pseudo Hirschsprung disease is another entity for which histopathological diagnosis is very difficult.[9] It shows clinical behavior similar to that of HSCR, but aganglionosis is difficult to demonstrate within a histological specimen. Recently, attempts have been made to categorize variants of HSCR based on histological findings, and these include internal neuronal dysplasia, intestinal ganglioneuromatosis, isolated hypoganglionosis, immature ganglia, absence of the argyrophil plexus, and megacystic microcolon intestinal hypoperistalsis syndrome.[10] In particular, syndromic cases of HSCR for which pathological diagnosis is difficult are suggested to have a basis of dysplastic ENS.[11] This difficulty means that conventional diagnostic methods, i.e. those based on morphologic features, are not adequate for evaluating the 'functional' aspects of ENS ganglion cells.

In the present study, we first examined several developmental markers of ENS ganglion cells using immunohistochemistry. As markers of immaturity, we selected transcription factors, since such factors are reported to be expressed only in immature ganglion cells. As maturation markers, we examined neuronal markers, since those that are commonly used would be expected to reflect functional maturity. If ganglion cells mature and become functional as a result of migration during ENS development, then immature ganglion cells would be identifiable oral to the aganglionic segment, i.e. in the transitional zone of HSCR.

MATERIALS AND METHODS

Case selection

First, to study the neuronal maturity of intestinal tissue, we reviewed two autopsy cases and 19 surgically resected cases. As shown in Table 1, these specimens were from patients ranging in age from 0 to 74 years, in whom the ENS was normal. Most of the surgically resected specimens were from patients with colon cancer, but we omitted cases that showed deep invasion of the enteric muscles, colonic obstruction, severe diarrhea, severe constipation, and perforation. As a reference for human fetal specimens, we used ileal tissue from early delivery patients. In addition, colon specimens from two Clawn miniature swine fetuses at 7 and 13 weeks of gestation were obtained, corresponding to the second and third trimesters in humans. These samples were divided into six groups according to age: (i) 2 human early delivery patients and 2

Table 1 Cases examined for ganglion cell maturation

				Specimen			Labeling index (%)			
Species	Age (Mo)	Group†	Gender	Location‡	Autopsy/Surgery	Disease§	calretinin	TH	SOX10	SOX2
Swine	7 weeks	Fetus	–	I	–	–	0.0	0.3	34.4	14.4
Swine	13 weeks	Fetus	–	I	–	–	0.0	19.5	32.2	23.1
Human	35 weeks	Fetus	M	I	Surgery	Adhesion	3.4	10.0	41.2	57.3
Human	39 weeks	Fetus	M	I	Surgery	Adhesion	5.6	1.7	49.0	0.0
Human	0 months	Little_infant	M	I	Surgery	Dupilicate	0.1	5.7	30.6	0.0
Human	2 months	Little_infant	F	I	Surgery	Adhesion	1.5	13.7	42.5	58.8
Human	5 months	Little_infant	M	S	Surgery	Anastomosis	1.1	16.5	40.0	47.6
Human	9 months	Little_infant	M	C	Surgery	Adhesion	0.0	35.2	29.4	0.0
Human	1	Infant	F	C	Autopsy	Brain tumor	0.0	17.2	14.4	0.0
Human	3	Infant	M	C	Autopsy	Congenital anomaly	0.0	22.8	10.9	0.0
Human	4	Infant	M	C	Surgery	Congenital anomaly	0.0	33.8	13.3	17.8
Human	8	Child	M	I	Surgery	Lymphangioma	11.7	42.1	14.8	0.0
Human	11	Child	M	I	Surgery	Adhesion	19.0	55.7	14.2	56.0
Human	12	Child	M	C	Surgery	Crohn disease	29.4	47.3	42.2	56.2
Human	16	Adult	F	S	Surgery	Ulcerative colitis	20.1	69.4	16.2	39.2
Human	30	Adult	F	S	Surgery	Colon cancer	31.5	52.0	1.9	38.9
Human	32	Adult	M	S	Surgery	Colon cancer	24.7	58.8	17.1	47.1
Human	41	Adult	F	S	Surgery	Colon cancer	12.0	76.6	30.3	61.4
Human	41	Adult	M	S	Surgery	Colon cancer	28.0	48.5	0.4	25.6
Human	44	Adult	M	S	Surgery	Colon cancer	22.2	50.0	0.5	54.0
Human	68	Aged	F	S	Surgery	Colon cancer	32.7	80.3	0.3	63.6
Human	72	Aged	M	S	Surgery	Colon cancer	31.7	65.5	1.0	51.3
Human	74	Aged	F	S	Surgery	Colon cancer	34.6	52.6	1.5	54.9

†Adult, ≥16 and <60 year old; Aged individual, ≥60 year old; Child, ≥6 and <15 year old; Fetus, before delivery or early birth; Infant, ≥1 and <5 year; Little infant, ≥0 and <1 year old.

‡C, Colon; I, Ileum; R, Rectum; S, Sigmoid colon.

§We selected the healthy region that was not affected by disease.

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd

Table 2 Clinicopathological characteristics of Hirschsprung disease (HSCR) patients and control patients

Case number	Age (Mo.)	Gender	Specimen Location†	Fecal incontinence	Constipation	Postoperative Ileus	Labeling index (%)		
							calretinin	TH	SOX10
HSCR 1	12	M	C	−	+	−	4.8	21.4	26.6
HSCR 2	10	F	R	−	+	−	3.7	22.6	40.8
HSCR 3	6	M	C	+	−	+	2.8	17.2	36.4
HSCR 4	7	M	C	−	−	−	5.3	20.0	13.2
HSCR 5	9	F	C	+	+	−	0.6	16.0	14.8
HSCR 6	6	M	R	−	−	−	3.0	35.0	18.2
HSCR 7	7	M	S	−	−	−	0.6	18.6	33.8
HSCR 8	7	M	C	−	−	−	3.6	49.7	37.6
HSCR 9	10	F	S	−	+	−	5.4	25.2	32.4
HSCR 10	4	M	C	−	−	−	3.4	30.7	38.0
HSCR 11	4	M	R	+	+	−	6.6	37.8	37.0
HSCR 12	2	M	R	−	−	−	5.4	37.4	44.2
HSCR 13	3	F	R	+	−	−	1.0	21.8	17.8
HSCR 14	4	M	R	−	−	−	5.8	35.8	36.2
HSCR 15	4	M	C	−	−	−	4.2	37.6	33.2
HSCR 16	4	M	R	−	−	−	0.0	18.1	21.2
HSCR 17	4	M	R	−	−	−	3.2	30.5	42.9
HSCR 18	9	M	C	−	+	−	2.3	19.8	36.2
HSCR 19	3	F	R	−	−	−	7.6	32.6	20.6
HSCR 20	3	M	C	−	−	−	4.2	18.1	35.2
HSCR 21	4	F	R	−	−	−	4.4	39.0	14.7
Control 1	12	M	R	−	+	−	4.4	41.4	44.2
Control 2	84	M	I	−	−	−	5.0	63.4	22.2
Control 3	10	M	R	−	+	−	8.2	67.0	35.0
Control 4	8	M	C	−	−	−	6.4	51.7	32.4
Control 5	1	M	R	−	−	−	11.5	32.8	45.2
Control 6	0	M	C	−	−	−	6.6	65.2	23.4
Control 7	7	M	C	−	−	−	7.0	38.0	35.2
Control 8	8	M	C	−	−	−	7.4	62.1	28.4
Control 9	6	M	R	+	+	−	7.8	40.3	28.2
Control 10	12	M	C	+	+	−	8.2	40.0	30.0
Control 11	12	M	C	−	+	−	10.4	40.1	18.1
Control 12	24	F	S	−	−	−	7.4	35.8	18.4
Control 13	36	M	C	−	−	−	8.6	44.4	39.5
Control 14	5	M	I	−	−	+	15.0	44.4	21.4
Control 15	8	M	R	+	−	−	17.8	54.4	15.6
Control 16	48	M	C	−	−	−	12.6	53.0	22.8
Control 17	12	M	R	−	−	−	18.6	46.6	34.0
Control 18	12	F	S	−	−	−	12.1	44.2	35.4

†C, Colon; I, Ileum; R, Rectum; S, Sigmoid colon.

miniature swine foetuses; (ii) 4 little infants (≥0 and <1 year old); (iii) 3 infants (≥1 and <5 year old); (iv) 3 children (≥6 and 15 year old); (v) 6 adults (≥16 and <60 year old); and (vi) 3 aged individuals (≥60 year old) (Table 1).

We also examined 21 patients with HSCR (age range 2 to 12 months) and 18 age-matched control patients (age range 0 to 84 months), all of whom had undergone surgical resection at Tsukuba University Hospital (Ibaraki, Japan) between 1997 and 2012. The 18 controls included 15 cases of anal atresia, 2 cases of inflammatory bowel disease, and 1 case of lymphoma. No histological anomalies of the ENS were evident in these control cases (Table 2). Written informed consent had been obtained from the patients or their guardians. All of the cases were anonymized and approved for use by the institutional review board of Tsukuba University Hospital.

Immunohistochemistry

For immunohistochemical analysis, we examined 17 neuron-associated proteins, including 11 that are routinely employed for pathological diagnosis as neuronal markers in our hospital: calretinin, CD56, chromogranin-A, c-kit, PGP9.5, N-cadherin, nestin, NeuN, p75NGFR, synaptophysin, and tyrosine hydroxylase (TH). In addition, the following six transcription factors reported to be associated with ENS development and thought to have a role in HSCR were investigated: HOXB5,[12] SOX2,[13] SOX10,[14–16] TCF4,[17] PHOX2B,[18] and ZEB2.[19–21] The antibodies against these markers (including their clone name) we employed, sources, dilution ratios, antigen retrieval methods, and secondary antibodies are shown in Table 3. According to the package instructions, all of these antibodies

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd

Table 3 The primary antibodies, dilution ratios, antigen retrieval methods, and secondary antibodies

Primary antibody	Clone‡	Source§	Dilution	Antigen Retrieval¶	Secondary antibody††
Calretinin	MM (DAK Calret1)	Dako	10	ProK, 5 min	ENVITION
CD56	MM (1B6)	Nichirei	–	CB, 120°C, 10 min	ENVISION
c-kit	RP	Dako	50	CB, 120°C, 10 min	ENVISION
Chromogranin-A	RP	Dako	500	CB, 120°C, 10 min	ENVISION
TH†	RP	Abcam	1000	TE, 105°C, 15 min	ENVISION
N-cadherin	MM (6G11)	Dako	50	TE, 105°C, 15 min	ENVISION
Nestin	MM (10C2)	Abcam	5000	TE, 105°C, 15 min	ENVISION
NeuN	MM (A60)	Millipore	100	TE, 105°C, 15 min	ENVISION
p75NGFR	RP	Abcam	2000	TE, 105°C, 15 min	ENVISION
PGP9.5	MM (13C4/I3C4)	Abcam	50	TE, 105°C, 15 min	ENVISION
Synaptophysin	MM (27G12)	Nichirei	–	TE, 105°C, 15 min	ENVISION
SOX10	GP	SantaCruz	50	TE, 105°C, 15 min	Histofine
PHOX2B	RP	Abcam	50	TE, 105°C, 15 min	ENVISION
SOX2	RP	Abcam	1000	TE, 105°C, 15 min	ENVISION
ZEB2	RP	Abcam	50	TE, 105°C, 15 min	ENVISION
TCF4	RP	Sigma-Aldrich	10	CB, 120°C, 10 min	ENVISION
HOXB5	RP	Abcam	50	TE, 105°C, 15 min	ENVISION

†TH, Tyrosine hydroxylase.
‡GP, goat polyclonal; MM, mouse monoclonal; RP, rabbit polyclonal.
§Millipore, Millipore, Billerica, USA; Nichirei, Nichirei Biosciences Inc., Tokyo, Japan.
¶CB, citrate buffer; ProK, Proteinase K (Dako, Glostrup, Denmark); TE, Tris-EDTA buffer.
††ENVISION, ENVISION + Dual Link Polymer (Dako); Histofine, Histofine MAX-PO(G) (Nichirei).

are reactive with human tissues. The antibodies against chromogranin-A, PGP9.5, SOX2, and HOXB5 react with swine tissue and that against TH reacts with all mammalian tissue. The other antibodies were not tested for their reactivity with swine tissue. All of the specimens had been previously fixed in 15% formalin and embedded in paraffin (FFPE). For HSCR cases, FFPE blocks from areas oral to the transitional zone were selected and examined. Sections from each block were cut at a thickness of 3 μm, then deparaffinized in xylene and rehydrated in an ethanol series. After antigen retrieval, the sections were incubated with ChemMate POD Blocking Solution (Dako, Glostrup, Denmark) for 5 min at room temperature, then incubated with each primary antibody diluted in Dako REAL Antibody Diluent (Dako) for 30 min at room temperature. They were then washed in Dako Wash Buffer (Dako) and incubated with the secondary antibody for 30 min. Visualization was performed by incubation with the DAB+ Liquid System (Dako) for 5 min. After rinsing in water, the sections were counterstained with hematoxylin and mounted. These immunohistochemical processes were performed with a histostainer (Nichirei Biosciences Inc., Tokyo, Japan).

Evaluation of labeling index

The labeling index (LI) for each primary antibody was evaluated. After counting at least 1000 ganglion cells in Auerbach's plexus, the percentages of positive cells were calculated. Ganglion cells were recognized and counted histologically using two differential blocks, which were cut into sections and stained with HE. During evaluation, if some small cells were positive (arrowhead in Fig. 1i SOX10), we also counted those cells as positive so as not to overlook any premature or small ganglion cells. However, any spindle cells that could be regarded as 'satellite cells' were omitted. All histological evaluations were judged by two pathologists (HK and KS) independently without access to any clinical information. The LI assessed by one pathologist (HK) was given priority as long as the difference in score was within 10%. If the evaluations differed by over 10%, two pathologists (HK and KS) discussed the case, and a consensus diagnosis was reached.

Statistical analysis

The Jonckheere-Terpstra trend test was performed to discriminate the maturation-associated markers. The Mann-Whitney U test was used to compare the patients' profiles and LI values between the transitional zone in HSCR cases and controls. For TH-LI and calretinin-LI, we also evaluated Cohen's κ coefficient to assess the degree of concordance between the immunohistochemical and clinicopathological diagnoses as a gold standard. The sensitivity and specificity of calretinin-LI and TH-LI for diagnosis of HSCR were determined by receiver operating characteristic (ROC) curve analysis. The optimal cut-off values selected were those that minimized the Euclidean distance from a point where specificity and sensitivity were 100%. The κ coefficient for HSCR diagnosis was also analyzed for calretinin-LI and TH-LI. Hierarchical cluster analysis was performed to evaluate the

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd

numbers of clusters and groups. The relationship of SOX10-LI with age was characterized using the Spearman rank-order correlation test. All statistical analyses were performed using R (The R Foundation for Statistical Computing, Vienna, Austria, version 3.0.1). Differences at P-values of 0.05 or less were considered statistically significant.

RESULTS

Profiles of patients with Hirschsprung disease and controls

The age of the HSCR patients at surgery ranged from 2 to 12 months (mean (± s.d.) 5.8 (±2.8) months), and that of controls ranged from 0 to 84 months (mean (± s.d.) 16.9 (±20.6) months) ($P = 0.004$). There were no significant differences between HSCR patients and controls in terms of gender (HSCR: 15 males and 6 females; controls: 16 males and 2 females, $P = 0.184$), the location of surgical resection (ileum, ascending to descending colon, sigmoid colon, and rectum, $P = 0.416$) and clinical presentation (fecal incontinence $P = 0.849$, constipation $P = 0.957$, postoperative ileus $P = 0.912$) (Table 2).

Immunohistochemistry

Among the two swine fetuses and two human early delivery patients, immunostaining for calretinin demonstrated no reactivity (Fig. 1a) and TH demonstrated little reactivity in the cytoplasm of ganglion cells (Fig. 1b), but SOX10 immunoreactivity was highly expressed in the nuclei of ganglion cells (Fig 1c). SOX2 also demonstrated reactivity in the nuclei of ganglion cells (data not shown). In adults, calretinin (Fig. 1g) and TH (Fig. 1h) showed strong reactivity in the cytoplasm of ganglion cells, but SOX10 immunoreactivity was negative (Fig. 1i). In specimens from children, there was moderate positivity for calretinin (Fig. 1d), TH (Fig. 1e), and SOX10 (Fig. 1f). SOX2 positivity varied from case to case. Specimens from little infants and infants demonstrated similar immunoreactivities to specimens from children.

Calretinin-LI and TH-LI showed a significant increase with age ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 2). The mean (± s.d.) calretinin-LI values were 0.0 (±0.0) for specimens from swine fetuses, 4.5 (±1.6) for preterm patients, 1.0 (±0.69) for little infants, 0.0 (±0.0) for infants, 20.0 (±8.9) for children, 23.1 (±6.8) for adults, and 33.0 (±1.5) for aged individuals. The corresponding values for TH-LI were 9.9 (±13.6), 5.9 (±5.9), 17.8 (±12.5), 24.6 (±8.4), 48.4 (±14.7), 59.2 (±11.5), and 66.1 (±13.9), respectively.

Of the two molecules (SOX10 and SOX2) known to be transcription factors associated with HSCR, only the SOX10-LI decreased significantly with aging ($P < 0.001$) but the SOX2-LI showed no particular trend with aging ($P = 0.965$) (Fig. 2). The mean (± s.d.) values for SOX10-LI were 33.3 (±1.6) in swine fetuses, 45.1 (±5.5) in preterm patients, 35.6 (±5.6) in little infants, 12.9 (±1.8) in infants, 23.7 (±16.0) in children, 11.1 (±12.2) in adults, and 0.9 (±0.6) in aged individuals. The corresponding values for SOX2-LI were 18.8 (±6.2), 28.7 (±40.5), 26.6 (±31.1), 5.9 (±10.3), 37.4 (±32.4), 44.3 (±12.6), and 56.6 (±6.3), respectively.

In addition, among the 23 samples (2 human autopsy cases, 19 surgically resected cases, and 2 swine samples), there were significant correlations between calretinin-LI and TH-LI ($\rho = 0.695$, $P < 0.001$), between calretinin-LI and SOX10-LI ($\rho = -0.459$, $P = 0.003$), and between TH-LI and SOX10-LI ($\rho = -0.608$, $P = 0.003$). The difference between human early delivery patients and miniature swine was not statistically comparable because of the limited sample size.

On the other hand, immunostaining using the remaining 13 antibodies was omitted from statistical analysis because they were not suitable as ganglion markers in this study. Antibodies against c-kit, N-cadherin, p75NGFR, PGP9.5, synaptophysin, and TCF4 were positively immunoreactive with almost all ganglia in all individuals of all ages, and also in miniature swine. Humans of all ages and miniature swine showed over 99% immunoreactivity in terms of c-kit-LI, N-cadherin-LI, p75NGFR-LI, PGP9.5-LI, synaptophysin-LI, and TCF4-LI. All ganglion cells in the miniature swine fetuses were negative for CD56, chromogranin-A, and nestin, whereas all human neurons were strongly positive for all three markers. Immunoreactivities for CD56, chromogranin-A, and nestin differed between humans and miniature swine. The ganglion cells were negative for NeuN, PHOX2B, HOXB5, and ZEB2. Humans of any age and miniature swine showed 0% immunoreactivity in terms of PHOX2B-LI, HOXB5-LI, ZEB2-LI and NeuN-LI. Finally, we accepted calretinin and TH as markers of maturation and SOX10 as a marker of immaturity. Then, in order to investigate the maturation status of HSCR, we examined HSCR cases imunohistochemically using these three markers. The cytological staining patterns for calretinin, TH, and SOX10 were similar to those in the former examination (Fig. 3). The calretinin-LI in the HSCR transitional zone was significantly lower than that in controls (median, 3.7 and 8.2; 25th percentile, 2.9 and 7.2; 75th percentile, 5.2 and 12.4, between HSCR cases and controls, $P < 0.001$) (Fig. 4a), similarly to the TH-LI (median, 27.9 and 44.4; 25th percentile, 19.8 and 40.1; 75th percentile, 35.6 and 53.7, between the HSCR transitional zone and controls, $P < 0.001$) (Fig. 4b). We then used receiver operating characteristic curve analysis to evaluate the immunohistochemical data for calretinin-LI and TH-LI. In terms of sensitivity, specificity, and κ-value, the cut-off points for discriminating between the HSCR transitional zone and controls were 89%, 91%, 0.795, and 6.0%

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd

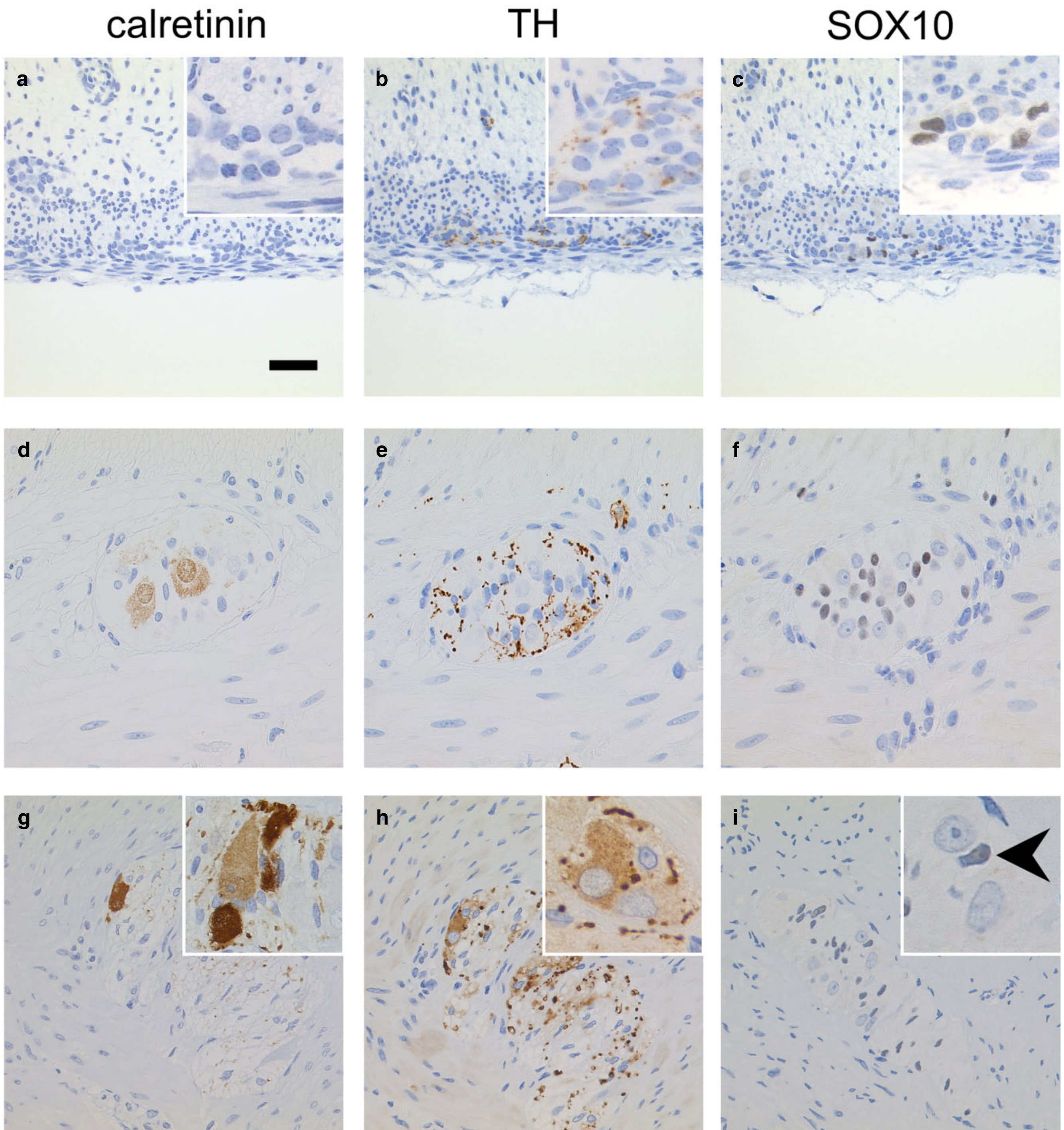

Figure 1 Immunohistochemical staining for calretinin, TH and SOX10 in samples from individuals of different ages. Representative images of the colon of a miniature swine fetus at 13 weeks of gestation (**a–c**), the ileum 8-year-old human (**d–f**) and the colon of a 44-year-old human (**g–i**). In swine fetus, immature ganglion cells showed no immunoreactivity for calretinin (**a**) and little immunoreactivity for TH (**b**), but immunoreactivity for SOX10 was evident (**c**). In contrast, in the 44-year-old human, mature ganglion cells were immunoreactive for calretinin (**g**) and TH (**h**), but negative for SOX10 (**i**). Some small cells were positive for SOX10 (arrowhead). We also counted those cells as positive so as not to overlook any premature or small ganglion cells. However, any spindle cells that could be regarded as 'satellite cells' were omitted. We also counted these cells as positive, but spindle cells that may have corresponded to 'satellite cells' were omitted. Scale bar, 50 μm. The inset figures represent high-power fields.

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd

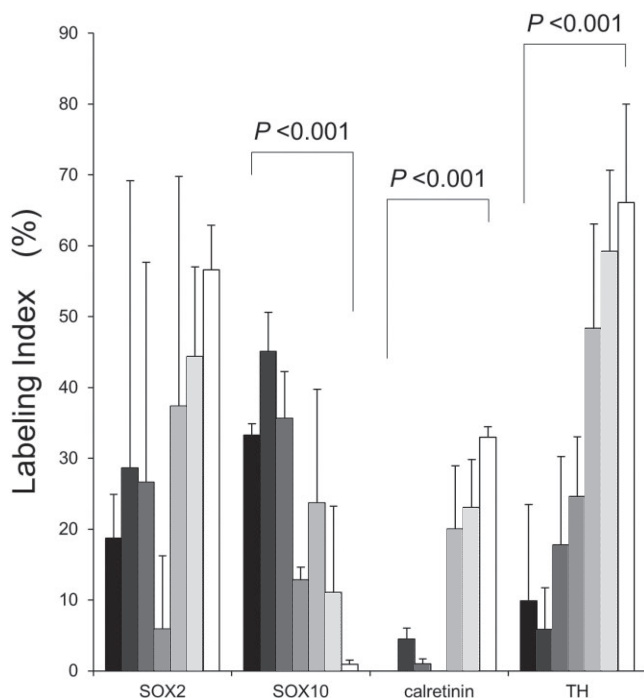

Figure 2 The quantitative immunohistochemical value of the labeling index (LI) in samples from individuals of various ages was assessed for SOX2, SOX10, calretinin, and TH. SOX2-LI showed no significant decreasing trend among the four age groups ($P = 0.965$). However, SOX10-LI showed a significant decreasing trend among these seven age groups ($P < 0.001$). Calretinin-LI and TH-LI showed significant increasing trends, respectively ($P < 0.001$). Data are expressed as means ± s.d. The Jonckheere-Trepstra trend test was used. ■, fetus_swine; ■, preterm; ■, little_infant; ■, infant; ■, child; □, adult; □, aged.

respectively, for calretinin-LI, and 95%, 79%, 0.745, and 40%, respectively, for TH-LI.

In contrast, the SOX10-LI was rather higher in the HSCR transitional zone than in controls, but the difference did not reach statistical significance (median, 33.7 and 29.2; 25th percentile, 20.8 and 22.4; 75th percentile, 36.9 and 35.2, between HSCR cases and controls, $P = 0.666$). Based on hierarchical cluster analysis, we divided the HSCR cases into two groups, and the threshold for SOX10-LI was 25%. In addition, the inter-group between the high SOX10-LI group (SOX10-LI ≥25%) and the low SOX10-LI-group (SOX10-LI <25%) was significant ($P < 0.001$). Fourteen HSCR cases were included in the high SOX10-LI group, and 7 in the low SOX10-LI group (Fig. 4c). We then investigated the correlation between age in months and SOX10-LI among HSCR cases. We hypothesized that even in HSCR, the SOX10-LI would decrease significantly with aging, but for HSCR patients as a whole, the SOX10-LI showed no particular trend with aging ($\rho = 0.475$, $P = 0.84$). We then analyzed the correlation between age in months and SOX10-LI in the high SOX10-LI group and the low SOX10-LI group, respectively. In the high SOX10-LI group, SOX10-LI was correlated with age in months ($\rho = 0.483$, $P = 0.034$) whereas in the low SOX10-LI group there was unexpectedly no such significant correlation ($\rho = 0.491$, $P = 0.132$), but rather a decreasing trend with age was observed (Fig. 4d).

DISCUSSION

This study investigated three maturation-associated markers of ganglion cell development using immunohistochemistry. First, calretinin-LI and TH-LI were selected as markers of colonic ganglion cell maturation. Calretinin is a calcium-binding protein that acts as a modulator of neuronal excitability, whereas TH is the enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine, which is a precursor of dopamine. These proteins mark commitment to a mature functional neuronal lineage, and therefore they can be considered markers of ganglion cell maturation. On the other hand, the SOX10-LI is an indicator of ganglion cell immaturity (Fig. 2). SOX10 is a transcription factor that acts as a nucleo-cytoplasmic shuttle protein and is associated with neural crest cell and peripheral nervous system development. In particular, SOX10 maintains the pluripotency of neural crest stem cells and suppresses neuronal differentiation.[22] For this reason, SOX10 was employed as a marker of ganglion cell immaturity in the present study.

Using these maturation-associated markers, we examined the maturation status of ganglion cells in the transitional zone of HSCR. As shown in Fig. 4, calretinin and TH were predictive markers of the HSCR transitional zone (Fig. 4a,b); the κ coefficient for calretinin-LI was 0.795, and that for TH-LI was 0.745. Although the results may not be directly comparable to ours, a previous study has revealed that calretinin-positive nerve fibers in the submucosa were an indirect marker of the HSCR transitional zone among rectal biopsy samples, with a κ coefficient of 0.98.[23] In that study, Guinard-Samuel *et al.* showed that calretinin immunohistochemistry was valuable for diagnosis of HSCR using suction rectal biopsy samples. The difference in the result between our study and the previous study would is likely to be due to the difference in the target evaluated for immunoreactivity. We studied ganglion cells directly using surgically resected specimens from the HSCR transitional zone, whereas the previous study evaluated simply biopsy specimens.

On the other hand, there was no significant difference of SOX10-LI between the HSCR transitional zone and controls ($P = 0.666$). There may not have been strict age-matching between HSCR patients and controls (Table 2, Fig. 4). In addition, the protein expression level may have differed according to genetic profile. However, among the transcription factors associated with ENS development and thought to

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd

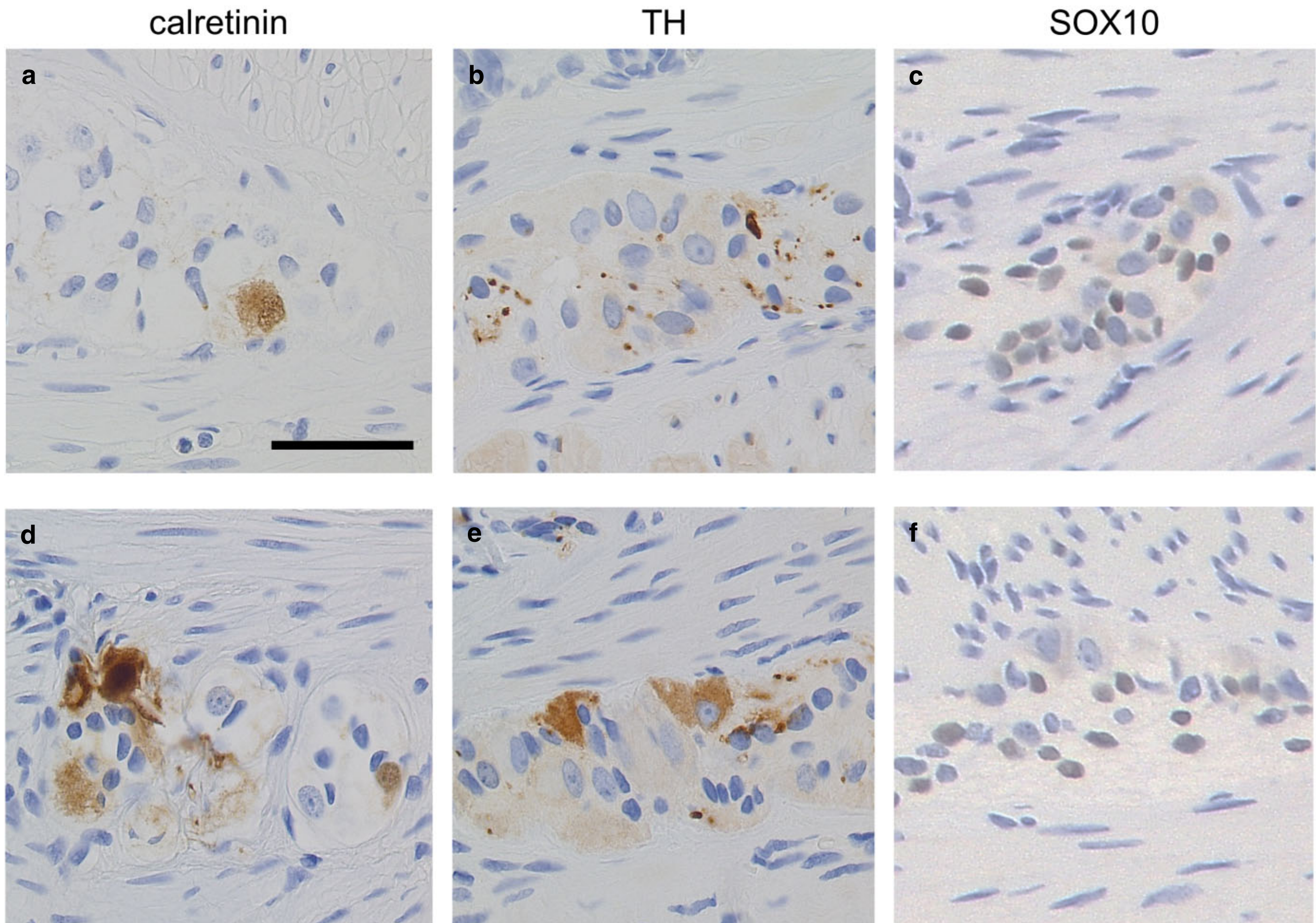

Figure 3 Immunohistochemical staining for calretinin (**a,d**), TH (**b,e**) and SOX10 (**c,f**) in patients with Hirschsprung disease (HSCR) and controls. Representative images show a HSCR patient (**a–c**) and a control patient (**d,e**). Ganglion cells in HSCR showed little immunoreactivity for calretinin (**a**) and TH (**b**). In contrast, ganglion cells from the control patient had higher reactivity for calretinin (**d**) and TH (**e**). Those from the HSCR patient (**c**) and the control patient (**f**) both showed immunoreactivity for SOX10. Scale bar, 50 μm.

have a role in HSCR, SOX10 is a reliable marker of genetic alteration. It has been reported that mutation of *SOX10* is associated with HSCR[24] and is also dysregulated by *PHOX2B* mutation.[25] Although these abnormalities may need to be investigated in future studies, it is of considerable interest that HSCR may have two different pathophysiological forms which are indicated as the high and low SOX10-LI groups (Fig. 4c,d). Experiments using mouse models of intestinal aganglionosis have indicated that ganglion cells may reach the colon, but then fail to develop or survive,[26,27] and another study has shown that ganglion cells develop via two pathways.[28,29] From a developmental point of view, these experimental mouse models prove that mature and immature stages of ganglion cells can both exist in a similar region of the intestine. This is for two reasons; they have two different origins, deriving from either vagal or sacral neural crest cells and can have two different migration routes of either across the mesentery or along the intestine. In this study, ganglion cells in the intestine of HSCR patients were divided into the high and low SOX10-LI groups based on cluster analysis (Fig. 4c,d). This result might suggest that human intestine of HSCR patient also contains ganglion cells at two different stages of maturation. Therefore, our findings suggest that the HSCR transitional zone involves not only mature ganglion cells but also immature ones.

This study had several limitations. First, for ethical reasons, we used swine fetuses as a reference material for human fetuses. Miniature swine are, of course, a commonly used mammalian experimental model, but obviously have limited homology to human protein, and thus in practice, we had to reject some candidate proteins. According to a recent bioinfomatics report, over 70% of the proteins annotated for humans are also present in swine.[30] Miniature swine fetuses can thus be representative of data for human fetuses. Secondly, the majority of control patients had anal atresia (15/19, 79%). To our knowledge, there have been no reports of neuronal immaturity in anal atresia, except for one report that demonstrated anomalies of the ENS in 60% of cases.[31] To

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd

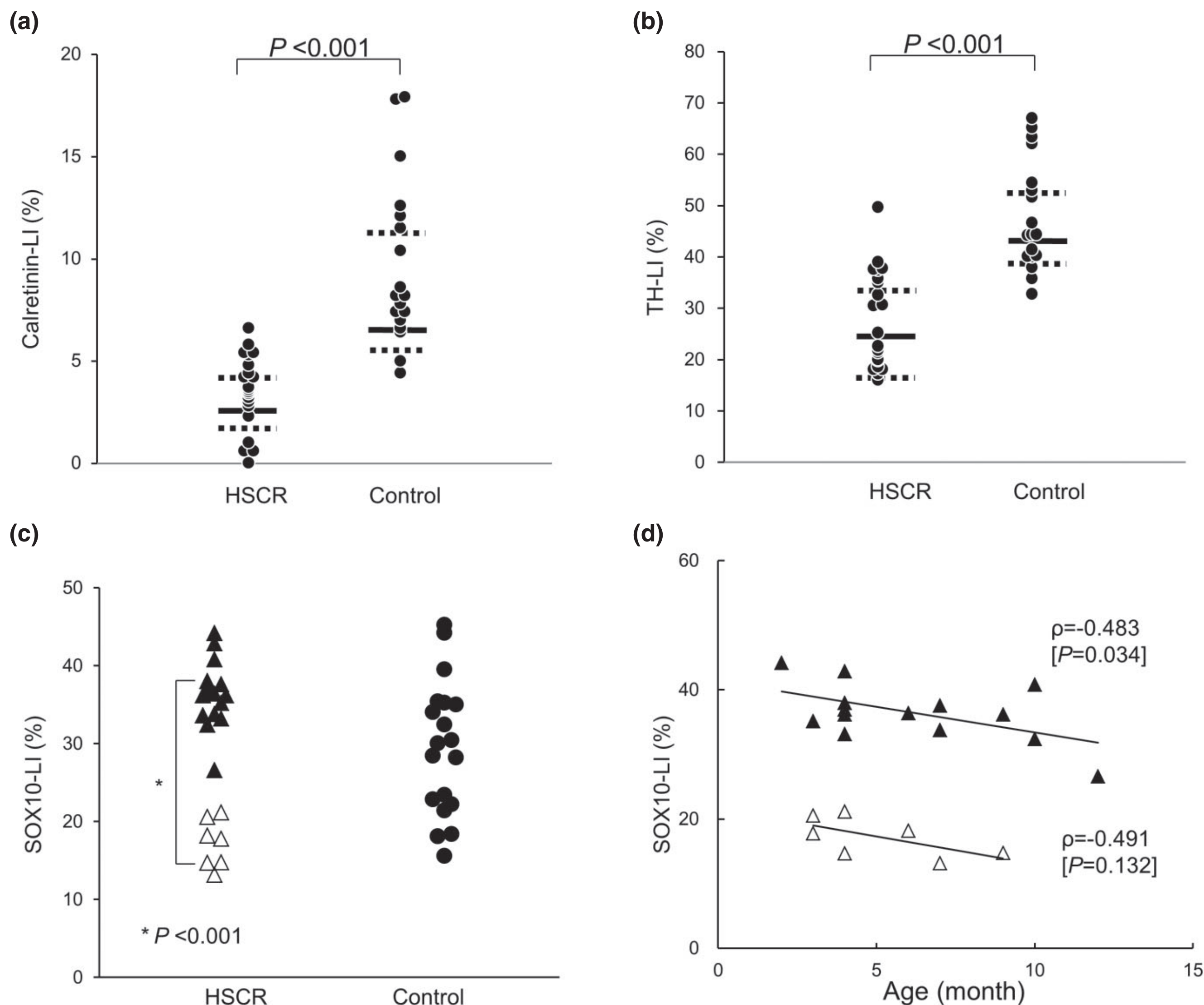

Figure 4 Quantitative immunohistochemical value of the labeling index (LI) in patients with Hirschsprung disease (HSCR) and controls. Both calretinin-LI and TH-LI differed significantly between HSCR and control patients ($P < 0.001$ and $P < 0.001$, respectively) (**a**), (**b**). However, SOX10-LI showed no significant difference between them (**c**). Based on hierarchical cluster analysis, HSCR cases were divided into the high SOX10-LI group (SOX10-LI ≥25%) and the low SOX10-LI-group (SOX10-LI <25%) and their difference of LI was significant ($P < 0.001$) (**c**). Only in the high SOX10-LI group, the SOX 10-LI was correlated with age in months (**d**). The Mann-Whitney U test was used for analysis in (**a**) to (**c**). Dependency of SOX10-LI on age was analyzed using Spearman's rank-order correlation test in (**d**).

avoid including such cases, we reviewed the histological findings at the time of first present study. Thirdly, it was difficult to continuously demonstrate temporal changes in the expression of the maturation-associated markers during development because we used autopsy cases and surgically resected cases. Finally, as this was a retrospective study, acetylcholine esterase staining could not be performed in some cases, even though this is a broadly accepted technique for diagnosis of HSCR. A further prospective study will be needed to verify the correlation between our immunohistochemical markers and the conventional diagnostic system that involves acetylcholine esterase staining. However, even in a prospective study, there would be difficulty in observing the muscle layer of the intestine, collecting an appropriate set of patients, and examining the correlation between clinicopathological features and clinical behavior.

Pseudo Hirschsprung disease is defined as a congenital functional intestinal obstructive disease resembling HSCR, in which the ganglion cells are maintained as far as the terminal rectum.[9] The disease has more than one pathogenesis, and its etiology is still under debate. Recently, there have been attempts to categorize HSCR into various types including internal neuronal dysplasia, intestinal ganglioneuromatosis, isolated hypoganglionosis, immature ganglia, absence of the

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd

argyrophil plexus, and megacystic microcolon intestinal hypoperistalsis syndrome.[10] The possible etiologies include myogenic abnormality,[32] neurogenic abnormality as shown in α3 nicotinic acetylcholine receptor-knockout mice,[33] and Cajal cell abnormality.[34] An understanding of its whole clinical picture, or a cure for patients based on their pathogenesis, still seems a long way off. However, some patients show morphological immaturity of ganglion cells, which appear small in size and have been described as showing 'immature ganglionosis' or 'ganglion cell hypogenesis'. The results of the present study may also suggest a novel approach for analysis of these features, perhaps leading to the development of a new therapeutic approach.

In conclusion, calretinin and TH have been shown to be markers of ganglion cell development in the ENS, and SOX10 appears to be a marker of ganglion cell immaturity. Immature ganglion cells are present in the transitional zone of HSCR and we propose that HSCR may have two different pathophysiological bases.

ACKNOWLEDGMENT

We thank Atsuko Nakazawa MD, PhD, for advice with pathological diagnosis and indispensable discussion in relation to this study. Kaishi Satomi has been nominated the winner of the Japanese Society of Pathology's Centennial Anniversary Award for Young Scientists in 2011.

REFERENCES

1 Furness JB. The enteric nervous system and neurogastroenterology. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 286–94.
2 Yntema CL, Hammond WS. The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. *J Comp Neurol* 1954; **101**: 515–41.
3 Hirschsprung H. Stuhlträgheit Neugeborener in Folge von Dilatation und Hypertrophie des Colons. *Jahrbuch für Kinderheilkunde und physische Erziehung (Berlin)* 1888; **27**: 1–7.
4 Obermayr F, Hotta R, Enomoto H, Young HM. Development and developmental disorders of the enteric nervous system. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 43–57.
5 Amiel J, Sproat-Emison E, Garcia-Barcelo M *et al*. Hirschsprung disease, associated syndromes and genetics: A review. *J Med Genet* 2008; **45**: 1–14.
6 So HB, Schwartz DL, Becker JM, Daum F, Schneider KM. Endorectal 'pull-through' without preliminary colostomy in neonates with Hirschsprung's disease. *J Pediatr Surg* 1980; **15**: 470–1.
7 Rintala RJ, Pakarinen MP. Outcome of anorectal malformations and Hirschsprung's disease beyond childhood. *Semin Pediatr Surg* 2010; **19**: 160–7.
8 Tsuji H, Spitz L, Kiely EM, Drake DP, Pierro A. Management and long-term follow-up of infants with total colonic aganglionosis. *J Pediatr Surg* 1999; **34**: 158–61. discussion 62.
9 Ravitch MM. Pseudo Hirschsprung's disease. *Ann Surg* 1958; **147**: 781–95.
10 Friedmacher F, Puri P. Classification and diagnostic criteria of cariants of Hirschsprung's disease. *Pediatr Surg Int* 2013; **29**: 855–72.
11 Matsuda H, Hirato J, Kuroiwa M, Nakazato Y. Histopathological and immunohistochemical study of the enteric innervations among various types of aganglionoses including isolated and syndromic Hirschsprung disease. *Neuropathology* 2006; **26**: 8–23.
12 Carter TC, Kay DM, Browne ML *et al*. Hirschsprung's disease and variants in genes that regulate enteric neural crest cell proliferation, migration and differentiation. *J Hum Genet* 2012; **57**: 485–93.
13 Jiang Q, Ho YY, Hao L, Nichols Berrios C, Chakravarti A. Copy number variants in candidate genes are genetic modifiers of Hirschsprung disease. *PLoS ONE* 2011; **6**: e21219.
14 Lang D, Chen F, Milewski R *et al.* Pax3 is required for enteric ganglia formation and functions with Sox10 to modulate expression of c-ret. *J Clin Invest* 2000; **106**: 963–71.
15 Harris ML, Baxter LL, Loftus SK, Pavan WJ. Sox proteins in melanocyte development and melanoma. *Pigment Cell Melanoma Res* 2010; **23**: 496–513.
16 Kapur RP. Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10 (Dom)/Sox10 (Dom) mouse embryos. *Pediatr Dev Pathol* 1999; **2**: 559–69.
17 Zweier C, Peippo MM, Hoyer J *et al*. Haploinsufficiency of TCF4 causes syndromal mental retardation with intermittent hyperventilation (Pitt-Hopkins syndrome). *Am J Hum Genet* 2007; **80**: 994–1001.
18 Amiel J, Laudier B, Attié-Bitach T *et al*. Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. *Nat Genet* 2003; **33**: 459–61.
19 Wakamatsu N, Yamada Y, Yamada K *et al*. Mutations in SIP1, encoding Smad interacting protein-1, cause a form of Hirschsprung disease. *Nat Genet* 2001; **27**: 369–70.
20 Zweier C, Albrecht B, Mitulla B *et al*. 'Mowat-Wilson' syndrome with and without Hirschsprung disease is a distinct, recognizable multiple congenital anomalies-mental retardation syndrome caused by mutations in the zinc finger homeo box 1B gene. *Am J Med Genet* 2002; **108**: 177–81.
21 Garavelli L, Donadio A, Zanacca C *et al*. Hirschsprung disease, mental retardation, characteristic facial features, and mutation in the gene ZFHX1B (SIP1): Confirmation of the Mowat-Wilson syndrome. *Am J Med Genet A* 2003; **116A**: 385–8.
22 Finzsch M, Schreiner S, Kichko T *et al*. Sox10 is required for Schwann cell identity and progression beyond the immature Schwann cell stage. *J Cell Biol* 2010; **189**: 701–12.
23 Guinard-Samuel V, Bonnard A, De Lagausie P *et al*. Calretinin immunohistochemistry: A simple and efficient tool to diagnose Hirschsprung disease. *Mod Pathol* 2009; **22**: 1379–84.
24 Shimotake T, Tanaka S, Fukui R, Makino S, Maruyama R. Neuroglial disorders of central and peripheral nervous systems in a patient with Hirschsprung's disease carrying allelic SOX10 truncating mutation. *J Pediatr Surg* 2007; **42**: 725–31.
25 Nagashimada M, Ohta H, Li C *et al.* Autonomic neurocristopathy-associated mutations in PHOX2B dysregulate Sox10 expression. *J Clin Invest* 2012; **122**: 3145–58.
26 Baetge G, Gershon MD. Transient catecholaminergic (TC) cells in the vagus nerves and bowel of fetal mice: Relationship to the development of enteric neurons. *Dev Biol* 1989; **132**: 189–211.
27 Young HM, Jones BR, McKeown SJ. The projections of early enteric neurons are influenced by the direction of neural crest cell migration. *J Neurosci* 2002; **22**: 6005–18.

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd

28 Erickson CS, Zaitoun I, Haberman KM *et al*. Sacral neural crest-derived cells enter the aganglionic colon of Ednrb-/- mice along extrinsic nerve fibers. *J Comp Neurol* 2012; **520**: 620–32.
29 Nishiyama C, Uesaka T, Manabe T *et al*. Trans-mesenteric neural crest cells are the principal source of the colonic enteric nervous system. *Nat Neurosci* 2012; **15**: 1211–8.
30 Dreher F, Kamburov A, Herwig R. Construction of a pig physical interactome using sequence homology and a comprehensive reference. *Evol Bioinform Online* 2012; **8**: 119–26.
31 Meier-Ruge WA, Holschneider AM. Histopathologic observations of anorectal abnormalities in anal atresia. *Pediatr Surg Int* 2000; **16**: 2–7.
32 Rolle U, O'Briain S, Pearl RH, Puri P. Megacystis-microcolon-intestinal hypoperistalsis syndrome: Evidence of intestinal myopathy. *Pediatr Surg Int* 2002; **18**: 2–5.
33 Richardson CE, Morgan JM, Jasani B *et al*. Megacystis-microcolon-intestinal hypoperistalsis syndrome and the absence of the alpha3 nicotinic acetylcholine receptor subunit. *Gastroenterology* 2001; **121**: 350–7.
34 Yamataka A, Ohshiro K, Kobayashi H *et al*. Intestinal pacemaker C-KIT+ cells and synapses in allied Hirschsprung's disorders. *J Pediatr Surg* 1997; **32**: 1069–74.

© 2014 Japanese Society of Pathology and Wiley Publishing Asia Pty Ltd