梨花醫大誌 : 第 26 卷 第 1 號 2003
Ewha Med J Vol. 26, No. 1, 2003

Analysis of Histopathological Findings of Hirschsprung' s Disease : Immunohistochemical Studies Including GDNF and Cathepsin D*

Heasoo Koo · Kum-Ja Choi*****

*Departments of Pathology** and Surgery,*** College of Medicine and Ewha Medical Research Center, Ewha Womans University*

= 국문초록 =

Glial Cell Line-Derived Neurotrophic Factor와 Cathepsin D 항체를 이용한 Hirschsprung병의 병리소견 분석

이화여자대학교 의과대학 병리학교실,** 외과학교실***

구 혜 수** · 최 금 자***

목 적 : 이 연구는 Glial cell line-derived neurotrophic factor(GDNF), cathepsin D, neuron specific enolase(NSE), S-100 단백에 대한 항체를 사용한 면역화학염색에서 관찰되는 Hirschsprung병의 병리소견을 분석하기 위하여 시행하였다. 태생기(16+6주, 25주, 30주, 35주)의 대장에서 같은 방법으로 신경절과 신경섬유의 발달과정을 관찰하였다.

방 법 : 임상소견과 병리소견에 의해 Hirschsprung병으로 진단된 34예를 신경절이 있는 정상부위, 신경절이 없는 부위, 이행부위로 분류하였다. 면역화학염색은 포르마린으로 고정하고 파라핀에 포매한 조직에서 peroxidase-antiperoxidase 방법을 사용하였다.

결 과 : 태생기의 신경절은 크기가 작고 세포막이 불분명하였지만 GDNF와 cathepsin D 항체에 대해 강한 양성 반응을 보여서 쉽게 구분할 수 있었다. 태생기 대장과 정상 부위에서 내인성 신경섬유에 비교해서 외인성 신경섬유는 GDNF 항체에 대해 더 강한 반응을 보였고 신경절이 없는 부위에서 증식하는 외인성 신경섬유는 S-100 단백과 NSE 항체에 대해 강한 양성 반응을 보였다. 점막과 점막내근육에 있는 증식된 신경섬유는 신경절이 있는 부위와 없는 부위에서 모두 관찰되었으며 대장창냄술을 시행한 부위에서 더 심해서 만성 자극과 관련이 있는 것으로 생각하였다.

결 론 : Hirschsprung병에서 신경절의 소실과 신경세포의 증식을 GDNF항체로 쉽게 파악할 수 있었고 태생기의 미성숙 신경절도 GDNF 항체에 강한 양성 반응을 보이므로 capthepsin D, S-100단백, NSE와 같은 항체와 GDNF 항체를 함께 사용하면 진단에 도움이 되리라고 생각한다. 그러나 Hirschsprung병에서 관찰되는 소견의 대부분이 부위에 따라 다양하게 나타나므로 신경절과 신경섬유의 분포와 변화를 함께 평가하여 진단해야 한다.

중심 단어 : Hirschsprung병 · 면역화학염색 · GDNF · Cathepsin D.

*This work was supported by the Ewha Womans University Tongdaemun Hospital Research Fund, 1997

Introduction

The Hirschsprung's disease (HD) is a congenital anomaly of enteric nervous system, which is characterized by absence of intrinsic ganglion cells in submucosal and myenteric plexuses of hindgut[1]. The absence of parasympathetic ganglion cells may be caused by either abnormal embryonic migration or defects in extracellular matrix components development of precursor cells from neural crest or immune-mediated neuronal necrosis[2-4]. It affects primarily neonates with incidence of one in 5,000 infants with 70−80% of male predominance.

The histopathological diagnosis of HD is based on the absence of ganglion cells in the myenteric and submucosal neural plexus and presence of hypertrophic nerve fibers. The biopsy material for the histopathological diagnosis of HD can be obtained from either a portion of intestinal wall taken during the operation or mucosal biopsy including mucosa and submucosa by suction rectal mucosal biopsy. The ganglion cells in submucosal nerve plexus are rare, with a density of $1/mm^2$, within 1cm of pectinate line compared to the normal density of $17/mm^2$ of rectum[5]. Detection of ganglion cells may be difficult because few may be present in small mucosal or muscle biopsies and the ganglion cells of young infants are morphologically immature and can be confused with endothelial cells, fibroblasts, macrophages, or Schwann cells. Immature ganglion cells are especially evident within the first few days or weeks of life when HD often presents clinically. For the correct diagnosis of HD in neonatal period, it is necessary to recognize the immature ganglion cells and characteristic patterns of aganglionic segment by standardized protocol including immunohistochemical studies. Previous studies with antisera to specific and nonspecific antigens[6-11] failed to find a single most important immunohistochemical method for the diagnosis of HD. In this study, we used glial cell line-derived neurotrophic factor (GDNF) antiserum to analyze the histopathological findings of HD as well as normal developmental stage of colon. Relatively well known markers of intestinal ganglion cells and nerve plexus such as neuron specific enolase (NSE), S-100 protein and cathepsin D were included in this study.

GDNF is a distant member of the transforming growth factor-β superfamily[12][13]. It has been known to act as a potent survival factor for motor neurons as well as numerous populations of peripheral nervous system neurons. Mice lacking GDNF show complete absence of neural crest-derived neurons in the small and large intestine, pyloric stenosis, and total renal agenesis as a result of failure of growth and arborization of the ureteric bud during early stages of kidney morphogenesis[14-21]. They showed remarkable similarities to mice defective for the gene encoding RET, which was recently found to be a component of the GDNF receptor. Several previous studies on GDNF and RET expression in normal developmental fetal intestine and HD cases showed inconsistent findings[12][13][19-21]. Cathepsin D is a family of lysosomal acidic proteinases that plays a crucial role in the intracellular catabolism of proteins[22]. The ganglion cells in bovine, rat, and human ocular tissue and rat dorsal root ganglia and spinal cord contain substantial amounts of this enzyme. Abu-Alfa et al. showed consistently positive reaction of submucosal and myenteric ganglion cells in colorectum, including the immature ganglion cells of children in the early postnatal period[23].

Material and Methods

A total of 34 cases of clinically and pathologically confirmed HD were included in this study, of which clinical findings are summarized in Table 1. The age of the patients ranged from 23 days to 4 years with mean age of 267 days. Five patients were less than 1 month of age and one patient was 4 years old, who had abdominal distention and constipation since birth. Thirty-seven sections of specimen were taken from 27 patients, who underwent postcolostomy closure and Duhamel pull through operation. Eight specimen of colorectal biopsy

Table 1. Clinical summary

	HD	Fetal intestine
Total cases	34	4
M : F ratio	28 : 6	2 : 2
Age distribution	23 days to 4 year	16+6, 25, 30, 35 weeks
Mean age	8.9 months	

HD : Hirschsprung's disease

were taken from 6 HD patients during colostomy. Resection of aganglionic segment was performed in one patient who was 15 years old. The sections were classified into normoganglionic (NG) segment, aganglionic (AG) segment, and transitional segment according to the presence or absence of ganglion cells in myenteric and submucosal plexus (Table 2). Thirty-seven sections of specimen taken from 27 patients, who underwent post-colostomy closure and Duhamel pull through operation consisted of 19 sections from intestinal segment and 18 sections from colostomy site. The fetal intestines at 16+6, 25, 30, and 35 weeks of gestation were obtained at elective termination of pregnancy with full ethical permission.

All studies were performed on formalin-fixed and paraffin-embedded intestinal tissues. The immunohistochemical reaction was accomplished using peroxidase-antiperoxidase method as described with some modification[24]. Briefly, each deparaffinized 5-μm section was reacted with primary antisera (see Table 3) for 60 min before reaction with the peroxidase-antiperoxidase complex by LSAB kit from DAKO (Santa Babara, CA, USA) The peroxidase reaction was carried by incubation with link antiserum and streptavidin for 20 min, for each and

Table 2. Sections used in this study

	Post-colostomy closure (27 cases)		Biopsy (cases)	Resection of AG
	Segment (sections)	Colostomy site (sections)		
NG	6	17	4	
AG	8		3	
Trans	5	1	1	1
Total	19	18	8	1

NG : normoganglionic segment, AG : aganglionic segment, Trans : transitional segment

Table 3. Antisera used in this study

Antisera	Raised in	Dilution	Company
GDNF	Rabbit	1 : 25	Santa Cruz Biotechnology
Cathepsin D	Rabbit	Prediluted	Dako (Carpinteria, CA)
NSE	Rabbit	1 : 50	Zymed (San Francisco, CA)
S-100 protein	Rabbit	1 : 200	Dakopatts (Glostrup, Denmark)

GDNF : glial cell line-derived neurotrophic factor, NSE : neuron specific enolase

Table 4. The immunohistochemical findings observed in HD cases

	GDNF	CD	S-100	NSE
Normoganglionic segment				
Ganglion cells	+++	+++	+/−	+++
Enteric nerve	+++	−	+++	++
Extrinsic nerve fiber	+/++	−	+++	+++
Enteric muscle	+/++	−	−	−
Coarse nerve fiber in mucosa and submucosa	++	−	++/+++	+++
Aganglionic segment				
Ganglion cells	0	0	0	0
Enteric nerve	++	−	+	++
Hypertrophic nerve bundles	++	−	+++	+++
Enteric muscle	+/++	−	−	−
Coarse nerve fiber in mucosa and submucosa	++	−	++/+++	+++

GDNF : glial cell line-derived neurotrophic factor, CD : cathepsin D, NSE : neuron specific enolase
Instensity of staining : − =negative : + =weak : ++ =strong : +++ =intensely strong

Table 5. The immunohistochemical findings observed in fetal colon

	GDNF	CD	S-100	NSE
16+6 weeks gestational age				
Ganglion cells	++	++	+/−	++
Enteric nerve	+	−	+	+++
Extrinsic nerve fiber	++	−	+	+++
Enteric muscle	++	−	−	−
>25 weeks gestational age				
Ganglion cells	+++	+++	+/−	+++
Enteric nerve	+	−	+	+++
Extrinsic nerve fiber	++	−	+	+++
Enteric muscle	+++	−	−	−

GDNF : glial cell line-derived neurotrophic factor, CD : cathepsin D, NSE : neuron specific enolase
Instensity of staining : −=negative : +=weak ; ++=strong : +++=intensely strong

subsequently with AEC(3-aminoethyl 9-carbasol). The sections were counterstained with Meyer's hematoxylin to visualize cell nuclei. Optimal antiserum concentration as determined by serial dilutions was used in all instances. Negative controls were obtained by exclusion of primary antiserum or by incubation with nonimmune bovine serum albumin at equivalent concentrations.

Results

The immunohistochemical findings of HD cases and fetal colon are summarized in Tables 4 and 5. At 16+6 weeks of gestational age, the immature ganglion cells in myenteric and submucosal enteric plexus had sparse cytoplasm without prominent nucleoli and formed rosette-like structures around a central neuropil type matrix. They showed clear positive reaction to GDNF and cathepsin D antisera(Fig. 1A and 1B). After 25 weeks of gestational age, the components of enteric nervous system showed increased evidence of differentiation with ganglion cells having more abundant cytoplasm, larger and less dense nuclei, and discrete nucleoli. Other cell types including enteric nerve fibers also become morphologically distinct as discrete coalescing enteric nervous system cellular plexus develop. Positive reactions in ganglion cells of myenteric and submucosal plexus to GDNF and cathepsin D antisera was stronger at 25, 30 and 35 weeks of gestational age(Fig. 1C and 1D). Enteric nerve fibers showed weak positive reaction to GDNF antiserum. Compared with intrinsic plexus, the extrinsic nerve fibers in pericolic soft tissue showed moderately strong positive reaction to GDNF antiserum.

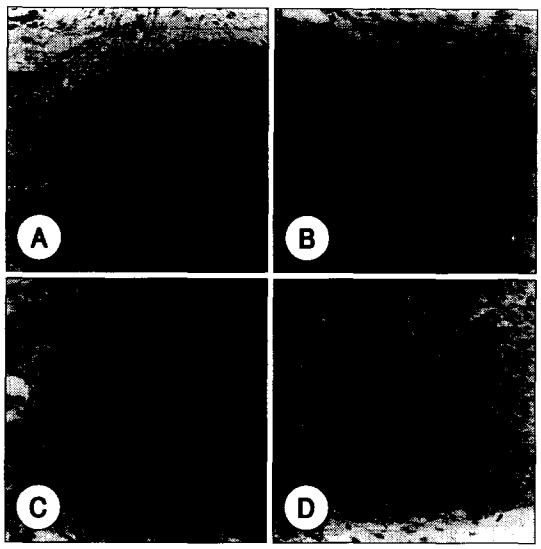

Fig. 1. Immunohistochemical findings of colon in fetus (peroxidase-antiperoxidase). At 16+6 weeks of gestational age, immature ganglion cells in myenteric and submucosal plexus show strong positive reaction to GDNF antiserum(A) and to cathepsin D antiserum(B) (×66). At 30 weeks of gestational age, the perikarya of immature ganglion cells in submucosal nerve plexus show strong positive reaction in ganglion cells and weak positive reaction in enteric nerve fibers to GDNF antiserum(C, ×100). The ganglion cells in myenteric and submucosal nerve plexus at 25 weeks of gestational age show strong positive reaction in perikarya to cathepsin D antiserum(D, ×50).

Ganglion cells in myenteric plexus were well defined at the earliest age examined after birth(23 days) com-

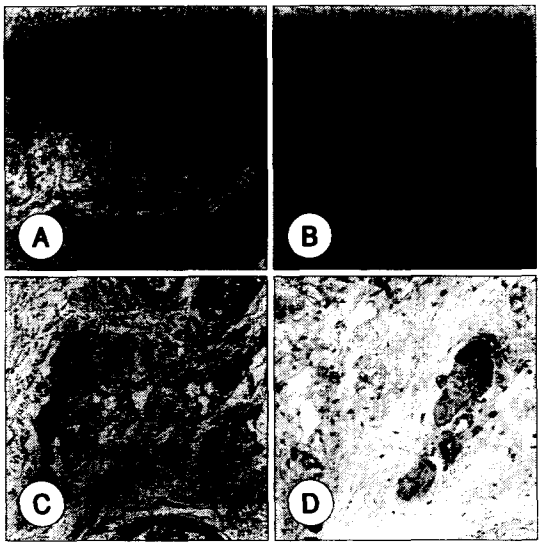

Fig. 2. Immunohistochemical findings of colon in infant (peroxidase-antiperoxidase, ×50). At 7 months of age, the polygonal or small round mature ganglion cells at the periphery of myenteric plexus show strong positive reaction to GDNF antiserum (A) and cathepsin D antiserum (B). Submucosal plexus shows strong positive reaction in ganglion cell perikarya and weak positive reaction in enteric nerve fibers to GDNF antiserum (C). Ganglion cells in submucosal plexus show intense, diffuse, granular cytoplasmic immunoreactivity to cathepsin D antiserum (D).

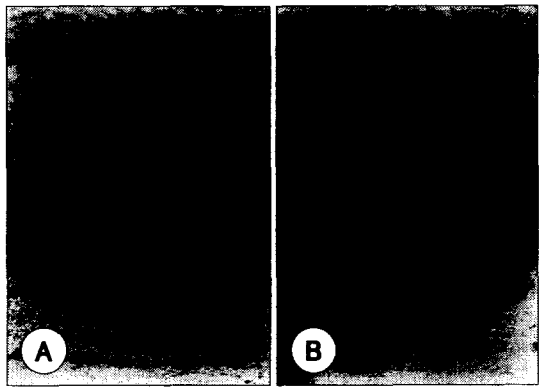

Fig. 3. NSE immunostain finding of normoganglionic segment (A) and aganglionic segment (B) of HD (peroxidase-antiperoxidase, ×16). The normoganglionic segment (A) shows diffusely distributed myenteric plexus and scattered submucosal plexus and numerous fine unmyelinated axons in bundles of muscularis propria. The aganglionic segment (B) shows proliferated extrinsic nerve bundles in intermuscular area and submucosa close to muscularis mucosae as well as in pericolic area. The unmyelinated axons in muscle layer are coarse, irregular, and decreased in numbers in aganglionic segment compared to normoganglionic segment.

pared to those at 35 weeks of gestational age, which were similar to adult ganglion cells. They were variable in size and shape and peripherally located surrounding loose nerve fibers. The ganglion cells showed similar strong positive reaction to GDNF and cathepsin D antisera (Fig. 2A and 2B). The nerve fibers showed strong reaction to GDNF antiserum and negative reaction to cathepsin D antiserum. Submucosal plexus was haphazardly scattered and some were abutting the inner surface of the muscularis propria and outer surface of the muscularis mucosae. Ganglion cells of submucosal plexus were large, polygonal with strong positive reaction to GDNF, cathepsin D, and NSE antisera (Fig. 2C and 2D). Extrinsic nerve fibers consisting of nerve fibers in pericolic soft tissue, subserosal nerve plexus, and peri- or paravascular nerve fibers in submucosa showed strong positive reaction to S-100 and NSE antisera (Fig. 3A).

The hypertrophic nerve bundles in aganglionic segment of HD were compact and solid and many of them were accompanying blood vessels (Fig. 3B). The hypertrophic nerve bundles were unevenly distributed and proportional to the amount of coarse, thick nerve fibers in muscle layer. The hypertrophic nerve bundles showed moderately strong positive reaction to GDNF antiserum and strong positive reaction to S-100 and NSE antisera.

The mucosa and muscularis mucosae of NG as well as AG segment of HD cases showed focally or diffusely distributed coarse nerve fibers, which were strongly positive by NSE antiserum (Fig. 4A). S-100 protein-positive fibers were much less than NSE-positive fibers (Fig. 4B). Those NSE- and S-100 protein-positive coarse fibers were found most prominently in sections from colostomy repair sites, of which incidence was not correlated with patient's age.

Transitional segment showed decreased numbers or absence of ganglion cells in myenteric and submucosal plexus and hypertrophic extrinsic nerve bundles. The ganglion cells and hypertrophic nerve fibers were unevenly distributed with focal aggregations of hypertrophic fibers. The myenteric plexus in transitional segment were smaller compared to those in NG segment. The amount

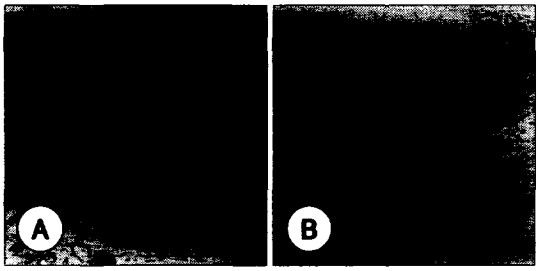

Fig. 4. Immunohistochemical findings of normoganglionic segment of HD. NSE antiserum(A) shows many strong positive thick nerve fibers in lamina propria and strong positive reaction in ganglion cells and nerve fibers of submucosal plexus(peroxidase-antiperoxidase, ×33). S-100 antiserum (B) shows scattered several positive fibers in lamina propria and negative reaction in ganglion cells in submucosal plexus(peroxidase-antiperoxidase, ×25).

of unmyelinated axons in muscle layers was different according to the presence or absence of ganglion cells. The muscle layers showed strong positive or weak positive GDNF reaction, which was not correlated with presence or absence of ganglion cells.

Discussion

The ganglion cells showed different shape and arrangement according to developmental stage. The ganglion cells in intermuscular myenteric plexus and submucosal nerve plexus were poorly defined and arranged in nests with rosettes-like appearance during fetal developmental period up to 35 weeks of gestational age and rather well defined with larger amount of cytoplasm at 27 days after birth. Compared with weak irregular positive reaction to NSE and S-100 protein antisera, those immature ganglion cells during fetal period showed strong positive reaction to GDNF and cathepsin D antisera. They showed granular localization of cathepsin D in perinuclear cytoplasm compared with mature ganglion cells with intense, diffuse, granular cytoplasmic immunoreactivity. Previous studies[23)25)] showed similar findings and Tatekawa et al.[25)] suggested the possibility to evaluate the maturation of ganglion cells by intracellular distribution of cathepsin D in meconium disease. Extrinsic nerve strands in developing intestine and NG segment from HD patients were poorly defined, but could be identified by their solid appearance and strong reaction to NSE and S-100 antisera. Hypertrophic extrinsic nerve strands in AG segment or oligoganglionic segment from HD patients also showed strong reaction to these antisera. The ganglion cells in myenteric plexus and submucosal plexus showed different morphology after birth. Compared with ganglion cells in myenteric plexus which are variable in size and shape and have peripheral location, the ganglion cells in submucosal plexus were large, polygonal with well defined cytoplasmic membrane and strong positive reaction to GDNF, cathepsin D, and NSE antisera. The recognition of markedly variable ganglion cells in myenteric plexus and difference in morphology of ganglion cells in two components of enteric nervous system are important for the diagnosis of HD. In NG segment of HD, the submucosal ganglion cells were unevenly scattered, but showed considerable abutting pattern to inner surface of muscularis propria and focally abutting on outer surface of muscularis mucosae. The ganglion cells closely abutting to muscularis mucosae as well as clear positive reaction of submucosal ganglion cells to GDNF and cathepsin D antisera could be helpful for the exclusion of HD in rectal mucosal suction biopsy.

Several previous studies on GDNF and RET expression in normal developmental fetal intestine and HD cases showed similar or inconsistent findings. Compared with the immunohistochemical studies showing GDNF immunostaining restricted in neural fiber-like structures across the gut wall[13)], other studies[19-21)] showed strong immunoreactivity in ganglion cells in myenteric plexus. Extrinsic nerve fibers also showed strong immunoreactivity[20)]. Muscle layer showed high GDNF immunoassay level[13)] or no immunoreactivity in both circular and longitudinal layers of NG as well as AG colon[21)] by immunofluorescence examination. Martucciello et al. also showed absence of GDNF expression in muscle layer by immunohistochemistry on frozen section[19)]. Full thickness specimen of AG bowel showed significantly reduced level of GDNF by ELISA[20)]. Mucosal epithelial cells showed strong GDNF immunoreactivity, which was significantly decreased in number in AG bowel compared with in NG bowel[20)].

The present study showed similar positive findings in ganglion cells and hypertrophic nerve trunk and moderate to strong positive reaction in muscle layer at NG

segment as well as in AG segment and transitional segment, where the ganglion cells are absent. The numbers of GDNF immunoreactive mucosal epithelial cells were variable and not consistently different according to the presence of ganglion cells by the method that we used. The fetal intestine showed strong positive reaction in muscle layer throughout the fetal period examined. The presence of GDNF immunoreactivity in fetal intestine as well as HD cases suggests that its role may not be restricted to development but rather additionally be associated with neuronal survival and function after birth.

This study showed focally or diffusely distributed NSE positive coarse nerve fibers in lamina propria of mucosa and muscularis mucosae with no significant difference between NG segment and AG segment of HD. The results were similar to previous studies showing NSE-positive unmyelinated axon-like structures in lamina propria of HD as well as control sections[6]. Mackenzie and Dixon[6] showed more evident unmyelinated axons in pediatric controls than in HD cases. The presence of more nerve fiber proliferation in lamina propria and muscularis mucosa in NG section from colostomy repair sites in this study suggest that nerve proliferation is a nonspecific finding occurring in the setting of tissue injury.

Since the diagnostic histopathological findings of HD are not always present in all of the sections from AG and transitional segments of HD and are variably expressed, diagnosis should depend on combination of changes in distribution and morphology of enteric nervous system as well as recognition of hypertrophic extrinsic nerve fibers. GDNF and cathepsin D antisera were very helpful to reveal the presence or absence of immature and mature ganglion cells as well as hypertrophic extrinsic nerve fibers. Combination of these antisera and other well-known markers for enteric ganglion cells and nerve fibers such as NSE and S-100 protein would increase the diagnostic accuracy of HD.

References

1) Rosai J : *Hirschsprung's disease and related disorders. In : Rosai J, ed. Ackerman's Surgical Pathology. 8th ed. St. Louis, Mosby, 1996 : 730-732*

2) Kapur RP : *Contemporary approaches toward understanding the pathogenesis of Hirschsprung's disease. Pediatr Pathol 1993 ; 13 : 83-100*

3) Parikh DH, Tam PKH, Van Velzen D, Edgar DH : *Abnormalities in the distribution of laminin and collagen type IV in Hirschsprung's disease. Gastroenterology 1992 ; 102 : 1236-1241*

4) Parikh DH, Tam PKH, Van Velzen D, Edgar DH : *The extracellular matrix components, tenascin and fibronectin, in Hirschsprung's disease : an immunohistochemical study. J Pediatr Surg 1994 ; 29 : 1302-1306*

5) Qualman SJ, Murray R : *Aganglionosis and related disorders. Hum Pathol 1994 ; 25 : 1141-1149*

6) Mackenzie JM, Dixon MF : *An immunohistochemical study of the enteric neural plexi in Hirschsprung's disease. Histopathology 1987 ; 11 : 1055-1066*

7) Deguchi E, Iwai N, Goto Y, Yanagihara J, Fushiki S : *An immunohistochemical study of neurofilament and microtubule-associated tau protein in the enteric innervation in Hirschsprung's disease. J Pediatr Surg 1993 ; 28 : 886-890*

8) Sams VR, Bobrow LG, Happerfield L, Keeling J : *Evaluation of PGP9.5 in the diagnosis of Hirschsprung's disease. J Pathol 1992 ; 168 : 55-58*

9) Vanderwinden J-M, de Laet M-H, Schiffmann SN, Mailleux P, Lowenstein CJ, Snyder SH, et al : *Nitric oxide synthase distribution in the enteric nervous system of Hirschsprung's disease. Gastroenterology 1993 ; 105 : 969-973*

10) Larsson LT : *Hirschsprung's disease-immunohistochemical findings. Histol Histopath 1994 ; 9 : 615-629*

11) Kobayashi H, Hirakawa H, O'Briain S, Puri P : *Nerve growth factor receptor staining of suction biopsies in the diagnosis of Hirschsprung's disease. J Pediatr Surg 1994 ; 29 : 1224-1227*

12) Koo H, Choi BH : *Expression of glial cell line-derived neurotrophic factor (GDNF) in the developing human fetal brain. Int J Devl Neuroscience 2001 ; 19 : 549-558*

13) Bar KJ, Facer P, Williams NS, Tam PKH, Anand P : *Glial-derived neurotrophic factor in human adult and fetal intestine and in Hirschsprung's disease. Gastroenterology 1997 ; 112 : 1381-1385*

14) Romeo G, Ronchetto P, Luo Y, Barone V, Seri M, Ceccherini I, et al : *Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. Nature 1994 ; 367 : 377-378*

15) Sanchez MP, Silos-Santiago I, Frisen J, He B, Lira SA, Barbacid M : *Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature 1996 ; 382 : 70-73*

16) Salomon R, Attie T, Pelet A, Bidaud C, Eng C, Amiel J, et al : *Germline mutations of the RET ligand GDNF are not sufficient to cause Hirschsprung disease. Nature Genetics 1996 ; 14 : 345-347*

17) Martucciello G, Favre A, Takahashi M, Jasonni V : *Immunohistochemical localization of RET protein in Hirschsprung's disease. J Pediatr Surg 1995 ; 30 : 433-436*

18) Angrist M, Bolk S, Halushka M, Lapchak PA, Chakravarti A : *Germline mutations in glial cell line-derived neurotrophic factor (GDNF) and RET in a Hirschsprung disease patient. Nature Genetics 1996 ; 14 : 341-344*

19) Martucciello G, Thompson H, Mazzola C, Morando A, Bertagnon M, Negri F, et al : *GDNF deficit in Hirschsprung's disease. J Pediatr Surg 1998 ; 33 : 99-102*

20) Ohshiro K, Puri P : *Reduced glial cell line-derived neurotrophic factor level in aganglionic bowel in Hirschsprung's disease. J Pediatr Surg 1998 ; 33 : 904-908*

21) Zhan J, Xiu Y, Gu J, Fang Z, Hu X : *Expression of RET proto-oncogene and GDNF deficit in Hirschsprung's disease. J Pediatr Surg 1999 ; 34 : 1606-1609*

22) Yamada T, Hara S, Tamai M : *Immunohistochemical localization of cathepsin D in ocular tissues. Invest Ophthalmol Vis Sci 1990 ; 31 : 1217-1223*

23) Abu-Alfa AK, Kuan S-F, West AB, Reyes-Mugica M : *Cathepsin D in intestinal ganglion cells. A potential aid to diagnosis in suspected Hirschsprung's disease. Am J Surg Pathol 1997 ; 21 : 201-205*

24) Hsu SM, Raine I : *The value of the avidin-biotin-peroxidase complex (ABC) in diagnosis and research pathology. In : Delellis RA, ed. Advances in immunochemistry. New York, Masson, 1984 : 31*

25) Tatekawa Y, Kanehiro H, Kanokogi H, Nakajima Y, Nishijima E, Muraji T, et al : *The evaluation of meconium disease by distribution of cathepsin D in intestinal ganglion cells. Pediatr Surg Int 2000 ; 16 : 53-55*