Immunohistochemistry for Intestinal Ganglion Cells and Nerve Fibers: Aid in the Diagnosis of Hirschsprung's Disease

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Abstract

Hirschsprung's disease is a disease of congenital abnormalities characterized by absence of the enteric ganglion cell of the colon. To make a definite diagnosis, biopsy of the aganglionic zones of colon is required. A specimen from submucosal biopsy of the colon is very small and difficult to identify submucosal ganglion cells. Our study reports an immunohistochemical technique to detect submucosal ganglion cells. Six antineural markers, peripherin, cathepsin D, PGP 9.5, synaptophysin, chromogranin and S-100 protein, were used. The best antibody for the detection of submucosal ganglion cells in our study was peripherin. The additional measurement of nerve fiber caliber using S-100 protein staining is a valuable aid in the diagnosis of Hirschsprung's disease. It can be applied to the suction submucosal biopsy in a patient suspected of having Hirschsprung's disease, therefore, the complicated full thickness colonic and rectal biopsy can be avoided.

Key word : Hirschsprung's Disease, Ganglion Cells, Immunohistochemistry

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Hirschsprung's disease or congenital colorectal aganglionosis is best described as congenital neural abnormalities. It is characterized by absence of the enteric ganglion cells of various lengths of the rectum and distal colon. Association of Hirschsprung's disease with other anomalies has been reported[1,2]. Down's syndrome was seen in 9 of 207 patients and may represent a real association, whereas, association with congenital heart defects was seen in 2 per cent (not including patients with Down's syndrome). A mortality of 16 per cent among the patients with Hirschsprung's disease emphasizes the extreme importance of early diagnosis. Current hypotheses on its pathogenesis re-

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volve around two schools of thought depending on whether one believes in the single or dual development gradient[3]. Okamoto et al[4], who demonstrated a cranial-caudal gradient of neuroblastic migration, concluded that aganglionosis of Hirschsprung's disease results from arrest of neuroblast migration. Hirschsprung's disease may also result from an abnormality in the intestinal microenvironment with failure of neuronal differentiation limited to a specific region[5].

Hirschsprung's disease occurs in one out of every 5,000 to 8,000 live births; 80 per cent of the patients are male[6]. Many patients have a positive family history for the disease. The classical presentation is that of a pot-bellied, malnourished 3 to 4 year old child with a history of intractable constipation and past episodes of enterocolitis. In some patients the diagnosis is delayed until adolescence[7]. Currently, the diagnosis is usually made in the neonatal period.

The five contiguous expressions of aganglionosis are the following: (a) limited segment (anus, rectum, rectosigmoid, and sigmoid colon), 60 per cent to 75 per cent; (b) ultrashort or short segment, 4 per cent to 15 per cent; (c) subtotal colonic (involvement beyond the rectosigmoid colon), 20 per cent; (d) total colonic, 1 per cent to 2 per cent and (e) extension into the small intestine, 2 per cent[8].

The diagnosis of Hirschsprung's disease is usually based on presenting symptoms[9] (Table 1), radiological appearance on barium enema, rectal manometry, and histology and/or histochemistry of rectal wall biopsy samples. Plain abdominal film shows air fluid levels in the distal small bowel, but X-ray contrast studies show that many of the changes are nonspecific. Some patients also have neurogenic defects of the urinary bladder. In the chronic form of the disease, the large bowel is hypertrophic and proximally dilated. Radiographic findings include a markedly dilated, feces-filled colon above the transitional zone, a narrowed rectum, a cone of funnel-shaped zone of transition and a mosaic colonic pattern caused by collapsed redundant mucosa after colonic cleansing. In adults, an abrupt, smooth transition zone in the rectum with an appropriate clinical history, suggests the diagnosis[10]. Biopsy diagnosis of Hirschsprung's disease commonly poses difficulties to the pathologist. Detection of frequently immature ganglion cells in the submucosal and myenteric neural plexuses of the large intestine marks the distinction between a positive and a negative diagnosis. Detection of ganglion cells may be difficult for several reasons particularly the scarcity of them in small mucosal biopsies. Also the ganglion cells of young infants (the population most often investigated for Hirschsprung's disease) are morphologically immature and can readily be confused with endothelial cells and plasma cells, among others. Acetylcholinesterase staining by enzyme histochemistry, which demonstrates hypertrophic nerves, was introduced as an aid in the diagnosis, however, it requires the use of fresh-frozen tissue, and its interpretation requires a certain level of expertise, especially with regard to equivocal results[11]. Subsequently, following the introduction of immunohistochemistry, several investigators have pursued immunohistochemical staining patterns of ganglion cells in routinely processed tissues using various antibodies to both neurospecific and nonspecific antigens[12-27]. Monforte-Munoz H studied the increased submucosal nerve trunk caliber in aganglionosis in Hirschsprung's disease to be used as a parameter in evaluating suction biopsies in morphologic diagnosis of Hirschsprung's disease in infancy and early childhood[28].

There are two basic methods of rectal biopsy for the diagnosis of Hirschsprung's disease, the full thickness longitudinal biopsy including the muscularis propia and the suction biopsy. Many centers in Europe and America no longer perform the deeper rectal biopsies as judged by the published studies[29]. Rees et al (30) reported their complications with rectal suction biopsy, which were very low and in fact, were less than the morbidity for the full-thickness biopsy. The microscopic evaluation of the mucosal biopsy is much more tedious and time consuming than the full-thickness biopsy since serial sections must be evaluated in most

Table 1. Symptoms and physical findings in Hirschsprung's disease.

Symptom	Physical finding
Constipation	Abdominal distention
Abdominal distention	Empty rectal ampulla
Delayed meconium	Fecal impaction
Vomiting	Dehydration
Failure to thrive	Enterocolitis
Diarrhea	Meconium plug syndrome

cases. One reason for this is the immaturity of the submucosal plexus in the neonate and the small size of the ganglion cells[29]. In comparison to the mature ganglion cells in the myenteric plexus, the cells of the submucosal plexus are diminutive, have darker nuclei, and lack a prominent nucleolus. These cells are also found individually or in small clusters in the submucosa, in contrast to the ganglion cells of the myenteric plexus, which are invariably, located in the plexus itself. The ganglion cells in the submucosal plexus are therefore difficult to identify under the best of circumstances with well-oriented and optimally stained histologic sections, and the problems are accentuated by frozen section examination.

The objective of the present study was to determine the usefulness of neural markers of submucosal ganglion cells and nerve fibers e.g. peripherin, chromogranin, cathepsin D, PGP 9.5, synaptophysin, and S-100 protein.

MATERIAL AND METHOD

Cases for study were retrieved from surgical pathology files at the Institute of Pathology, Medical Service Division, Ministry of Public Health, Thailand. Twenty-six cases of confirmed Hirschsprung's disease in the year 1999 were reviewed and studied by immunohistochemistry staining in both ganglionic and aganglionic zones. The extent of involvement of the patients was limited to the rectosigmoid region and/or colon distal to splenic flexure except two patients who were diagnosed as total colonic aganglionosis. Their specimens were taken from the jejunum. All specimens were received from patients admitted to the Queen Sirikit Childrens Institute. The surgical procedures included pull through procedure (8/26 cases), rectal biopsy (8/26 cases), sigmoid colon biopsy (4/26 cases), colon biopsy (4/26 cases), and jejunal biopsy (2/26). Incidental appendectomies were done in 13 patients and the appendixes were included in this study as the positive control of ganglion cells. Seventeen patients had more than one biopsy for the diagnosis. The patients' ages ranged from 1 month to 19 months at the time of surgery. All specimens of the 26 cases had been fixed in 10 per cent formalin, embedded in paraffin, sectioned at 3-5 microns for a total of 54 glass slides. They were stained with hematoxylin and eosin. An additional 318 slides were cut and prepared for labeled streptavidin – biotin immunoperoxidase technique using antibody to peripherin, chromogranin, cathepsin D, PGP 9.5, synaptophysin and S-100 protein (Table 2). The positive control for each antibody is shown in Table 2. Negative controls were prepared by adding bovine serum albumin (BSA) to the slides instead of primary antibody. The slides were finally counter stained with hematoxylin, mounted in Crystalmount, coverslipped and examined by light microscope for the ganglion cell of submucosal and myenteric neural plexuses.

RESULT

All positive control slides showed positive staining. All negative slides showed negative immunoreactivity for all antibodies tested. Immunoreactivity of the ganglionic segments is shown in Table 3.

Table 2. Antibodies used in the immunohistochemical technique.

Antibody	Detail/Clone	Source	Positive control	Negative control	Dilution
Peripherin	PGM-50	Novocastra	Rectum	BSA*	1: 500
Chromogranin	LK2H10	Novocastra	Islet cells of pancreas	BSA	1: 250
Cathepsin D	1C11	Zymed	Ganglion cell of appendix	BSA	1: 50
PGP 9.5	-	DAKO	Rectum	BSA	1: 500
Synaptophysin	SY38	DAKO	Medullo-blastoma	BSA	1: 250
S-100 protein	-	DAKO	Melanoma	BSA	1: 450

* BSA = Bovine serum albumin.

Table 3. Immunoreactivity of antibody in the ganglionic segment of the intestine.

Antibody	Ganglion cells	Nerve fiber	Histiocytes Inflammatory cells	Neuroendocrine cells
Peripherin	2+-3+	1+	Negative	Negative
Chromogranin	1+	Negative	Negative	3+
Cathepsin D	3+	Negative	3+	Negative
PGP 9.5	3+	3+	Negative	1+
Synaptophysin	1+	1+	Negative	1+
S-100 protein	Negative	3+	2+	Negative

Negative staining = No staining at all.
1+ staining = Faint staining in part of the cytoplasm.
2+ staining = Weak or moderate staining of the entire cytoplasm.
3+ staining = Strong staining of the entire cytoplasm.

The ganglion cell bodies in the submucosal and myenteric neural plexuses of the ganglionic zones of the intestine showed intense granular cytoplasmic immunoreactivity for cathepsin D (3+) and PGP9.5 (3+), and variable staining for peripherin (2+-3+) (Fig. 1), faint immunoreactivity for chromogranin (1+) (Fig. 2) and synaptophysin (1+). Immunoreactivity for the ganglion cell body was negative for S-100 protein. Nerve fibers in the submucosal and myenteric neural plexuses showed intense immunoreactivity for PGP9.5 (3+) and S-100 protein(3+) but showed faint immunoreactivity for peripherin (1+) and synaptophysin (1+). Immunoreactivity for the nerve fiber was negative for cathepsin D and chromogranin. Histiocytes and inflammatory cells also immunoreacted for cathepsin D (3+). In three cases of associated colitis, the background of the slide was loaded with inflammatory cells and histiocytes that interfered with the interpretation of the ganglion cells (Fig. 3). The nuclear features of the ganglion cells, such as open fine chromatin and large central nucleoli may be helpful in the differentiation. Neuroendocrine cells of the intestinal epithelium reacted for the chromogranin. Distribution of these cells in the mucosa can distinguish them from the ganglion cells. Immunoreactivity of PGP9.5 to both ganglion cells and nerve fibers was intense (3+). So it was difficult to distinguish these two types of tissue (Fig. 4). S-100 protein showed intense immunoreactivity (3+) for nerve fiber but was negative for ganglion cells. (Fig. 5).

The aganglionic zone revealed no false positive staining of the ganglion cells with all 6 antibodies. Measurements of submucosal nerve

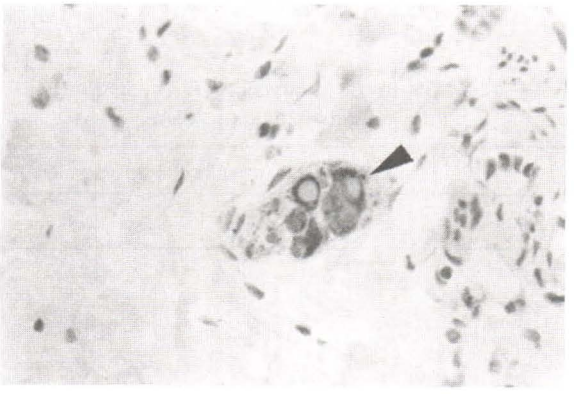

Fig. 1. Peripherin immunostaining patterns in ganglion cells (arrowhead). Showing a clean background. (original magnification x 400).

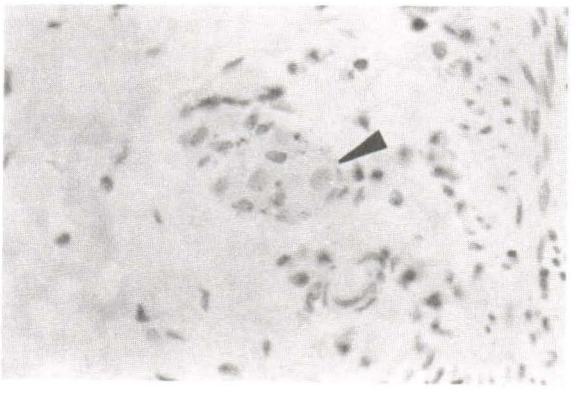

Fig. 2. Chromogranin immunostaining patterns in ganglion cells (arrowhead). Showing faint immunostaining with clean background. (original magnification x 400).

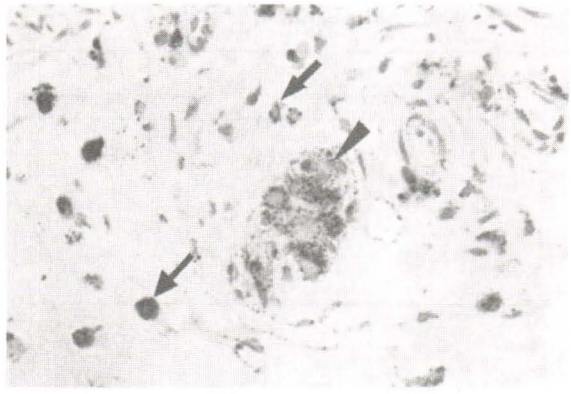

Fig. 3. Cathepsin D immunostaining patterns in ganglion cells (arrowhead). The intestinal histiocytes (arrow) are seen in the background. (original magnification x 400)

Table 4. Measurement of nerve fibers in the ganglionic and aganglionic zone.

	Submucosal nerve fiber diameter (micron)	
Case No.	Ganglionic zone	Aganglionic zone
S42-324	12.5	75.0
S42-734	25.0	50.0
S42-775	7.5	32.5
S42-812	37.5	100
S42-2814	12.5	55.0
S42-3058	12.5	55.0
S42-8990	25.0	50.0
S42-9371	17.5	62.5
Mean	18.75	60.00

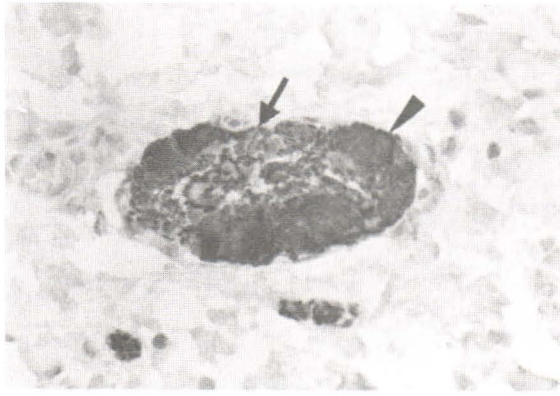

Fig. 4. PGP 9.5 immunostaining patterns in both ganglion cells (arrowhead) and nerve fiber (arrow). (original magnification x 400)

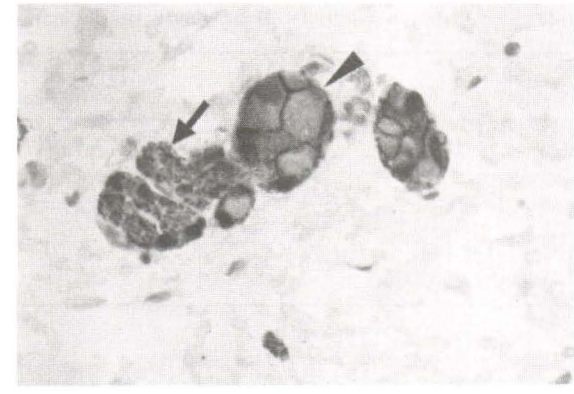

Fig. 5. S-100 protein immunostaining pattern in the nerve fibers (arrow). The ganglion cells show negative shadow (arrowhead). (original magnification x 400)

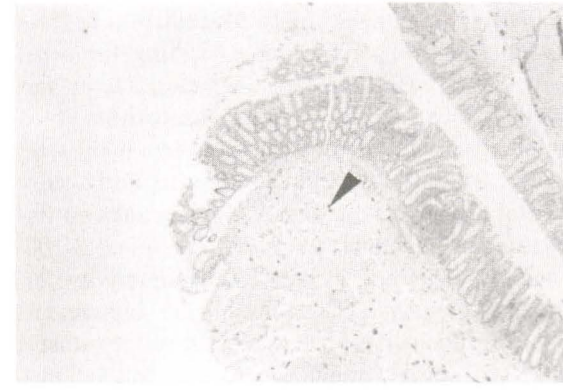

Fig. 6. Submucosal plexus with inconspicuous thin nerve fibers (arrowhead) in the ganglionic zone. (S-100 protein, original magnification x 40)

fibers demonstrated by S-100 protein of both ganglionic and aganglionic zones were done in 8 patients with pull through procedures (Table 4). Most nerve fibers in the ganglionic zone appeared inconspicuous and simple. The vast majority had a diameter between 12.5 and 25 microns (mean 18.75, SD 9.8198) (Fig. 6). The largest single nerve fiber measured 37.5 microns. Nerve fibers in the aganglionic zone were more numerous, in part due to their marked tortuousity and their caliber varied greatly, ranging from 32.5 to 100 microns (mean 60, SD 20.1335) (Fig. 7). A paired sample *t* test was

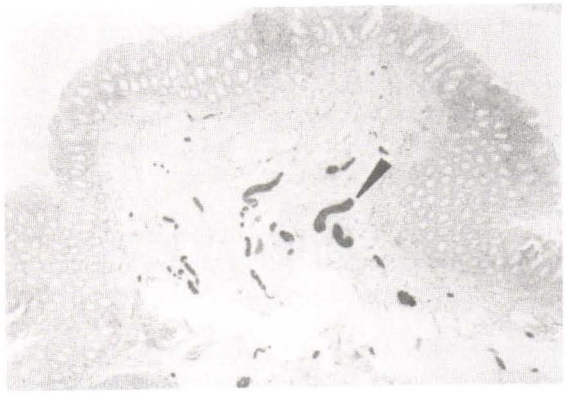

Fig. 7. Hypertrophic and tortuous submucosal nerve fibers (arrowhead) in the aganglionic zone. (S-100 protein, original magnification x 40)

performed to determine whether the mean of nerve fiber caliber was statistically different for the ganglionic zone and aganglionic zone. Significant correlation was observed (p ~ 0.0000).

DISCUSSION

Difficulties in the diagnosis of Hirschsprung's disease in the rectal or colonic mucosal biopsies depend on an increase in cholinergic nerve fibers and absence of ganglion cells in the submucosal plexuses which are not as easily identified as the ganglion cells in the myenteric plexuses. Moreover, in the age group most commonly investigated for this diagnosis, the ganglion cells of the submucosa are immature and may be difficult to distinguish from endothelial cells and other cells present there. Therefore, a multitude of methods ranging from enzyme histochemistry to the evaluation of various immunohistochemical markers has been investigated. Acetylcholinesterase staining is a useful histochemical procedure, however, this method is compromised by the need for frozen sections of biopsies oriented under a dissecting microscope, positive and negative control frozen section, and a pathologist experienced in interpreting the result of the stain[11].

With the introduction of immunohistochemistry and its expanding application in pathology, several investigators have turned to this technique to search for markers useful in detecting ganglion cells[12-27]. Amer K et al[22] used cathepsin D as a single antibody to identify the intestinal ganglion cells. This study showed strong positive immunostaining in intestinal ganglion cells, both mature and immature. But the histiocyte was the most potential confusion in their study, because the histiocyte showed no significant difference from ganglion cells in immunostaining intensity or pattern of granular cytoplasmic distribution. Details of cytology and nucleoli of histiocytes may be helpful in identifying the characteristics. However, we found that it may sometimes be difficult to differentiate them from immature ganglion cells.

According to our study, the most useful antibody for detection of ganglion cells is peripherin. In this study, we found a very clean background of the submucosa and muscular layer of the intestine with immunostaining for peripherin (Fig. 1). It was positive for both ganglion cells and nerve fibers but more strongly positive for ganglion cells than nerve fibers. Staining of chromogranin was a weaker positive immunoreactivity (1+) compared to 3+ immunostaining of neuroendocrine cells of the intestinal mucosa. Another useful antibody is S-100 protein. Measurement of S-100 protein immunostained nerve fibers greater than 40 microns in diameter is of value in the diagnosis of Hirschsprung's disease. According to the study of submucosal nerve fiber caliber of Monforte-Munoz at al in Hirschsprung's disease[29], they found that the aganglionic zone contained many distinct nerve fibers greater than 40 microns in diameter. The ganglionic zone showed no nerve fibers larger than this threshold value (p~ 0.0000). In our study, we had a similar result. Using the cut off point at 40 microns, the sensitivity was 100 per cent and specificity was 88.89 per cent.

In conclusion, the best antibody for the detection of submucosal ganglion cells is peripherin. The additional measurement of nerve fiber caliber using S-100 protein staining is a valuable aid in the diagnosis of Hirschsprung's disease. It can be applied to suction submucosal biopsy in a patient suspected of having Hirschsprung's disease. Therefore the complicated full thickness colonic and rectal biopsy can be avoided.

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การใช้เทคนิคทางอิมมูโนฮิสโตเคมีในการวินิจฉัยโรคเฮิร์ซปรุง

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โรคเฮิร์ซปรุงเป็นความผิดปกติที่เกิดจากการขาดเซลล์ประสาทบริเวณลำไส้ซึ่งพบบ่อยที่ลำไส้ใหญ่ส่วนปลาย เป็นสาเหตุให้ลำไส้ส่วนนั้นเคลื่อนไหวไม่ได้ ผู้ป่วยส่วนใหญ่จะมีอาการตั้งแต่แรกเกิด คือมีอาการท้องผูกเรื้อรัง การวินิจฉัยที่แน่นอนของโรคนี้ต้องทำการตรวจตัวอย่างชิ้นเนื้อที่ตัดจากลำไส้ส่วนที่สงสัยว่าไม่มีเซลล์ประสาทและส่วนที่ปกติด้วยวิธีทางพยาธิวิทยากายวิภาค ก่อนจะทำการผ่าตัดรักษาต่อไป การตรวจทางพยาธิวิทยากายวิภาคจะตรวจหาเซลล์ประสาทที่อยู่ในชั้นใต้เยื่อบุลำไส้และเซลล์ประสาทระหว่างชั้นกล้ามเนื้อจากชิ้นเนื้อของลำไส้ใหญ่ที่ศัลยแพทย์ส่งตรวจ ซึ่งการตรวจในปัจจุบันมักทำโดยการย้อมด้วยวิธีธรรมดา ซึ่งบางครั้งให้การวินิจฉัยได้ยากเนื่องจากชิ้นเนื้อมีขนาดเล็กและเซลล์ประสาทของผู้ป่วยเฮิร์ซปรุงจะมีขนาดเล็กด้วย นอกจากนี้เซลล์ประสาทดังกล่าวยังคล้ายกับเซลล์อื่นๆที่ปะปนอยู่ในชิ้นเนื้อบริเวณนั้นด้วย บางครั้งในกรณีที่มีการอักเสบบริเวณลำไส้ใหญ่ด้วยจะทำให้การตรวจหาเซลล์ประสาทยากยิ่งขึ้น บทความนี้รายงานการศึกษาทางอิมมูโนฮิสโตเคมีของเซลล์ประสาทด้วยแอนติบอดี้ 6 ชนิด คือ เพอริเฟอริน, โครโมแกรนิน, คาเทปซิน ดี, พีจีพี 9.5, ซินแนพโตไฟซิน และ เอส-100 โปรตีน ซึ่งพบว่าการย้อมด้วย เพอริเฟอรินจะเห็นเซลล์ประสาทชัดเจน โดยที่พื้นสไลด์สะอาดไม่มีเซลล์อื่นๆรบกวน ทำให้สามารถวินิจฉัยโรคเฮิร์ซปรุงได้แม่นยำและง่ายยิ่งขึ้น ดังนั้นแม้ชิ้นเนื้อที่ส่งตรวจที่ได้จากการตัดชิ้นเนื้อบริเวณเยื่อบุและใต้เยื่อบุลำไส้จะมีขนาดเล็กมากก็สามารถวินิจฉัยได้โดยวิธีนี้ อันจะเป็นการลดผลแทรกซ้อนจากการตัดชิ้นเนื้อขนาดใหญ่ที่มักทำกันอยู่ทั่วไปได้ด้วย และจากการวัดขนาดเส้นผ่าศูนย์กลางของเส้นประสาทใต้เยื่อบุลำไส้ใหญ่บริเวณปกติเทียบกับบริเวณที่ไม่มีเซลล์ประสาท พบว่าขนาดของเส้นประสาทบริเวณที่ไม่มีเซลล์ประสาทจะมีขนาดใหญ่กว่าบริเวณปกติ ซึ่งจะเป็นข้อมูลที่ทำให้การวินิจฉัยโรคเฮิร์ซปรุงได้แม่นยำยิ่งขึ้น

คำสำคัญ : โรคเฮิร์ซปรุง, เซลล์ประสาท, อิมมูโนฮิสโตเคมี

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