Original Article
Calretinin, S100 and protein gene product 9.5 immunostaining of rectal suction biopsies in the diagnosis of Hirschsprung’s disease

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Abstract: Evaluation of rectal suction biopsies for the ganglion cells and neural hypertrophy is the basic modality for the diagnosis of Hirschsprung’s disease (HD). However, the traditional hematoxylin and eosin staining coupled with acetylcholinesterase histochemistry remain challenging, especially in newborns. Thus we conducted a prospective study to evaluate the usefulness of calretinin combined with S100 and protein gene product 9.5 (PGP9.5) immunostaining of rectal suction biopsies for the diagnosis of HD. A total of 195 patients were enrolled in our study. Of the 195 patients 69% had ganglion cells on the initial diagnostic protocol. Sixty cases were devoid of ganglion cells, and of these, 90% and 91% showed submucosal neural hypertrophy on S-100 staining and PGP9.5 staining, respectively. Eighty-one patients underwent a colonic resection, and of these, 59 had confirmed aganglionic segment, the other 22 patients were diagnosed as intestinal neuronal dysplasia type B (n=13) and isolated hypoganglionosis (n=9). Of the rest 114 patients, 51 cases underwent a full-thickness biopsy, and HD was excluded; sixty-three patients were thoroughly followed-up with no evidence of HD. We encountered two false-negatives and they were proved to be short segment HD after the surgery. The sensitivity and specificity rates of our diagnostic protocol was 96.49% (95% CI, 0.88-0.99) and 100% (95% CI, 0.97-1.00), respectively, excluding 5 patients with inconclusive results. Our findings demonstrated that calretinin coupled with S100 and PGP9.5 immunostaining on suction rectal biopsies is sensitive and specific for diagnosing HD.

Keywords: Calretinin, S100, PGP9.5, Hirschsprung’s disease

Introduction

Hirschsprung’s disease (HD) is a congenital disorder characterized by the absence of the enteric ganglia along a variable length of the intestine, which can lead to tonic contraction of the affected segment and massive distension of the bowel [1]. HD can be attributed to a failure in the migration of enteric neural crest-derived cells into the intestine during embryonic development. According to the extent of aganglionosis, patients can be classified as classical segment HD (80%) when the aganglionic segment does not extend beyond the upper sigmoid, long-segment HD (15%) when aganglionosis extends to the splenic flexure or transverse colon, total colonic aganglionosis (TCA, 5%) when the aganglionosis extending from the anus to at least the ileocecal valve and a short segment of terminal ileum. The incidence of HD varies greatly by gender and race, and is most prevalent among Asians (2.8 per 10,000 live births) [2].

Since 1948 when Swenson described the first surgical approach to HD [3], the pull through procedure has gradually become the standard treatment strategy. The goals of surgical treatment are to remove the aganglionic bowel, followed by reconstruction of the intestinal tract with an end-to-end anastomosis [4]. It needs to be stressed that precise diagnosis is vital for
accurate treatment, for there are several enteral nervous system malformations with clinical symptoms resembling HD despite the presence of ganglion cells in the bowel [5]. For HD patients, the first choice is to remove the aganglionic bowel. However, for other different causes of intestinal neuronal dysplasias, colectomy is indicated only when the bowel symptoms fail to improve after 12-24 months’ conservative treatments [6]. Preoperative histologic evaluation of rectal suction biopsy (RSB) a safe, simple and inexpensive method is recommended in most centers [7, 8]. Compared with the other two widely used methods of contrast enema and anorectal manometry, it was demonstrated to be the most accurate test in diagnosing HD [9].

The golden criterion for diagnosing HD is the absence of ganglion cells in the myenteric or submucosal nerve plexus of the intestine and the presence of hypertrophic nerve trunks in the submucosa. Due to limited submucosal sampling as opposed to full-thickness tissue from surgical specimens, histopathological examination of suction biopsies with hematoxylin and eosin (H&E) staining is theoretically challenging for pathologists. Since 1972, when it was first reported by Meier-Ruge et al. [10], acetylcholinesterase (AChE) staining on frozen rectal suction tissue has been performed in many institutions to detect the hypertrophic submucosal nerve trunks as an adjunct to the diagnosis of aganglionosis.

However, AChE histochemistry is not universally employed due to its relying on specialist facilities and staff, time-consuming and inadequate sensitivity [5, 11]. Several other immunohistochemical markers have been introduced to identify ganglion cells and nerve fibers in formalin-fixed and paraffin-embedded specimens [12], which are less demanding technically and frozen specimens can be avoided. For example, calretinin, MAP2 and peripherin were used to identify ganglion cells and nerve fibers in formalin-fixed and paraffin-embedded specimens [12], while GLUT1, S100 and PGP9.5 were employed to stain the nerve fibers [16, 17].

Although mounting evidence have demonstrated the usefulness of particular antibodies to diagnose HD, few of them are exempt from some common shortcomings including (1) retrospective analysis of full-thickness specimens or suction biopsies from patients with proven HD or non-HD; (2) use of proximal ganglionic bowel from HD resections as a “normal” control; and (3) relatively small sample size [12]. As a tertiary referral hospital, our center cares for a large proportion of HD in central China and has accumulated plentiful experience in the transanal endorectal pull-through procedure [18, 19], which necessitate precise preoperative diagnosis. Based on the potential effectiveness in the diagnosis of HD in previous studies, we conducted a prospective study to evaluate the value of calretinin combined with S100 and PGP9.5 immunohistochemistry (IHC) of rectal suction biopsies as a primary diagnostic tool for the diagnosis of HD. We also applied the same IHC method to the surgical specimens and full thickness biopsies, and compared the results to the final diagnosis of our tertiary pathology center.

Materials and methods

Study design

This was a prospective, single-center-based study, and it was approved by the Research Ethics Board of the Union Hospital of Huazhong University of Science and Technology.

Patients

Between February 2011 and January 2015, we enrolled a consecutive series of patients who were suspected of having HD in our study. The purpose of the trial and the potential risks were fully explained to each subject. The primary chief complaint was severe defecation problems from birth during the neonatal period, or chronic intractable constipation in childhood. The children’s general information, including gender, ethnicity, age at presentation, onset of symptoms as well as time of diagnosis were carefully documented. After careful history and physical examinations, those patients who had obvious sources for constipation (e.g., metabolic disorders and functional constipation) were excluded. In addition, the patients were also excluded if the parents did not provide informed consent.

Rectal suction biopsy

Once the patient was suspected of having HD, we conducted an RSB after informed consent was obtained from the guardian. The instru-
ment used in our institution for suction biopsy is Suction Rectal Biopsy System (Aus Systems Pty Ltd, South Australia, Australia), which consists of a blunt ended tube with a side hole two centimeter from the tip [20]. To minimize the risk of insufficient tissue that is commonly resulted from the presence of stools inside the mucosa, we performed bowel preparation with cleaning enemas two days before the procedure. In all children, we carried out 4 suction biopsies at 3 cm and 6 cm above the dentate line anteriorly and posteriorly. A well-trained pediatric surgeon performed all the RSBs. The operative technique was detailed in a previous published literature [20]. Bowel specimens were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. All children were observed carefully after the procedure to rule out some complications such as rectal bleeding and rectal perforation.

**Immunohistochemistry**

Immunostaining was performed on representative replicate sections (0.4 μm-thick) of all available samples. Sections were dewaxed and rehydrated at first. Details of antibodies against the antigen of calretinin, S100 and PGP9.5 were listed in Table 1. Antigens were retrieved using heat treatments (95°C, 8 min in Ultra-cell-conditioning solution 1 for calretinin, S100 and PGP9.5). A Tris-based buffer solution (pH 7.6 ± 0.2) was used. Primary antibody reactions were performed after the inactivation of endogenous peroxidase by UV-inhibitor, then the sections were counterstained with hematoxylin before dehydration and coverslipping.

A qualitative assessment was made during microscopic examination (Thermo & 3DHISTECH, Budapest, Hungary) to score the positive or negative immunostaining in ganglion cells and hypertrophic nerve trunks. Calretinin immunostaining was considered positive if characteristic ganglion cell was identified within a submucosal plexus. The maximum diameter of submucosal nerve fibers was evaluated on PGP9.5 and S100 immunostaining using visual micrometry. Nerve fibers that with a diameter of ≥ 40 μm were defined as hypertrophic nerve trunks. An RSB was considered to be HD positive when the hypertrophic nerve trunks were recognized combined with aganglionosis. When ganglion cells exist, HD was excluded.

**Treatment and follow-up**

The preoperative diagnostic scheme is shown in Figure 1. If the diagnosis of HD was supported by the RSB, we performed transanal endorectal pull-through procedure to remove the aganglionic bowel segment after been consented. If the RSB results were negative or not conclusive, conservative therapies such as colonic irrigation and high dose lactulose were recommended at least 6 months. In case of persistent constipation, the patients were verified through a full-thickness biopsy under general anesthesia. In all other children, the disappearance of symptoms was considered to be an evidence of non-HD. All patients were followed-up at least 1 year according to a standardized questionnaire to monitor the symptoms.

### Table 1. Details of antibodies used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Source</th>
<th>Dilution</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calretinin</td>
<td>Polyclonal</td>
<td>Zytomed Systems</td>
<td>1:2</td>
<td>CA, USA</td>
</tr>
<tr>
<td>S100</td>
<td>4C4.9</td>
<td>Zytomed Systems</td>
<td>1:3000</td>
<td>CA, USA</td>
</tr>
<tr>
<td>PGP9.5</td>
<td>Polyclonal</td>
<td>Abcam</td>
<td>1:50</td>
<td>MA, USA</td>
</tr>
</tbody>
</table>

![Preoperative diagnostic scheme.](Figure 1)
Calretinin, S100 and PGP9.5 for diagnosing Hirschsprung disease

Table 2. Clinicopathologic features of the patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total group</th>
<th>HD</th>
<th>Non-HD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=195</td>
<td>N=59</td>
<td>N=136</td>
<td></td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>78/117</td>
<td>14/45</td>
<td>64/72</td>
<td>0.002</td>
</tr>
<tr>
<td>Median age at intake (months)</td>
<td>5.5</td>
<td>3.8</td>
<td>6.0</td>
<td>0.03</td>
</tr>
<tr>
<td>&lt; 1 (%)</td>
<td>20%</td>
<td>46%</td>
<td>9%</td>
<td></td>
</tr>
<tr>
<td>1-6 (%)</td>
<td>32%</td>
<td>20%</td>
<td>37%</td>
<td></td>
</tr>
<tr>
<td>6-12 (%)</td>
<td>17%</td>
<td>30%</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td>≥ 12 (%)</td>
<td>31%</td>
<td>4%</td>
<td>43%</td>
<td></td>
</tr>
<tr>
<td>Delayed passage of meconium</td>
<td>37%</td>
<td>91%</td>
<td>13%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median defecation frequency/week (range)</td>
<td>3.0 (0-41)</td>
<td>0.5 (0-20)</td>
<td>3.0 (0-43)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

HD: Hirschsprung disease.

Figure 2. Immunostaining of rectal suction biopsies for calretinin. A. Calretinin was expressed in mucosae and submucosa of normally innervated tissue (× 40). B. Nuclear and cytoplasmic granular staining in ganglion cells and in intrinsic nerve twigs in the lamina propria and submucosa of normally innervated tissue (× 400). C. Total absence of calretinin immunostaining in mucosae and submucosa of HD tissue (× 40). D. Neither ganglion cells nor intrinsic nerve fibers were stained in the lamina propria and submucosa of HD tissue (× 400).

Final diagnosis

Surgical and full thickness biopsy specimens were handled in our laboratory and the tertiary pathology department of our center, respectively. In our laboratory, the full thickness specimens underwent the same immunostaining procedure as the rectal suction biopsies, but myenteric plexus were included in the analysis. The final diagnosis was made by a senior pathologist of the pathology department, and the diagnostic criterion of HD was the total absence of ganglion cells in full thickness sections; or HD was excluded by thorough clinical follow-up to confirm the disappearance of symptoms.

Statistical analysis

Sensitivity and specificity rates with 95% CIs for the test were calculated with the method of
Wilson [21]. Evaluation of the absence or presence of hypertrophied nerve trunks and ganglion cells were performed using the $\chi^2$ tests. A $P$-value of $< 0.05$ showed statistical significance. The SPSS 13.0 software (SPSS, IL, USA) was used for data analysis.

**Results**

**Clinical features**

During the 4-year period between February 2010 and January 2015 we approached the guardians of 210 infants and children for participation in the study, of whom 195 (93%) consented, enrolled, and were included in our final analysis. The clinicopathologic features of all patients were summarized in Table 2. Of the 195 children included in the study, 117 (60%) were boys. The median age of the total population was 5.5 months (range, 15 days-14 years). All patients received rectal suction biopsies for evaluation of the presence of ganglion cells and hypertrophic nerve fibers. Two patients had mild rectal bleeding after the RSB procedure, but no surgical re-examination was required to stop it. No perforations or any other complications occurred.
Staining of ganglion cells for calretinin

In normally innervated tissue, calretinin antibody stained ganglion cells in both the submucosal (Figure 2A and 2B) and myenteric (Figure 3A and 3B) plexuses reliably and allowed us to interpret the result easily. Immunostaining of ganglion cells presented as chromogen deposition in the cytoplasm and nucleus (Figure 2B). In addition, positive calretinin staining of intrinsic nerve fibers were also observed in the muscularis mucosa and lamina propria (Figure 2A and 2B). On the other hand, calretinin immunostaining was not detected in any area of the

Figure 4. Immunostaining of rectal suction biopsies for S100 and PGP9.5. (A and B) S100 staining highlighted negatively stained ganglion cells as a cell-shaped “blank” area surrounded by glia cells and intrinsic nerve fibers in the submucosa of normal tissue (× 100 in A and × 400 in B). (C and D) Dense and prominent S100 immunostaining showed hypertrophic nerve trunks in the submucosa of HD-affected tissue (× 100 in C and × 400 in D). (E and F) PGP9.5 staining in normal tissue showed granular staining of small nerve twigs in contrast to strong staining of ganglion cells within the submucosa (× 100 in E and × 400 in F). (G and H) PGP9.5 staining in HD tissue showed dense hypertrophic nerve fibers in the submucosa (× 100 in G and × 400 in H).
HD tissue (Figures 2C, 2D, 3C and 3D). The qualitative assessment of calretinin staining is outlined in Table 3. Difference between HD-affected tissue and normal innervated tissue is highly significant (P < 0.001).

**Staining of nerve fibers for S100 and PGP9.5**

In normal tissue, S100 staining revealed intrinsic nerve fibers and negatively stained ganglion cells surrounded by positive glia cells and nerve fibers in the submucosa (Figure 4A and 4B). Whereas in HD-affected tissue, dense and prominent S100 immunostaining highlighted hypertrophied nerve trunks in the submucosa (Figure 4C and 4D).

PGP9.5 stained the hypertrophic nerve fibers intensely in HD affected tissue (Figure 4G and 4H). While in the normally innervated intestine, only granular staining of small nerve twigs presented in the submucosa, in contrast to strong staining of ganglion cells (Figure 4E and 4F). The qualitative assessment of the neural hypertrophy is displayed in Table 3.

**Patient cohort**

The diagnosis based on our initial diagnostic scheme was typical HD in 55 cases, non-HD in 135 cases and suspicious for HD in 5 cases (Table 4). Ganglion cells were identified in 135 patients on initial diagnostic protocol. Follow-up was available of all these patients. Their follow-up durations ranged from 12 to 52 months. Seventy-two patients were further evaluated by a full-thickness biopsy at our institution for persistent severe constipation and 21 of them underwent a Duhamel procedure. Two of the 21 cases were finally diagnosed as short segment HD, 13 were diagnosed as intestinal neuronal dysplasia type B and 6 were diagnosed as isolated hypoganglionosis. For the other 51 of the 72 patients we confirmed the presence of ganglion cells in the full thickness biopsies. Sixty-three patients were clinically cured by conservative therapies, and the HD was excluded.

Ganglion cells were absent in suction biopsies of 60 patients, and of these, 90% and 91% showed submucosal neural hypertrophy on S-100 staining and PGP9.5 staining, respectively. Fifty-five patients showed as typical HD-nerve hypertrophy combined with aganglionosis and were confirmed by followed colonic resection. Five patients were suspicious of HD for neither ganglion cells nor hypertrophic trunks were identified. After pull-through procedure due to persistent symptoms, two of them were confirmed as TCA, the other 3 were classified as isolated hypoganglionosis.

A final diagnosis of HD was made in 59 children (30.2%) and 55 of these were diagnosed by initial RSB. Of the 59 patients, 45 were male and 14 were female. As is shown in Table 4, the sensitivity and specificity rates of our initial diagnostic protocol was 96.49% (95% CI, 0.88-0.99) and 100% (95% CI, 0.97-1.00), respectively. As for immunostaining of full-thickness specimens, we experienced neither false positive results nor false negative ones compared with the final diagnosis.

**Discussion**

The mainstay in the diagnosis of HD is to confidently exclude the presence of ganglion cells in H&E stained sections from a colonic biopsy [12], which is still challenging for even seasoned pathologists, especially in the neonatal cases. There are several reasons that lead to the diagnostic challenge in neonates. On the one hand, immature ganglion cells in newborns may be missed by inexperienced pathologists. On the other hand, submucosal ganglia in neonates is sparser than older children, which necessitate more careful tissue preparation.

| Table 4. Results of initial diagnosis according to our diagnostic protocol and the final diagnosis based on surgery and clinical follow-up |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Initial diagnosis | Final diagnosis  | Sensitivity (95% CI) | Specificity (95% CI) | Accuracy (95% CI) | Inconclusive (%) |
| HD: 55 | HD: 59 | 96.49% (88.08%-99.03%) | 100% (97.19%-100%) | 98.95% (96.24%-99.71%) | 5 (2.56%) |
| Non-HD: 135 | Non-HD: 136 | 55/(55+2) | 133/(133+0) | 55+133/(55+0+2+133) |
| Suspicious: 5 | | | | |
| TP = true positive test result; FN = false negative test result; TN = true negative test result; FP = false positive test result; CI = confidence interval; HD= Hirschsprung’s disease. |
For several decades, researchers have sought reliable and simple specific methods to simplify HD diagnosis basing on suction biopsies. Pediatric surgeons have realized that in addition to the absence of ganglion cells, another striking and diagnostically useful phenomenon is the presence of hypertrophic nerve trunks in the submucosal plexuses [22]. The most widely utilized technique of identifying the hypertrophic nerve fibers is AChE histochemistry, which was initiated by Meier-Ruge et al. [10]. The advantage of this method is that it enables an adjunctive diagnosis with small biopsies contain little submucosa, and it requires inexpensive materials. However, several drawbacks have limited the universal application of this method. First, the procedure requires sophisticated techniques and frozen sections, and is time-consuming [16]. Particularly, the results of the staining are not always easy to interpret, it is documented to have high rate of interobserver disagreement as well as false positive results [23, 24].

The limitations associated with AChE histochemistry have fueled efforts to seek immunohistochemical markers that can facilitate the diagnosis of HD. Among several immunohistochemical makers, calretinin proved to be very valuable in identifying ganglion cells, even in suboptimal or inadequate RSB samples. Calretinin is known as a vitamin D-dependent calcium-binding protein, which was first advocated to be a potential HD marker by Barshack et al. in 2004 [13]. In this research the authors revealed that calretinin antibody stained nearly 80% of ganglion cells and small intrinsic nerve fibers in both myenteric and submucosal plexus throughout the normal intestine. By contrast, calretinin was not expressed in HD affected segments. This immunohistochemical staining pattern was confirmed by a later research conducted by Kapur et al. [24], in which the investigators concluded that calretinin immunostaining was superior to AChE staining as an adjunctive method in diagnosing HD. Additionally, calretinin was also proved to be of high value in the context of inadequate rectal suction biopsies and TCA [25, 26].

In our study, calretinin highlighted cytoplasm and nuclei of ganglion cells in both submucosal plexus and myenteric plexus. The distinct “black and white” staining patterns made them quite easy to interpret. In keeping with some previous studies [24, 27, 28], we also observed positive calretinin staining of small intrinsic nerve twigs in the muscularis mucosa and lamina propria. But such nerve fibrils were not stained in Yang’s study [14], in which only ganglion cells were positively decorated. In our opinion this discrepancy did not influence the diagnosis of HD, although this need further investigation.

Much like combined H&E and AChE staining strategy, we used another two immunohistochemical stains (S100 and PGP9.5) coupled with calretinin to offer double markers for ganglion cells and nerve trunks.

S100 is a low molecular calcium-binding protein and is normally present in cells derived from the neural crest such as Schwann cells. In our study S100 staining revealed negatively stained ganglion cells surrounded by normally stained glia cells and nerve fibers in the submucosa of normal intestine, in contrast to prominent staining of nerve trunks in submucosa of HD-affected tissue. This is consistent with the findings reported by Robey et al. [29]. Similarly, Barshack et al. [13] found that S100 immunostaining effectively highlighted the proliferation of the nerve fibers. Two other reports have contributed to the growing evidence that S100 immunostaining can specifically show neural hypertrophy in the submucosa of HD affected tissue [15, 16].

The nerve fibers can also be highlighted using immunohistochemical stain of PGP9.5. Sams et al. [30] have evaluated the value of PGP9.5 and S100 immunostain in the diagnosis of HD. They found that S100 staining assessed alone gave a higher false-negative rate in diagnosing HD than PGP9.5 used alone, so the combination of the two makers were recommended for the immunohistochemical diagnosis of HD. That’s why we selected PGP9.5 as an adjunctive marker for the neural hypertrophy at the beginning of our study. Unlike S100, which expressed only in nerve fibers and glial cells, PGP9.5 reliably stained nerve fibers as well as ganglion cells in the mucosal and submucosal plexuses. Intriguingly, the immunostaining pattern is quite different between normal and HD-affected tissue and is easy to discriminate (as shown in Figure 4). What is more important is that PGP9.5 immunostaining can be act as a supplement to calretinin staining of ganglion
Calretinin, S100 and PGP9.5 for diagnosing Hirschsprung’s disease

According to previous studies, we may expect that immunopositivity for calretinin in the submucosa is strictly correlated to the exclusion of HD. On occasion, this hypothesis seemed partially wrong [27]. For in short-segment HD, a slight calretinin positivity may be an indication of the beginning of a transitional zone [13]. In our study, calretinin immunostaining was positive in two false negative patients with authentic HD. Surgery revealed that only 1.5 cm and 1.8 cm of aganglionosis in the two patients, respectively. Attention should also be paid in case of TCA, for two such patients in our study showed submucosal nerve trunks less than 40 μm (29 μm and 33 μm, respectively) in maximum diameter. A previous study [17] has compared the nerve innervation between TCA and common-segment-type HD in 18 patients and found that the nerve trunks were indeed thicker in the latter group. To avoid these exceptional pitfalls, we must interpret the results of the stain in the context of the entire case.

In conclusion, RSB is a reliable and effective test that provides a histological diagnosis of HD. We can get an accurate result when the biopsy is obtained from the correct site. Our data suggest that the adjuvant immunohistochemical stains of calretinin, S100 and PGP9.5 is sensitive and specific for the identification of ganglion cells and hypertrophic nerve trunks in suction rectal biopsies. Each of them offer specific advantages, and the complimentary differences could contribute to a reliable diagnosis of HD.

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Disclosure of conflict of interest

None.

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