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When is the optimal diagnostic biopsy timing of acetylcholinesterase staining in Hirschsprung disease?

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Abstract

Purposes The preoperative diagnosis of Hirschsprung disease (HD) requires a rectal mucosal biopsy and acetylcholinesterase (AchE) staining. However, the optimal timing for performing these procedures remains unclear. In this study, we assessed the most effective timing for a rectal biopsy and AchE staining to diagnose HD.

Methods We retrospectively collected data from 57 patients who underwent radical surgery for HD at Mie University Hospital between January 2008 and April 2024. We reviewed all rectal biopsies, including those with multiple samples, and analyzed the biopsy date and the results of AchE staining (positive or negative).

Results Among the 57 children with a confirmed HD diagnosis, 36 were included in the study. Fifty-two rectal biopsy sessions were performed in these 36 patients with HD, including 15 with multiple biopsies. A receiver operating characteristic analysis showed that the optimal age for diagnosing HD was older than 21 days. Biopsies performed at ≤ 3 weeks of age had a low diagnostic accuracy rate of 30.8%, whereas rectal biopsies performed at > 3 weeks of age showed a high positive diagnostic rate of 87.5%.

Conclusions Rectal mucosal biopsies performed after 3 weeks of age show a higher diagnostic accuracy for HD.

Clinical trial number Not applicable.

Keywords Rectal biopsy, Acetylcholinesterase staining, Hirschsprung disease

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Introduction

Early diagnosis of Hirschsprung disease (HD) is crucial because serious complications, such as severe enterocolitis, toxic megacolon, and intestinal perforation, can arise without a definitive diagnosis and appropriate management. In a systematic review, Zhang et al. reported that preoperative enteritis was a risk factor for the development of postoperative Hirschsprung disease-associated enterocolitis (HAEC) [1]. Furthermore, the development of HAEC before radical surgery affects postoperative bowel function [2]. Based on this background, radical surgery should be performed as soon as possible after a definite diagnosis of HD disease is made.

An accurate diagnosis of HD relies on a combination of a rectal mucosal biopsy and acetylcholinesterase (AchE) histochemistry, which has become the established gold standard [3]. Nevertheless, premature infants, neonates, and total colonic aganglionosis cases show false negative results because of insufficient AchE staining [4–7]. Several studies have reported that the intensity of AchE staining is enhanced with age and growth [4, 8, 9]. Even if the initial AchE staining result is false negative, a repeat multiple rectal biopsy and AchE staining should be performed if HD disease is strongly suspected from clinical symptoms [4]. However, there is a risk of complications, such as rectal perforation and bleeding due to rectal biopsy, and this risk is higher in neonates and infants [10].

The above-mentioned findings suggest that HD should be diagnosed and treated as early as possible, before HAEC or other problems develop. However, early AchE staining of rectal biopsy specimens can not only lead to false negative results, but also increases the risk of bleeding and perforation because multiple additional biopsies may be required later. This dilemma has always affected clinicians. To overcome this hurdle, in this study, we aimed to evaluate the optimal timing of a rectal biopsy and AchE staining for the definitive diagnosis of HD disease in a retrospective manner. The findings from this study may provide useful clinical information.

Methods

Ethics statement

This study protocol using medical information of pediatric patients with HD was reviewed and approved by the ethics review board at Mie University Graduate School of Medicine (approval no. H2023-245). Informed consent was obtained in the form of opt-out on the web-site. Neonates and infants whose guardians or parents did not provide consent were excluded from this study.

Patient enrollment

Data of patients who underwent radical surgery for HD at Mie University Hospital from January 2008 to April 2024 were collected retrospectively. Patients with suspected HD first underwent a contrast enema and recto-anal manometry (including recto-anal reflex). A rectal mucosal biopsy was performed on the basis of the following criteria: the presence of symptoms suggestive of intestinal obstruction (including bilious vomiting, chronic constipation, and abdominal distension), radiographic evidence of intestinal dilatation, and identification of a "caliber change" indicative of the transitional zone associated with HD during contrast enema. Furthermore, recto-anal manometry showed decreased basal rhythmic contractions below 10 times/min and the absence of a recto-anal reflex. Figure 1 shows enrollment of the patients in this study. Patients with a confirmed diagnosis of HD who underwent radical surgery were selected during the study period. All of these patients were definitely diagnosed with HD by a pathological analysis of resected intestinal specimens. In addition, to investigate the optimal timing (days of age) of the rectal mucosal biopsy, patients who had a stoma created at the first operation before the diagnosis of HD ($n = 9$) and those who were older than 1 year of age at the time of the first biopsy ($n = 12$) were excluded from this study. Patients whose initial rectal mucosal biopsy did not confirm the diagnosis of HD, but who were strongly suspected of HD on the basis of other test results, had a repeated biopsy at least 2 weeks after the initial biopsy.

Rectal mucosal biopsy and AchE staining

Rectal mucosal biopsies were performed using at least two samples between 1 and 2 cm above the dentate line without general anesthesia. All of the samples were frozen immediately by liquid nitrogen and embedded in a cryostat. Sliced Sect. (10 μm) were placed on microscopic slides, and AchE histochemistry and hematoxylin and eosin staining were conducted. AchE staining was performed according to the method of Karnovsky and Roots, modified by El-Badawiand and Schenk [11, 12].

Diagnostic criteria for HD

The diagnosis of HD was determined by the results of AchE, and the following method reported by Nakao et al. was used to determine whether the results of AchE staining were sufficient for the diagnosis of HD [4]. Briefly, we categorized the grade of proliferation of AchE-positive fibers as follows: (-), no proliferation of AchE-positive fibers in the lamina propria mucosa; (±), slight proliferation of thin fibers only at the bottom of the lamina propria mucosa; (+), obvious proliferation in the lower part of the lamina propria mucosa; (++), AchE-positive fibers extended to the tip of the lamina propria mucosa; and (+++), AchE-positive fibers diffusely proliferated, running transversely, and creating a network. We diagnosed

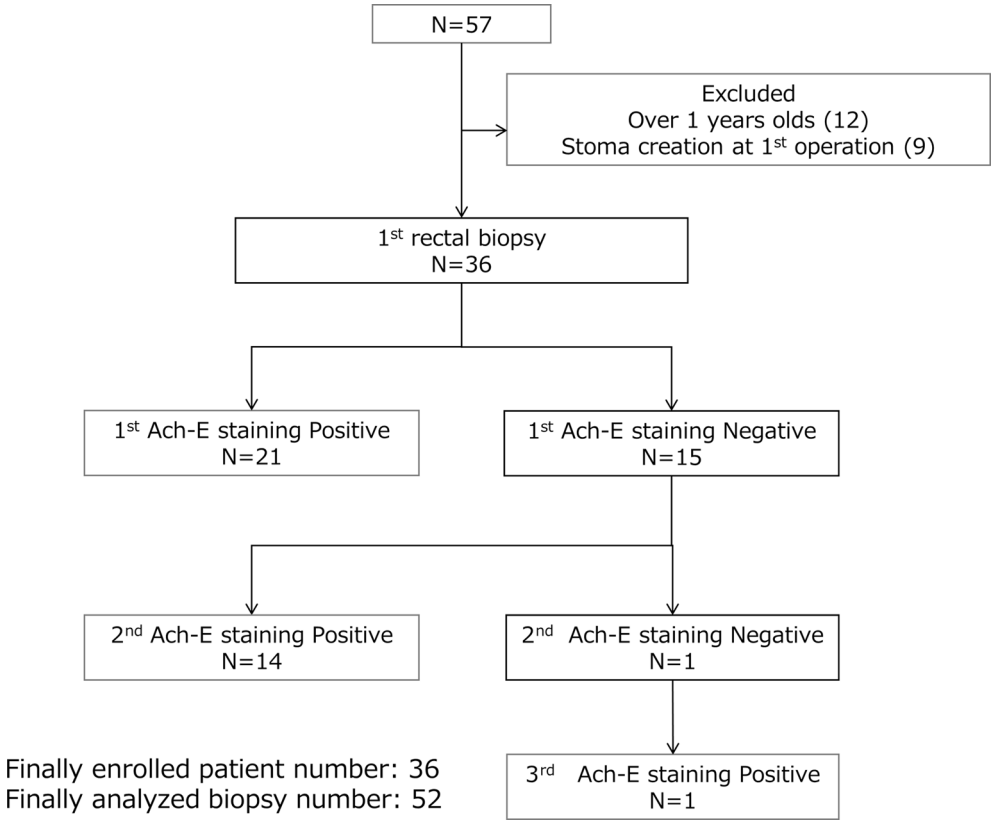

Fig. 1 Patient enrollment. Fifty-seven patients with a confirmed diagnosis of HD who underwent radical surgery were selected during the study period. All of these patients were definitely diagnosed with HD by a pathological analysis in the resected intestinal specimens. Fifty-two sessions of rectal biopsies were performed in the 36 patients with HD enrolled in this study

HD when there was proliferation of AchE-positive fibers(± to +++) [4].

Statistical analysis

Data were analyzed using JMP Pro 16 (SAS Institute, Inc., Cary, NC, USA). Continuous variables are expressed as the median ± interquartile range. The F-test was performed to compare the variance of each variable between groups. Subsequently, because of the non-normal distribution of the data, the Mann–Whitney U test was used to analyze the differences between groups. Categorical variables were compared using the chi-square test or Fisher's exact test if the expected counts were less than five. Receiver operating characteristic (ROC) curves with Youden's index were established to identify the timing of positive AchE staining (age in days). The sensitivity and specificity of a definite HD diagnosis in two groups of patients (positive HD diagnosis and negative HD diagnosis by a rectal mucosal biopsy) were calculated. All p values were calculated using a two-tailed test, and $p < 0.05$ was considered statistically significant.

Results

Summary of patients enrolled and rectal biopsies

Of 57 children with a confirmed diagnosis of HD, 36 were finally enrolled in the study, after excluding those older than 1 year of age and those who had a colostomy at first surgery. Multiple rectal biopsies were required in 15 patients. Of the 15 patients who required two biopsies to diagnose HD, one patient underwent a third biopsy because the second biopsy did not result in a definitive diagnosis of HD. Fifty-two sessions of rectal biopsies were performed in the 36 patients with HD who were enrolled in the study (Fig. 1). The HD diagnosis rate at the first rectal biopsy was 58.3% (21/36) and that at the second rectal biopsy was 93.3% (14/15). Table 1 shows the 36 patients' characteristics. Of these, 31 were boys and 5 were girls. The median age at radical surgery was 89 days (50.2–129.5 days). The median gestational age was 39.2 days (37.6–40.0 days), and the median birth body weight was 3079 g (2800–3364 g). The median age at the first biopsy was 20.5 days (10.5–72.5 days) and that in the second biopsy was 80.0 days (34.8–120 days).

Table 1 Patient characteristics

Clinical variables	(*n* = 36)
Sex (male / female)	31 / 5
Gestational age* (weeks)	39.2 (37.6–40.0)
Birth body weight* (g)	3079 (2800–3364)
Chromosomal abnormality (Y/N)	3 / 33
Operation age* (days)	89 (50.2-129.5)
Multiple rectal biopsies (Y/N)	15 / 21
Day of age at 1st rectal biopsy* (days)	20.5 (10.5–72.5)
Day of age at 2nd rectal biopsy* (days)	80 (34.8–120)

*Data are presented as median & interquartile range

Relationship between AchE results and days of age at the rectal biopsy

Figure 2 A shows a plot of the days of age at rectal biopsies that led to the diagnosis of HD (positive) and the days of age at rectal biopsies that did not lead to the diagnosis of HD (negative) based on the results of AchE staining in 52 rectal biopsies in this study. The positive group was significantly older than the negative group ($p = 0.012$). The median difference in days between the first and second rectal biopsies was 41.5 days (17.5–98.85 days).

ROC curve analysis for the days of age when AchE was positive

Figure 2B shows the ROC curve analysis of the days of age with positive AchE staining results. In this analysis, the sensitivity and specificity were 77.8% and 75%, respectively. The area under the curve was 71.9%, and the cutoff value was 21 days of age for the best timing for diagnosing HD.

Comparison of positive and false negative diagnostic rates between ≤ 3 weeks and > 3 weeks of age at the rectal biopsy

On the basis of the results of the ROC analysis, two new groups were created: patients with aged ≤ 3 weeks at the biopsy and patients aged > 3 weeks at the biopsy. At ≤ 3 weeks, the false negative rate was as high as 69.2% in children with a final diagnosis of HD, whereas at > 3 weeks, this rate was significantly lower at 12.5% ($p < 0.01$, Fig. 3). However, at ≤ 3 weeks, the rate of an accurate HD diagnosis was as low as 30.8%, whereas a high positive rate of 87.5% was obtained for a rectal biopsy performed at > 3 weeks of age.

Discussion

To the best of our knowledge, this is the first study to analyze the optimal timing of a rectal mucosal biopsy and AchE staining for diagnosing HD. Hirsig et al. first hypothesized that the number of AchE-positive nerve fibers in HD gradually increases after birth, and thus a negative result of AchE staining in the neonatal period should not exclude the diagnosis of HD [13]. De Brito

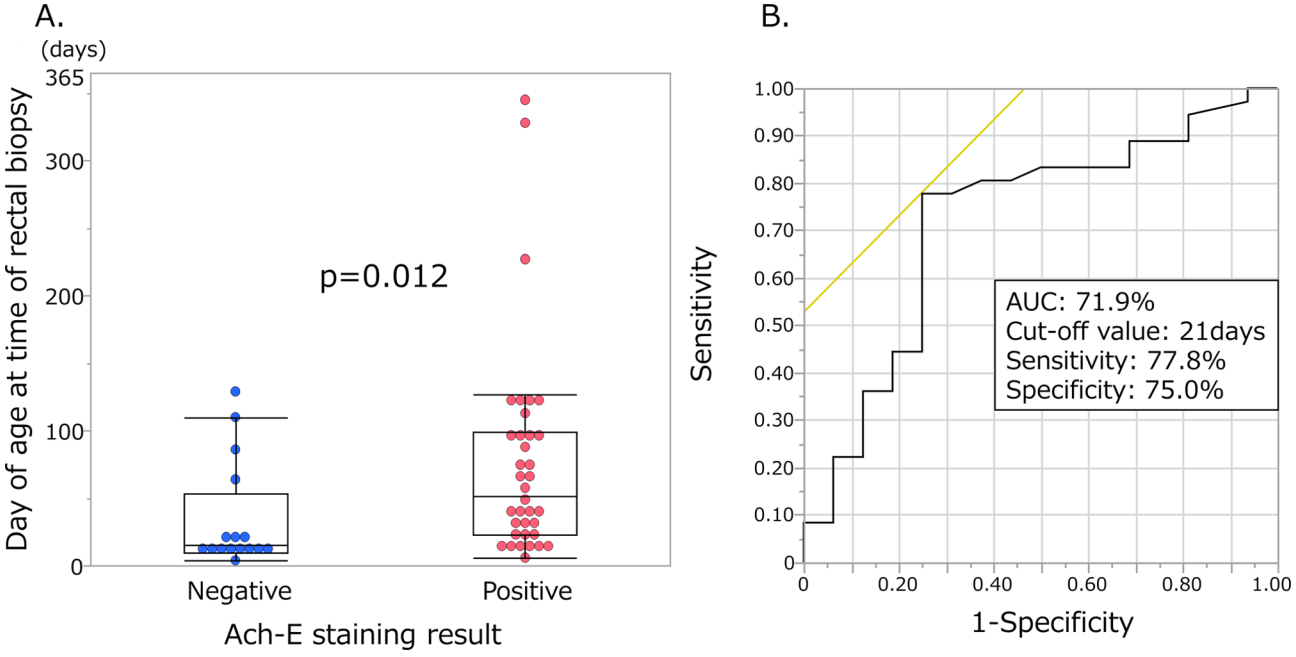

Fig. 2 **A.** Relationship between AchE results and days of age at rectal biopsies. A plot of the days of age at rectal biopsies that led to the diagnosis of HD (positive) and the days of age at rectal biopsies that did not lead to the diagnosis of HD (negative) based on the results of AchE staining ($n = 52$ rectal biopsies) is shown. The positive group was significantly older than the negative group ($p = 0.012$). **B.** ROC curve analysis of the days of age when AchE staining was positive. The ROC curve analysis to evaluate the performance of AchE staining in relation to the days of age. The sensitivity and specificity were 77.8% and 75.0%, respectively. The area under the curve (AUC) was calculated to be 71.9%. The optimal cutoff value for diagnosing HD was identified as 21 days of age

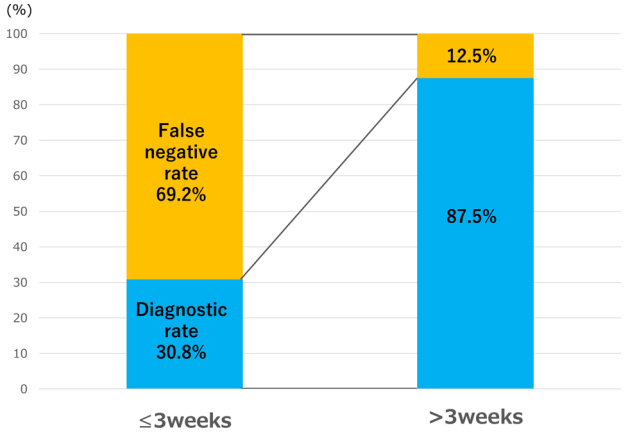

Fig. 3 Comparison of positive and false negative diagnostic rates between ≤ 3 weeks and > 3 weeks of age at rectal biopsies. At ≤ 3 weeks of age, the false negative rate was higher than that at > 3 weeks of age. However, at ≤ 3 weeks of age, the rate of an accurate HD diagnosis was low, whereas a high positive rate was obtained in rectal biopsies performed after 3 weeks of age

et al. observed that AchE activity underwent changes with age [14]. In newborns, AchE-positive reactions are primarily expected in the submucosa and muscularis mucosa, while the absence of an AchE reaction in the lamina propria should not be considered a false negative result. Goto et al. compared the results of AchE staining in neonatal cases, which were divided into three groups (days 0–10, 11–20, and 21–30), and reported that 21–30 days was safe for performing a biopsy to diagnose HD [9]. Interestingly, the age range of 21–30 days reported by Goto et al. is almost identical to the cutoff value of > 3 weeks of age, which was the optimal timing found for a rectal mucosal biopsy in this study. Goto et al.'s study and our study suggest that the most appropriate timing for the first rectal biopsy and AchE staining is > 3 weeks after birth in cases of suspected HD.

Nakao et al. reported that although the diagnosis of HD by AchE staining was ambiguous in the neonatal period, the diagnosis of HD was almost certainly made at 100 days of age or older [4]. Goto et al. also reported that the diagnosis of HD by AchE in the neonatal period was likely to be false negative [9]. However, their results are mostly consistent with the results of the present analysis [4, 9]. Unfortunately, these studies did not use a statistical approach, and were thus not able to adequately analyze the results to identify the optimal rectal biopsy timing in HD. In this study, the best cutoff value for when to conduct a rectal biopsy was calculated using a statistical approach. We found that the positive diagnostic rate increased from 30.8% at ≤ 3 weeks of age to 87.5% at > 3 weeks of age, thus enabling a more definitive analysis of the optimal timing of performing a rectal mucosal biopsy and AchE staining.

This study has some limitations. First, we did not compare our results with those of new staining methods such as immunostaining with calretinin, and the optimal timing of a rectal biopsy with other staining methods may differ from that with AchE staining [15]. However, because calretinin stains only the nerve plexus in the submucosa, we were not always able to obtain sufficient submucosal tissue in biopsy samples, and whether calretinin or AchE is generally superior is still debatable. Second, since this study did not include patients who remained AchE negative after their biopsy, it remains unclear in the current study whether a negative biopsy at this 3-week postnatal timing can exclude HD. Third, the level of evidence was not high owing to the small number of patients and the retrospective nature of the study. Based on the findings of this study, we recommend conducting a prospective, multicenter study with a larger patient cohort to obtain data with a higher level of evidence.

Using statistical methods in this study, this study suggests that the optimal timing for a rectal mucosal biopsy to diagnose HD should be performed after 3 weeks of age. This approach allows for an accurate diagnosis of HD with the fewest necessary biopsies. The dilemma that preoperative enterocolitis can easily lead to sepsis and postoperative defecation can be impaired is faced by many pediatric surgeons. Therefore, HD should be diagnosed as early as possible. However, an early postnatal biopsy does not provide a definitive diagnosis of HD because AchE-positive nerve fibers have not sufficiently grown yet. We believe that the interesting results from our study will resolve this dilemma.

In conclusion, this study shows that rectal mucosal biopsies performed after 3 weeks of age result in a higher diagnostic accuracy for HD than those performed before this time. Clinicians may find this information useful as an indicator for when to perform a rectal biopsy in their routine practice.

Abbreviations

HD	Hirschsprung disease
AchE	Acetylcholinesterase
HAEC	Hirschsprung disease-associated enterocolitis

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Author contributions

Y. K.: Designed the study, operated on the patients, acquired data, analyzed the data, and wrote the manuscript. K. H.: Acquired the data. Y. S.: Acquired the data. S. Y.: Acquired the data. Y. N.: Acquired the data. K. M.: Operated on the patient and provided surgical information. T. S.: Acquired and analyzed the data. T. K.: Acquired and analyzed the data. Y. O.: Provided expert advice. Y. T.: Designed the study and wrote the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Human ethics approval and consent to participate
This study protocol using medical information of pediatric patients with HD was reviewed and approved by the ethics review board at Mie University Graduate School of Medicine (approval no. H2023-245). Informed consent was obtained in the form of opt-out on the web-site. Neonates and infants whose guardians or parents did not provide consent were excluded from this study. Because the need for written informed consent to participate was waived by an Institutional Review Board [Mie University Graduate School of Medicine (approval no. H2023-245)], written informed consent for publication of identifying images or other personal or clinical details was not obtained from the parents or legal guardians of any participant under the age of 18.

The ethics declaration
In accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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