Original Research Article

Reliability of S-100 and Tuj-1 immunofluorescence markers in the diagnosis of Hirschsprung's disease

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Received: 02-11-2021 / Revised: 18-12-2021/ Accepted: 09-01-2022

Abstract

Background: Hirschsprung's disease (HSD) is a congenital malformation of the hindgut characterised by absence of intrinsic ganglion cells. Acetyl cholinesterase (AChE) staining is used as a gold standard test in HSD. Intramucosal neuroglial cells (INCs) are present in aganglionic colonic mucosa of HSD patients, challenging our current understanding.**Objectives:** To evaluate for the presence of INCs using S100 and Tuj 1 and to evaluate for the presence of ganglion cells (GCs) using Tuj1 immunofluorescence in clinically suspected cases of HSD.**Materials and Methods:** This study was carried out in the Department of Pathology, Bangalore Medical College and Research Institute, Bangalore on 35 colorectal biopsies of patients suspicious of HSD. Direct immunofluorescence (DIF) was done on rectal biopsies using Tuj1 and S100 markers.
Results: Out of 35 biopsies, 24 showed absence of Tuj 1 and S 100 expressing cells in the biopsy and were diagnosed as HSD and 8 cases showed presence of Tuj 1 and S 100 expressing cells in the biopsy and were diagnosed as not suggestive of HSD. 3 were excluded as they were low rectal biopsies. Tuj 1 and S 100 DIF showed sensitivity of 100% and specificity of 72.7%.**Conclusion:** DIF markers Tuj 1 and S 100 were found to be more sensitive than AChE stain and these markers are helpful in detecting false negative cases missed by AChE. A larger cohort is necessary to consider these DIF markers as an additional tool in evaluatingand ruling out HSD in cases where AChE stain is equivocal.

Keywords: Hirschsprung's disease; Direct Immunofluorescence; Intramucosal neuroglial cells; Acetylcholinestarase.

Introduction

Hirschsprung's disease (HSD) is a congenital malformation of the hindgut diagnosed on the basis of absence of intrinsic ganglion cells in the submucosal and myenteric plexus.The disease occurs in approximately 1 of 5000 live births. The definitive diagnosis of the disease is entirely based on histological results.[1,2] The diagnosis can be made when no ganglion cells are seen in rectal biopsies on histology. However unlike full thickness rectal biopsies where the submucosal and myenteric plexuses are available for a detailed study, reporting on minute mucosal biopsies is challenging.

The standard histology obtained from rectal mucosal biopsies demonstrating aganglionosis requires analysis of 100 or more histological sections, moreover difficulties in analysis may arise in situations when

- Sample is too superficial with not enough sub mucosa and
- There is difficulty in identifying ganglion cells with confidence, particularly in patients of neonatal age group.

For this particular reason standard histology is frequently supplemented with Acetylcholinesterase histochemistry (AChE). This technique provides quick results but requires additional technical expertise and Cryostat facilities.Once the diagnosis is established, the definitive treatment is operative intervention like primary pull-through procedure.[3]Although Acetylchlolinesterase (AChE) staining is used as a routine test in the diagnosis of HSD, it sometimes becomes very difficult to interpret.Therefore, there is a need for a new, easy and rapid method to diagnose HSD.

Intramucosal neuroglial cells (INCs)

The current understanding of HSD is based on two fundamental concepts:

- All intrinsic enteric neurons and glia are derived from the neural crest; and
- HSD is caused by a defect in the rostrocaudal migration, survival, or differentiation of these neural crest-derived cells.Enteroglial cells (EGC) are described as stellate cells expressing specific glial markers such as S100 and glial fibrillary acidic protein (GFAP), while neurons are described as dendritic cells with a prominent cell body expressing specific neuronal markers such as neuron-specific class III β-tubulin (Tuj1) and calcium-binding protein calretinin.Intramucosal neuroglial cells (INCs) are a group of cells which express both the S100 and Tuj 1 markers indicating neurglial differentiation.

Kamran Badizadegan et al detected an intramucosal network of interconnected dendritic cells with overlapping neuronal and glial differentiation. They designated this cellular network as INCs, which were distinct in morphology and in number from the occasional intramucosal ganglion cells. Confocal imaging of colon mucosa in formaline fixed paraffin embedded tissue sections from the proximal ganglionated colon of patients with HSD showed Tuj1 and S100

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coexpression. Aganglionic mucosa from the same patients with HSD revealed persistence of these double-expressing cells, though with a reduction in Tuj1 and S100 expression. These cells also expressed the neural crest cell marker HNK1.[4]Bassotti G et al, in their review on Enteric glial cells and gastrointestinal motility have concluded that enteric glial cells are essential for maintaining the homeostasis of enteric neurons, these cells support and stabilize the Enteric nervous system (ENS) through continuous adaptations to the structural and metabolic impairments of the gut wall.[5]

Study conducted by Wilkinson DJ et al, shows isolation of enteric nervous system progenitor cells in 12 patients with short segment HSD and 1 patient with long segment form HSD. The fact that neuronal progenitors can be isolated from aganglionic HSD gut raises the possibility of using aganglionic tissue, normally discarded at surgery, to provide a source of cells for future autologous transplants in the treatment of HSD. Additionally, the existence of these cells raises the intriguing possibility to design future therapeutic modalities to stimulate neurogenesis in the aganglionic region in vivo, removing the need for surgery.[6]

A study conducted by Benjamin N Rollo et al, on colon tissue obtained immediately after surgery from 31 HSD patients, which included 24 patients aging less than 4 months and 4 were familial forms of HSD, showed that neural crest lineage cells can be obtained from HSD patient colon and can form enteric nervous system like structures in aneuronal colonic muscle from the same patient.[7]

A study conducted by Gabsang Lee et al, showed that transplantation of human neural crest stem cells into the developing chick embryo and adult mouse hosts demonstrates survival, migration and differentiation compatible with neural crest identity. The availability of unlimited numbers of human neural crest stem cells offers new opportunities for studies of neural crest development and for efforts to model and treat neural crest-related disorders.[8]

The identification of INCs in the setting of aganglionosis lead to new hypotheses.

- INCs may not reach the distal bowel via the rostrocaudal wave of crest cell migration, which appears to be the path taken by ganglion cells.
- Perhaps HSD represents a selective defect affecting only ganglion cells, rather than all intrinsic cells of the ENS.

Although many studies have been conducted on the sensitivity and specificity of AChE and compared it with the routine H&E stain and other markers like calretinin, none of the studies so far have evaluated the usefulness of DIF markers like S100 and Tuj 1 in diagnosing HSD. Also the study by Kamran Badizadegan et al[4] evaluated for the INCs in only a case series of surgical resections in three children with confirmed HSD.There is need for a detailed evaluation of the presence of INCs and their role in the rectal biopsies of HSD cases. Therefore, this study was aimed at detecting these INCs in the colorectal biopsies taken from suspected cases of HSD.

Material & methods including Statistics

Sample size calculation

It was a prospective study.At 95% confidence level. Design effect of 1. According to the study conducted by Kamran Badizadegan et al[4] the proportion of suspected cases of Hirschsprung's disease showing positive for Tuj 1 and S100 is 99%=p. At 20%, Absolute precision, Sample size estimated is 24. Taking 10% extra for sample loss=26, which is rounded off to 30.

Formula used n= [DEFF*Np(1-p)]/ [(d2/Z21-α/2*(N-1)+p*(1-p)].

After obtaining approval and clearance from the institutional ethical committee, a total of 35 biopsies were received in our department during the study period of 18 months. Patients presenting with clinical features (delayed/non passage of meconeum or chronic constipation) were included in the study. Suction rectal biopsies were received in gauze piece soaked with 0.9% saline. The tissue was oriented on the chunk containing a base of Optical clear transparent media (OCT). The tissue is frozen at -25^{o}C for 20 minutes and 10 µm thickness sections were cut and were taken on frosted slides.

One section was stained with rapid Haematoxylin & Eosin staining method and was analysed for the orientation of the mucosa and submucosa, to ascertain the adequacy and check for ganglion cells and hypertrophic nerve twigs (Figure 1-4). One section was stained with Acetylcholinesterase staining as a routine for the detection of abnormal activity of neurons in the submucosa and mucosa. Additional two sections were taken, one of which was stained with S100 DIF marker to look for intramucosal neuroglial cells and the other section was stained with Tuj1 DIF marker to look for ganglion cells and neuroglial cells. The remaining tissue was kept fixed in formalin and processed for routine histopathological examination.

Interpretation of AChE histochemistry

In the present study, the rapid modified AChE stainingtechnique of Kini et al[9] was used. The original AChEstaining method of Karnovsky and Roots[10] as detailed by Meier-Ruge[11] was further modified by Kini et al[9] to suit a general histopathology laboratory in a developing country like India. The nerve fibers/RBC's are stained black and serve as inbuilt controls. Cytoplasm of the ganglion cell stains positive (black) while its large nucleus is negative. The nucleus appears as negative shadow in a black background.

Pattern A.

Nerve fibers in the muscularis mucosae, submucosa and in between the crypts in the lamina propria stain positive and appear black. The nerve fibers travel in between the crypts and can reach the surface. The pattern resembles a tree. This is also known as the mature pattern as it is usually seen in infants > 3–6 months of age.[12]

Pattern B.

Nerve fibers in the muscularis mucosae, submucosa and at the base of the crypts stain positive and appear black. Nerve fibers do not travel up along the crypts. The pattern is similar to a tree pruned just above the trunk. This is also known as the immature pattern as it is usually seen in neonates and infants < 3 months.[12]

Equivocal pattern.

Staining of nerve fibers occurs in the submucosa only. This pattern can be associated with or without hypertrophied nerve bundles in the submucosa, which is important to assess. Equivocal pattern with hypertrophied nerve trunks, with clinical scenario and radiological findings suggests a diagnosis of HSD. Equivocal pattern without hypertrophied nerve trunks is truly equivocal and a diagnosis of HSD cannot be suggested. It can be usually seen in cases of constipation due to other reasons.[12]

Negative pattern.

No stained nerve fibers are seen in the muscularis mucosae and lamina propria, but small twigs are seen in the submucosa. The myenteric plexus or the submucosal ganglion cell however stains positive with negative shadows of the nuclei of the ganglion cells, which if present, strongly suggests the presence of ganglion cells and negates the diagnosis of HSD. In a suction biopsy however this advantage is not there and a negative pattern may also indicate that the stain has not worked and demands a repeat.[12]

A diagnosis of HSD was made when no ganglion cells were seen on H&E stained sections and a positive AChE pattern (A or B) was noted. The tissue is kept in cryostat for 24 hours at 25 ^{0} c and later given for routine histopathology processing.

Immunoflorescence staining protocol

2 cryostat sections of 10 micrometer thickness were taken on a slide. The sections were fixed by dipping in Methanol and then washed with Phosphate buffered saline (PBS) pH 7.5 for 10 min. Later one section was incubated with optimal concentration of labelled mouse anti-beta III tubulin (Tuj1), isotype IgG2 was used from Medaysis Enable Innovation and the other section was incubated with mouse anti-S100 (isotype IgG2a/k) from Medaysis Enable Innovation for a period of 45 minutes at 37°C. The slides were washed gently in PBS pH 7.5 and then mounted in buffer glycerin mixtures. These stained

sections were screened in an incident light under Olympus research microscope fitted with an HBO 50 ultraviolet lamp.

Results

35 rectal biopsies from suspected cases of HSD were received in our institution during the study period. Of the 35 cases, rapid H&E showed stratified squamous epithelium suggesting the diagnosis of low rectal biopsy and DIF was not done in these 3 cases. Thus they were eliminated from the study. Remaining 32 biopsies were screened for presence of ganglion cells on rapid H&E and showed absence of ganglion cells in all the 32 biopsies. AChE staining and DIF was performed and the results were compared in these 32 cases.

AChE staining results

On the basis of AChE staining pattern 18 (56.2%) cases out of 32 were diagnosed as HSD and remaining 14 (43.8%) cases were reported as not favouring HSD. Out of the 18 cases that were diagnosed as HSD, Pattern A was seen in 5 (15.6%) cases (Figure 5 and 6) and Pattern B was seen in 13 (40.6%) cases (Figure 7). Rest of the 14 (43.8%) cases were reported as equivocal and not favouring HSD (Figure 8). The total number of cases showing different patterns of AChE staining has been shown in Table 1.

Out of 18 cases that were diagnosed as HSD on the basis of AChE staining, 13 (72.3%) cases were male patients and remaining 5 (27.7%) were females. Most common presenting complaint in cases diagnosed as HSD was delayed passage of meconium in 12 (66.6%) cases, followed by chronic constipation in 6 (33.3%) cases.

Immunofluorescence stainingresults

Frequency of expression of INCs using Tuj 1 and S 100 DIF markers in all the 32 cases is shown Table 2. When correlated with the diagnosis made on AChE staining interpretation pattern, 18 cases that were diagnosed as HSD on AchE stain showed absence of INCs in the mucosa with Tuj-1 and S-100 markers (Figure 9 and 10), which favours a diagnosis of HSD concurring with AChE diagnosis. These cases were considered as true positive for HSD.

Whereas out of the 14 cases that showed equivocal pattern on AChE and were diagnosed as not HSD, only 8 cases showed presence of INCs with our DIF markers, not favouring HSD (Figure 11 and 12)concurring with AChE diagnosis. These cases were considered to be true negative for HSD. Remaining 6 cases showed absence of INCs with Tuj-1 and S-100 markers, still favouring a diagnosis of HSD. Table 3 shows correlation between AChE and DIF markers expression.Out of these 6 non-concordant cases, on routine formalin fixed paraffin embedded tissue 3 cases showed presence of ganglion cells in the submucosa. Hence it appears that these 3 cases were false positive for HSD. Remaining 3 cases did not have sufficient amount of tissue to be processed in formalin and were followed up clinically. On following up we came to know that these 3 cases were treated as HSD on the basis of high clinical and radiological suspicion. These patients underwent levelling biopsy which concurred with clinical opinion and endorectal pull through was done. Hence it appears that AChE stain failed to pick up these 3 cases and were false negative for HSD but were detected by our DIF markers. Table 4 shows relation between final clinical diagnosis and diagnosis based on AchE and DIF markers. When statistical analysis for sensitivity and specificity of both AChE staining and DIF markers was performed sensitivity of AchE stain was found to be 85.71% and specificity was 100%. Whereas sensitivity of Tuj-1 and S-100 was 100% and specificity is 72.7%.

Discussion

This study was aimed at analysing the utility of DIF markers S 100 and Tuj1 to detect the presence of INCs in rectal biopsies in suspected cases of HSD. In the present study, of the 35 suspected cases of HSD, 3 biopsies were eliminated because of the presence of overlying stratified squamous epithelium. If the biopsy is taken from the normal physiological hypoganglionic area of the anorectal region, an erroneous diagnosis of HSD will be made, as ganglion cells may not be found. Therefore, AChE and DIF could not be done in these 3 cases. AChE and DIF staining was done in remaining 32 cases.

The incidence of HSD in the present study was more in males compared to females with males being 72.3% of cases and females were remaining 27.7% of cases. The M:F ratio is 2.6:1 in our study which is in concordance with Agarwal et al study which showed M:F ratio of 2.5:1 and Schofield DE et al study which showed M:F ratio of 4:1.[12,13] In the present study the most common symptomatic presentation was delayed passage of meconium (66.6%), which is in in concordance with the study conducted by Zamir N et al.[14] Whereas the study conducted by M Izadi et al showed that the most common presentation of HSD was abdomen distension.[15] This could be due to the age of the patients as most of our study group were comprised of neonates.In concordance with studies conducted by Agarwal et al,[12] Guinard et al[16] and Serafini S et al,[17] our study also showed that AChE stain can be a useful aid along with the H &E in diagnosing HSD. Table 5 shows results of AChE diagnosis in different studies. Whereas the study by Kamran Badizadegan et al[4] evaluated for INCs in a case series of surgical resections in only 3 children with confirmed HSD, in our study we evaluated for the presence/absence of INCs and their reliability in diagnosing HSD in 32 colorectal biopsies and compared it with the AChE diagnosis and final clinical diagnosis. In the present study we found that out of the 6 non-concordant cases, 3 were false positive for HSD by the DIF markers. This usually happens due to technical issues during staining procedure or during the evaluation of the immunofluorescent slides.

Also we found that 3 cases were false negative for HSD by AChE staining which is widely used and considered to be the gold standard. False negative results in AChE stain can be obtained in neonates, ultra shortsegment HSD, Total Colonic Aganglionosis, technical factors, poor orientation of theminute biopsy, focal increase in AChE activity that maybe missed and inexperienced hands.Rectal biopsies taken proximal to splenic flexure does notgive typical staining pattern in a case of HSD. Also AChE staining requires handling of toxic reagents.Due tocomplex technology of preparation and lack of pathologists' experience, AChE histochemistry seems cumbersome and thus is used in specialized pathology centersonly. We found that DIF markers Tuj 1 and S 100 are specific reliable immune markers for ruling out diagnosisof suspected HSD. Our DIF markers detected the 3 false negative HSD cases that were missed by the AChE stain and the sensitivity of Tuj-1 and S-100 was 100%. Hence these markers are technically simple and easy to interpret and can be used whenever AChE stain is equivocal.Our study had several limitations, largely due to the size of the study sample and constraints posed by the use of human tissue. As HSD is a rare congenital disease we were only dependent on the samples we received in our laboratory during the study period of 18 months. We had to rely on the quantity of the tissue received, which was a small suction rectal biopsy and we didn't have the liberty to perform repeated tests as it was an intra-operative testing procedure. Also we had little control over clinical variables, such as patient demographics, clinical presentation, surgical approach, or quantity or quality of tissue available for study. DIF markers as we know will not be stable and usually get faded away. Because of this we could not store the stained slides and had to rely on the images.

Conclusion

Hirschsprung's disease is a developmental disorder seen more commonly in males than females. Delayed passage of meconium is the most common mode of presentation followed by chronic constipation. Acetylcholinesterase staining is the established standard technique for diagnosing HSD with a specificity of 100%, in the present study sensitivity of AchE was found to be 85.7%. The low sensitivity of the stain can be attributed to absence of well established pattern and lack of technical expertise in interpreting the pattern. In our study sensitivity and specificity of Tuj 1 and S 100 DIF markers were 100% and 72.7% respectively.The 3 false negative cases on AchE in our study when subjected to Tuj 1 and S 100 DIF markers showed absence of INCs suggesting a diagnosis of HSD which were in concordance with clinical opinion. Hence these

markers would give additional information in evaluating HSD when AchE stain is equivocal.

DIF markers Tuj 1 and S 100 staining and interpretation is a relatively simple method and needs less technical expertise in the evaluation of HSD when compared to AchE stain. In view of high sensitivity, but low specificity of the DIF markers Tuj 1 and S 100, a larger cohort study is necessary to consider these markers as an additional tool in the evaluation of HSD.

Table 1: Showing Acetylcholinesterase staining pattern

AchE staining pattern	Frequency	Percent
Equivocal	14	43.8%
Pattern A	05	15.6%
Pattern B	13	40.6%
TOTAL	32	100%

Table 2: Showing frequency of expression of INCs using Tuj 1 and S 100 DIF markers

Remarks	Frequency	Percent
Negative for neuroglial cells in the mucosa	24	75.0%
Positive for neuroglial cells in the mucosa	08	25.0%
TOTAL	32	100.0%

Table 3: Correlation between AChE and DIF markers expression

DIF markers	Diagnosis on AchE stain		Total
	HSD	Not HSD	
Negative for neuroglial cells in the mucosa	18	6	24
Positive for neuroglial cells in the mucosa	00	08	08
TOTAL	18	14	32

Table 4: Showing relation between final clinical diagnosis with AchE and DIF markers in HSD and not HSD

Stain	Final Clinical Diagnosis		Total
	HSD (21)	Not HSD (11)	
AchE stain	18	14	32
Tuj 1 and S 100 markers	24	08	32

Table 5: Comparing AChE interpretation in different studies.

	Present study (n-35)	Agarwal et al (n-73) [9]	Guinard et al (n-131) [13]	Serafini S et al (n-50) [14]
HSD	18(51.5%)	17(24%)	40(30.5%)	26(52%)
NON HSD	14(40%)	56(76%)	91(69.4%)	24(48%)
Low rectal biopsies	3(8.5%)			

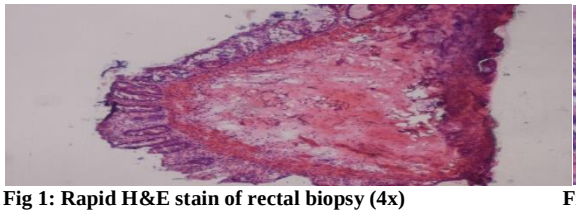

Fig 1: Rapid H&E stain of rectal biopsy (4x)

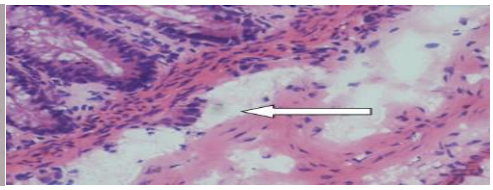

Fig 2: Rapid H&E stain showing sustentacular cell in submucosa (40x)

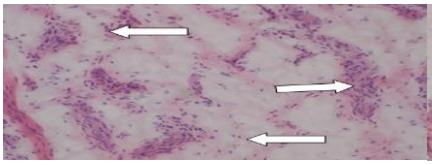

Fig 3: Rapid H&E stain showing hypertrophic nerve fibres in submucosa (10x)

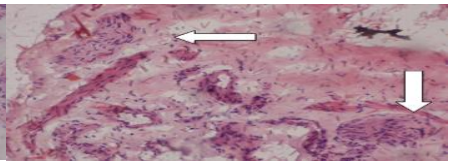

Fig 4: Rapid H&E showing hypertrophic nerve fibres (20x)

Fig 5: AchE stain showing Pattern A (10x)

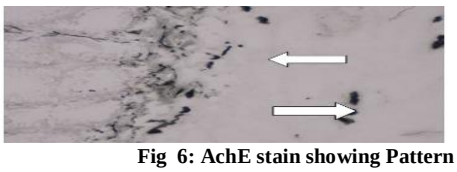

Fig 6: AchE stain showing Pattern A (40x)

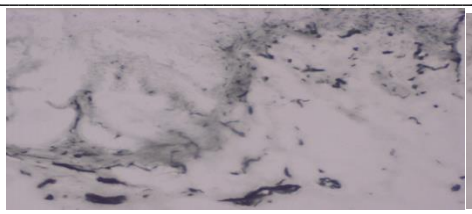

Fig 7: AchE stain showing Pattern B (40x)

Fig 8: AchE stain showing equivoval pattern (40x)

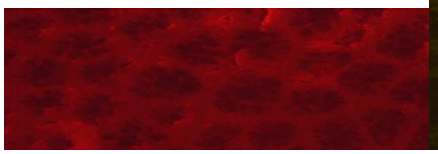

Fig 9: S100 DIF marker negative in mucosa (40x)

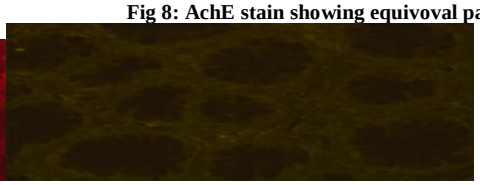

Fig10: Tuj 1 DIF marker negative in mucosa (40x)

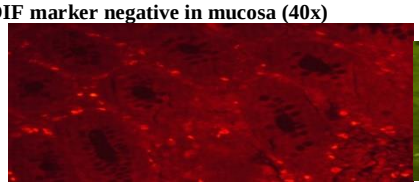

Fig 11: S100 DIF marker positive cells in mucosa (40x)

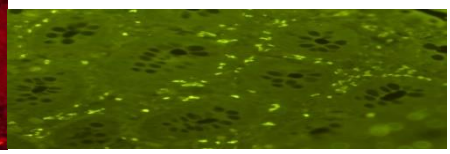

Fig 12: Tuj1 DIF marker positive cells in mucosa (40x)

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Conflict of Interest: Nil Source of support:Nil