Frequency of loss of calretinin expression in clinically susceptible cases of Hirschsprung disease in rectal biopsies

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ABSTRACT

Objective: To determine the frequency of loss of calretinin expression in clinically susceptible cases of Hirschprung disease in rectal biopsies.

Material and Methods: This descriptive, cross-sectional study was conducted at Department of Histopathology, Combined Military Hospital, Multan for a period of 6 months (17th Oct 2021 to 16th April 2022). A total 93 suspected cases of Hirschprung disease were taken. Specimens received in 10 % buffered formalin and fulfilling inclusion criteria were included in the study. The cases diagnosed as Hirschprung disease were taken and calretinin immunohistochemical stain was applied and result recorded after evaluation by consultant histopathologist. Data was analyzed using (Statistical Software for Social Sciences (SPSS) version 19).

Results: Out of 93 cases, 67 (72.0%) were male patients, whereas 26 (28.0%) were female patients. The average age of the study participants was 7.95 months, with a minimum age of 1 month and a maximum age of 12 months. Twenty-eight (30.1%) of the 93 study cases were from rural areas, while 65 (69.9%) were from metropolitan areas. Twenty-five families (26.9%) reported monthly incomes of up to Rs. 35000, while 68 families (73.1%) reported incomes of over Rs. 35,000. Of these 93 study cases, failure to pass meconium was noted in 24 (25.8%) patients, constipation was noted in 75 (80.6%) and abdominal distension was noted in 62 (66.7%) patients. Loss of calretinin expression was noted in 34 (36.6%) of our study cases.

Conclusion: According to the findings of our investigation, calretinin is very helpful in identifying suspicious cases of Hirschsprung disease. When acetylcholinesterase enzyme histochemistry is not accessible, it can be a helpful, cost-effective diagnostic assistance in centers with limited resources. It is a reliable and efficient diagnostic tool for early diagnosis of the illness. Loss of Calretinin expression was significantly associated with age and residential status.

Keywords: Calretinin expression, Frequency, Hirschsprung disease, Immunohistochemistry

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INTRODUCTION

The enteric nervous system is the part of the autonomic nervous system that controls the coordination of gastrointestinal tract [1]. It originates from a population of multipotent migratory cells known as neural crest cells. Neurocristopathies are conditions that occur from abnormal neural crest cell development. One of such disorders is Hirschprung disease. Hirschprung illness, which affects 1 in 5000 live births, causes the patient to present with abdominal distension and an inability to pass meconium; this condition typically necessitates surgical removal of the aganglionic intestine [1]. A frequently encountered congenital illness, Hirschsprung disease (HD) is brought on by a lack of parasympathetic ganglion cells in the intramucosal and submucosal plexuses [2]. It is

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mostly diagnosed during first year of life in children. The definitive diagnosis of HD is made by histologic demonstration of absence of ganglion cells in both the myenteric (Auerbach) plexus which lies in longitudinal and inner circular smooth muscle fibers layers in intestine and the sub-mucosal (Meissner) plexus, even though anorectal manometry or radiologic investigations are also employed to establish the diagnosis [3]. Hirschsprung disease is identified when there are no ganglion cells present in rectal biopsies. There may also be hypertrophied submucosal nerve trunks that are larger than 40 microns in diameter [4].

There are several reasons for the development of HD. It can both run in families or manifest spontaneously. Boy babies have been observed to be more susceptible than girls to develop this illness. The symptoms of HD can range from a persistently increasing constipation in older children to a sudden intestinal blockage in newborn. In the first few months of life, over 80% of patients show growing abdominal distention, poor feeding, and problematic bowel movements.

Because it necessitates specific tissue handling, acetylcholinesterase histochemistry (AChE) helps diagnosis but is not widely accepted [5]. This technique requires frozen tissue samples and there are chances of false-negative results mostly due to the young age of patients [3]. Since the loss of calretinin immunoreactive neurons is correlated with aganglionosis, calretinin immunohistochemistry (IHC) may be a beneficial alternative [5]. A study conducted in India indicated loss of calretinin expression in 40.45% cases [6]. In this study, any issues arising due to the combination of histology and acetylcholinesterase labeling are resolved by calretinin immunohistochemistry, which reliably detected almost all HD cases without producing any false positive results [6]. It indicated calretinin sensitivity as 91.4% and a 100% specificity in HD cases.

In this study loss of calretinin expression will be observed in rectal biopsy specimen using immunohistochemistry. This study is conducted with the purpose of diagnosing clinically suspicious cases of Hirschsprung disease and providing a reliable diagnostic tool. As very little information at the national level is available on this topic, the findings of this study may lead to establishing a baseline data of local population.

MATERIAL AND METHODS

This descriptive cross-sectional study was conducted at the Department of Histopathology, Combined Military Hospital, Multan, from October 17th 2021 to April 16th 2022 for a period of 6 months, following approval of the ethics review committee. Using the WHO sample size calculator, a sample size of 93 was determined with a confidence level of 95, an anticipated population proportion of 40.45%, a margin of error of 10%, and a sample size of 93. After receiving informed consent patients were chosen using a non-probability consecutive sampling technique.

Biopsy samples received in 10% buffered formalin taken from patients of both genders of any age were included in the study. Inadequate sample (Biopsy not including submucosa and muscularis mucosae), unfixed tissues and poorly stained slides were excluded from this study.

The samples were processed after being embedded in a cassette and fixed in 10% buffered formalin. Hematoxylin and eosin (H&E) were used to stain the sections after they were cut at a thickness of 3-5 microns. The cases diagnosed as Hirschsprung's disease were taken and calretinin immunohistochemical stain was applied and the result was recorded after evaluation by a consultant histopathologist. The bias for inter-observer variation was minimized by showing all cases to two different consultant histopathologists. Confounding variables were minimized by strictly adhering to inclusion and exclusion criteria. Endpoint of the study was the presence or absence of calretinin immunohistochemical stain.

Data was analyzed by using Statistical Software for Social Sciences (SPSS) version 19. Mean $\pm$ SD were calculated for quantitative variables like age. Frequency and percentages were calculated for qualitative variables like gender, age groups, residential status, socioeconomic status, failure to pass meconium,

abdominal distension, constipation and diagnosis. Chi-square test after stratification was used for analysis with a significant p-value of <0.05.

RESULTS

A total of 93 patients who met the inclusion criteria for our study were included. Out of these patients, 67 (72.0%) of the patients were male and 26 (28.0%) were female (Table-I). Mean age of the patients was 7.95 ± 3.00 months (ranging from 1 month till 12 months). Mean age of the male patients was 7.45 ± 2.96 months, while female patients was 9.23 ± 2.77 months.

Twenty-eight (30.1%) of the 93 study cases were from rural areas, while 65 (69.9%) were from urban areas. Twenty-five families (26.9%) reported monthly income of up to 35000.00 PKR, while 68 families (73.1%) reported income of over 35,000.00 PKR. Of these 93 study cases, failure to pass meconium was noted in 24 (25.8%), constipation was noted in 75 (80.6%) and abdominal distension was noted in 62 (66.7%) as shown in Table-I.

In 34 (36.6%) of the study cases, there was a loss in calretinin expression (Table-II). Association and significance of calretinin expression was checked with gender, age, monthly family income, residential status, inability to pass meconium, constipation, and abdominal distension. A significant association (p-value <0.05) was seen with age and residential status. There was not any significant association of loss of calretinin expression with gender, monthly family income, residential status, failure to pass meconium or constipation.

Table-I: Frequency and percentages of qualitative variables.

Variables		Frequency	Percentage (%)
Age	< 6 months	38	40.9
	>6 months	55	59.1
Gender	Male	67	72.0
	Female	26	28.0
Monthly income	< 35000	25	26.9
	>35000	68	73.1
Residential status	**Rural**	28	30.1
	Urban		
Failure to pass meconium	Yes	24	25.8
	No	69	74.2
Constipation	Yes	75	80.6
	No	18	19.4
Abdominal distention	Yes	62	66.7
	No	31	33.3
Loss of Calretinin	Yes	34	36.6
	No	59	63.4

Table-II: Association of loss of calretinin with regards to gender, age, monthly family income, residential status, failure to pass meconium, constipation and abdominal distension.

Variables		Yes (loss of calretinin) n=34	No (No loss of calretinin) n=59	p-value
Gender	Male (n=67)	23	44	0.483
	Female (n=26)	11	15	
Age	Up to 6 months (n=38)	30	08	0.000
	More than 6 months (n=55)	04	51	
Residential status	Rural (n=28)	16	12	0.010
	Urban (n=16)	18	47	
Monthly family income	Up to 35000.00 (n=25)	07	18	0.341
	More than 35000.00 (n=68)	27	41	
Failure to pass meconium	Yes (n=24)	11	13	0.328
	No (n=69)	23	46	
Constipation	Yes (n=75)	27	48	1.000
	No (n=18)	07	11	

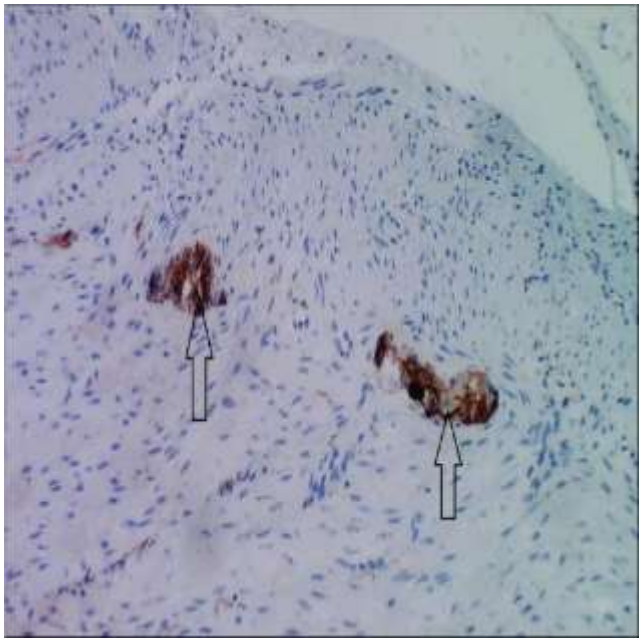

Figure-I: Calretinin positivity in rectal biopsy (IHC x40).

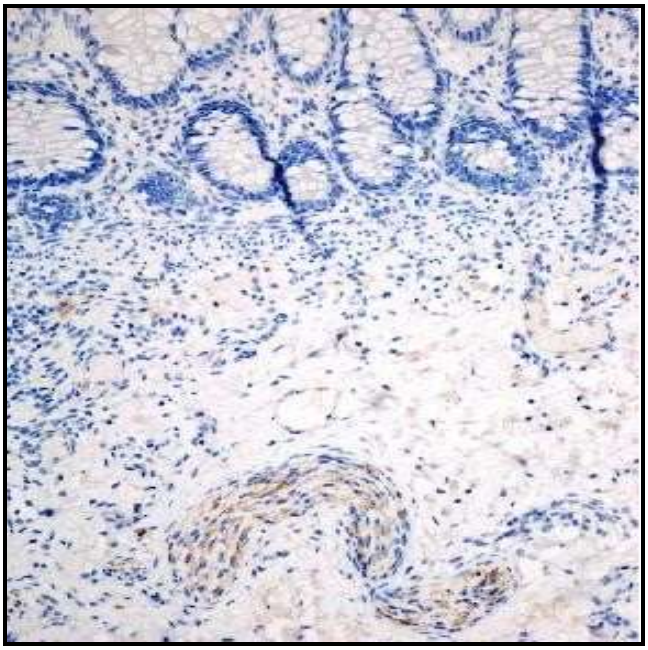

Figure-II: Calretinin negative in Hirschsprung disease (IHC x40).

DISCUSSION

Hirschsprung disease, often referred to as congenital megacolon or congenital colonic aganglionosis, is a developmental illness that is characterized by a lack of ganglion cells in the submucosal (Meissner's) and myenteric (Aurbach's) plexuses in the distal bowel extending proximally for various distances. This leads to a functional intestinal blockage brought on by the afflicted segment's dysmotility. With a frequency of around 1 in 5,000 live births, it is one of the most prevalent surgical diseases in children. Because they are unable to migrate cephalocaudally via the neural crest between the fourth and the 12th week of pregnancy, ganglion cells are lacking in all or part of the colon as a result of Hirschsprung disease. The aganglionic segment typically projects proximally from the anus. The recto-sigmoid portion of the colon is where short-segment disease is most prevalent. Beyond this area, long-segment illness can infect the entire colon. The large and small intestines are infrequently involved.

The main modalities of diagnosis include radiographic examinations, anorectal manometry, and histological investigation of rectal wall samples [18]. A total of 93 biopsy samples were examined in this study. Among the 93 study cases, 67 (72.0%) patients were male and 26 (28.0%) were female. Calretinin positivity was also observed in male patients predominantly. Numerous other researches have also revealed similar male gender predominance. A study from Peshawar by Khan *et al* [19] similarly revealed a 75% male gender predominance, which is comparable to our research's findings. Another study by Henna *et al*. from Lahore also showed a 4:1 male to female ratio. A substantial male gender predominance was also found by Hussain *et al* [21] from Karachi. Additionally, Zamir *et al* [22]. reported a 3:1 male to female ratio, which is in line with the outcomes of the study we conducted.

Mean age of this study´s cases was 7.95 ± 3.00 months. About 55 of the study's patients, or 59.1%, were older than 6 months. Similar age group was observed in a study conducted by Henna *et al*. Though Mabula *et al* from Tanzania has reported a mean age of 24 months in their study population which may be due to the fact that study population in this study did not exceed the age of 12 months. Calretinin positivity was observed more in patients up to 6 months of age as compared to patients older than 6 months. Of the 93 study cases, 28 (30.1%) were from rural areas and 65 (69.9%) were from metropolitan areas. Twenty-five households (26.9%) reported monthly family income of up to Rs. 35,000, while 68 families (73.1%) reported monthly family

income of beyond Rs. 35,000. The same results were also observed by Zamir *et al*.

Of these 93 study cases, the most common symptom observed was constipation, noted in 75 (80.6%) patients followed by abdominal distension in 62 (66.7%) patients. Failure to pass meconium was noted to be the least frequent symptom, observed in 24 (25.8%) patients. A study conducted at Lahore by Henna *et al* [20] has also reported similar frequencies of these associated symptoms. They also reported constipation to be the most common symptom, in 88.9% patients, abdominal distension in 77.8 % and delayed passage of meconium in 33% of patients. Mabula *et al* [23] from Tanzania has reported constipation in 94.5 %, abdominal distension in 92.7% and failure to pass meconium in 61.8% patients.

Twenty-eight rectal biopsy samples from 2010 to 2011 were examined in a prospective study at the University of Texas in Houston [4]. In the muscularis mucosae, superficial submucosa, and lamina propria, thin nerve fibrils that were positive for calretinin were consistently linked with the presence of ganglion cells establishing positive predictive value of this IHC stain. Another study conducted retrospectively between 2008 and 2010, tissue samples from children with a histological diagnosis of HD who underwent radical surgery at the Dr. Sheikh Children Hospital in Iran were examined [3]. In this study, there were 20 blocks in the control group, 30 blocks from the ganglionic zone, and 30 blocks from the aganglionic zone. Based on immunostaining for calretinin in the submucosa, there were no false-negative or false-positive results in this investigation. Loss of calretinin expression was noted in 34 (36.6%) patients in this study. In India, a study carried out 2013 on 131 suspected cases of HD, loss of expression of calretinin was noted in 53 (40.45%) patients [6]. These findings are closer to current study´s outcome.

CONCLUSION

In conclusion, this study indicated that calretinin is an extremely useful IHC stain which can be very reliably used in diagnosing suspicious cases of Hirschsprung disease. It can serve as a valuable as well as cost-effective diagnostic aid in resources-limited centers where acetylcholinesterase enzyme histochemistry is not available. We do encourage that more studies be conducted using this IHC stain for better predictability of diagnosis as well as an aid to start timely treatment of affected patients.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Declared none

AUTHORS CONTRIBUTION

Ayesha Haider: Entire research work, sample collection and data analysis

Unaiza Jamil: Literature review, drafting and analysis

Iqra Ahmad Shah: Sample collection, data analysis and revision of manuscript

Maria Aslam: Result interpretation, data analysis and revision of manuscript

Kiran Mumtaz: Sample collection and data analysis

Syed Naeem Raza Hamdani: Concept, design and overall supervision

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