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Comparison of Immunohistochemical Expression of Calretinin, Map2, S-100 and Glut1 in Rectal Biopsies from Suspected Hirschsprung's Disease

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Abstract

Background: This study aimed to compare the immunohistochemical expression of calretinin, Map2, S-100, and Glut1 in rectal biopsy samples from patients suspected of Hirschsprung's disease (HD).

Methods and Results: Rectal biopsy samples from 40 patients with suspected HD were analyzed using hematoxylin and eosin (H&E) and immunohistochemistry (IHC). Immunohistochemical stains were assessed after previous routine histology interpretation, which was classified as "Positive or in favor for HD" and "Equivocal or negative for HD." The staining patterns for calretinin, Map2, S-100, and Glut1 were analyzed regarding the following structures: lamina propria small nerve fibrils, submucosal small nerve fibrils, submucosal nerve fibers, and submucosal ganglia. The IHC stains for calretinin and Map2 were score-ranked as 0 – negative and 1 – positive. The IHC stain for S-100 was score-ranked as 0 – normal and 1 – hypertrophic. The IHC stain for Glut1 was ranked as 0 – normal perineural and 1 – conspicuous perineural accentuation

Calretinin had 92% accuracy, the highest sensitivity (80%) and specificity (92.00%). Map2 also had the same accuracy as calretinin but lower sensitivity (46.67%). Regarding S-100 and Glut1, these two markers did not support a conclusive diagnosis. The accuracy for S-100 and Glut1 was 66.7% and 60%, respectively.

Conclusion: Calretinin remains the currently most valuable single IHC marker in diagnosing difficult cases of HD.**(International Journal of Biomedicine. 2024;14(1):153-158.)**

Keywords: Hirschsprung's disease • diagnosis • biopsy • immunohistochemistry

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Abbreviations

AChE, acetylcholinesterase; **GC**, ganglion cells; **Glut1**, glucose transporter 1; **H&E**, hematoxylin and eosin; **HD**, Hirschsprung's disease; **IHC**, immunohistochemistry; **MAP-2**, microtubule-associated protein-2.

Introduction

Hirschsprung's disease (HD) is a congenital disease of the enteric neural system characterized by the absence of GC in submucosal and myenteric plexuses of the distal digestive tract due to failure to migrate of the neural crest cells during embryonic development. Usually, this occurs around the 12th week of embryogenesis. Migration and differentiation occur from the proximal to the distal segment. As GC are responsible for normal peristalsis, their absence results in functional

obstruction of the aganglionic segment, followed by bowel dilatation proximally from the affected zone. In approximately 80% of cases, aganglionosis involves the recto-sigmoidal segment. The disease-related mortality in the first year of life has dropped from 25%-30% to 1% due to early diagnosis and successful treatment methods.[1-4]

The gold standard for diagnosis of HD remains the histological examination of biopsy samples obtained from the affected narrow segment. These tissue samples exhibit a lack of GC with hypertrophy and disorganization of the nerve fibers in the submucosal and muscular plexus.[5] Adequate and well-oriented tissue samples, coupled with the experience of the pathologist, are the most important elements in the diagnosis of HD.[6] H&E staining in formalin-fixed and paraffin-embedded tissue is generally used for histological diagnosis. The acetylcholinesterase (AChE) stain in frozen samples obtained by aspiration rectal biopsy is also a well-established method.[7] However, histochemistry with AChE is not universally used due to difficulties related to technical aspects and interpretation.[5,8] In recent decades, various markers have been tested using immunohistochemistry (IHC) to increase diagnostic accuracy in HD.[9-12] According to studies, calretinin and Map2 showed sensitivity in identifying GC even in insufficient or non-optimal samples.[13] Additionally, Glut1 and S-100 were shown to be valuable in detecting nerve fibers.[13] Such an immunohistochemical diagnostic panel provides dual accuracy in terms of conclusion for the absence of GC and the evaluation of nerve fibers and their thickness and distribution in tissue samples.

This study aimed to compare the immunohistochemical expression of calretinin, Map2, S-100, and Glut1 in rectal biopsy samples from patients suspected of HD.

Materials and Methods

This study was conducted at the Pediatric Surgery Clinic, Institute of Pathology at the University Clinical Center of Kosovo, and NUCLEUS Pathology Diagnostics & Research Laboratory. Rectal biopsy samples from 40 patients with suspected HD were analyzed using H&E and IHC, respectively, from 2017 to 2023. Patients had been previously evaluated clinically and with imaging studies. Twenty-three cases were prospectively analyzed, and 17 cases were obtained from our archive and medical records. Two independent general pathologists examined biopsy samples. Since the study was morphological, partly retrospective, and unrelated to any additional clinical interventions, patient consent was not explicitly obtained.

Routine Histology

After optimal fixation for 24 hours in neutral buffered formalin, tissue samples were processed in a tissue processor (Leica TP 1020), where they underwent an additional fixation procedure in 10% neutral buffered formalin (NBF), gradual dehydration in 70%, 80%, 96% and absolute ethanol, xylene cleansing and immersion in liquid paraffin at 60°C. Subsequently, the labeled specimens were molded into paraffin blocks, sectioned at 3–4-micron-thick sections, and applied on microscope glass slides. After deparaffinization and gradual rehydration in decreasing solutions of ethanol and distilled water, tissue sections were stained with hematoxylin and eosin (H&E), covered by the mounting medium, and coverslipped. The H&E-stained sections were analyzed to evaluate the sample's diagnostic adequacy and the presence, distribution, and morphological features of the neural ganglia and nerve fibers in the submucosal layer. Immunohistochemical stains were assessed after previous routine histology interpretation, which was classified as "Positive or in favor for HD" and "Equivocal or negative for HD."

Immunohistochemistry

IHC analysis was carried out for calretinin, Map2, S-100, and Glut1. Antigens were retrieved by placing the slides in a target retrieval solution for 45 minutes at 95-98°C. After the peroxidase block, the slides were incubated with the primary antibody against calretinin, Map2, S-100, and Glut1 for 30 minutes. In the next step, dextran polymer conjugated with peroxidase and a secondary antibody (EnVision+, DAKO, K534011) were applied for another 30 minutes. The visualization was carried out with DAB and chromogen. The vendor, clone, and dilution of the antibodies are presented in Table 1. The slides were counterstained by Harris's hematoxylin, washed, covered by the mounting medium, and coverslipped.

Table 1.

Antibody, Vendor, Clone, and Dilution of the Employed Immunohistochemical Markers

Antibody	Vendor	Clone	Dilution
Calretinin	DAKO	DAK-Calret1	RTU
Map2	Milipore	Polyclonal	1:1000
S-100	DAKO	Polyclonal	RTU
Glut1	Milipore	Polyclonal	1:300

The staining patterns for calretinin, Map2, S-100, and Glut1 were analyzed regarding the following structures: lamina propria small nerve fibrils, submucosal small nerve fibrils, submucosal nerve fibers, and submucosal ganglia. The IHC stains for calretinin and Map2 were score-ranked as 0 – negative and 1 – positive. The IHC stain for S-100 was score-ranked as 0 – normal and 1 – hypertrophic. The IHC stain for Glut1 was ranked as 0 – normal perineural and 1 – conspicuous perineural accentuation (Fig. 1).

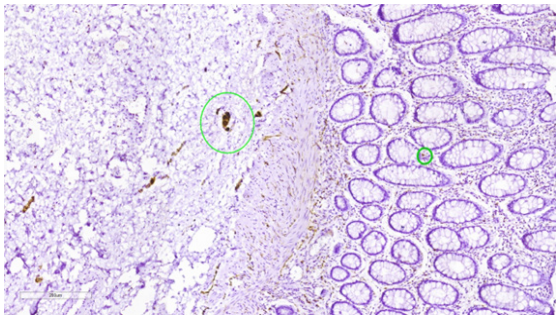

Fig. 1a. *Calretinin stain in a normal subject highlights the neural ganglion (big circle) and small nerve fibers of submucosa and lamina propria. Cross reaction with mast cells (small circle) serves as an internal "built-in" positive control.*

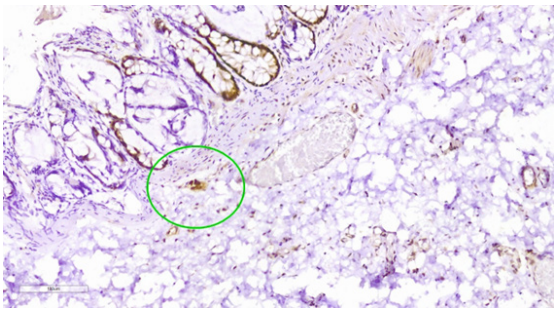

Fig. 1b. *Map2 stain in a normal subject highlights the neural ganglion (circle). Cross-reaction with nuclei of mucosal epithelial cells and lymphatic tissue (not shown) was consistently observed in our stains.*

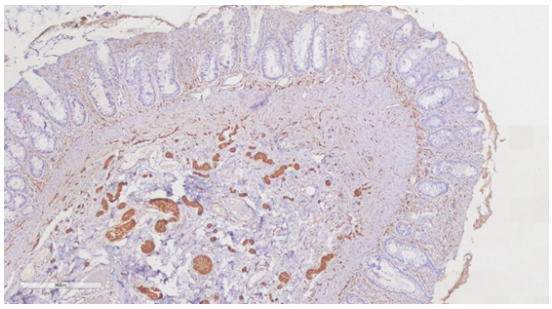

Fig. 1c. *S-100 stain in HD marks the hypertrophic and disorganized nerve fibers in the submucosal layer.*

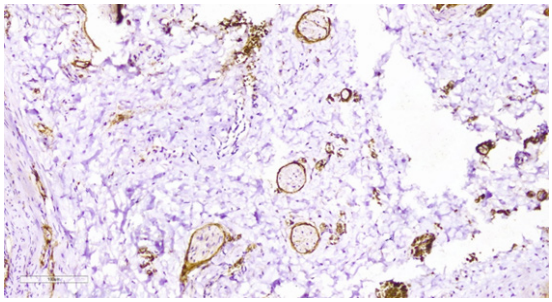

Fig. 1d. *Glut1 stain in HD shows increased, hypertrophic, and disorganized nerve fibers with characteristic perineural accentuation in the submucosal layer. Cross-reaction of Glut1 with red blood cells was observed (not shown).*

Statistical Analysis

Statistical analysis examined the relationship between H&E and immunohistochemistry groups. Descriptive statistics were calculated using the chi-square test or Fisher's exact test. Furthermore, each immunohistochemistry group's accuracy, sensitivity, and specificity were calculated. Descriptive statistics were employed to summarize the main characteristics of the data. The chi-square or Fisher's exact tests are used for categorical data analysis. These tests determine whether there is a significant association between two categorical variables. These tests were applied to explore the relationship between different staining methods (H&E and immunohistochemistry groups). Immunohistochemistry tests are used in pathology to detect the presence, abundance, and localization of specific proteins within tissues. This study also assessed the accuracy, sensitivity, and specificity of immunohistochemistry tests. Accuracy represents the proportion of true results (both true positives and true negatives) among the total number of cases examined. It provides an overall measure of how well the immunohistochemistry tests identify specific proteins. Sensitivity, also known as the true positive rate, measures the proportion of actual positives correctly identified by the test. It is a crucial metric in medical diagnostics, indicating the test's ability to correctly identify individuals with the condition (true positives). Specificity, also known as the true negative rate, measures the proportion of actual negatives correctly identified by the test. It signifies the test's ability to correctly identify individuals without the condition (true negatives). A P value of <0.05 was chosen as the threshold for statistical significance. This indicates that if the P value obtained from the statistical tests is less than 0.05, the results are considered statistically significant, suggesting that the relationship observed between HE and immunohistochemistry groups is unlikely to have occurred by chance. IBM SPSS Statistics® version 22 was used to perform descriptive analysis, and MedCalc® was used to perform a detailed analysis of the relationship between different staining methods.

Results

Statistical significance between histology interpretations and corresponding IHC results was observed when routine histology interpretations were clustered in Group 1 (n=25 – Positive or in favor of HD) and Group 2 (n=15 – Equivocal or negative for HD). However, during the accuracy calculation, calretinin was shown to be the most useful test for the two histological subgroups. Regarding Group 1, all four markers confirmed the presence or absence of HD by their positive or negative reaction, respectively. However, in Group 2, calretinin proved to have the highest accuracy. Out of 25 patients with an HE interpretation of HD, 23(92%) were confirmed by calretinin staining (Table 2).

Table 2.

Comparison between Routine Histology Interpretation and Immunohistochemical Expression of Calretinin, Map2, S-100, and Glut1

		H&E Interpretation		*P*-value
		Positive or in favor for HD [n (%)]	Equivocal or negative for HD [n (%)]	
Calretinin	Positive	2 (8)	12 (80,0)	<0.01[a]
	Negative	23 (92.0)	3 (20,0)	
Map2	Positive	2 (8)	7 (46.7)	<0.01[b]
	Negative	23 (92)	8 (53.3)	
S-100*	Positive	22 (88)	5 (33.3)	<0.01[b]
	Negative	3 (12)	10 (66.7)	
Glut1	Positive	22 (88)	6 (40)	<0.01[b]
	Negative	3 (12)	9 (60)	

**S-100 hypertrophy. The chi-square test or Fisher's exact test was appropriately used to explore the relationship between H&E and immunohistochemistry groups (IBM SPSS Statistics® version 22). The immunohistochemistry tests' accuracy, sensitivity, and specificity were calculated using MedCalc® statistical software.*

Also, out of 15 patients in Group 2, 12(80%) were confirmed as negative. Calretinin had 92% accuracy (Table 3). Calretinin staining also showed the highest sensitivity (80%) and specificity (92%). This was not the case with the other three markers. Map2 also had the same accuracy as calretinin but lower sensitivity (Table 3). Regarding S-100 and Glut1, in Group 2, these two markers did not support a conclusive diagnosis (Table 2). The accuracy for S-100 and Glut1 was 66.7% and 60%, respectively (Table 3).

Table 3.

Sensitivity, Specificity, and Accuracy of Calretinin, Map2, S-100, and Glut1 Stains in the Study Groups.

	Statistics	Value (%)	95% CI
Calretinin	Sensitivity	80.00	51.91 - 95.67
	Specificity	92.00	73.97 - 99.02
	Accuracy	92.00	78.94 - 98.20
Map2	Sensitivity	46.67	21.27 - 73.41
	Specificity	92.00	73.97 - 99.02
	Accuracy	92.00	78.94 - 98.20
S-100 (*)	Sensitivity	88.00	68.78 - 97.45
	Specificity	66.67	38.38 - 88.18
	Accuracy	66.67	50.02 - 80.75
Glut1	Sensitivity	88.00	68.78 - 97.45
	Specificity	60.00	32.29 - 83.66
	Accuracy	60.00	43.33 - 75.14

Discussion

IHC for calretinin remains the most valuable single IHC marker in diagnosing difficult cases of HD. Our study had a statistical accuracy of 92% and a high sensitivity and specificity of 80% and 92%, respectively. In contrast, S-100, Glut1, and Map2 did not support a conclusive diagnosis. The diagnosis of HD is complex and requires multidisciplinary management involving neonatologists, pediatric gastroenterologists, pediatric surgeons, radiologists, and pathologists. The gold standard for diagnosing this disease is histological examination of tissue samples obtained by rectal biopsy.[14,15] Histological diagnosis consists of determining the absence of GC and hypertrophy, as well as disorganization of nerve fibers, in the submucosal layer of the biopsy sample.[16] The muscular layer is usually not present in the biopsy samples. Therefore, the submucosal layer should be thoroughly evaluated. In practical terms, obtaining a sufficient sample with a representative submucosal layer very much depends on the expertise and experience of the pediatric surgeon. Hence, small samples with limited amounts of submucosal layer and consequent need for a repeat biopsy are common in our practice. In these situations, IHC as a valuable diagnostic tool may overcome the need for repeat biopsy and avoid unnecessary interventional complications for the patient. Historically, hypertrophic and disorganized nerve fibers in the submucosal plexus were identified through histochemical staining of frozen tissue samples with AChE.[7,17] However, in practical terms, assessing nerve fibers with AChE may be difficult due to cross-interaction with red blood cells and smooth muscle. Also, identifying small nerve fibers extending into the lamina propria is subjective and depends on the pathologist's experience.[18] Recently, choline transporter IHC has been introduced as an alternative to AChE histochemistry with a similar reaction pattern but a much simpler interpretation.[19] Surgical treatment for HD in recent decades has evolved from 3-stage surgery to a single operation. By this method, a significant number of patients undergo surgery in the neonatal period. Thus, the preoperative diagnosis should be rendered at neonatal age despite the difficulties in identifying GC and nerve fibers by H&E and AChE stains in this period.[20-23] In recent decades, various immunohistochemical markers have been tested to increase diagnostic sensitivity and specificity for HD. These markers have shown great potential in accuracy and ease of applicability, given that they are suited for formalin-fixed, paraffin-embedded tissue. Hence, there is no need to depend on difficult-to-perform and difficult-to-interpret frozen techniques.

Barshack et al.[24] evaluated the applicability of calretinin in identifying intrinsic nerve fibers and GC in samples from patients suspected of having HD. Also, Kapur et al.[21] demonstrated the importance of combining calretinin with H&E and AChE in cases of total colonic aganglionosis and ultra-short HD. Yang et al.[25] concluded that calretinin and Map2 are useful markers in identifying HD in aspiration rectal biopsies. The presence of GC in the submucosal layer by calretinin stain excludes HD. Still, caution should be exercised in cases with ultra-short HD where biopsy specimens may show slight positivity for calretinin in the transition zone.[24,26] Guinard-Samuel et al.[26] found that calretinin visualized specimens in the "black and white" model and had excellent consistency between experienced and inexperienced pathologists, avoiding the need for repeat biopsies.[26]

In the study by Musa et al.,[27] samples with HD showed an absence of expression for calretinin in lamina propria of mucosa and submucosa. In contrast, all samples negative for HD showed positive expression by ganglia as well as nerve fibers in these layers. This was also observed in our study. Calretinin stained the nucleus and cytoplasm of GC and the cytoplasm of fine nerve fibrils and nerve fibers of the lamina propria and submucosa. In our study, the interpretation of calretinin IHC was straightforward. Cross-reaction with mast cells was used as a "built-in" internal control for negative cases. In the study by Burtelow and Longacre,[28] Map2 was successfully applied to identify GC. Map2 successfully marks GC without staining other neural elements of the neuroenteric system.[28] In our study, Map2 stained a smaller number of GC than did calretinin. However, the difference between the groups was significant. Map2 showed the same accuracy as calretinin. In contrast to calretinin, it showed a lower statistical sensitivity. We believe that in a larger study group, higher sensitivity may be observed.

Monforte-Muñoz et al.[17] investigated the efficacy of S-100 in highlighting nerve fibers and found that about 90% of samples from HD had hypertrophic fibers of over 40 microns

in diameter. Our study also observed this even though no measurements have been taken. Lim et al.[29] recorded two false negative results from 27 patients with HD. In our study, S-100 identified nerve fiber hypertrophy in cases with HD but was less reliable in the equivocal or negative study group. Hence, the accuracy and specificity of S-100 were lower than calretinin and Map2. Glut1, like S-100 and AChE, identifies hypertrophic fibers in HD patients.[30]

In our study, Glut1 stained the hypertrophic nerve fibers and the perineurium in the submucosa in HD but was less sensitive than the S-100 stain. Like S-100, this marker was less reliable in the equivocal or negative study group and had lower statistical accuracy and specificity than calretinin and Map2.

The study's main strengths are the robust methodology, including the staining techniques and interpretation criteria, which are well-detailed. The study incorporates multiple IHC markers, providing a comprehensive analysis. This approach increases the reliability of the findings and allows for a more nuanced understanding of the disease. The study also combines retrospective and prospective analyses, enhancing the robustness of the results by considering a diverse set of cases over several years. The study employs appropriate statistical tests to analyze the relationship between different staining methods, providing a quantitative basis for the conclusions. The study compares IHC results with routine histology interpretations, allowing for validation of the IHC markers against established diagnostic methods.

Limitations of the Study

The main limitation of the study is a relatively small sample size. While the results are significant within this sample, a more extensive and more diverse sample could enhance the generalizability of the findings. Furthermore, the study is conducted in a single center, potentially limiting the diversity of cases and diagnostic challenges encountered. Multi-center studies might provide a broader perspective on the applicability of these markers. The study does not provide information on the long-term outcomes of patients diagnosed using IHC markers. Long-term follow-up data could validate the accuracy of the diagnoses made based on these markers. Finally, the interpretation of IHC stains involves a certain level of subjectivity, which could introduce observer bias.

Conclusion

Calretinin remains the currently most valuable single IHC marker in diagnosing difficult cases of HD. The pediatric surgeon's or gastroenterologist's role in assessing the clinical features and biopsy site cannot be overstated.

Competing Interests

The authors declare that they have no competing interests.

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