Peer Review File

Article information: https://dx.doi.org/10.21037/tp-22-592

Reviewer Comments

The manuscript by Alatas et al describes immunohistochemical findings in 5 patients with intestinal hypoganglionosis in comparison with findings in a single control. The following concerns should be considered:

Major Concerns

The manuscript fails to acknowledge a recent publication by Kapur et al. (Am J Surg Pathol 2021;45:1047-60). This publication includes several relevant subjects, including immunohistochemical observations which should be addressed in the current study. Examples include

The patchy nature of myenteric hypoganglionosis with a mix of normal and abnormal biopsy findings in different intestinal locations from the same patient.

No appreciable abnormality of submucosal innervation.

Near complete absence of myenteric NeuN and calretinin-immunoreactive ganglion cells.

Reduced intramuscular innervation.

Inadequacy of rectal biopsies to establish the diagnosis.

The authors should compare their own results with those published in the prior paper, many of which are similar. They should also perform NeuN and calretinin immunohistochemistry to determine whether they confirm the previous published findings. They should include their own findings with regard to points (b) and (d).

Methods: Much more detailed description is required to understand exactly how data was collected for this study. Consider the following:

Table 1 should be expanded or supplemented to explicitly state which specimens were used in the study

Reply: Thank you for your valuable comment. We agree that it is important to add those to the manuscript.

Changes in Text: Supplemental Table 1.

What size biopsies were obtained from each resection? How many and from which resections? How were they oriented (longitudinal or transverse)?

Reply: Thank you for your valuable comment. We agree that it is important to add those to the manuscript.

Changes in Text: Supplemental Table 1.

What criteria were used to identify ganglion cells (e.g., any portion of cell body? Only cell bodies with nucleus in plane of section?)

Reply: Thank you for your kind comment. For the initial diagnosis of hypoganglionosis, ganglion cells were already counted by experienced pathologist. However in this study, ganglion cells were not counted again, we utilized S-100 staining to measure the area stained in order to identify the enteric nervous system

(ganglion cells and nerve fibers).

The criterion of <1.52 ganglion cells per mm is based on published work from another group (reference 7). The reference provided is not the primary basis for this value, which appears to derive from prior studies cited in reference 7. The prior studies were published in a relatively obscure journal, which I am unable to access. I suggest that the authors consult these primary papers and determine whether the value seems justified and whether the methods used to quantify ganglion cells/mm were the same as in the current study. They Kapur et al study cited above (see concern #1) also has some normative data that may be useful.

Reply: Thank you for your kind comment. We would like to clarify that our diagnosis of hypoganglionosis based on morphological full-thickness biopsy of the resected intestine (paucity and immature ganglion cells on myenteric plexus) in combination with clinical manifestations from the patients, as there is still no international consensus to specify this disease.

Change in Text: "Hypoganglionosis was diagnosed based on morphological full-thickness biopsy of the resected intestine (paucity and immature ganglion cells on myenteric plexus) in combination with clinical manifestations from the patients, as there is still no international consensus to specify this disease." Page 6

Description of the quantitative measurements is insufficient. What were the boundaries of the histological field used to establish the background surface area used to quantify the fraction of surface area occupied by positive immunoreactive labeling?

Reply: Thank you for you kind comment. Quantification was performed based on the methods from our previous study.(1)

Change in Text: -

Was the entire bowel wall included or were portions excluded. Was muscle include in fields for S-100 and ICC quantification? If so, what layers and how much of the field? Were random fields chosen in some way to avoid observer bias? How did they correct for distension or other changes that might result from chronic pseudo-obstruction?

Reply: Thank you for you kind comment. Muscle was not included during S-100 and ICC quantification. Random fields were chosen to be observed qualitatively as well as quantitatively. As we have shown in our study, there wasn't any significance change of the muscular layer.

Change in Text: -

The claim that this study includes 5 patients with hypoganglionosis seems unjustified because immunohistochemical data (which is the core of the research) was only collected from 3 patients.

Reply: Thank you for you kind comment. Full-thickness biopsy of intestinal samples from the other 2 patients were already stained with hematoxylin-eosin and acetylcholine esterase staining, which showed the diminished amount of ganglion

cells as well as smaller ganglion cells. However the slides from both patients were not available for further staining with S-100, c-Kit and α-SMA.
Change in Text: "However, only three intestinal samples from patients with hypoganglionosis (patient 2, 3, and 4) were available for further staining in this study." Page 6

More information is required about the single control used in this study including demographic details (age, sex), reason for resection, and intestinal site. The figures should be modified to include representative images from the control for each antibody.
Reply: Thank you for your valuable comment. We agree that it is important to add those to the manuscript.
Change in Text: Intestinal sample (jejunum, ileum, appendix and colon) from an autopsy of a 30-day-old boy without gastrointestinal disease was used as control. Page 7

The study contains a bit of circular reasoning, which is difficult to justify, especially since only one control was included. A diagnosis of hypoganglionosis has been rendered, seemingly based on clinical findings and the sole histopathological diagnostic criterion of >1.52 ganglion cells/mm. If this is sufficient, what is the evidence that "international consensus criteria are urgently needed"? If this is not sufficient, how do the authors know their patients truly have hypoganglionosis or that the other findings they describe are actually abnormal? Perhaps the single control has hyperganglionosis and abnormal immunohistochemistry. More controls are required to strengthen the study.
Reply: Thank you for you kind comment. We admit that the biggest limitation of this study is the use of only 1 control patient. Furthermore, we do agree that more than 1 control are needed to compare the specimens with age-matched control as biopsies were performed at various ages. However, at our center, control specimens from children autopsy were difficult to be obtained, due to the reluctancy from the parents.
Change in Text: -

It would be helpful to have a listing of each site and an indication of which sites had normal findings. The frequency of such normal biopsies is extremely relevant to decisions about how many biopsies should be done to exclude hypoganglionosis.
Reply: Thank you for your valuable comment. We agree that it is important to add those to the manuscript.
Change in Text: Supplemental Table 1.

Figures (especially Figure 3): The contrast between specific and non-specific labeling in some of the figures is relatively poor. This raises concern about how well the quantitation of positive vs. negative surface area was achieved. It would be helpful to have a supplemental figure that shows side-by-side comparison of the actual image and the areas deemed "positive" by ImageJ analysis for a challenging image like that shown for c-kit in case 4.

Reply: Thank you for your valuable comment. We agree that it is important to add those to the manuscript as the stained area did not have good contrast. However, we are terribly sorry as we cannot provide the ImageJ analysis as we did not save those data. We are terribly sorry for this.
Change in Text: -

Title: A better title might emphasize what seems to be the key conclusion from the study, which is that hypoganglionosis is difficult to diagnose even with immunohistochemistry because the abnormal findings irregularly distributed.
Reply: Thank you for your valuable comment. We already revised the manuscript based on your comment.
Change in Text: "Diagnostic Challenges of Hypoganglionosis Based on Immunohistochemical Method"

Abstract: The concluding sentence, which begins "Each segment of intestine …" is awkward because the ending phrase "… ranging from severely decreased to normal" cannot be applied to the preceding phrase "… pattern of musculature …". This identical sentence with its awkward construction is repeated under Key Findings and in the Discussion.
Reply: Thank you for your valuable comment. We already revised the manuscript based on your comment.
Change in Text: Each segment of intestine in hypoganglionosis had different numbers of ICCs, sizes and distributions of ganglions, as well as patterns of musculature, which may range from severely abnormal to nearly normal.

Abstract (and elsewhere): "S 100" should be replaced with "S-100".
Reply: Thank you for your valuable comment. We already revised the manuscript based on your comment.
Change in Text: S 100 have been change to S-100 throughout the manuscript.

Introduction: Citation "(1)" is located inside the period at the end of the first sentence, but all other citations are located outside the punctuation mark.
Reply: Thank you for your valuable comment. We already revised the manuscript based on your comment.
Change in Text: The reference has been changed accordingly.

Introduction: 1st paragraph: The abbreviation "HG" is introduced in the first sentence, but not used consistently through the paper until the Results section.
Reply: Thank you for your valuable comment. We already revised the manuscript based on your comment.
Change in Text: HG have been changed to hypoganglionosis.

Introduction: 1st paragraph: This paragraph would benefit from a sentence two describing the fundamental differences between Hirschsprung disease and HG.

Reply: Thank you for your valuable comment. We already revised the manuscript based on your comment.
Change in Text: However, unlike Hirschsprung's disease, hypoganglionosis is characterized by the presence of myenteric and submucosal ganglions along the gastrointestinal tract, but with sparse distribution and low number of myenteric ganglions.(3) As such, diagnosis of this disease requires a different diagnostic approach from that of Hirschsprung's disease. Page 5

Introduction, line 97 (and elsewhere): The text frequently uses the preposition "on" incorrectly, when the proper term is "of" (e.g., "… particularly on ganglia …; line 97) or "in" (e.g., "… layer in some intestinal segments …; line 271). The figure legends all contain a similar error and should read "… in hypoganglionosis patients …".

Reply: Thank you for your valuable comment. We already revised the manuscript based on your comment.
Change in Text: We have revised the preposition usage of this manuscript based on your review.

Methods, line 125: The sentence reads "Samples were cut into 4-um-thick slices …". I think that the paraffin-embedded tissues were sectioned this thin during slide preparation. Surely the embedded tissues were thicker than 4 um.
Reply: Thank you for your valuable comment. We already revised the manuscript based on your comment.
Change in Text: All samples were then formalin-fixed, embedded into paraffin and cut into 4 μm thickness in preparation for immunohistochemical staining. Page 7

Re-review Comments

The revised version of this manuscript addresses many of the concerns raised in my original review and provides some necessary clarifications. The following issues represent some original concerns which have been incompletely addressed, as well as new concerns which were only evident after the responses to my original review.
1. The most serious issue relates to how the diagnosis of hypoganglionosis was established for the patients included in this study. In their response to one the concerns raised in my original review, the authors clarified that the criterion of <1.52 ganglion cells per mm which was intimated as the basis for the diagnosis of hypoganglionosis, was not in fact used. Rather they state that "our diagnosis of hypoganglionosis was based on morphological full-thickness biopsy of the resected intestine (paucity and immature ganglion cells on myenteric plexus) in combination with clinical manifestations). This is a subjective assessment. The study appears designed to determine whether immunohistochemistry can provide objective support for their subjective impression. I believe this could be stated more clearly in the Introduction and Abstract and the text could be modified to avoid confusion about this point.

Reply: Thank you for your kind comment. We are sorry that we did not state clearly our objective in previous version of our manuscript. In this study we aim to validate our subjective evaluation with the use of objective support.
Change in Text: Abstract page 3: " This study aims to evaluate the use of immunohistochemistry to provide objective support for our initial subjective impression of hypoganglionosis as well as to describe the morphological features of this study."
Introduction page 6 : "Therefore, this study aims to evaluate the use of immunohistochemistry to provide objective support for our initial subjective impression of hypoganglionosis. Furthermore, we also aim to describe the morphological features of the distribution of the enteric nervous system, muscular layer thickness, and the presence of interstitial cells of Cajal (ICCs) in various sections of the intestines from patients with hypoganglionosis as well as to match those data with patients' clinical features."

P7, Tissue Specimens, 2nd sentence: I recommend modifying to read "Hypoganglionosis was diagnosed subjectively based on …" (adding the word "subjectively" emphasizes an important concern raised in my original review, which is that since objective diagnostic criteria are lacking, it is unclear whether the patients in this study have the same condition)
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Page 6 Methods Tissue Specimen : " Hypoganglionosis was diagnosed subjectively based on morphological full-thickness biopsies of several parts of resected intestine (paucity and immature ganglia on myenteric plexus) in combination with clinical manifestations of the patients, as there is still no international consensus to specify this disease."

Supplementary Table 1 references the "amount and size of ganglion cells". I would modify this to indicate that the assessment was made subjectively from H&E-stained sections.
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Footnote was added: "*Results were based on subjective evaluation of hematoxylin and eosin-stained sections"

A second significant issue relates to the response to a concern raised in my original review, to which the authors clarified that samples from only 3 of the 5 patients mentioned in the paper were available for immunohistochemical staining. Since relevant data are only provided from these 3 patients (those shown in Supplementary Table 1) it seems inappropriate and unnecessary to mention the other 2 patients at all. It is more appropriate to revise the manuscript to be a description based on 3 patients and 1 control.''

Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Five patients were changed to three patients in the entire manuscript.

The limitation of using only 1 “normal control” as the basis for comparison to the patients in this study should be explicitly noted in the Discussion. If quantitative data are to be used for the diagnosis of this condition certainly they will require robust control data.
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment. We agree that the use of only 1 normal control should be clearly stated in this manuscript.
Change in Text: Page 15: " In addition, our study included only 1 control patient which may affect the validity of our quantitative analysis. Therefore, more control samples from various age groups should be included in order to match the intestinal samples with each specific age group as well as to increase the validity of the study."

The plural of “ganglion” is “ganglia”. Throughout the entire manuscript, the term “ganglion” is used, when “ganglia” is required
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Ganglion was changed to ganglia in the entire manuscript.

P6, last paragraph, 2nd sentence: should read “Disruption of these systems, particularly in ganglia, …” (note replacement of “on” with “in” and “ganglion” with”ganglia”). The same grammatical error (using “on”, when “in” is appropriate) occurs elsewhere many times in the manuscript. Some editing will be required to address these types of errors.
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Page 5: " Disruption of these systems, particularly in ganglia within the ENS and ICCs, is suggested to be the etiology of many gastrointestinal motility problems such as hypoganglionosis"

No ganglion cells were identified in any of the appendices reviewed in this study. This seems remarkable and suggests a potentially novel diagnostic feature of myenteric hypoganglionosis. Were entire appendices evaluated or just biopsies? Doesn’t this finding warrant discussion?
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment. We do not recommend the use of aganglionosis appendix as a diagnostic feature of hypoganglionosis as this finding can also be found on other diseases. Furthermore, the structure of myenteric plexus in the appendix tends to be irregular and may differ in each patient which eventually can complicate the evaluation.

Change in Text: Page 13: "Furthermore, even though appendix samples from all our patients were aganglionosis, the appendix by itself should not be used as a sole specimen to diagnose hypoganglionosis as this finding can also be found in other conditions such as total colonic aganglionosis and long segment Hirschsprung's disease"

The claim that all patients provided consent for inclusion in the study (page 8) cannot be correct, as all were young infants at time of surgery and two died before adulthood. Perhaps the parents were consented?
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Page 6: "This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Written ethical approval and informed consent were waived by the Kyushu University Ethics Committee because this research was conducted on already preexisting tissue samples. Parents from each patient provided verbal consent for inclusion in this study."

The Figure Legends for figures 1-3 need to be modified to read “hypoganglionosis patients (plural) as well as a normal patient” (insert “a”). They should also specify from what part of the bowel each image was obtained (e.g., jejunum).
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Figure legends have been adjusted accordingly

P13, first sentence: should read “… hypoganglionosis is still difficult to make due to …”
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Sentence has been adjusted accordingly

P14, 2nd paragraph, 2nd sentence: The newly added text which reads “… are confirmed by the same previous study …” is ambiguous because several previous studies were cited in preceding text. The specific citation should be repeated for clarity.
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Reference was added

Reference 3 should have lower case lettering for the first letters of the words in the title, apart from “Congenital”.
Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Reference has been adjusted accordingly

Reference 4 should not have the title entirely in upper case lettering.

Reply: Thank you for your kind comment. We have revised our manuscript based on your valuable comment.
Change in Text: Reference has been adjusted accordingly

Reference

1. Alatas FS, Masumoto K, Esumi G, et al. Significance of abnormalities in systems proximal and distal to the obstructed site of duodenal atresia. J Pediatr Gastroenterol Nutr. 2012;54(2):242-7.