Peer Review File

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Reviewer A

This case report of a 7 month old male describes his clinical features, the biochemical findings and the heterozygous mutations in the PEX26 gene found by whole exome sequencing.

In the introduction, the authors provide background information about peroxisomes including the metabolic functions of the PEX26 protein and description of the range of phenotypes of ZSDs within the PEX genes comprising 14 complementation groups The PEX26 gene functions are detailed. And the range of phenotypes in patient with PEX26 mutations are described.

The case description contains information regarding the abnormalities found on clinical exam. The bloodwork results are detailed and include normal and abnormal findings. The abnormal findings include elevated plasma very long chain fatty acids, abnormal urine organic acids, elevated aspartate aminotransferase and slightly increased total bile acids. The clinical and biochemical findings suggested that the child had a peroxisomal disorder and whole exome sequencing was performed and two novel heterozygote mutations in the OPEX26 gene were found.

This paper provides the information necessary for publication of a case report.

This reviewer has a few suggestions that would improve the report.

1). Here is the link to the 75 mutations listed for PEX26 (the mutations in PEX26 for the case reported in this paper are novel as they are not listed in this database.), https://databases.lovd.nl/shared/genes/PEX26

Reply1): We checked the database. To date, the total number of public variants reported in the "lovd" database are 76, and the unique public DNA variants reported are 46.

2). The authors should consider either removing Table 3 which lists only 25 mutations in PEX26 and their corresponding phenotypes, or adding and including in Table 3 the 75 mutations that are reported for PEX26 in the "lovd" database.

Reply2): To date, the total number of public variants reported in the "lovd" database are 76, and the unique public DNA variants reported are 46. Thus, we should have list 46 unique variants in Table 3.

In the unmodified version, Table 3 lists 29 unique public DNA variants which from 25 mutation phenotype, 4 of the 29 variants are not listed in the "lovd" database, including 256T>C(Cys86Arg), 153C>A (Phe51Leu), 506T>C(Leu169Pro), 347T>A(Leu116Gln). Thus, the other 21 unique variants (see Supplementary File 2) should be added to the Table 3. While we noticed that the 21 unique variants have no reported cases. Thus, we mentioned them in the Supplementary File 2 instead of listing them in table 3.

Changes in the text: Line 172-173.

3) There are a few English edits that this reviewer found. Line 83: Which account, change to which accounts

Line 142 the C26:0/C22:0 ratio should be only expressed as a number and not 0.42umol/L in line 143, the C24:0/C22:0 ratio should be a number and not umol/L

Line 143 "isolated from whole blood spots" Suggest removal as the measurements were most likely done in plasma and not whole blood spots.

Lines 147 to 153 including the findings on imaging could be part of the physical findings and moved to line 125 in the case description.

Line 234, replace there with therefore.

Figures 1 and 2 are excellent. The label for Table 1 should be CGs and PEX gene defects in ZSDs

Reply3): The text has been revised based on the aforementioned suggestions. Additionally, Table 1 was deleted following the advice of another reviewer.

Reviewer B

This manuscript presents a case report describing a novel heterozygous variant in the PEX26 gene associated with Zellweger's Spectrum Syndrome. This report is useful for improving diagnosis and understanding of the manifestations of Peroxisome Biogenesis Disorders. I have several suggestions for improvement for manuscript clarity that are listed below.

Additionally, I have the following misgivings about ethical considerations in this paper. I note that I am not trained in matters of patient consent or treatment, so I leave these ethical concerns to the discretion of the editor.

1 – In the footnote, it is stated that the patient and parents have not consented to the publication of the medical information. It is also stated that the patient and parents refused a skin biopsy, which perhaps indicates a lack of support for this research effort. It seems ethically tenuous to publish without their informed consent. At the very least, I might state parent 1 and parent 2 instead of mother and father while publishing sequence variations so as to further remove any identifying information.

Reply 1: The reviewer's comments are greatly appreciated. The patient's family was contacted once again, and the written informed consent from the patient has been obtained and submitted to the editorial office. Additionally, we modified the terms "father" and "mother" to "parent 1" and "parent 2" to further remove any identifying information.

Changes in the text: Figure 1 and Table 1.

2 - Line 166 – "The oral administration of bifendate, a pharmaceutical compound derived from the investigation of Schisandra chinensis in traditional Chinese medicine, was conducted with the aim of reducing liver enzymes." This statement raises ethical concerns about the prescribed treatment options for this patient, as this appears to be an experimental treatment, there is no report on whether or not it was efficacious/had side effects, and there is no description on if this was for research purposes. If this statement is included, there should be a citation to studies on the effect of bifendate on liver enzymes; however, I would advise removing all discussion of the prescribed care for the patient (lines 166-172), as there are no scientific results presented about their efficacy or outcome.

Reply 2: we have removed all discussion of the prescribed care for the patient (unrevised version lines 166-172) as the reviewer advised.

Changes in the text: The last paragraph of **Part 2. Case Description.**

Suggestions for manuscript improvement:

Major:

The main conclusion of this paper is that the novel variants of PEX26 cause the disease symptoms of the patient. While it is likely that the authors are correct that the patient's symptoms arise from the mutations in PEX26, the results are correlative. It would be helpful if the authors could either make available the exome sequences or, at the very least, make a table of SNPs (or lack thereof) identified in the introns and exons of other peroxisome associated genes, so researchers can rule out other contributing factors.

Reply: We have provided the table that presents information on mutations in the coding and shearing regions of ZSD-related genes (see Supplementary File 1). The PEX26 gene variant is considered to be the only pathogenic one.

In an ideal scenario, the authors would show some or all of the following to support the conclusion that the mutations in PEX26 cause symptoms due to a peroxisome disorder: immunofluorescence of peroxisome markers (anti-PMP70 and anti-catalase) in patient and control fibroblasts to verify a peroxisome defect, an immunoblot showing protein levels of PEX26 in patient and control fibroblasts, and complementation of peroxisome defects with re-expression of canonical PEX26 in patient fibroblasts.

Reply: We fully agree with the reviewer 's viewpoint. We made further attempts to communicate with the patient's family and reiterated the significance of conducting a skin biopsy for accurate disease diagnosis. However, despite our efforts, the family remained adamant in their refusal to undergo a skin biopsy procedure. Consequently, studies based on fibroblasts are currently unavailable.

In all cases where medical measurements are given, it should also be clear what the normal range is for those values. For example, the measurements on Lines 126-128 – please state the 'normal' or 'anticipated' range for these measurements. For Line 149 – "The inner diameters of gallbladder cross section were measured as 5.2 cm x 0.9 cm, the spleen thickness measured at 2.6 cm." - please indicate what is considered the normal range.

Reply: The normal ranges have been added to the measurements. Changes in the text: Line 132-134.

The text has been revised to indicate that the inner diameters of the gallbladder cross section and the thickness of the spleen are within normal range. Changes in the text: Line 127-128.

The information in Table 1 is already in the literature (Fujiki et al 2014; https://www.frontiersin.org/articles/10.3389/fphys.2014.00307/full), and it is not necessary to present it.

Reply: We have deleted the Table 1 as the reviewer advised.

Line 89 – "Pex26 directly interacts with Pex14 through its N-terminal binding domain, known as the Pex6-binding domain, acting as a scaffold to recruit Pex1-Pex6 and form a heterooligomeric complex called the AAA ATPase complex." The wording is confusing, and needs citations. In Guder et al, Pex26 does not interact with Pex14 through its Pex6 binding domain, but rather HR2. In Tamura et al, Pex26 interacts with Pex14 through its 'Pex6 binding domain'. This domain should be named by amino acid numbering rather than 'Pex6 binding domain', given that it might interact with multiple proteins.

Reply: We have modified the sentence as advised and cited relevant reference (Tamura et al).

Changes in the text: Line86-87.

Line 91 – "acting as a scaffold to recruit Pex1-Pex6 and form a heterooligomeric complex calledthe AAA ATPase complex." A 'AAA-ATPase complex" is a general class of motor protein that is not specific for Pex1/Pex6 or peroxisome biology. The term often used to describe the Pex1/Pex6/Pex26 complex at the peroxisome is the "exportomer".

Reply: We have modified the word "AAA ATPase complex" as "peroxisome exportomer complex".

Line 165 – "we concluded that the patient may have suffered from NALD". In the intro it wasn't clear what distinguishes NALD from ZS or IRD, so it isn't clear why the authors made the diagnosis of NALD instead of just ZSD. The name suggests leukodystrophy as a defining feature, but the cerebral MRI was normal. Can the authors further elaborate on their diagnosis?

Reply: The historical terms "Infantile Refsum (IRD)" and "neonatal adrenoleukodystrophy (NALD)" have been excluded based on the recommendation of another reviewer, as these two categories have now been lumped under the umbrella term of “Zellweger spectrum disorders (ZSDs)”

The phenotype of the *PEX26* variant is classified into two subtypes, namely Peroxisome biogenesis disorder 7A (Zellweger) and Peroxisome biogenesis disorder 7B based on the documented severity of clinical manifestations in the OMIM database. The patient's mild clinical presentation classifies him under the latter subtype.

Changes in the text: Line 93-96; Line 166-167.

Minor:

Line 115 “on standardized growth curves” Please cite the source of the growth curves, for example WHO or CDC?

Reply: We have cited “Capital Institute of Pediatrics” as the source of growth curves.

Changes in the text: Line 113-114.

“however, he had not yet attained independent sitting ability” Please specify the age by which he had not yet attained sitting ability.

Reply: We have added the age by which he had not yet attained sitting ability.

Changes in the text: Line 119.

Line 135 – define AFP.

Reply: We have defined AFP.

Changes in the text: Line 142.

Line 137 – define EB

Reply: We have defined EB.

Changes in the text: Line 144.

Line 159 – “while the latter is a nonsense mutation and suspected to be pathogenic variant as it

mediates mRNA degradation leading to downregulate of gene expression." Since the authors did not measure mRNA levels, this is speculation, and it should be made clear that it is speculation. It could also be a pathogenic variant due to the truncated form of the protein.

Reply: We added the word "may" to make it clear that it is speculation.

Changes in the text: Line 159.

Line 176 – "To date, a total of 24 genotypes and 29 mutations have been documented in the PEX26 gene." This needs a citation. Other citations mention 32 pathogenic variants - https://dx.doi.org/10.21037/tp-21-103

Reply: Thanks for the reviewer's recommendation. Some mutations listed in this mentioned literature are duplicated, such as "c.265G>A. p. (Gly89Arg)", "c.153C>A. p. (Phe51Leu)", "c.292C>T. p. (Arg98Trp), c.131T>C. p. (Leu44Pro)", etc. (see below). The number "32" may refer to the number of cases instead of unique variants.

Figure 1 - There is a typo in "Mather", which should be "Mother"

Reply: The terms "Father" and "Mother" in Figure 1 have been replaced with "Parent 1" and "Parent 2"

Figure 1 - It is difficult to read the colored letters indicating the sequence calls.

Reply: The quality of the figure has been enhanced.

Figure 1 - It would be helpful to be able to easily compare the nucleotide calls (colored letters above peaks) with the canonical sequence, which is not indicated. At first, I assumed the canonical sequence was the top row of nucleotide calls, but for the c.347T>C mutation, the patient genotype and father's genotype are switched indicating a C to T mutation in the patient while the Father's genotype indicates a T to C mutation. You could correct this and label the top row as canonical.

Reply: The canonical sequences have been incorporated into Figure 1.

Figure 1 - Sanger sequencing following whole exome sequencing was not mentioned in the text, but is shown in Figure 1. Please mention in the text.

Reply: The Sanger sequencing has been mentioned in the text as advised.

Changes in the text: Line: 163.

Figure 2 - This figure would be improved by references of where these mutations come from. I suggest citing Table 2 in the figure legend.

Reply: We have modified the figure legend as advised.

Changes in the "Legends" section.

Line 183 – "However, it is not necessary for Pex26 to be localized in peroxisomes for its function, as the physiological splice variant PEX26Δex5 lacking a transmembrane domain remains functional (17)." This statement "it is not necessary for Pex26 to be localized in peroxisomes for its function" warrants more discussion. Weller et al clearly shows that Pex26 when targeted to the mitochondria is still functional, as is Pex26ex5, which is mostly cytosolic. However, these experiments don't rule out that some Pex26 is present on peroxisomes. Additionally, Guder et al shows that Pex26ex5 still binds Pex14, so it is reasonable that Pex26 could still be at peroxisomes even if the majority is elsewhere.

Reply: The controversial sentence was omitted without impacting the speculation that "variants involving other domains may only cause minor or negligible phenotypic abnormalities".

Changes in the text: Line 180-184.

Line 186 - 188 – "mutations affecting the formation of AAA ATPase complex" and "variants involving other domains". Please be more specific than "AAA ATPase complex" and "other domains". Do you mean Pex1/Pex6 or Pex1/Pex6/Pex26? By other domains, do you mean the heptad repeats and transmembrane helix? Also, there is a typo in "will be result in PBD CG8".

Reply: "AAA ATPase complex" and "other domains" were replaced with "peroxisome exportomer complex" and "transmembrane domain and heptad repeats"

Changes in the text: Line 182-183.

Line 188 – "This could explain why mutation sites tend to concentrate within the Pex6-binding domain (Figure 2)." The logic of this statement relies on selection bias that is not clearly explained. Mutations should occur with the same frequency throughout the PEX26 gene due to errors in DNA repair, etc. The observed mutations are listed because they were identified from symptomatic

patients – so not deleterious enough to kill the patient, but enough to cause symptoms, which is further muddled by heterozygosity. Please edit your point for clarity.

Reply: In response to the reviewer's correction, this point was omitted.

Line 234 – typo – there should be therefore

Reply: We have corrected the typo.

Changes in the text: Line 235.

Reviewer C

In this manuscript, the authors report a case of Zellweger spectrum disorder caused by PEX26 pathogenic variants. Although the clinical and laboratory findings are not atypical of this condition, the PEX26 variants are novel and potentially helpful in characterizing the protein functional domains. Moreover, in these rare disorders, case reports correlating clinical features to genotype and other laboratory test results are critical to appriciate the full phenotypic spectrum of the disease. I would suggest addressing the following:

1) Did the whole-exome sequencing (WES) analysis exclude any other variant from other PEX genes? Because of the overlap in clinical presentation and laboratory findings, I think that it is important to clearly state that no other gene was implicated in this case. This was implied but not clearly stated.

Reply 1): We have modified our text to clearly state that no other gene was implicated in this case.

Changes in the text: Line 163-164.

2) The dicarboxylic aciduria observed by gas chromatography–mass spectrometry (GC–MS) analysis could have been dietary in origin. I think that suggesting a dysregulated fatty acid metabolism based on a fairly common and non-specific finding is a stretch. I would re-word or take out this section

Reply 2): The phrase ", indicating dysregulated fatty acid metabolism" was omitted as advised.

Reviewer D

This case report describes a patient diagnosed with Pex26 deficiency causing peroxisomal biogenesis defects. Abnormalities of Pex26 are rare and there are limited numbers of individuals described with this subtype of Zellweger spectrum disorder. I appreciate the authors' efforts to publish additional cases and new variants associated with characteristic biochemical and clinical features.

Minor Revisions

1) Key findings: "…dysfunction of the Pex26 protein result…" should be "results" to match the single dysfunction as the subject of the sentence.

Reply 1): We have corrected the typo.

Changes in the text: Key findings.

2) I recommend changing word choice for a couple of specific phrases. The clinical genetics community at large is trying to change the identification of large-scale sequencing to "exome sequencing" rather than "whole exome" since most platforms don't truly cover the entire exome and the terminology risks misinterpretation and misunderstanding.

Reply 2): The term "whole exome" was substituted with "exome sequencing" as recommended.

3) The genetics community is avoiding the term "mutation" when describing human genetics. "Variant" or "genetic change" are helpful alternatives. These word choices are not requirements of this journal but are becoming more normative in clinical genetics dialog and journals. Minor edits of word choice can impact the tone and reader impressions.

Guide for authors - Genetics in Medicine - ISSN 1098-3600 (elsevier.com) (see section on Terminology for efforts to provide clarity and inclusion when discussing human genetics)

Reply 3): The term "mutation" has been substituted with "variant" whenever feasible.

4) Recommend simplifying terms and MIM identifiers used in the introduction to describe peroxisomal disorders. "Infantile Refsum" and "neonatal adrenoleukodystrophy" are historical terms that are phenotype-based before the genetic causes were identified. These two categories have now been lumped under the umbrella term of "Zellweger spectrum disorders." For clarity,

I'd recommend removing the historical terms and sticking with exclusively Zellweger spectrum disorder for this manuscript. I'd also recommend removing the current MIM numbers and only using the one specific to Pex26: MIM 608666.
Entry - *608666 - PEROXISOME BIOGENESIS FACTOR 26; PEX26 – OMIM
Reply 4): We removed the historical terms "Infantile Refsum" and "neonatal adrenoleukodystrophy".

5) Tables 1 and 3 include the historical terms for phenotypic information highlighted in item 4 above. Recommend removing the NALD and IRD terms from Table 1 and deleting the phenotype column from Table 3 as it does not add much information. Most of the variants in Table 3 have single patient cases which is insufficient evidence to establish a phenotypic spectrum.
Reply 5): The entire Table 1 was removed based on the recommendation of another reviewer.
The phenotype column from Table 3 was removed as advised.

Reviewer E

The authors here describe the clinical outcome of a 7-month old boy with complex heterozygous mutations in the PEX26 gene. They have identified new mutations in the gene, expanding the spectrum of known mutations in the PEX26 gene. This is definitely an interesting case study with valuable information useful for researchers in the field. In particular, the table 3 summarising the various PEX26 mutations recorded so far is a useful resource. However, the manuscript could benefit from some more information or changes as outlined below.

Major comments:
Is there something known regarding what time of the gestation period the patient was delivered, with information on the birth weight of the patient, etc.?
Reply: The birth information has been incorporated into the Part 2 Case Description, Line 111-112.

ZSD patients often suffer from retinal abnormalities. The authors also mention this in their introduction. Was this studied in the current patient? It would be useful to note this (even the lack

of it) in the study if possible, especially considering that previous reports have suggested retinal problems in PEX26 mutant patients (PMID: 34430430, PMID: 33912394).

Reply: Thanks for the reviewer's advice, fundus examination was absent while we have mentioned that the vision should be further conducted to monitor disease progression in Part 3 Discuss, Line 245.

The authors simply mention that the parents are "heterozygous carriers for both mutations". However, looking at Figure 1, it appears that each parent is heterozygous to one of the 2 mutations, which is unclear from the wording in the text. It would be ideal if the authors would make it clear in the text.

Reply: We have modified the sentence make it clear as advised.

Changes in the text: 161-163.

Line 149-150: While the dimensions of spleen and gall bladder are mentioned, it would be useful to know for a non-clinician whether these dimensions are normal or not.

Reply: We modified the sentence to indicate that these dimensions are normal.

Changes in the text: Line 127-128.

Line 178-179: "Among these genotypes, more than half are homozygotes and present as ZS, which is consistent with the conclusion of Yue et al (15)." It appears that the authors borrow the conclusion of He et al. from ref. 15. So it is confusing when the authors say their conclusion is consistent with the conclusions from He et al. Moreover, the authors seem to refer to the first name of the author of this study, which deviates from usual conventions and is confusing.

Reply: The sentence "which is consistent with the conclusion of Yue et al (15)" has been removed.

Line 195-195: The observations of ref. 18 from Peter Kim group claiming a potential rescue of peroxisomal numbers, etc. upon inhibition of autophagy, are challenged by other observations from the group of (PMID: 33869228).

Reply: Thanks for the recommendation. The concepts presented in the aforementioned article have been incorporated for discussion.

Changes in the text: Line 193-196.

The relevance of the paragraph in lines 202-224, describing the biochemical alterations and their consequences in ZSD, to this study is unclear, given that not much is known regarding the biochemical alterations in PEX26 patients. The levels of DHA or plasmalogens were not determined.

Reply: The purpose of this paragraph is to elucidate the biochemical basis underlying clinical symptoms associated with ZSD and to advise clinicians on the importance of monitoring these corresponding symptoms for disease management and appropriate treatment interventions. The complete assessment of biochemical abnormalities was not conducted; however, we think it is important to bear in mind the specific biochemical tests that should be pursued during subsequent follow-up. Thus, the paragraph has been retained, and we have made necessary revisions based on the reviewer's suggestions (Line:204-216).

Minor comments:

The complementation groups refers to PBDs and not ZSDs.

Reply: We checked the test again and found that we used PBD CG8 instead of ZSDs CG8 in the text.

If possible, the levels of plasmalogens would also be an interesting parameter to examine in these patients, given that their levels are seemingly not yet known in patients with PEX26 mutations.

Reply: We totally agree with the reviewer’s perspective, while the measurement of plasmalogen levels remains unavailable within our medical facility.

Line 73-74: suggestion to use “which represent a heterogeneous group of mostly autosomal recessive disorders.” An autosomal dominant ZSD was recently reported (PMID: 37493040).

Reply: We have modified the sentence.

Changes in the text: Line 72-73.

Line 204-205: “Tissue accumulation of VLCFA leads to damage in the brain, nerves, and adrenal glands” needs the right specific reference.

Reply: The section discussing the potential clinical significance of the biochemical abnormalities (Line 204-214) was cited from Klouwer et al. (PMID: 26627182). We have included an introductory sentence at the beginning of this section and referenced this source.

Line 206-207: “Impaired shortening of VLCFA chains results in a defect in the synthesis of docosahexaenoic acid (DHA), which is an essential component for the brain and retina”. It is not the most easy sentence to comprehend. The wording “VCLFA chains” is not the most accurate. Moreover, factually, VLCFA generally represents saturated fatty acids, while DHA is synthesized from the breakdown of C24:6 n-3 which can be considered a VLC-PUFA (very long chain polyunsaturated fatty acid). So it is in general difficult for someone to put together how impaired VLCFA shortening can affect DHA synthesis.

Reply: We appreciate the reviewer's correction and have made the necessary revisions to the text. Changes in the text: Line 207-208.

Line 207-208: “The excessive presence of pristanic acid can impair cerebral function.” Can the authors provide a specific reference for this?

Reply: The section discussing the potential clinical significance of the biochemical abnormalities (Line 204-214) was cited from Klouwer et al. (PMID: 26627182). We have included an introductory sentence at the beginning of this section and referenced this source.

Line 208-211: “The presence of bile acid intermediates, namely 3α,7α,12α-trihydroxycholestanoic acid (THCA) and 3α,7α-dihydroxycholestanoic acid (DHCA), has been observed in the brain and liver and is believed to contribute to central nervous system damage and liver toxicity.” References are missing. Moreover, is there evidence that bile acids damage the nervous system?

The section discussing the potential clinical significance of the biochemical abnormalities (Line 204-214) was cited from Klouwer et al. (PMID: 26627182). We have included an introductory sentence at the beginning of this section and referenced this source.