Peer Review File

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Reviewer A

This paper has little novelty. Discussion in this paper is poor.

The number of LOPD patients in this paper is very small. The present version is not accepted for publication if they do not desperately revise their paper.

Reply: We added some content to enrich the discussion, see Page 13 line 273-279, Page 14 line 296-307. This study concluded genetic features and clinical manifestations of LOPD in Eastern Chinese patients. I recognized this paper is short of innovation. In the future, we will concentrate on the research of Pompe disease, for example, conduct comprehensive nutritional and metabolic assessment of LOPD patients, explore the influence of diet and rehabilitation management, and so on.

Reviewer B

This manuscript focuses on the phenotypic and GAA variant spectrum within a group of late-onset Pompe disease patients. The authors have done well detailing the phenotypes and management of the patients from mainland China. While many details are provided, further revisions are needed before the manuscript can be accepted for publication. They are outlined below:

Enzyme activity from dried blood spots is noted. Were these dried blood spots ascertained through newborn screening? Additionally, did any of these individuals undergo newborn screening for Pompe Disease prior to this report? Lastly, were any confirmatory enzyme assays performed on isolated lymphocytes from whole blood in addition to testing on dried blood spots? If not, can the authors comment on why enzyme testing on isolated lymphocytes was not performed?

Reply: These dries blood spots didn't ascertain through newborn screening and nobody underwent newborn screening for Pompe disease prior to this report, it was used to diagnose the disease when the patients came to our department. Because of the low awareness of disease and other reasons, until now, newborn screening for Pompe disease is not carried out in Jiangsu Province.

We measured the activity of the enzyme GAA in dried blood spots (DBS) and didn't confirm enzyme assays performed on isolated lymphocytes. Literature reports the determination of GAA activity in DBS is sensitive and time-saving, and is suitable for high throughput analysis and newborn screening for Pompe disease. Furthermore, the

blood collection and storage of DBS is convenient for transportation, and the activity of GAA is stable for storage in several weeks. The method in DBS is a mature method and widely used to detect the GAA activity. However, about enzyme testing on isolated lymphocytes, the method operates complexly, and low purity of lymphocyte separation will result in false negative test.

Within the NGS methods paragraph (lines 122 - 140), what orthogonal method was used to confirm the variants before returning results to the patients (e.g. Sanger sequencing)? Additionally, NGS does not have uniformity of coverage across all exons for some genes. What was the average NGS depth of coverage across the GAA exons? For any exons with reduced coverage (e.g. less than 100x), what method was used to ensure adequate coverage of those exons that failed to have adequate coverage by NGS (e.g. Sanger sequencing to fill in gaps in coverage)? These details need to be included in the methods. Lastly, was the common exon 18 deletion tested?

Reply: We use sanger sequencing to confirm the variants before returning results to the patients, and now we included these details in the methods Page line 155-165. Besides, most patients underwent pedigree verification, the results were added in Supplementary Table S1.

Table 1 needs to include proper HGVS nomenclature, including the RefSeq transcript used for the cDNA nomenclature as well as the predicted protein change (e.g. NM_000152.5(GAA):c.2238G>C (p.Trp746Cys)). Alternatively, if only the cDNA and predicted protein change are to be described in Table 1, the RefSeq transcript that the variant impacts need to be referenced somewhere within the manuscript (e.g. below Table 1 or in the methods section). Additionally, some labs will include the genomic coordinates of the variant as well.

Reply: We added proper HGVS nomenclature in Supplementary Table S1.

Can the cut-off or reference range for GAA enzyme activity be listed in Table 1? This will help readers determine the % GAA enzyme activity relative to a reference/control.

Reply: We have listed the reference range for GAA enzyme activity in Table 1 and in Page 6 line 131-132.

HGVS nomenclature does not use X for nonsense variants. p.E721X in Line 62 needs to be updated to Ter or *.

Reply: We have updated p.E721X to p.E721* in line 212.

In Table 1, a column needs to be included next to each allele to denote the ACMG interpretation of the variant according to the guidelines set forth in Richards et al. Genet Med. 2015 May;17(5):405-24. doi: 10.1038/gim.2015.30. PMID: 25741868. This should be noted as pathogenic (P), likely pathogenic (LP), or variant of uncertain significance (VUS).

Reply: The results about the ACMG interpretation of each allele were added in Supplementary Table S1.

Is the CRIM status known for the patients in the manuscript (see PMID: 22252923, Bali et al. Am J Med Genet C Semin Med Genet. 2012 Feb 15;160C(1):40-9. doi: 10.1002/ajmg.c.31319.)? If so, it needs to be indicated in Table 1. If it is not known, can the authors comment on why it is unknown?

Reply: We knew that the CRIM status was related on the effect of enzyme replacement therapy, but in the early years of learning Pompe disease, we were short of the experience of the research of Pompe disease. The detection of CRIM status needs the sample of patient fibroblast cells which is invasive and the turn-around time is several weeks owing to culture time for fibroblasts. Besides, in our department, we don't have mature detection technology.

Were parents of the patients tested for the respective GAA variants in the proband? If not, how can one know the phase of these variants? If phase is unknown, that fact needs to be stated in the results.

Figures 2B-E appear to be Sanger sequencing traces. If Sanger sequencing was performed, the methods need to include details about how the Sanger sequencing was conducted and Sanger sequencing needs to be indicated in the figure 2 legend Also, were only the four variants listed in figure 2 confirmed by Sanger sequencing? The other variants in Table 1 need to be confirmed by Sanger sequencing as well.

Reply: Most patients underwent pedigree verification, the results were added in Supplementary Table S1. Some patients could not be verified by pedigree for some reasons. We thought that depending on clinical features, reduced GAA enzyme activities and mutations of the GAA gene, we can confirm the diagnosis, even though we lacked some genetic information about family.

On line 185, a citation is needed referencing the Richards et al. ACMG Interpretation

guidelines for sequence variants (PMID: 25741868).

Reply: We added the reference the Richards et al. ACMG Interpretation guidelines for sequence variants (PMID: 25741868) on line 216.

In reference to lines 175 - 179, what other evidence exists to indicate pathogenicity of the two novel missense variants besides in silico data and protein modeling? Are the variants absent from gnomAD? Even if the variants are absent from gnomAD, in-silico programs predict the variants to be pathogenic, and the enzyme activity is low in these patients; altogether this evidence is not sufficient for the variant to be deemed pathogenic. Unless further detailed justification can be provided in the manuscript for these variants to be interpreted as pathogenic, they need to be classified as variants of uncertain significance in the manuscript.

Reply: In our study, we indicated pathogenicity of the two novel missense variants in silico data and protein modeling. We didn't perform cell and animal experiments to verify the pathogenicity of two novel missense variants. In future, we will carry out relevant basic research. The two novel missense variants need to be classified as variants of uncertain significance in the manuscript.

In line 182: c.2832delA (p.E945Sfs*78) occurs in the last exon of GAA according to figure 2A. According to the Richards et al. ACMG interpretation guidelines (PMID: 25741868), one should be careful in applying PVS1 as a criterion to variants in the last exon of a gene since it is unknown if the protein will merely be truncated, but still functional without undergoing nonsense-mediated decay. What other evidence exists supporting a classification of pathogenic for this variant? Using the Richards et al. ACMG interpretation guidelines (PMID: 25741868), if there is not enough evidence to support this variant as likely pathogenic or pathogenic, it needs to be classified as a VUS.

Reply: Thank you for your suggestion, there is not enough evidence to support c.2832delA (p.E945Rfs*78) as likely pathogenic or pathogenic, so this variant needs to be classified as a VUS.

Were any of the pseudodeficiency alleles detected in any of the patients reported in the study (NM_000152.5(GAA):[c.1726G>A;c.2065G>A] or c.271G>A p.(Asp91Asn))? This needs to be indicated in the text of the manuscript whether these alleles were detected or not in any of the individuals. If they were detected, which individuals had the alleles need to be indicated in table 1.

Reply: We didn’t detect any of the pseudodeficiency alleles in the study.

Lastly, there are grammatical errors in many places in the manuscript that need to be corrected prior to publication.

Reply: We correct some grammatical errors in the manuscript, meanwhile, we seek the help of medical writing service to polish our article.