Peer Review File

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Reviewer A:

The article presents the data from 573 genes panel NGS analysis in 1173 Chinese newborns. I have following questions and remarks to the authors:

1. There are 1173 newborns mentioned in the title, however 1127 samples were in fact analyzed due to quality issues. Hence, I think that referring to 1173 in each part of the manuscript is misleading

Reply 1: We thank the reviewer for the suggestion. We have modified our title and text as advised.

Changes in the text: Please see page 1, line 2&17; page 2, line 78; page 5, line 211, page 6, line 350.

2. Was the project approved by ethical committee?

Reply 2: We thank the reviewer for the comment. Residual dried blood spots were reused in this study. The project was approved by the Xinhua Hospital Ethics Committee Affiliated to Shanghai Jiao Tong University School of Medicine (XHEC-C-2017-021-2). Please refer to Page 7, line 375.

3. English needs to be carefully corrected, also in diseases names eg. lactase, not lactose deficiency. Line 172 p9 I assume that the authors did not find any variants in those genes, but the genes themselves were present? This error is also repeated in other parts of the text.

Reply 3: We thank the reviewer for the advice. We have modified our text as advised. We have carefully checked the manuscript and corrected some inappropriate expression. Candidate variants of those genes were not identified in the cohort. We have modified as advised.

Changes in the text: Please see Page 10, line 743.

4. In the abstract, the authors say, that this study was intended to investigate whether NGS can increase the detecting rate of genetic disorders. The authors identified 5 affected children using their approach. In one case they clearly showed that biochemical NBS was false negative, but I cannot find information about the general rate of children diagnosed using standard biochemical and MS/MS approach in China. This part should be included and discussed in the text. Furthermore, this aim of the project is not clearly defined in main body of the manuscript. In this part we can read that this study investigated the carrier frequencies of IMD. Hence, I think that the aim should be redefined and unified.

Reply 4: We thank the reviewer for the suggestion. We have modified our text as advised.

We identified 5 affected children, 4 of whom carried hemizygous *G6PD* causative variants, and the other one carried compound heterozygous mutations of *SCL22A5* (ID 84123).

MS/MS screening for individual 84123 in 2019 showed reduced free carnitine deficiency (C0 value: 4.3μmol/L, reference: 10-60μmol/L), which was consistent with the results of the genetic analysis, indicating a false-negative MS/MS finding at birth (C0 value: 11.6μmol/L, reference: 10-60μmol/L). For inherited metabolic disorders that were tested by MS/MS, the diagnostic rate

was between 1:10165 to 1:2363 according to previous NBS studies of large cohorts in three provinces of China (1-3).
The aims of the study have been redefined and unified. We compared the biochemical results with genetic variants in these newborns and attempt to investigate whether NGS could identify neonates with severe inherited disorders that were confirmed by current biochemical screening effectively and whether NGS screening could be used as a supplement to improve the detection rate of biochemical screening. This study also aimed to investigate the carrier frequencies of mutations in genes related to amino acid metabolism, organic acid metabolism, and fatty acid oxidation disorders in this cohort.
Changes in the text: page 5, line 212-216, page 6, line 336-341.

5. The general aim of NBS is presymptomatic testing of those diseases, where rapid and presymptomatic treatment is available. Hence, my next question about this aspect and, precisely why those genes were selected for the panel? And is it justify with respect to ethical and financial issues to perform such wide analysis in every child and not to focus on treatable disorders in NBS only and perform WES in children manifesting any symptoms?
Reply 5: We thank the reviewer for the comment. Currently, genomic screening has been gradually recognized, but the panel used in genomic screening varies among different organizations. Some projects targeted on treatable inherited disorders (PMID: 29961769) while some projects like the BabySeq project in USA, WES was performed on individuals including healthy newborns (PMID: 30609409). This is a pilot study that residual dried blood spots were reused, and the cost of wide genetic analysis has decreased greatly with the wide application of NGS.

6. The authors should add the paragraph describing the limitation of NGS procedure. For example, there are genes such as DMD, where typically large rearrangements – deletions and duplications occur or H19, whereas Silver –Russel syndrome is frequently caused by epimutations.
Reply 6: We thank the reviewer for the comment. We have modified our text as advised. Several diseases of high clinical importance are technically challenging, making them difficult to assess with target sequencing. For example, Duchenne muscular dystrophy is typically caused by exonic rearrangements (4, 5), whereas Silver-Russell syndrome is frequently caused by epimutations of *H19/IGF2* imprinted domain (6, 7). Some diseases such as spinal muscular atrophy have high homology (e.g., pseudogenes), special techniques are needed as pseudogenes are highly identical to the causative genes (8).
Changes in the text: page 14, line 1043-1050.

7. I have some doubts about the variant filtration procedure. Does filtering out variants with freq >0.2% prior to ClinVar pathogenicity status evaluation not lead to omission of any pathogenic variant? Why missenes were not among candidate mutations when absent from ClinVar or HGMD.
Reply 7: We thank the reviewer for the comment.
For the first question, we have corrected the description of criteria in this version.
Considering there are false positive variants in HGMD, we set the criteria of upper frequency limit to reduce the false positive rate. For candidate variants that were absent in ClinVar

database and only appeared in HGMD, the variants with >0.2% frequency in this cohort or in the population variant databases - Genome Aggregation Database (gnomAD), and 1000Genomes of East Asia database were filtered. Data from our NBS center revealed that hyperphenylalaninemia caused by *PAH* mutations is the most common inherited metabolic disease except for G6PD deficiency that could only be triggered by exposure to exogenous primaquine or fava beans. Hence, we referred to the criteria for BS1 (0.2%) of *PAH* as the upper frequency limit for variants that only appeared in HGMD (9).
Furthermore, in case two variants were identified in an autosomal recessive disorder that both met any piece of the three criteria in a subject, or one variant was identified in an autosomal dominant or X-linked disorder that met any one of the three criteria, the variants were exempted from the frequency limitation and further evaluated. For example, we identified two other newborns with variants that met the inclusion criteria (a variant on *EYA1* for one case, and a variant on *MYO1A* for another case), which were not mentioned in the manuscript. Later we excluded them after further assessment as the evidences of pathogenicity for the variants were not strong enough and the parents of the two children also described their well-growth.

For the second question: We considered evaluating the pathogenicity of variants according to ACMG guideline. However, many items of evidence were not applicable for novel missense variants in a newborn screening study without phenotype information, including the evidences listed below.
I. PVS1: Null variant (nonsense, frameshift, canonical +/−1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function is a known mechanism of disease. PVS1 is not applicable for missense variants.
II. PS2: De novo (both maternity and paternity confirmed) in a patient with the disease and no family history. We reused residual dried blood spot without parents' samples. Therefore, PS2 was not applicable in this situation.
III. PS3: Well-established functional studies supportive of a damaging effect on the gene or gene product. The HGMD professional version was used to interpret variants with updated variants in literature. Therefore, PS3 is not applicable for a novel missense variant that had not been studied.
IV. PS4: The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls. PS4 is not applicable for a novel missense variant because the prevalence of the variant is unknown.
V. PM3: For recessive disorders, detected in trans with a pathogenic or likely pathogenic variant, which requires testing of parents to determine phase. PM3 is not applicable for a novel missense variant in a screening study if another causative variant had not been identified in trans phase.
VI. PM4: Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants. PM4 is not applicable for missense variants.
VII. PM6: Assumed de novo in a patient with the disease. Just like PS2, PM6 was not applicable in this situation.
VIII. PP1: Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease. PP1 is not applicable for a novel missense variant without co-segregation information either in literature or in this study.

IX. PP4: Patient's phenotype or family history is highly specific for a disease with a single genetic etiology. PP4 is not applicable for a novel missense variant without phenotype information either in literature or in this study.
X. Besides, there are some other evidences that are not applicable for some candidate genes. For example, PM1 (located in a mutational hot spot and/or critical and well-established functional domain) and PP2 (missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease) are not applicable for PAH according to ClinGen *PAH* Expert Panel Specifications to the ACMG/AMP Variant Interpretation Guidelines.
The evidences applicable for a novel missense are very limited. According to the ACMG criteria, even some causative missense variants reported in literature could not be rated as pathogenic/likely pathogenic variants if the number of patients is not enough or the information of patients had not been described fully. A novel missense variant is very likely to be rated as a variant of uncertain significance (VUS). Some missense variants that are rated as VUS might be proven to be causative variants later if more evidences of pathogenicity are supplemented
Since this is a screening study without phenotype information, potential causative variants are indistinguishable among novel missense variants until more evidences of pathogenicity are applicated. It is hard to avoid the omission as we need to balance the rate of newborn recalling. Besides, NGS screening is the supplement of biochemical screening, we will evaluate both the biochemical values and genetic variants.

Changes in the text: page 8.

8. Which version of HGMD was used by the authors?
Reply 8: The HGMD 2018-04 professional version was used to interpret variants.

9. In the discussion, the part describing the 17-OHP (page 10) needs to be more profoundly discussed and needs some conclusions.
Reply 9: We thank the reviewer for the comment. We have modified our text as advised.
Changes in the text: page 11, line 777-783.

10. The fig1 lacks description
Reply 10: We thank the reviewer for the comment. The figure legend has been added on the last page of the manuscript.
Changes in the text: page 19.

Reference

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