Peer Review File

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Reviewer A

The Authors present results of retrospective DMD screening on already available exome sequencing data of 10,481 newborns admitted to the intensive care unit between June 1, 2016 to June 30, 2020.
We really appreciate the constructive suggestion and comments from you.

Comment 1: While reading the manuscript the suggestion is made that no DMD experts (both clinical and genetic) were involved. The DMD information provided in the manuscript has to be carefully checked for correctness.

Reply 1: Thank you for your advice and comments. The suggestions of DMD experts are very important in our study.
We further discussed these cases with Prof. Xihua Li and Prof. Yunafeng Zhou.
Prof. Xihua Li is a molecular geneticist of DMD and works on the DMD management during her career.
Professor. Yuanfeng Zhou who is a neurologist and works on the management and follow-up of the DMD patients.
Because we cannot add their names in the authorship, we appreciate their suggestions to our study.
We thoroughly discussed these cases about the types of mutations, the current phenotypes, the family history, the previously reported cases, the clinical assessment based the 2010 DMD recommendation supported by the US CDC and the updated version, and also how to consult these families. We revised our manuscript thoroughly.

Comment 2: The genetic description of the variants detected has to be updated to the most recent version of HGVS nomenclature, especially the SNVs. For CNVs HGVS nomenclature is more difficult to use and less clear for the reader, it can be considered to use HGVS nomenclature together with the already used description of the exons deleted/duplicated. The latter description of CNVs is more clear to the reader, thus has to be kept in the manuscript. Also information about the impact of the CNV on the reading frame of the DMD gene has to be provided in the manuscript.

Reply2: Thank you for your comments and very useful information which help to improve our paper.

We check the most recent version of HGVS nomenclature, and revised the SNVs of Neo_18 and Neo_19.

Changes in the text: Neo_18: changes to "c.5452G>T (p.Glu1818Ter)";Neo_19: changes to "c.6408G>A (p.Trp2136Ter)"

We check the CNVs HGVS, as you said CNVs HGVS nomenclature was more difficult to use and less clear for the reader, also the reading frame of the *DMD* gene was important for the DMD diagnosis. Therefore, we referred to the HGVS nomenclature and predicted mutation type of from Leiden muscular dystrophy database website.

For example: Neo_2: c.(6614+1_6615-1)_(6912+1_6913-1)del/exon 46-47 del/Out-of-frame del

Comment 3: But what worries most is that some CNVs have been classified as pathogenic, while it is really not clear that these CNV are pathogenic.

Reply 3: Thank you for your advice and comments. Because we had limited clinical phenotype currently, we agree with you that the definition of the pathogenic variations of *DMD* gene is important in our study.

In order to get more supportive information, we consulted these cases with two DMD experts as stated above. We further checked the DMD locus specific databases including Leiden Open Variation Database, LOVD, the UMD-DMD, the eDystrophin, and the TREAT-NMD DMD Global database and HGMD. Combined with the persistent elevated CK, family history, previously reported cases and reported cases in our home database, we though these variations were pathogenic.

Comment 4: On the contrary, it is quite possible that these variants are not pathogenic but benign. Also discrimination between CNVs most likely resulting in Duchenne muscular dystrophy and CNVs most likely resulting in Becker muscular dystrophy is lacking in the manuscript.

Reply 4: Thank you for advice. We totally agree with you that variable phenotypic expression relates mainly to the type of mutation and its effect on the production of dystrophin. We discussed these cases with two experienced DMD experts. Therefore, we further classified these cases as DMD cases, BMD cases, and uncertain cases based on the reading frame rule.

Among eight cases with DMD, six cases (Neo_2, Neo_4, Neo_5, Neo_11, Neo_14, Neo_15), they had the out-of-frame deletions during the 45 to 50 exons. Neo_9 had the out-of-frame duplications of exon31-43 and high CK level(50000IU//L). Neo_16 had out-of-frame deletions of exon 22-37 and family history of DMD.

Six cases (Neo_6, Neo_8, Neo_5, Neo_10, Neo_12, Neo_13) were diagnosed with BMD based on the reading frame rule. And they had normal or mild elevated CK. However, the further management could not only depend on the types of mutations. There are variable phenotypes in BMD patients. Some patients can present severe form of BMD, which the clinical symptoms are similar to the DMD, they will be managed as DMD patients.

We also had five cases (Neo_1, Neo_7, Neo_17, Neo_18, Neo_19) who were difficult to make a diagnosis of DMD or BMD, based on current information.

We have added this information in the result part and discussion part.

Comment 5: I am concerned that the results have been interpreted in this way and then discussed with the parents as well. The data must be reinterpreted by a clinical laboratory geneticists with expertise in DMD, followed by correction steps where necessary.

Reply 5: Thank you for your comments and advice. This is one place where we did not make ourselves clearly, and we apologize for any misunderstanding you may have had. We did not consult patients directly. We updated these genetic report and referred them to the DMD experts or neurologists.

According to your valuable comments, we discussed these cases with the geneticists of DMD and neurologist, we classified these cases as stated above.

Fourteen patients with diagnosis of DMD or BMD referred to the DMD clinic for the scheduled follow-up plans and treatment guidance, and others without definitive diagnosis of DMD or BMD suggested for further follow-up in a genetic clinic. Two DMD experts as stated above will closely monitor these infants.

Reviewer B

This paper investigates clinical utility of next-generation sequencing-based DMD screening. Genomic screening for DMD would identify patients who might not come to clinical attention prior to disease manifestation. It is known that early targeted intervention of DMD is important. But I have some general comments:

We really appreciate the constructive suggestion and comments from the editor and reviewers,

which will improve our revised manuscript. And we answered all the questions point by point.

Comment 1. Table 1: It can be interesting to include the open reading frame of mutations. The correlation of deletions with clinical severity usually depends on their effect on the open reading frame. Deletions that disrupt the translational reading frame generally lead to complete or near-complete absence of dystrophin protein in muscle, thereby resulting in a severe DMD phenotype in males. In contrast, those that preserve an open reading frame, generally give rise to a milder BMD clinical presentation.

Reply 1: Thank you for your advice and valuable suggestions. This question is similar to the comment 3 and 4 from reviewer 1.
We further discussed these cases with two DMD experts. According to the reading frame rule, we classified these nineteen patients into three group: DMD group included Neo_2, Neo_4, Neo_5, Neo_9, Neo_11, Neo_14, Neo_15, Neo_16; BMD group included Neo_6, Neo_8, Neo_5, Neo_10, Neo_12, Neo_13; uncertain cases: Neo_1, Neo_7, Neo_17, Neo_18, Neo_19. We have added the mutation type, previously reported cases, and clinical assessment at last phone call follow-up to table 1.
For the further management, some patients with BMD can present severe form of BMD, which the clinical symptoms are similar to the DMD, they will be managed as DMD patients. Therefore, for the BMD group, they still required further follow-up and assessment by neurologists.

Comment 2. Previous reports in association with dystrophinopathy, including checking the DMD locus specific databases (Leiden Open Variation Database, LOVD, which includes information on whether a given variant has been associated with DMD, BMD and/or other phenotypes). I recommend that the authors go through the literature and DMD variants that have been previously reported may be detailed in the paper.

Reply 2: Thank you for your advice and valuable information
In order to get more supportive information for predict the DMD or BMD, as you suggested, we
checked the DMD locus specific databases including Leiden Open Variation Database, LOVD, the UMD-DMD, the eDystrophin, and the TREAT-NMD DMD Global database and HGM. We added the mutation type, previously reported cases, and clinical assessment at last phone call follow-up to table 1.
However, previously reported cases have limitation. For example, Neo_12 had in-frame deletions of exon 48-51. Some previously reported cases were diagnosed with DMD. We

further check the reference and found genetic tests used in some previous study was Multiplex PCR which is widely available and the least expensive, but only detects deletions and does not cover the whole gene, so that a deletion might not always be fully characterized. Therefore, the further follow-up for our six cases with BMD and five uncertain cases are necessary.

Comment 3: Points 1 and 2 can help to explain the association between genotype and phenotype and clinical course. Most of patients were less than 4 years old and asymptomatic at the end of the study. BMD usually present with clinically heterogeneous and onset is usually in childhood, typically after 7 years of age, but can be later. Application of this reading-frame rule to the coding regions of the DMD gene thus makes it possible to predict whether a male is likely to develop BMD or DMD. While this holds true for the majority of cases, there are several exceptions. However, the reading frame rule should be applied with caution for duplications.

Reply 3: Thank you for your comments. We totally agreed with you.
As stated above, the three classification of these cases can help to guide the follow-up plan and treatment. As you said BMD usually present with clinically heterogeneous, according to the recent recommendation and two experts' advice, those patients with BMD still required further follow-up and assessment by neurologists.

Reviewer C

This study reports on an NGS-based (clinical exome sequencing) screening of ~10000 newborns who were admitted in the NICU in an academic hospital setting, resulting in early diagnosis of about 15~20 DMD cases. The resulting incidence, in the order of 1:5000 ~ 1:10000, is in line with the expected figures from the existing epidemiological literature. This screening effort is an impressive feat from a strictly genetic/technical standpoint, and one that may add to the increasing literature about DMD newborn screening (NBS), but I find that the manuscript leaves much room for improvement from the standpoint of the interpretation of findings, as detailed in the observations below.
We really appreciate the constructive suggestion and comments from you.

Major comments

Comment 1：The study design is original in the context of DMD NBS because it "jumps" directly to NGS, while most if not all NBS programs so far rely on CK dosing as a first line of screening. The obvious observation here is that the cost of CK assays is cheaper by at least 2 orders of magnitude compared to NGS (few dollars vs. a few hundred dollars). When scaling up to population-wide screening, this difference is crucial for policy-makers who need to implement NBS into health systems. The authors argue that CK has false positives due to birth trauma, which however may be avoided by correct CK dosage timing, and false negatives. Their arguments in support of CK false negatives are not convincing, which leads to my second observation.

Reply1: Thank you for your careful review. This is one place where we did not make ourselves clearly, and we apologize for any misunderstanding you may have had.

We further discussed these nineteen cases with two DMD experts. Prof. Xihua Li is a molecular geneticist of DMD and works on the DMD management during her career.

Professor. Yuanfeng Zhou who is a neurologist and works on the management and follow-up of the DMD patients.

Our study design is based on the reanalysis of NGS data. These data of the individuals were from our Chinese neonatal Genomic project. They were patients admitted in NICU and highly suspected genetic disease. In this study, we reanalyzed the data between June 1, 2016 to June 30, 2020, focusing the *DMD* gene pathogenic variations. We did not apply the NGS to do the population-based screen.

Therefore, we revised the title as "Genetic identification of pathogenic *DMD* gene variation", which is better than "genomic screening".

We totally agree with you that CK levels can be avoided by correct CK dosage timing, and false negatives. We just pointed out the limitation of the CK levels. But as you said, CK is an economic screening tool. Currently, we did not think the NGS can be instead of CK as a screening tool of DMD.

Comment 2: The authors argue that 3 patient with normal CK (DMD-1, DMD-6, and DMD-17) would have been missed by CK screening, but were correctly diagnosed by their NGS approach. A lot of caution is needed here. DMD-1 and DMD-17 have a duplication of exons 1-7, that is they probably transcribe an intact open reading frame from exon 1-79, possibly leading to expression of a normal dystrophin protein. This genetic finding is NOT diagnostic of DMD and these patients should undergo a muscle biopsy to check or muscle histology and dystrophin IHC and Western Blot with N-terminal, core, and C-terminal directed antibodies.

DMD-6, on the other hand, similar to DMD-12 who in fact also has slightly elevated, close-to-normal CK, has a deletion of exons 48-51 which classically leads to a very mild form of Becker (NOT Duchenne) muscular dystrophy with intact muscle strength, normal-to-elevated CK, and myalgia.

Reply 2: Thank you for your comments. We totally agreed with you.
We further discussed these nineteen cases with two DMD experts.
We thoroughly discussed these cases about the types of mutations, the current phenotypes, the family history, the previously reported cases, the clinical assessment based the 2010 DMD recommendation supported by the US CDC and the updated version, and also how to consult these families. We revised our manuscript thoroughly.
According to the reading frame rule, we classified these nineteen patients into three group: DMD group included Neo_2, Neo_4, Neo_5, Neo_9, Neo_11, Neo_14, Neo_15, Neo_16; BMD group included Neo_6, Neo_8, Neo_5, Neo_10, Neo_12, Neo_13; uncertain cases: Neo_1, Neo_7, Neo_17, Neo_18, Neo_19)
Neo_1and Neo_17 had exon1-7 duplication, as you said this mutation did not follow the reading frame rule. However, this mutation was previously reported in PMID 21515508 but no information of patient. Therefore, to be cautious, we classified them into uncertain cases. They will be follow-up in clinic. These two patients were suggested to reassess in clinic.

Comment 3: Have the parents of these boys been told that their boys have Duchenne muscular dystrophy, a devastating disease leading to severe disability and early death? I hope not, but if so, this is probably WRONG (and possibly leading to psychological trauma to these families).

Reply 3: Thank you for your advice and valuable suggestion. This is one place where we did not make ourselves clearly, and we apologize for any misunderstanding you may have had.
We did not consult patients directly. We updated these genetic reports and referred them to the DMD experts or neurologists or some patients were suggested to genetic clinic for further counselling.

Comment 4: What about other diseases? NGS may lead to diagnosis of hundreds of other genetic diseases, were these included in the program and consent?

Rely 4: Thank you for your advice and valuable suggestions. This is one place where we

did not make ourselves clearly, and we apologize for any misunderstanding you may have had. But it will be a very good idea to discuss other genetic disease in NGS data. These data of the individuals were from our Chinese neonatal Genomic project. They were patients admitted in NICU and highly suspected genetic disease. In this study, we reanalyzed the data between June 1, 2016 to June 30, 2020, focusing the *DMD* gene pathogenic variations. We did not discuss other genetic diseases in this study.

Comment 5: The fact that recruitment was in the NICU may somehow skew results, although the reasons leading patients to the NICU are probably unrelated to DMD; this should be discussed more thoroughly.

Reply 5: Thank you for your comment. This is one place where we did not make ourselves clearly, and we apologize for any misunderstanding you may have had.
As stated above, these data of the individuals were from our Chinese neonatal Genomic project. The recruited individuals were patients admitted in NICU and highly suspected genetic disease. Our study is based the NGS data from this population. Therefore, our study aims to perform a genetic identification of *DMD* gene pathogenic variations in newborns from NICU using the clinical exome sequencing data, then investigate the impact of the early identification of pathogenic variations of *DMD* gene on the clinical decision.

Comment 6: Last, but not least in importance, there is a widespread feeling that ethical concerns regarding DMD NBS are not only limited to questions of sensitivity/specificity, but also to the ethical question: is a diagnosis of DMD at the age of 1 month, 6 months, 12 months desirable? May not the psychological consequences of this burden for the mother, father, and other relevant figures in the boy's life, surpass the actual need for medical intervention at the present state of available medications? Of course the availability of effective gene therapies or molecular therapies would change this balance. While the focus of this manuscript may not be ethical, still the opportunity and cost/effectiveness of an NGS approach is influenced by these considerations, which should be discussed.

Reply 6: Thank you for your comments and kindly suggestions.We totally agree with you that ethical issues should be careful considered.
In fact, we faced ethical issues during the re-analysis of these data and we discussed these ethical issues with two DMD experts as well.
For example, eight patients with DMD were normal development and at stage 1 of

disease and six patients with BMD were unclear about the severity of BMD, the potential harm or anxiety to families should be dealt with carefully. Currently, there is no curative treatment. Also, these infants may have social stigmatization of persons with disabilities and feel negative in their later life. Another two deceased newborns with pathogenic DMD gene variations, those families may feel negative about having another baby.

All above these, we have added in the discussion part.

As you said, NGS approach is influenced by these considerations. And currently, we think that with the more widespread realization of benefits of NGS identification of DMD gene pathogenic variations in practice, muscle-directed therapies and organized multidisciplinary care, we would have the ability to deal with the ethical issues.

Minor comments and edits:

Comment 7: MLPA results in S2 are unreadable

Reply 6: Thank you for your comments. We revised the Figure S2.

Comment 8:“Natural history” instead of “Nature history”

Reply 7: Thank you for your correction

Changes in the text: “Natural history”

Comment 8 “Figure shows” instead of “Figure showed”

Reply 8: Thank you for your correction

Comment 9 “Pathogenic DMD gene mutations” (the word “mutations” is missing)

Reply 9: Thank you for your correction.

Changes in the text:We change to DMD gene pathogenic variations or pathogenic variants of DMD gene.

Comment 10“relevance” is a noun not an adjective (i.e. “relevant”)

Reply 10: Thank you for your correction.

We deleted this sentence.