Author’s response to reviews

Title: A heterozygous duplication variant of the HOXD13 gene caused synpolydactyly type 1 with variable expressivity in a Chinese family

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**Version:** 1  **Date:** 26 Aug 2019

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Response to editor and reviewers

**Editor Comments:**

1. Please change the main text headings to: Background, Methods, Results, Discussion, Conclusions.

-------We thank the editor for the suggestion. We have changed the main text headings accordingly in our revised manuscript and highlighted in yellow. We have also modified the title as “A heterozygous duplication variant of the HOXD13 gene caused synpolydactyly type 1 with variable expressivity in a Chinese family”, thank you for your consideration.

2. Please indicate the role of the funding body in the design of the study and collection, analysis, interpretation of data and in writing the manuscript in the Funding section.

-------Thank you for the comments, our work was supported by the National Key Research and Development Program of China (No. 2016YFC1000504, to SF). It provided valuable suggestions in the design of the study, and provided fully financial supports in sample collection, experimental costs, data analysis, and interpretation of data, etc.

The role of the funding body has been indicated in the FUNDING section in our revised manuscript, and highlighted in yellow.

3. We note that you have not included a ‘Consent for publication’ section in Declarations. If identifying images or other personal or clinical details of participants are presented that compromise anonymity, a statement of consent to publish from the patient should be included. As you have included details of the family, along with a pedigree and images of the family, we would expect Consent for publication in written form from all family members, and consent for minors from parents/guardians for the publication the minor's information. Please note, Editors can request to see consent forms at any stage.

-------We thank editor very much for the comments. We have added CONSENT FOR PUBLICATION section in Declarations in our revised manuscript, and highlighted it in yellow. “All the participants provided the signed informed consent, while consent for the individual under 18 years-old was given by his parent. The study was approved by Institutional Research Board of Harbin Medical University.” Thank you for your consideration.

**Reviewer #1:**
Sajid Malik, PhD (Reviewer 1): BMC Medical Genetics
A 24-base pair duplication in exon one of HOXD13 gene caused synpolydactyly type 1 in a Chinese family
Manuscript Number: MGTC-D-19-00018
Zaib et al. study a Chinese family with synpolydactyly and identify a 24 bp duplication in HOXD13. It is an interesting study, however, the manuscript would benefit from the following mandatory changes/amendments:

1. Page 2, line 15. 'Were collect' should be 'were collected'.

--------We thank the reviewer very much for pointing out the mistake. We have corrected the mistake and made modification accordingly in our revised manuscript, and highlighted in yellow.

2. Introduction: page 4, paragraph 3, 'In vertebrates HOX genes have.....' This paragraph may be omitted.

--------Thank you very much for the suggestion. We consider the suggestion and have omitted the mentioned paragraph (In vertebrates HOX genes have.....) from the BACKGROUND section of our revised manuscript.

3. There should be consistency in the use of abbreviation, for instance, the authors use ExAc and ExAC.

--------We thank the reviewer very much for pointing out the typo. We have corrected and used the abbreviation “ExAC” consistently in our revised manuscript, and highlighted in yellow.

4. The phenotype in the affected family members may be presented in a tabulated form showing major and minor symptoms in hands and feet separately.

--------Thank you very much for the suggestion. We have presented the phenotypes of the affected family members in Table 1 in our revised manuscript, and highlighted in yellow.

5. The basic demographic details of the family should be provided.

--------Thank you very much for the suggestion. Clinical information and peripheral blood were obtained from 12 persons which include seven affected members (V-1 (proband), IV-2, IV-11, III-1, III-3, III-7, II-3, ) and five unaffected members (V-2, IV-3, III-5, II-4, II-5) of the family. The basic demographic details including gender and age of the family members have been included in Table 1 in our revised manuscript.

6. Fig. 2. The subject IV-11 is not asymptomatic. Indeed she has camptodactyly of right 5th finger, clinodactyly of left 5th finger, camptodactyly of toes and contracture in right hallux. These changes are also reminiscent in the radiographs (though the quality of radiographs is low). Accordingly, the symbol of IV-II should be changed in Fig. 1.

--------We thank the reviewer very much for pointing out these deformities in subject IV-11 of SPD1 family. We have added the text about deformities of subject IV-11 and made modification accordingly in our revised manuscript, and highlighted in yellow. We also have changed the symbol of IV-11 in Figure 1 accordingly.

7. Please remove the text mentioning lack of penetrance in the family.
We thank the reviewer very much for the suggestion. We considered the suggestion and removed all the text mentioning about lack of penetrance in the family in our revised manuscript.

8. Page 7, clinical findings. Clinical detail in the text may be omitted and can be presented in a table instead.

Thank you very much for the suggestion. We have omitted the clinical details in the text, and have presented the phenotypes of all the affected family members in Table 1 in our revised manuscript and highlighted in yellow.

9. Methods: The WGS data filtration strategy is not described in adequacy. Please give detail of analyses scheme; total number of variants, how many variants were removed, how many rare variants were left in the final round, etc.

Thank you very much for the suggestion. We have added the information from WGS data in RESULTS section, and made a list of mutation of syndactyly/limb developmental genes found in the WGS data, and presented it in Supplementary Table 1 in our revised manuscript and highlighted in yellow, as follows:

“Pathogenic analysis of WGS data revealed 16 pathogenic, 1 likely pathogenic, 2511 variants of uncertain significance (VUS) and 24 319 benign variants. Further analysis of WGS results revealed total of 10 883 missense mutations, 57 frameshift insertions, 183 non-frameshift insertions, and 194 non-frameshift deletions. We have checked all the rare variants falling in syndactyly/limb developmental genes which have been presented in Supplementary Table 1. We found their population frequencies were higher than 0.01. The literature related to SPD1 disease was reviewed, and we found that mutation in HOXD13 gene is the most widely reported for SPD1 disease. Comprehensive analysis of WGS results revealed the duplication of 24-base pair at chr2:176957801-176957823 (GRCH37/hg19), which caused a non-frameshift mutation in HOXD13.”

10. The genomic/UCSC coordinates of the variant should be provided.

Thank you very much for the suggestion. We have added the genomic coordinates of the variant in RESULTS section in our revised manuscript and highlighted in yellow, as follows:

“Comprehensive analysis of WGS results revealed the duplication of 24-base pair at chr2:176957801-176957823 (GRCH37/hg19), which caused a non-frameshift mutation in HOXD13.”

11. All the rare variants falling in syndactyly/limb developmental genes (Shh, GLI3, LMBR1/ZRS, GJA1, LRP4, BHLHA9, APC, etc.) should be presented in a supplementary table.

Thank you very much for the suggestion. We have made a list of syndactyly/limb developmental genes found in the WGS data, and presented it in Supplementary Table 1 in our revised manuscript.

12. The consequence of mutation/variant has not been reported.
Thank you very much for the comment. The 24-base pair duplication mutation of HOXD13 is predicted to result in addition of eight extra alanine (A) residues. There are 15 alanine (A) residues present in N-terminal domain p.57-71 of wildtype HOXD13 protein. Due to 24-base pair duplication mutation produced eight alanine (A), the number of alanine (A) residues increases to 23 at the location of p.57-79 of mutated HOXD13. We have added the consequence of the 24-base pair duplication variant in RESULTS and DISCUSSION sections in our revised manuscript and highlighted in yellow.

“It encodes additional eight alanine (A) residues in the polyalanine tract at N-terminal domain of HOXD13 protein. There are 15 alanine residues present in N-terminal domain of wildtype HOXD13 protein, due to 24-base pair duplication mutation consequently the number of alanine residues increases to 23 (15 aa plus 8 aa).

We have also used Insilco prediction tools to evaluate the impact of variant on protein structure, protein function and its evolutionary conservation. Furthermore, conservation analysis of the mutation locus was patterned through Aminode webtool. We have also used SWISS-MODEL modeling server to build the protein structures for both wildtype and mutant type of HOXD13 to see the effect of our 24-base pair duplication variant on the structure and size of HOXD13 protein. We have added it in Figure 6 in our revised manuscript and highlighted in yellow, as follows:

“3.3.3. Evolutionarily constrained region of HOXD13

Evolutionarily constrained regions (ECRs) of HOXD13 according to Aminode revealed that the polyalanine tract (p.57-71) of the HOXD13 protein was conserved among different species, such as Mus musculus and Rattus norvegicus, etc (Fig. 6A).

“3.3.4. Homology model for the HOXD13 protein

A homology model of wildtype and mutant HOXD13 proteins revealed that the 24-base pair duplication variant encoding eight extra alanine (A) residues in the polyalanine tract locally affected the shape and results in increase in size of the protein (Fig. 6B).

In addition, we have added a small literature review about the consequence of 24-base pair duplication mutation/variant of HOXD13 in DISCUSSION section in our revised manuscript and highlighted in yellow, as follows:

“HOXD13 encodes highly conserved DNA binding transcription factors that helps other genes to initiate their transcription. Hyperexpansion of DNA-triplet repeats as a result of 24-base pair duplication in exon 1 of HOXD13 may lead to altered transcription or translational activities which can result in defective mRNA and ultimately defective proteins. Polyalanine expansions in other transcription factors had been reported to be linked with human birth defects such as deformities of digits and other structures [6].”

Reviewer 2 (Reviewer 2): PEER REVIEWER ASSESSMENTS:

GENERAL COMMENTS: The study is not done intentionally; just during diagnostics a family with SDT1 was studied by WGS.

The authors need to address the following items:
1) It remains unclear why the authors did not directly sequence HOXD13 or at least used a panel.

--------We thank to reviewer very much for the comment. There are many genes (SHH, GLI3, LMBR1/ZRS, GJA1, LRP4, BHLHA9, APC, FGF8, WNT7A, etc.) which has important role in limb development. Genetic alteration in any of these genes could lead to limb deformities. To know about the specific gene mutation that caused SPD1 in the five generation Chinese family WGS was performed. The other reason to perform WGS was to know about genetic alterations that may have contributory role in variable expressivity.

We have added it in BACKGROUND, METHODS and RESULTS sections in our revised manuscript, and highlighted in yellow.

2) Why they started with the only patient in analyses showing 'both symptoms as only one of family'.

--------Thank you very much to reviewer for the comment. We started this study by collecting the blood samples from the affected and unaffected members of the family. We send the blood samples of only one patient IV-2 for WGS to the company, since patient IV-2 has typical symptoms of SPD1. After comprehensive analysis of WGS data we found 24-base pair duplication mutation in HOXD13 gene. To confirm cosegregation of 24-base pair duplication variant of HOXD13 in all affected members of the family we performed direct Sanger sequencing.

3) Authors found a duplication as potential reason for SPD1 - that is somehow of interest and should be described.

--------We thank the reviewer very much for the suggestion. We consider the suggestion and accordingly have described the duplication as potential reason for SPD1 in the DISCUSSION section of our revised manuscript and highlighted in yellow.

“HOXD13 encodes highly conserved DNA binding transcription factors that helps other genes to initiate their transcription. Hyperexpansion of DNA-triplet repeats as a result of 24-base pair duplication in exon 1 of HOXD13 may lead to altered transcription or translational activities which can result in defective mRNA and ultimately defective proteins. Polyalanine expansions in other transcription factors had been reported to be linked with human birth defects such as deformities of digits and other structures [6].”

4) Best practice was not met as panel or direct Sanger sequencing of HOX-genes would have been better way to do this studied in a directed way.

--------Thanks to reviewer for the comment. There are numerous genes (SHH, GLI3, LMBR1/ZRS, GJA1, LRP4, BHLHA9, APC, FGF8, WNT7A, etc.) which has important role in limb development. Genetic alteration in any of these genes could lead to limb deformities. To know about the specific gene mutation that caused SPD1 in the five generation Chinese family WGS was performed. The other reason to perform WGS was to know about genetic alterations that may have contributory role in variable expressivity.
5) The only objective of the study was as follows: we have a family with SPD1 and have no idea which gene could be the reason, so we did WGS. The authors need to describe why the objective was important.

Also there is no final interpretation of the family. The only thing found, was a new variant in a mutation. There is no discussion on different phenotypes in family and also it is unclear what we learn from this new mutation or comparison with other cases.

--------We thank the reviewer very much for the comments.

Why the objective was important: The main objective of the study was to identify the causative gene in proband and its cosegregation in other affected family members. There are many genes (SHH, GLI3, LMBR1/ZRS, GJA1, LRP4, BHLHA9, APC, etc.) which has important role in limb development. Genetic alteration in any of these genes could lead to limb deformities. To know about the specific gene mutation that caused SPD1 in the five generation Chinese family we perform WGS. That's why our objective of performing WGS was very important. We described the importance of our objective in our revised manuscript and highlighted in yellow.

“There are many genes (SHH, GLI3, LMBR1/ZRS, GJA1, LRP4, BHLHA9, APC, etc.) which has important role in limb development. Genetic alteration in any of these genes could lead to limb deformities. To know about the specific gene mutation that caused SPD1 in the five generation Chinese family, WGS of the blood sample from the typical SPD1 patient IV-2 (affected with both hands and both feet) was performed by Novogene technology limited-liability Company (Beijing, China). “

Discussion on different phenotypes: We have discussed in detail about the variable phenotypes DISCUSSION section of our revised manuscript and highlighted in yellow.

“In case of variable expressivity or gene expression, some family members show severe complications of synpolydactyly (II-3, III-1, III-7, IV-2, V-1) while other did not show the same complications (III-3, IV-11) (Fig. 2). The five generation Chinese family showed from minor to more severe limb deformities, which are beneficial for further understanding of SPD1 clinical features in future. Camptodactyly and clinodactyly of 5th finger and toes and contracture in right feet’s hallux have been added to the list of possible phenotypes caused by HOXD13 polyalanine expansion mutations.

The variable phenotypes of SPD1 patients in this family predicts that some other factors (environmental or genetic) can contribute to the expression of HOXD13, as some genetic factors (e.g. gene modifiers) and epigenetic factors also found to play an important role in controlling the expression of any specific gene. Phenotypic heterogeneity of any disorder is because of interaction of responsive gene with other associated genetic factors or modifier genes. Raj et al. also showed that mutation in that locus or gene which is critical for developmental process could lead to variable expressivity [35].”

Final interpretation of the family: We found 24-base pair duplication mutation in exon 1 of HOXD13 which is predicted to cause addition of eight extra alanine residues in HOXD13 protein. Our findings are critical for future perspective of SPD1 disease. Our results increases the phenotypic spectrum of SPD1. Our results also increase the genotypic spectrum of HOXD13. In comparison with other studies it was clear that the variable expressivity is the common phenomena in SPD1. More research is demanding in this area to
“We successfully identified duplication mutation (c.183_206dupAGCGGCGGCTGCNGGCCGCGGCNGGCC) in exon 1 of HOXD13 [NM_000523.3]. Based on clinical data, cosegregation analysis, in silico predictions and ACMG assessment, we classified the HOXD13 24-base pair duplication variant as likely pathogenic and the main cause of SPD1 in this family. Our results widen the genotypic spectrum of HOXD13 mutations that are responsible for SPD1. The phenomena of variable expressivity was quite obvious in this family. In comparison with previous studies it is established that the variable expressivity is the common phenomena in SPD1. More research is required in the area to find out the genetic factors behind phenotypic heterogeneity.”