Peer Review File

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Replies to Reviewer A

In this presented study the authors report detecting two known variants, a SNP (g.1170C>T and a missense (c.1025C>T, p.R342Q), in 4 relatives affected with FD. These variants were absent in the unaffected relatives examined in this study. The authors used WES to screen their cases for variants in GLA gene. They also assessed the effect of the non-coding SNP on GLA transcription using Luciferase reporter assay.

General comments:
1) Why did the authors choose to do WES instead of doing direct sequencing (Sanger) for the GLA gene, especially that they already have established the diagnosis clinically and GLA is a short gene with only 7 exons?

Replay: Thank you for your professional question. The cause of Fabry disease is the mutation of the GLA gene. More than 900 sites of this gene may cause abnormal expression or activity of GLA. In addition. There were 10 members in this family underwent genetic testing. If Sanger sequencing was used for sequencing firstly, the cumulative cost is higher than WES. In addition, Sanger sequencing takes a long time, so we finally adopted the strategy of Sanger sequencing verification after WES sequencing.

WES is normally used for:
i) new gene discovery studies
ii) trying to identify the genetic underpinnings for genetically heterogeneous conditions
iii) When trying to solve cases with uncertain clinical diagnosis (cases with overlapping phenotypes, for example primary erythromelalgia mimicking FD)
So far, no published reports in FD have suggested the involvement of genes other than GLA in this disease so the justification provided by the authors for using WES is not convincing. The reasonable and more feasible workflow is first to exclude any potential clinically significant variants in GLA by standard Sanger sequencing before moving to WES.
Also, a possible justification for using WES is to identify additional new genes that may explain the phenotypic variability observed in these cases. However the authors have not stated this as an objective nor did they follow the correct workflow for identifying new candidate genes or variants. The appropriate workflow (analysis pipeline) is by applying variant filtering criteria that takes into account many aspects such as gene expression/function, variant effect, variant frequency, etc… None of that was done in this study.

Replay: As you pointed out, no published reports in FD have suggested the involvement of genes other than GLA. As a nephrologist, before definitive diagnosis of FD, we were not sure those were caused by a single GLA gene, or by a single site mutation of GLA cause, but we very much hope to make an accurate diagnosis for patients as soon as possible. Start in multiple areas at the same time. Therefore,

we start to diagnose from the clinical manifestations, renal pathology, genetic testing and other aspects at the same time. In addition, if Sanger sequencing is performed on hundreds of GLA sites, the accumulated time and cost will exceed the cost of WES. Therefore, we have selected WES as the detection method in this study firstly.

2) Few important points were not covered in the discussion
i) Discussing the findings in light of other published genetic studies of FD in Chinese patients (which mutations or SNPs were detected and how variable was the clinical presentation?)
Replay: We found one report about Chinese FD double site mutation: the frameshift mutation c.273_276del TGAT (p.I90MfsTer25) with the missense mutation (c.281G>T, p.Cys94Phe). Familial episodic painusing was the only clinical manifestation of the affected menbers. We also added the discuss about the double site mutation worldwide.

Changes in the text: Page15 line279-282

ii) Providing a hypothesis or speculation as to why there is an apparent phenotypic variation even among the (males) form the same family.

Replay: We modified our text as advised. There were phenotypic differences between male members in this family. Hemizygote male members have severe clinical symptoms and wild-type male members have mild clinical symptoms. It was affected by gene mutations, and also related to gene expression. It may be affected by the individual ’s external environment such as age, other diseases, and other genetic variations .

Changes in the text: Page 12-13 line 224-237.

iii) Discussing the study limitations,
- for example, copy number variants (CNVs) could be another type of genetic defect underlying FD that has not been investigated here.
- The pathological/histological investigation was only done for the proband. Renal/skin biopsies from the other (available) affected individuals can be very informative for correlating all three aspects (genetic/clinical and histological findings)
- Enzyme activity was not assessed in these patients.

Replay: Thank you for your professional advice. We have modified our text. Added the study limitations in the text.

Changes in the text: Page 17 line 331-337.

iv) The impact of g.1170C>T on GLA transcription has been previously performed in HEK cells by Ferreira et al., 2015 (DOI 10.1007/8904_2015_424) where they demonstrated an opposite effect of on GLA transcription. The authors can cite this study and discuss possible explanations of the discrepancy.
Replay: Your guidence is very import. Oliveira et al. indicated that the g.1170C>T polymorphism was associated with decreased enzyme expression. We have modified our text as advised, and added the article as reference.

This article used 4 kinds of cells to detect the effect of 1170 mutation on the expression of Lac Z, showing that HEK-293 increased the expression of downstream genes. But for other 3 kinds of cell, the results were opposite. On the one hand, it showed that the mutation may have different effects in different cells. On the other hand, the control used in the article is different from that we used. As discussed in the article, it needs to be further verified by the dual luciferase experiment, and it happens that we use the dual luciferase system. This may be the cause of the inconsistent results. More detailed functional verification needs to be studied in the future using EMSA and other experiments.

Changes in the text: Page 14 line 263-272.

Specific comments

1) The g.1170C>T variant is not a mutation (a mutation has to be nearly absent in controls and has to have a clear functional consequence). The correct terminology for this variant is SNP, as this is how it has been regarded in published studies.

Replay: We have modified our entire text as advised.

2) In the abstract the authors report identifying a total of 1375 mutations in their cases. This could be very misleading and confusing for the readers for a number of reasons:

i) The correct term to use when describing genetic variants in NGS studies is “variant(s)” not “mutation(s)” as the latter may incorrectly imply causality

Replay: Thank you for your professional guide. We have modified our entire text as advised.

ii) The authors did not explain how they narrowed down the number of variants from 1375 to 2 ? what was their inclusion/exclusion criteria? The authors basically used WES to perform targeted gene analysis (i.e. GLA only) however, they did not provide a concise method on how they selected their candidate variants from the 1375. I therefore suggest removing this number and specifically stating that only GLA was analyzed.

Replay: We have added the part “2.5data analysis for bioinformatics annotation after WES” in our text.
Changes in the text: Page 8 line 133-142.

iii) Page2/line 24-25 “For the first time, two simultaneous missense mutations of the GLA gene, c.1025C>T and g.1170C>T, were verified….”
- Only c.1025C>T is a missense mutation the g.1170C>T is not a coding variant therefore it is not a “missense mutation”

Replay: Your guidance is very professional. We have modified our text as advised.
Changes in the text: Page 2 line 48-50.

3) Page3/line 44-46 “Does FD have other possible pathogenic genes in addition to the GLA gene? WES technology can not only identify the pathogenic genes of single gene genetic diseases but also has superior suggestive significance for the diagnosis of potential polygenic diseases”

See point (1) in general comments, also revise the sentence the message is not clear.

Replay: Thank you for your professional advise. We deleted the sentence “Does FD have other possible pathogenic genes in addition to the GLA gene? ” and explained in point (1).

Changes in the text: Page 5 line 68-69.

4) Page3/line 49 “and some patients do not have FD but have the GLA gene mutation(4, 5).”
A typo mutation is missing an (s)

Replay: Thank you for your care and help us avoid an error. We have modified.

Changes in the text: Page 5 line 74.

5) Page 3/line 52 “Here, we report two simultaneous pathogenic transversions, c.1025C>T (p. Arg342Gln)and g.1170C>T (-)”
Two errors here, the nucleotide change from C>T is a transition (bases from the same group, pyrimidine to pyrimidine) not a transversion.

Replay: We have modified all the places in the article that deal with this issue.

The second error is g.1170C>T (-)? The variant nomenclature here is not correct. The correct nomenclature according to HGVS guidelines is either g.1170C>T or c.-10C>T, so one of these formats has to be used throughout the manuscript.

Replay: Thank you for your advise. We have modified the error throughout the manuscript.

6) Page 4/line 54-55 “Confirm the g.1170C>T mutation affects transcription of GLA gene, presumably the transcription start site.”
This sentence is very ambiguous! Also several lines of evidence are required before confirming the effect of this SNP on GLA expression. The reporter assay is suggestive not conclusive.

Replay: For rigorous description, we deleted this sentence.

Changes in the text: Page 6 line 82-83.

7) The authors did not mention the number of independent experiments (n) from which the reporter assay results were derived nor did they provide information on the type of statistical test carried out.

Replay: We have provide information on the type of statistical test carried out in the new part “2.7 Statistical Analyses” and added the the number of independent experiments.

Change in the text: Page 9 line 146-149, and Page 11 line 208-209.

8) “Bioinformatics analysis of the mutations” this section looks like it was reused from another draft/manuscript were WES was applied in cancer studies. For example description of germline and somatic mutations. Somatic mutations were not explored here and are not relevant in FD. Also the application of GeneFuse, is specific for gene fusion (inversions-translocations). These events are specific to cancer genetics not monogenic diseases such as FD.

Another thing is that the authors state in the methods that CNV was called from the WES, however no results from this type of analysis was presented. It seems that as mentioned earlier this part was not written specifically for the manuscript at hand.

Replay: We have deleted the events that are not specific to FD .

Changes in the text: Page 7-8 line 120-122.

9) In WES, the standard practice requires checking variants frequency in public DNA databases (preferably ethnically matching). This is missing here.

Replay: We have modified our text. Functional annotation of variants was performed using ANNOVAR, which contains more than 40 databases, such as 1000g2014, ExAC, esp6500, etc.

Changes in the text: Page 7-8 line 120-122.

10) The variant impact prediction tools used in this study are applicable only to DNA changes (SNVs) , mainly substitutions, located only in the coding region. But not applicable to variants in DNA non-coding regions (c.1170C>T)
Therefore the following sentence is not accurate and has to be revised:
Page9/lines 173-175 “In our study, the mutation was predicted to be “probably damaging” by PolyPhen-2, “damaging” bySIFT, and “disease causing” by MutationTaster”

Replay: Thank you for your advise. We have deleted the sentence and explained this in the part of “methods”.

Changes in the text: Page 13 line 237-239.

11) Page8/line 142 “Ten family members of the pedigree were involved in this study, including five affected individuals (III:5, III:7, IV:2, IV:3, and…”
There is a discrepancy in the affection status of IV:2, here it is reported as an affected individual while in the pedigree it is indicated as normal?

Replay: We rechecked the information, IV:2 should be an affected individual in this family as showed in the text. In the pedigree, we only marked “N/M” for IV:2, but not blacked out. We have modified the pedigree in Fig1.

12) The terms first or second generation sequencing could be replaced with more specific and commonly used terms such as Sanger sequencing and Next-generation sequencing.

Reply: Thank you for your professional advise. We have replaced follow your instructions throughout the manuscript.

13) Page8/line 148-150 “All five affected individuals from two generations in this family had two simultaneous missense mutations, c.1025C>T (p. Arg342Gln) and g.1170C>T(-), in the GLA gene. All of them had classical phenotypes of FD.”
This statement is not accurate for two reasons:
- Classical FD is characterized by very low or complete loss of α-galactosidase A activity. Enzyme

activity was not assessed in these patients, can the authors explain how they reached this conclusion.
- Also there is a large degree of phenotypic variability among the affected individuals, so they definitely do not all have classical FD.

Reply: Appreciate your professional textbook-level guidance. This guidance is valuable to clinicians. Affected individuals showed some typical clinical manifestations of FD such as neuropathic pain, angiokeratoma, and renal impairment, we thought those were classical phenotypes but ignore the important laboratory testing such as α-galactosidase A activity. We have modified our text.

Changes in the text: P9 line163-164.

14) Page 8/ line 150-153 “Our data indicate that the c.1025C>T variant (p. Arg342Gln) in the GLA gene was the disease-causing mutation in the family. g.1170C>T(-) mutations occur in noncoding regions of the 5’ untranslated region (UTR) of the human α-Gal gene but also have pathogenicity.”
- This sentence is very ambiguous.
- Can the authors include references to the source of this information “mutations occur in noncoding regions of the 5’ untranslated region (UTR) of the human α-Gal gene but also have pathogenicity.”

Reply: We provided reference to the source of this information “mutations occur in noncoding regions of the 5’ untranslated region (UTR) of the human α-Gal gene but also have pathogenicity” .

Reference 9: Oliveira JP, Ferreira S, Barcelo J, Gaspar P, Carvalho F, Sa Miranda MC, et al. Effect of single-nucleotide polymorphisms of the 5' untranslated region of the human alpha-galactosidase gene on enzyme activity, and their frequencies in Portuguese caucasians. J Inherit Metab Dis. 2008;31 Suppl 2:S247-53.

15) The missense mutation reported here (Arg342Gln) is sometimes referred to as (R342Q). variant nomenclature has to be unified either use p.Arg342Gln or p.R342Q throughout.

Reply: We changed “R342Q” to “Arg342Gln” to ensure consistency throughout the text as you advised.

Changes in the text: P13 line244-246.

16) Page 10/line 184-185 “This extra hydrogen bond of arginine can be of major importance for the conservation of the tertiary structure or may play a role in substrate binding.”
- Citation of the source of this information is missing

Reply: We provided reference (15) to the description “This extra hydrogen bond of arginine can be of major importance for the conservation of the tertiary structure or may play a role in substrate binding.”

Reference (15): Ploos van Amstel JK, Jansen RP, de Jong JG, Hamel BC, Wevers RA. Six novel mutations in the alpha-galactosidase A gene in families with Fabry disease. Human molecular genetics. 1994;3:503-5.

Changes in the text: P13 line251.

17) Page 10 line 192 -195 “To confirm this hypothesis, we demonstrated that the g.1170C>T mutation can cause subsequent transcriptional expression of the GLA gene using the dual luciferase reporter system, presumably the transcription start site of the gene.”

- This sentence is very ambiguous. What do the authors mean by “transcriptional expression” the SNP can alter transcription or in other words gene expression but the term “transcriptional expression” is not correct? Also it is not clear here what exactly is the subsequent effect ?

Reply:We have modified our text as advised.

Changes in the text: P14 line 262-267.

18) Page 11/ line 215-218 “Considering that FD has progressive effects on various organs, vital organ damage would have already begun regardless of the activity of the GLA enzyme. This implies that biopsy of the involved organ could help us identify the extent of FD and start therapy accordingly.”
- Citation of the source of this information is missing
- Perhaps the authors can say that GLA activity when measured in blood may not reflect the true level of the enzyme activity in the affected organs. Therefore the critical threshold of GLA activity is hard to be determined. (Schiffmann et al 2016, https://doi.org/10.1038/gim.2016.55)

Reply:Thank you for your kind and professional guidence. We have modified our text as advised and added the mentioned reference above.

Changes in the text: P16 line308-312.

19) Page 11 line 226-227 “Due to the limitations of the test conditions, our family members did not detect α-Gal activity”
- This sentence is very ambiguous. Revision is required. Did or did they not test the enzyme levels in their patients? If they tested the enzyme activity was it detectable or not ?

Reply: Family members did not do the α-Gal activity test. We have modified our text.

Changes in the text: P17 line327.

Figures
Figures 2-4: The abnormal findings in the biopsies are not clearly indicated
(using an arrow or a box)

Reply:We have added the black arrows in the Fig.2-4.
Figure 5: The chromatograms are not clear. The called bases are not visible.

Reply:We have replaced the the clear chromatograms.

Replies to Reviewer B

This paper describes a four generation pedigree with Fabry disease where four affected and six unaffected individuals were analysed by exome sequencing. A missense mutation (Arg342Gln) was identified which has been previously reported in association with disease. A 5' untranslated region variant (g.1170C>T) was also identified which was in cis with the missense variant. All four affected individuals, who were sequenced carried both variants. One of the asymptomatic individuals, a 24-year old female, also carried both variants. Luciferase assays demonstrated that the g.1170C>T variant was associated with decreased expression. It is unclear as to why this team performed the luciferase assay given that it has been previously shown that this variant is associated with decreased expression (Oliveira JP, Ferreira S, Reguenga C, Carvalho F, Månsson JE. The g.1170C>T polymorphism of the 5' untranslated region of the human alpha-galactosidase gene is associated with decreased enzyme expression--evidence from a family study. J Inherit Metab Dis. 2008 Dec;31 Suppl 2:S405-13)
Allelic variants are of interest as we try to understand clinical variability between and within families. As the two variants in this family co-segregated (presumably because they were linked), we do not know whether the promoter variant has an impact on disease severity or whether the decreased expression is dwarfed by the effect of the missense mutation. Therefore, we don't know if it is a modifier. That said, the case report is worth publishing but the manuscript needs to be extensively revised before that time.

Title:
The title could be reworded to improve clarity. Perhaps a title like this would be clearer:
GLA missense and promoter variants co-segregating in a Chinese family with Fabry Disease
Reply: Thank you for your professional advice. We have modified the title and the running title as advised.

Changes in the text: P1 line1-5.

Abstract:
Line 13-14 – please reword the portion of the sentence beginning with "only partial genotype-phenotype…" as it is currently confusing

Reply: We modified the description to make it clearer. Thank you for your kindly reminder.

Changes in the text: P3 line33-36.

Over 900 GLA gene mutations are currently known; only partial genotype-phenotype relationships were verified by the pedigree, most with doubtful clinical significance.

Reply: We have modified in the text.

Changes in the text: P3 line33-36.

Line 18 – Please avoid using the word "mutations" and instead describe them as variants as you have no evidence to show that 1375 variants are disease causing. Please explain to the reader how you arrived at 1375 variants. How was the data filtered?

Reply: We changed the word "mutations"as advised , added the criterias for the methods of narrowed

down the number of variants from 1375 to 2, in the part of “2.5. Data analysis for bioinformatics annotation after WES”.

Changes in the text: P8 line128-137.

Line 19 – When describing the c.1025C>T variant, state it has been previously reported in association with disease and is known to be pathogenic. Add p.Arg342Gln.

Reply: We have modified the description according to your professional advice.

Changes in the text: P3 line 42.

Line 20 – mention the age and gender of the “asymptomatic carrier”.

Reply: The “asymptomatic carrier” actually had hypohidrosis as showed in the table. However the symptom was mild. The desciption of “asymptomatic carrier” was not suitable. So we modified the description into “five patients”. Sorry again for the error.

Changes in the text: P9 line 160-162.

Line 24 – although this may be the first time that double variants were reported in a Chinese kindred, they have been reported in other ethnicities previously. Suggest to reword for clarity.
Reply: Appreciate your professional guidance, which help us avoid an error. We have modified the description.

Changes in the text: P3 line 48.

Background:
Paragraph two is conversational – suggest deleting it as it doesn’t add anything.

Paragraph three – give a more concrete synopsis for the penetrance in males and females which will help your reader to be less surprised that you have an asymptomatic 24-year old female who carries both variants.

Reply: As mentioned above, the patient had mile symptom hypohidrosis, so we modified the description into “five patients”.

Changes in the text: P9 line 160-162.

Paragraph four (beginning line 52) – This is very specific. Simply state “Here we performed WES on affected and asymptomatic members of a four generation Chinese Han kindred with Fabry disease and filtered the data to identify coding and non-coding variants in GLA.”

Reply: We have modified the description. We appreciate your patience and professional guidance.

Changes in the text: P6 line 78-80.

Methods:
Section 2.3 needs additional work. Was it just WES which was performed or was an array performed also to detect the promoter variant? This is not clear from the existing methods.

Replay: As you pointed out, before making the diagnosis of FD, we were not sure that the clinical manifestations were caused by FD, or by site mutations of GLA cause. In addition, if Sanger sequencing is performed on hundreds of GLA sites, the accumulated time and cost will exceed the cost of WES. Therefore, we have selected WES as the detection method in this study firstly. In order to confirm that the founded mutation sites were not false positives, sanger sequencing was used for verification.

Results:
With the exception of the c.1025C>T variant which has been shown to be pathogenic, please use the word “variant” instead of “mutation”.

Reply:Thank you for your advice. We have used the word “variant” instead of “mutation” when mentioned c.1025C>T.

Section 3.3 As per the abstract, please explain the filtering which led to the identification of the 1375 variants (line 139) and then clarify that these variants were further filtered to identify all GAL variants etc. Was the non-coding variant captured by exome sequencing or through microarray.

Reply: We Added the criterias for the methods of narrowed down the number of variants from 1375 to 2, in the part of “2.5. Data analysis for bioinformatics annotation after WES”.

The whole exon sequencing we performed covered all the exon regions of all genes and the 200 bp intron regions upstream and downstream of the exons.

Changes in the text: Page 8 line 133-142.

Line 141 please include the minor allele frequency and any evidence supporting the pathogenicity of the variant (preferably citing papers or databases like ClinVar and LOVD).

Replay: We added 4 reference(6-9) to support the pathogenicity of the variant.

Line 144/145 The sentence about the second variant is confusing. It would be simpler to state that “All five also carried a g.1170C>T variant in cis”.

Replay: We have modified our text as advised.

Changes in the text: P9 line162-163.

Line 147-149 This is an x-linked disorder so comparing the family to an autosomal recessive pedigree is confusing. Line 149 states that both mutations are missense, which is incorrect.
Replay: We modified our text according to your professional advice.

Changes in the text: P11 line189-190.

Discussion:
Line 171 – the variants were identified in four affected individuals, not five. The fifth individual was asymptomatic.

Replay: Sorry again for the error. The patient had mile symptom hypohidrosis, so we modified the description into “five patients”. IV:2 should be an affected individual in this family as showed in the text. In the pedigree, we only marked “N/M” for IV:2, but not blacked out. We have modified the pedigree in Fig1.

Changes in the text: P9 line 160-162.

Line 173 – It would be preferable to describe it as two variants in cis as opposed to a double-site mutation.

Reply: Thank you for your advice. We have describe it as “two variants in cis”.

Line 173-176 – In silico predictions would usually be mentioned in the results rather than the discussion.
Line 200 – a reference is needed after the word “mutation”. The sentence that follows doesn’t make sense.
It would be helpful to summarise other incidents of double variants in GLA e.g. Yasuda M, Shabbeer J, Benson SD, Maire I, Burnett RM, Desnick RJ. Fabry disease: characterization of alpha-galactosidase A double mutations and the D313Y plasma enzyme pseudodeficiency allele. Hum Mutat. 2003 Dec;22(6):486-92.

Reply: Thank you for your professional guidance, which helped me avoid an error. We deleted the desciption about silico predictions, added references and explaination of the statement. We have summarized the literature on double mutations in FD and added a description in the discussion section.

Changes in the text: P14-15, line267-286.

Replies to Reviewer C

This is a nice case study of a 5 generation, 26 member Chinese family with Fabry Disease. Exomic sequencing is used to identify the causative mutations in the GLA gene on the X chromosome. Genetic testing was done and clinical history was obtained for 10 members of the family including all 4 living individuals who had symptoms consistent with Fabry Disease.

The findings of note in this study are: 1) The authors found two mutations in the GLA gene that appeared in all of the symptomatic family members that were studied and only one out of six asymptomatic family members that were studied. The authors report that this is the first time that two potentially pathogenic mutations in the GLA gene have been found in Fabry Disease patients. 2) The authors use a functional assay to show that one of the two mutations, g.1170C>T, which is in the 5' UTR of the GLA gene, decreases transcription of the gene. Per the authors, this mutation has been observed before but its phenotypic importance has not been explored using a functional assay. In addition to these two major points, the authors pose the question of whether damaging mutations in genes other than GLA could be involved in Fabry Disease. In the end, they find two mutations in the GLA gene that appear in all symptomatic family members, but no mutations in other genes that had the same distribution in the family tree. While evidence of the involvement of another gene in Fabry Disease was not found in this case, it is interesting to consider whether mutations in a gene other than GLA could produce Fabry Disease symptoms.

The content of the study is interesting and the results are straightforward. My main concern about the manuscript is the quality of the writing. There were several places where I was completely unable to discern the authors meaning. There are numerous other smaller grammatical or proofreading mistakes. I think the article would greatly benefit from either more careful proofreading by the authors or perhaps the services of an English language editor/proofreader.

Comments/Recommended Changes

Line 12 – 14 I don't understand the second part of this sentence (the part after the semi-colon). Do the authors mean that the phenotypes of only some of the 900 known GLA gene mutations are known and of those most are thought not to be clinically significant? Or are they referencing the family pedigree they used for the study?

Reply: We modified our text to make it more accurate. We mean that the phenotypes of only some of the 900 known GLA gene mutations are known and of those most are thought not to be clinically significant.

Changes in the text: P3 line33-36.

Line 25 g.1170C>T isn't a missense mutation since it does not affect the amino acid sequence.

Reply: Thank you for helping us avoid an error. We have changed it into g.1170C>T SNP throughout the manuscript.

Line 39 tThe -> the

Reply: Thank you for your patience. We have modified our text.

Changes in the text: P5, line63.

Line 54 – 56 This sentence has no subject. Perhaps the authors meant “We confirm the……”?

Reply: Thank you for your reminder. For smoothness, we deleted this sentence.

Line 68 “Heart Doppler ultrasound” -> Do the authors mean Echocardiogram?

Reply: Yes, we mean Echocardiogram. We switched to “echocardiogram”.

Changes in the text: P6 line93.

Line 103 The comma after pGL4-GAL-WT show be a period.

Reply: Thank you for your patience. We modified it as advised.

Changes in the text: P8 line139.

Line 122 The authors could state for clarity that the 10 individuals included in the study included all 4 living symptomatic persons in the family.

Reply: Here is a situation here that needs to be explained. The subject IV:2 actually had hypohidrosis as showed in the table, the symptom was mild, she was the symptomatic person. So we modified the description into “five patients”.

Changes in the text: P9 line 160-162.

Line 132 – 133 I’m not sure what is meant by “flaky and severely fibrotica”.

Reply: The previous description is not professional. We changed it into “severely fibrosis”.

Changes in the text: P10 line174.

Line 139 Could the authors clarify what they mean by “mutations”? Are these SNPs among the 10 individuals they did WES for? Or were they differences between the sequenced exomes and the human reference?

Reply: A total of 1375 variants were found among 10 individuals by the whole-exome sequencing. In this study, high-throughput sequencing was used to further explore new possible pathogenic genes of FD in addition to the GLA gene by performing WES on the family to find more new mutation sites. They were different between the sequenced exomes and the human reference.However the next-generation sequencing results found several other abnormal genes related to kidney, eye, heart and other symptoms, but they were not consistent with the family symptoms, and no sanger sequencing was performed. We modified in the text to make it more clear.

Changes in the text: Page10 line188-189.

Line 142 Authors should clarify difference between “symptomatic” individuals (those with clinical FD) and “affected” individuals (those with the two GLA mutations).

Reply: As you pointed out that “symptomatic” individuals and “affected” individuals are the same subjects (III:5, III:7, IV:2, IV:3, and IV:4). “Symptomatic” persons emphasized that they were subjects without clinical symptoms, while “affected” persons emphasized those with the two GLA mutations.

Line 147 I think the authors mean X-linked recessive here instead of autosomal recessive.

Replay: Yes, here we modified. Thank you for your kind help.

Changes in the text: P11 line189.

Line 150 Here it says that all individuals with the two GLA mutations had FD phenotypes. However earlier in the text and in Figure 1, the authors state that one of the individuals with the mutations was not symptomatic.

Reply: We admire your care and professionalism. As explained above, the subject IV:2 actually had hypohidrosis as showed in the table, the symptom was mild, she was the symptomatic person. So we modified the description and fig 1.

Changes in the text: Page 12-13 line 224-237.

Line 150 – 151 I don’t understand this sentence. Elsewhere the authors seem to imply that both of the mutations they describe in the GLA gene are pathologic.

Reply: Yes, both of the mutations we described in the GLA gene are pathologic.

Line 160 – 163 I think that this should be two sentences instead of one with a comma in the middle.

Reply: Yes, we have modified.

Line 178 Why do the authors refer to the 1025C>T mutation as a G – A transition here but as a C – T change everywhere else?

Reply: We have made changes to be consistent with other places.

Changes in the text: P13 line243-245.

Line 188 gen -> gene

Reply: We have made modified, thank you for your attentive.

Changes in the text: P13 line253.

Line 189 The word “polymorphism” should be removed.

Reply: We removed as advised.

Changes in the text: P13 line254.

Line 191 The authors make a reference here to a second UTR mutation, g.1150G>A, but they don’t introduce it. It’s not clear to me how this mutation relates to the study. Is it one of the 3 mutations they

mention in line 186?

Reply: g.1150G>A is one of the 3 UTR mutations mention in line 251. Reference mentioned that like g.1150G>A, g.1170C>T would operate at transcriptional or translational control. g.1150G>A have no relationship with our research. So we deleted “g.1150G>A”.

Changes in the text: P14 line 256.

Line 192 Period missing after “(9, 10)”

Reply: We added the period.

Changes in the text: P14 line 257.

Line 193 – 195 Do the authors mean that the g.1170C>T mutation can cause decreased transcriptional expression?

Reply: Yes, the g.1170C>T mutation can cause decreased transcriptional expression as you said.

Line 198 The word “comparing” should be removed.

Reply: We have deleted the word “comparing” according to your professional guidance.

Changes in the text: P15 line286.

Line 318 (Table) I’m a bit confused about which of the family members the authors consider to be symptomatic. In this table, 5 out of the 10 individuals have at least one symptom whereas elsewhere in the paper, the authors state that there are only four living symptomatic family members. Perhaps the authors meant that there are 5 living symptomatic family members but only 4 of them were previously diagnosed with FD? This should be clarified.

Reply: Thank you for your advice. The subject IV:2 actually had hypohidrosis as showed in the table. However the symptom was mild. She was a symptomatic person. So we modified the description into “five patients”.

Changes in the text: P9 line159-161.

Replies to Reviewer D

Fabry disease (FD) is an X-linked recessive inheritance lysosomal storage disorder due to mutations in the GLA gene leading to deficiency of lysosomal α-galactosidase A (α-Gal A) and has a wide range of clinical presentations. The present investigation studied the potential mutation in GLA and the clinical phenotype of Fabry disease in a Chinese large family gave evidence to explore the relationship better.

However, there is several issues need to be further addressed.

1. In the family, there is no patient in generation I and V. Therefore, there are only three generations investigated. In statement in the abstract and method should be revised.

Reply: We deleted the "five generations" in the abstract.

Changes in the text: P3 line 40.

2. The authors stated that this is the first report of double mutaion in fabry disease. Hwoever, there are previous double mutation reported even in Chinese patient population, this is not the first report. Please conduct a comprehensive literature search before statement.

Reply: Thank you for your guidance of literature search, which helped me avoid an error. We have summarized the literature on double mutations in FD and added a description in the discussion section.

Changes in the text: P3 line48, P14-15 line 267-286.

3. What is the possible mechanisms to explain the phenotype in male is more severe than female patients?

Reply: Women with random inactivation of both X-chromosomes may cause the different phenotype.The skewed X-inactivation may also be responsible for clinical manifestations in female carriers of X-linked diseases. Normal heterozygotes could present skewed X-inactivation in favour of the normal allele. We have modified our text as advised.

Changes in the text: P12 line 218-222.

4. The functional study in Figure 6 stated the impact of g.1170C>T down-regulated the expression level. However, the extent of down regulation is relative small （From 1 to 0.6-0.7）. The author need to conduct celluar biological functional study to prove the small extent change could lead to biological behaviour changes.

Reply: Oliveira et al. indicated that suggest that the g.1170C>T SNP may be co-dominantly associated with a relatively decreased GLA expression at the transcription and/or translation level. We have modified our text as advised, and added the article as reference.

This article used 4 kinds of cells to detect the effect of 1170 mutation on the expression of Lac Z, showing that HEK-293 increased the expression of downstream genes. But for other 3 kinds of cell, the results were opposite. On the one hand, it showed that the mutation may have different effects in different cells. On the other hand, the control used in the article is different from that we used. As discussed in the article, it needs to be further verified by the dual luciferase experiment, and it happens

that we use the dual luciferase system. This may be the cause of the inconsistent results. More detailed functional verification needs to be studied in the future using EMSA and other experiments.

Changes in the text: Page 14 Line 257-259, 264-266.

5. In addition, the author need to prove double mutation is more severe than single mutaiton in Fabry Disease.

We provided two articles explaining this problem, respectively, in two cases where only g.1170C> T SNP and c.1025C>T mutations occurred. None of them had kidney damage. While our case was mainly kidney damage. We speculated that double mutations may have a greater impact on protein structure and function than single mutations, which requires further computer simulation of protein structure and function.

Changes in the text: P18 Line 289-298.

6. Please summarize previous double mutations in Fabry disease.

Reply: We summarized the literature on double mutations in FD and added a description in the discussion section.

Changes in the text: P14-15 line267-285.

7. Although the finding is relatively new, but this is only in a single family. The author still need to explain why this study could have a impact on our understanding or clinical practice in Fabry Disease? Otherwise, this manuscript should be submitted as a case report rather than an original article.

Reply: There are many and complicated GLA gene mutation sites in Fabry disease. What we found for the first time was that g.1170C> T and c.1025C> T variants occurred at the same time. Newly discovered genetic mutation sites are still increasing with the widespread application of next-generation sequencing technology. But the research on the correspondence of clinical phenotypes and functional studies of these variants sites has been carried out slowly, which requires the judgment of clinicians and the verification of molecular biology. What our study does is the clinical phenotypic research and functional verification that are most helpful for clinical diagnosis and treatment, which can promote our clinical practice of FD. Thank you for your suggestions, it is very instructive for clinical work. We sincerely hope that this article can be published as an original article to help more clinicians and patients.

Changes in the text: P17 Line337-339.