Author’s response to reviews

Title: Variable expressivity of FGF3 mutations associated with deafness and LAMM syndrome

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Author’s response to reviews: see over
Responses to reviewers’ comments

Thank you for reviewer’s helpful suggestions and comments. We provide point by point responses to them.

Referee 1: Dr. Tom Walsh

1. Can authors comment on the possibility that the nucleotide alteration (c.283 C>T), underlying p.R95W, may affect splicing of the FGF3 exon in which it is located. For example, could the C>T mutation create a cryptic splice site mutation or perturb a potential exonic splice enhancer site that would lead to a ‘leaky’ splice mutation?

~Thank you for your comment. In silico programs indicate that c.283 C>T is not predicted to affect splicing. We have added the following sentences on pages 10 of the Results section, which reads

“The variant (c.283C>T) associated with the variable phenotype has no predicted effect upon splicing using ESEfinder v3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) or BDGP (http://www.fruitfly.org/se_tools/splice.html) programs. Therefore, it is unlikely that this variant creates a cryptic splice site or perturbs an exonic splice enhancer site that would lead to a leaky splice mutation.”

Referee 2: Dr. Frei Klemens

Major compulsory revisions

1. “Although this frequency is moderately higher than the Somali family reported previously, the results are essentially identical.”

~We agree and did not claim that our radiologic phenotype associated with p.R95W is different from that reported by Ramsebner et al., 2010. In terms of the radiologic phenotype, our results confirmed the data reported by Ramsebner et al., 2010. The similar radiologic findings in two ethnically different families suggests that the phenotype is probably not due to genetic background/modifiers but rather a manifestation of the mutant allele itself. Without a second unrelated family segregating p.R95W, such a suggestion would not have been possible.

2. “The dental results reported in the paper should be reevaluated and statements such as ‘absent syndromic features’ should be more carefully drafted as ‘minimal features’, if appropriate.”

~We have revised the abstract accordingly and now state
“The manifestations of recessive FGF3 mutations range from fully penetrant LAMM syndrome to deafness with residual inner ear structures and, by extension, with minimal syndromic features”

Given the ongoing environmental catastrophe and relentless violence in Pakistan, it is not now possible to obtain additional dental records.

3. “Conclusion of ‘semi-dominance’ drawn from three heterozygous carriers with comparable, bilateral hearing loss should be re-evaluated since there can be a variety of other reasons for bilateral hearing loss.”

— The reviewer is correct. We originally carefully worded our statement about this to take into account several possible explanations. All of the family members live in rural areas of Pakistan. We have already asked family members to travel to Lahore, Pakistan several times. Initially, an audiologist in Lahore tested one heterozygote from each of the three families (PKDF295, PKDF887 and PKDF817) to determine if heterozygotes have normal hearing as we expected. However, the heterozygous carrier with the p.R95W showed isolated mild conductive hearing loss. To confirm this observation, we requested that a different audiologist evaluate the same person as well as an additional two heterozygous carriers of p.R95W who agreed to visit Lahore. All of the three heterozygous carriers consistently showed conductive hearing loss, unlike carriers from the other two families. We were unable to obtain measurements of the status of the tympanic membrane, and ossicles from the three heterozygous carriers of p.R95W (PKDF817 IV-5, V-6 and V-9).

As suggested, we have added one sentence in the result section (p9) which states

‘However, we can not rule out otitis media in these carriers due to a lack of tympanometric data.’

We added another sentence in the discussion section (p14) which states

“These data warrant careful interpretation, since we were unable to obtain information about the status of the tympanic membrane, and ossicles in the carriers.”

We have also softened our position on this matter by deleting the following sentence on page 15.

“This is also possible that a subtle semi-dominant phenotype of carriers in the Somali family was not noticed.”

4. The molecular modeling methodology employed can deliver no definite information on the effects of mutations on the binding of ligands to glycosylated receptors. The conclusion should be modified appropriately.

— As suggested, in the discussion section (p14), we have added the following sentences that read “While these models provide a plausible rationale for the effects of the
mutations identified here, there remains the caveat that available structural information does not account for the role that receptor glycosylation might play in FGF interaction.”

5. “The authors did not state which mutation should be screened with regard to CI candidacy.”

– Only eight mutations of FGF3 have been reported that are associated with hearing loss. Data in our manuscript imply that some FGF3 mutations detected in the future may also show phenotypic variability similar to that for p.R95W. Therefore, we suggest to clinicians that screening affected family members with regard to cochlear implant candidacy should be undertaken regardless of the mutant allele of FGF3 and even if CLA has already been documented in one member of a particular family.

6. “Some of the images presented also appear to have been duplicated.”

– Our intention was to be perfectly clear in the original legend that panels 3d and 3n are in fact taken from the same subject (PKDF817 V-3). Panel 3d is a representative image of individual V-3 from family PKDF817 and was shown for comparison to the same level TBCT cuts from as many affected subjects as possible. In contrast, panel 3n includes all of the contiguous TBCT cuts from subject PKDF817V-3 in order to show the details of partial development of labyrinth.

We have clarified the relevant text in the result section (p11) to read

“Partial development of the labyrinth was observed in two p.R95W homozygotes, including one (PKDF817 V-3) with a cochlear basal turn, vestibule, and posterior semicircular canal (Figure 3D and 3N from PKDF817 subject V-3).

Minor essential revisions

“P3, background line 2/3: it would be better to order the symptoms for LAMM in the order CLA, microtia and microdontia”

– We have ordered the symptoms as the reviewer suggests.

“P3, background line 3/4: The erroneous and confusing statement about Fgf3 knockout mice should be removed from the abstract.”

– As the reviewer suggested, we have removed this sentence from the abstract.

“P3, Methods Line 3: the origin of the large families should be stated.”

– We stated the origin of the large families on page 3. We said “three large Pakistani families”
“P3 results Line 4/5: CLA in more than of the half inner ears is not ‘less severe’ but different.”

-We changed ‘less severe’ to ‘variable’.

“P4 conclusions: One long sentence is too confusion: Construct two sentences, and rephrase ‘absent syndromic features’”

-We constructed two sentences and rephrased ‘absent syndromic’ to ‘minimal syndromic’.

“P5; Line 2: References 1,2. Morton CC described a frequency for congenital deafness of 1.33 per 1,000 new born in the United Kingdom and ‘1.86 per 1000 seems to be a reasonable overall estimate for the incidence at birth’. Smith stated: Thus at least one child in 1,000 is born with bilateral SNHL of at least 40dB.’ If the authors have no specific data for the Pakistani population, they should amend the quoted figures.”

-We amended the figure from ‘500’ to ‘1,000’ in the first sentence in p5, which reads “Sensorineural hearing loss is one of the most common congenital disorders, affecting at least 1 in 1,000 births.

“P5: Line 9/10: ‘The etiology of CLA is usually unknown except for thalidomide exposure’. Why are FGF3 mutations not mentioned?”

-We rephrased the sentence according to the reviewer’s suggestion. It now reads “Until recently the etiology of CLA was unknown except for cases associated with thalidomide exposure.”

“P5: Line 22: ‘revealed no cochlear or internal’….Complete labyrinthine aplasia is better.”

-We rephrased the sentence according to the reviewer’s suggestion. It now reads “The previously reported families revealed complete labyrinthine aplasia.”

“P6, third paragraph: less severe is a relative, because labyrinthine aplasia is not less severe, P6 Line 15: how is labyrinthine aplasia a mild phenotype.”

-We revised the wording. The sentence now reads “A recessive p.R95W mutation was associated with a variable inner ear and auricular phenotype”.

P7 Line 1: What is ‘the IRB’

-IRB is Institutional Review Board (IRB) which is equivalent to the European ethics committees. We have clarified this in the text.
“P7 Mutations screening of FGF3+10: other methodology, PCR conditions, double strand sequencing etc are not shown.”

-We have added the following sentence in the method section.

“Methods for direct sequencing of PCR products were described previously [22]. BigDye terminator reaction products were resolved on an ABI3730 instrument. Sequencing traces were analyzed with the SeqMan Pro tool of DNASTAR Lasergene software (www.dnastar.com).”

“P8; third paragraph: Which examinations were performed?”

-Pure tone audiometry and temporal bone CT/MRI were performed as described on page 8.

“P8: CT and MRI also reveal middle ear structures and nerves not only inner ear structures.”

-We have changed the wording. It now reads

“Computed tomography (CT) and magnetic resonance imaging (MRI) of the temporal bones were performed, when possible, to examine middle and inner ear structures, and the internal auditory canal and its contents in affected individuals.”

“P8: Is there any word recognition, function of the nerve, hearing aids as criteria for CI”

-We did not perform speech audiograms of affected individuals from family PKDF817. This is a good idea but not feasible given the problems in Pakistan at the moment.

“P9; Line ½ It should be mentioned that PKDF537 and 702 have no mutation in FGF, if this is the case.”

-On page 9, we added a sentence that families PKDF537 and PKDF702 did not segregate FGF3 mutations.

“P9; Line 6: 162 ethnically matched….Are the Pakistani population intended here or which origin do they have”

-The controls were Pakistani normal hearing controls. We state on page 9 that “These mutations were not found in 162 ethnically matched Pakistani normal-hearing control individuals.”

“P9; Line 16: Conductive HL the statement that a heterozygous mutation causes mild to moderate degree of bilateral conductive HL cannot be taken seriously.”
-We have added a sentence which reads “However, we cannot rule out otitis media in these carriers due to a lack of tympanometric data.”

“P9; Line 18/19: three heterozygotes are mentioned: IV5, V6 and V9.
V6 her or homo? V2 is shown as heterozygous in figure 2”

-The reviewer is correct. V6 is a heterozygous carrier. We have corrected this in table 1. V2 could not visit Lahore for further audiologic evaluations at that point in time.

“P10; Line 24: in four of six members”
Table 1: V:1 Bi-CLA
V:4 Bi-CLA
V:7 Lt-CLA
V:8 Bi-CLA
V:10 Bi-CLA
So there are at least 5 affected: 4 Bi-CLA and 1 Uni-CLA

-What we meant by CLA is bilateral CLA. To minimize confusion, we have added ‘bilateral’ ahead of CLA. The sentence (p11) now reads “The images demonstrated bilateral CLA in all four deaf members of PKDF295 and PKDF887 and in four of the six deaf members of PKDF817 (Table 1 and Figure 3)”

“P10/11: The description is very confusing. Why didn’t the authors continue in alphabetical order? They should write that 3d and 3n is V-3(V:3), but I’m guessing.”

-We have clarified this in the text as stated above.

“P11: Line 3+15: MRI: 3i-j and 3l-m are which patients in which family”

-This information is provided in the figure legend 3: PKDF295 IV-2 (I and L) and PKDF887IV-3 (J and M) and we have clarified this again in the text (p11).

“P11: Line 3/4: not only pneumatization should be described but also ossicles of middle ear, exclusion of cholesteatoma and other diseases”

-We have added the sentence “All affected individuals show normal middle ear development.”

“P11: line 13: V7 is mentioned in text, is there also an image?”

-Yes, there is an image and our radiologist and co-author Dr. John Butman reviewed the image. We did not provide these data since it requires a series of images to effectively show the residual structures.
“P13: Line 7: Equivocal teeth are described in V-8 and V10, but this is really equivocal. Are there any measurements of dental and parameters for the Pakistani populations for reference?”

-As stated above, we have revised the abstract accordingly and now state “the manifestations of recessive FGF3 mutations range from fully penetrant LAMM syndrome to deafness with residual inner ear structures and, by extension, with minimal syndromic features”

“In V-10, it might be that these ears are conspicuous for Weerda I. Frontal images would be more informative to see the position of the ears in correlation to the head.”

-V-10 does not show clinically significant dysmorphism in helix/ antihelix, although the helical rim looks a little bit folded over in Figure 2.

“P14; Line 4: Conductive HL: Have other reasons for conductive HL definitely been excluded to support the argument that a heterozygous mutation can lead to this?”

-Please refer to our response stated above.

“P14: Line 11 Reference 36 & 37 for the mild conductive hearing loss in LADD syndrome”

-In reference 36 (in page 1310), the authors said that “the affected boy’s middle sister, 19 years old, had discolored teeth, hypodontia, aplasia of both submandibular glands (Fig. 6b and 7b), shortened fifth digit, and mild hearing loss in the left side. She had also abnormality of middle ear ossicles in the left shown in high resolution CT.”

We think that reference 36 is appropriate. We have deleted reference 37 since it just reported an example of isolated mild conductive hearing loss in autosomal dominantly inherited diseases.

“P14: Line 16 Reference 38 for high frequency hearing loss?”

-The reason why we cited reference 38 (now reference 37) is that we wanted to show that the dental phenotype of LAMM syndrome is closer to that of autosomal dominant LADD syndrome (associated with mutations in FGFR2) than to Globodontia of oto-dental syndrome (associated with large deletion encompassing FGF3). We suggested that semi-dominantly inherited conductive hearing loss associated with a heterozygous carrier of p.R95W may reflect an interaction between FGF3 and FGFR2. However, data from our molecular modeling argued against this hypothesis.

“In table 1, why PKDF295 p.R104X is not examined in this study?”
In the reference column in table 1, we cited the first paper that reported the variant. We have changed the reference of p.R95W from PKDF817 ‘this study’ to ‘Ramsebner et al., 2010’.