Author's response to reviews

Title: Two novel missense substitutions in the VSX1 gene: Clinical and Genetic analysis of families with Keratoconus from India

Authors:

Rohit Shetty Dr (drrohitshetty@yahoo.com)
Rudy Nuijts Dr (rudy.nuijts@mumc.nl)
Soumya Ganesh Nanaiah Dr (soumya.kaveri@yahoo.com)
Venkata Ramana Anandula Dr (ramanagene@gmail.com)
Arkasubhra Ghosh Dr (arkasubhra@narayananethralaya.com)
Chaitra Jayadev Dr (drchaitra@hotmail.com)
Natasha Pahuja Dr (dr.natashapahuja@gmail.com)
Kumaramanickavel Govindasamy Dr (gkumarmvel@gmail.com)
Nallathambi Jeyabalan Dr (drnallathambi@narayananethralaya.com)

Version: 4 Date: 5 December 2014

Author's response to reviews: see over
Dear Editor,

We submit the revised version of the manuscript entitled “Two novel missense substitutions in the VSX1 gene: Clinical and Genetic analysis of families with Keratoconus from India”. We sincerely appreciate the review provided by the Journal and the invaluable comments from the reviewers. This has immensely improved the quality of our manuscript. In the revised manuscript, we have made the necessary revisions as per the comments raised.

Below are our replies to the reviewer’s comments. Our revisions are in red. All the modifications and corrections in the manuscript text have been highlighted in yellow. As suggested by the editor, a sentence about the Ethics Committee approval has been added with the Approval Number.

We hope our revised version meets the high standards of the journal. Thank you for your kind consideration.

Best regards,
Sincerely

Dr Nallathambi
GROW Research Laboratory,
Narayana Nethralaya Foundation,
Bangalore, India
Referee-1
Reviewer: Dr James Fielding Hejtmancik
Reviewer's report:
In this manuscript the authors describe a probably causative mutation and a probably benign sequence variant in VSX1 found by screening 8 families with keratoconus. While the results are generally believable, some addition as to the analysis would help increase the significance of the findings. Specific comments follow:
1. Abstract, Results 'In silico analysis revealed that L268H is a pathogenic variant affecting the protein coded by VSX1, whereas S251T showed a neural effect on functional properties of VSX1.': In silico analysis cannot reveal the pathogenic nature of a sequence change, but only suggest it. Also, what is a 'neural effect'?

We have changed the text as per the comments; it is a “neutral effect”. We regret the error.

2. Results, mUTATION SCREENING..., P. 6, "This mutation was not present in 50 normal controls and the unaffected family members.". The comments about the various databases (1,000 Genomes, NHLBI ESP, BGI Complete Genomics, etc.) should probably be given here rather than below, with a little additional description. Similarly, were the sequence changes present in dbSNP or the Biobase web site?

We thank you for the suggestion and have included the public database analysis in the results section.

3. Results and Discussion, "we screened 20 patients of eight unrelated families with KC...: It would be informative to see whether the two families with the L268H mutation were not actually related. One way to accomplish this would be to genotype intragenic VSX1 SNPs in both families and compare the haplotypes.

We thank the reviewer for pointing out the haplotype analysis in two families. The analysis is done and included in results section with an additional Figure 4. This has certainly added more merit to our paper.

4. Discussion, "One of the variants is a missense mutation and the other a missense substitution.": It is unclear just what the difference between these two is. The authors should be careful about calling the sequence changes a 'mutation' based on bioinformatic analysis--maybe a caveat should be added.

We agree with your point and have modified it accordingly.

5. Figure 2C: While inclusion of zebra fish helps, it would be good to add more
diverse species to the alignment (maybe chicken, Xenopus, etc.) to the alignment to increase evidence of conservation rather than the mostly mammals currently shown.

As suggested we have included diverse species (chicken, Xenopus, dog, chimp) for the protein conservation analysis.

6. Discussion, "However, leucine 268 amino acid residue was located in the C-terminal region of the CVC CVC (Chx10/Vsx-1 and ceh-10) domain of VSX1 and has been previously reported in familial KC patients [16, 24].": The meaning here is unclear. Are the authors saying that the L268H variant is not novel?

We have changed the sentence to bring in more clarity.

7. General: While the English in this article is generally understandable, there are a number of usage and phrasing difficulties that make the manuscript difficult to read and impede the meaning in some instances.

We regret the same and have done an English and Grammar edit of the whole article.
Reviewer: Dr Forbes Manson
Reviewer's report:
1. Major Compulsory Revisions
I. In Figs. 3A and 3B identify the family member who is shown in the sequence and compare it to the unaffected family member.

We have labeled the family members (IDs) in the Figure 3

II. Need at least 105 controls (210 chromosomes) for sufficient power (80%). 340 chromosomes would be preferable (95%). See Collins & Schwartz (2002). AJHG 71; 1251-2.

We have screened 105 unrelated individuals (controls) for the variations in exon 4.

III. Variants in VSX1 are an extremely rare cause of keratoconus. However in this study 2/8 families (25%) were found to have a putative mutation. This high 'success' rate needs discussed, as does the fact that the novel mutation was the same in both families. Considering the number of studies and KC patients that have been screened previously, finding the same novel mutation in 2/8 families strongly suggests these families are related. If this cannot be determined from family histories it should be addressed by genotyping flanking markers.

We have discussed the reason for the high mutation rate in the study subjects. Additionally haplotype analysis is included.

Haplotype analysis demonstrated a sharing of common SNPs around the missense change, (p.Leu268His) in two unrelated KC families, suggesting the possibility of founder effect, which requires further exploration. The rate of variants identified in this study was not really higher than compared (2 out of 8) to the previous reports, but could be due to the reason that our families originated from an endogamous community.

2. Minor Essential Revisions
I. P.3 ?Genetic analysis of KC patients with different ethnic backgrounds has revealed multiple mutations in the VSX1 gene.? This suggests a lot of mutations that in turn implies the locus is more important than it is. Probably only 4 confirmed mutations (5 at a push). Rephrase accordingly.

We have modified the sentence as suggested.
II. P.3. Locus is known more accurately than 20p11-q11 (20p11.21). Correct.

Changed as per the suggestion, thank you.

III. P.5. The text identifying the probands does not match the indicated probands on Figs. 1A and 1B. Correct.

We have modified the text.


We have modified the sentence

V. The level of English is poor in several places and needs corrected.

We have done the necessary language correction in the manuscript

VI. At position c.767 in VSX1 the residue is a ?C? in the codon GCC (Ala). The correct codon for p.Leu268 is c.802-804.

We regret the error and the same has been rectified.

VII. P.6. ?c.767 T>A mutation, CTC (Leu 268) to CAC (His 268)?. This is clumsy. Better to list amino acid change after the cDNA change as p.Leu268His.

Thank you for the suggestion, the same has been incorporated.

VIII. Table 2 shows the change as at c.767 as T>C instead of T>A. cDNA numbering is wrong as well, as noted above.

Numbering has been changed, thank you.

IX. P.7. In silico analysis are predictions (as acknowledged in first line), so cannot then say in next line that a variant ?is pathogenic?. This has to be demonstrated empirically. Based on in silico analysis a variant is no more than a putative mutation. This terminology must be corrected throughout the manuscript.

We have modified the sentence as suggested.

X. P.7. ?However, leucine 268 amino acid residue was located in the C-terminal region of the CVC CVC (Chx10/Vsx-1 and ceh-10) domain of VSX1 and has
been previously reported in familial KC patients [16, 24]? This reads as if the variant in Leu268 has been reported twice before in references 16 and 24. This is not the case and is probably not what is meant. Re-write for clarity.

We have modified the sentence for clarity.

However, leucine 268 amino acid residue was located in the C-terminal region of the CVC domain of VSX1 protein, only two mutations were reported previously in this region associated with familial KC patients and posterior polymorphous dystrophy.

XI. P.7. Moreover, the pathogenic nature of this mutation also comes from leucine?. This does not make sense. Rephrase for clarity.

The sentence has been rewritten.

XII. P.8. Furthermore, in our study, this potentially damaging mutation was detected in all affected individuals with a dominant inheritance of KC without PPCD?. This is inaccurate. You screened 20 KC patients and found it in two families consisting of 5 affected individuals.

We regret the error and it has been rectified.

XIII. P.8. This is consistent with previously identified mutation studies in an Iranian family [24]? It is not clear what you think is consistent.

We have modified the sentence for the clarity

XIV. P.8. This is in agreement with previous studies suggesting that missense substitutions in the VSX1 may or may not be a disease-causing variant [16, 36]?. This gets to the crux of the matter with VSX1 variants. They are a very rare cause of KC and some authors have dismissed VSX1 as a causative gene. More needs to be given to this discussion point highlighting the difficulties in being certain that VSX1 is causative. Many studies find no putative pathogenic variants or find that previously reported mutations are actually polymorphisms.

We agree with you on the ambiguous role of VSX1 in KC and have included a paragraph discussing the same.

At this stage, it is difficult to conclude about the pathogenic nature of variants p.Leu268His,p.Ser251Thr, which is in agreement with previous studies shows that missense substitutions in the VSX1 may or may not be a disease-causing variant. It is controversial and
unclear that contribution of VSX1 coding variants found in the KC patients, since few studies finds that previously reported mutations were actually non-pathogenic or polymorphism [36]

XV. P.8. ?In this study we were able to identify genetic changes in three out of eight families?. This is close to over-playing what has been found. A single, putative mutation, was found in 2/8 families. A second sequence variant of unknown significance (but probably a benign polymorphism) was found in a third family.

We have modified the explanation our findings.

In this study, we screened 20 patients of eight unrelated families with KC for mutations in the VSX1 gene. Among these, five patients from two families had a novel coding variant (p.Leu268His), another variant (p.Ser251Thr) was identified in a KC family with three affected individuals.

XVI. P.8. ?In summary, we add two novel missense variations in the coding region of VSX1 to the existing spectrum of VSX1 mutations observed in Indian patients with the characteristic phenotype of KC?. As previously noted, from your data you have identified one putative mutation and one VUS. The mutation can only be considered as such because it has been found in two families. However if the families cannot be shown to be unrelated than this variant will also have to be considered as a VUS.

Families (KC-01,KC-02) were belonging to an endogamous community origin, based on the haplotype analysis, (p.Leu268His) might be a common variant among the population or founder mutation which requires further analysis.

3. Discretionary Revisions
I. P.5. ?Another missense substitution (S251T) was identified in both patients and their family members? would be clearer as ?both affected siblings and their affected father?

We thank you for you suggestion and incorporated the same.

II. P.6. Suggest ?Amino acid conservation analysis revealed that the wild type leucine 268 was highly conserved among the VSX1 orthologs in other species? would be better as ?Amino acid conservation analysis revealed that leucine at
position 268 is conserved in 9 vertebrate orthologs? (a residue is either conserved or it is not).

The sentence has been modified as per your suggestion and sounds definitely better.

III. P.7. Only need to expand CVC on first use.

Changes have been done.
This manuscript describes two novel mutations in the VSX1 gene in 3 Indian families with keratoconus. Mutations in the VSX1 gene have been previously implicated in keratoconus with rare non-synonymous variants identified in a small percentage of keratoconus patients. It does not appear that VSX1 accounts for a high proportion of keratoconus, although highly penetrant rare variants may make a contribution. In this paper the sequencing of the VSX1 gene in 20 keratoconus patients from 8 families from India was undertaken in order to determine if VSX1 mutations could explain disease in any of the patients. Two novel variants were detected. One of these was found in 2 apparently unrelated families and is predicted to be pathogenic by a number of algorithms. The other was found in a single family and is predicted to be a neutral variant. The study described is relatively straightforward and appears to have been conducted appropriately. The new knowledge gained is incremental and only a small number of families were assessed. It does add to the mutation spectrum observed in keratoconus patients.

Minor essential revisions
Changes have been done.

Methods: Were the sequence chromatograms visualized to identify the mutations? The chromatograms are presented, but the methods only discuss the use of Blast alignment on the sequence to find the mutations.
We have now included details about the chromatogram viewer

Results: Were any other previously reported polymorphisms found?
We found other polymorphism which are included in the haplotype analysis with SNP IDs in Figure-3

Table 1: Please define the abbreviations
Abbreviations have been defined

Table 2: SIFT classification is usually notated as Deleterious (not damaging).
Thank you, changed appropriately

Table 3: Please indicate how the pathogenicity classifications in this table were determined. From the original report, or from modern bioinformatics predictions? Perhaps the evidence for pathogenicity or not could be summarized? How can the same mutation be both pathogenic and not pathogenic? Is this a case of it first being reported one way, then a later paper having a different conclusion? Does this table only include mutations in KC patients, or also in PPCD patients as implied in the text?

We have modified the table as per your valuable comments.

Major compulsory revisions

Methods:
Which individuals were sequenced? All 20 affecteds? 1 affected from each of the 8 families? All family members? Were controls sequenced? There is no description of how the mutations were assessed in the 50 controls. Were they sequenced for the whole gene, or was a targeted assay used?

We have modified the methods section to include all the above details.

Which PolyPhen2 algorithm was used? HumDiv or HumVar?

We included the PolyPhen2 algorithm in table-2

The AAMSPSM results should be noted in the results section and the tool should be described in the methods section.

The methods and results have been modified as per the reviewer comments.

The rate of variants in this study is 3 out of 8 families, although probably only 2 out of 8 have a potentially pathogenic change. This seems quite high compared to other studies which typically find VSX1 mutations are quite rare. Please discuss this mutation rate and how it compares to other studies.

We have explained the mutation rate in the discussion section.

Haplotype analysis demonstrated a sharing of common SNPs around the missense change, (p.Leu268His) in two unrelated KC families, suggesting the possibility of founder effect, which requires further exploration. The rate of variants identified in this study was not really higher
than compared (2 out of 8) to the previous reports, but could be due to the reason that our families originated from an endogamous community.

It is stated that the two families with the L268H mutation are not related. How was this determined? Are they from the same geographical region or ethnic group? Was any genetics (haplotype analysis) done to confirm the same mutation has arisen twice? It seems improbably that two unrelated families in the same research study would have the same novel rare variant.

Haplotype analysis was done and details included in the manuscript.

The last sentence of the conclusion (also re-iterated in the abstract) is perhaps overstated and should be removed. There may be some utility for genetic counselling in the 2 families segregating the pathogenic variant, but this study does not shed much light on the role of VSX1 in keratoconus. In fact, it adds to the general confusion over this gene with a likely neutral polymorphism segregating, probably by chance.

This sentence was removed from the abstract as well as from the conclusion.

Discretionary revisions
Results: The first paragraph is difficult to follow. It refers to S251T being identified in both patients and their family members?. Which patients? It becomes clearer later on that this is a third family, but it could be clarified here

We have clarified the same in the results

Discussion: First paragraph; Among these, eight patients from three families had two coding variants?? is ambiguous. It sounds like all 8 patients had the same 2 variants.

The sentence has been modified.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: I declare that I have no competing interests