Author's response to reviews

Title: LOXL1 gene variants and their association with pseudoexfoliation glaucoma (XFG) in Spanish patients

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

We highly appreciate the constructive comments and critiques made by the reviewers of our manuscript (Ms). We have taken them very seriously and addressed them point-by-point.

We hope that the changes introduced in the revised Ms meet with the approval of the reviewers, as well as of the editorial board for publication in BMC Medical Genetics.

Sincerely yours,

Miguel Coca-Prados

Note: The page and line numbering is referred to the revised version of the manuscript.

Responses to Reviewer #1:

1.- Reviewer’s comment: “The manuscript needs to be shortened. I suggest to substantially shorten molecular methods (next generation sequencing and PCR-RFLP genotyping) section and to include such information as supplementary material”.

Response to reviewer’s comment: As suggested by the Reviewer’s comment, the following paragraphs have been deleted from the “Methods” section of the manuscript, and now they have been included as supplemental material (see Additional file 1):

“Hs_LOXL1_01a_LR for fragment 1 amplification (10464bp; Chr15: 74214119-74221883), Hs_LOXL1_02a_LR for fragment 2 amplification (10800bp; Chr15: 74221523-74232323), Hs_LOXL1_03a_LR for fragment 3 amplification (9965bp; Chr15: 74231398-74241363) and Hs_LOXL1_04a_LR for fragment 4 amplification (9993bp; Chr15: 74240655-74250648)”

“Amplification of fragments 1, 2 and 3 were run with a four-stage program with an increased extension time in stages three and four: 93 °C for 3 min; 93 °C for 15 s, 62 °C for 30 s, 68 °C for 10 min over 10 cycles; 93 °C for 15 sec, 62 °C for 30 sec, 68 °C for 10 min over 15 cycles in which extension time is increased by increments of 20 sec in each cycle; 93 °C for 15 sec, 64 °C for 30 sec, 68 °C for 15 min over 10 cycles in which extension time is increased by increments of 20 sec in each cycle. Addition of 7.5% DMSO was required for fragment 1 amplification. The PCR program for fragment 4 was a three-stage program with an increased extension time in stage three: 93 °C for 3 min; 93 °C for 15 sec, 62 °C for 30 sec, 68 °C for 10 min over 10 cycles; 93 °C for 15 sec, 62 °C for 30 sec, 68 °C for 10 min over 25 cycles in which extension time is increased by increments of 20 sec in each cycle”
“…under the following settings: i) Run type: Single Read, Multiplexing, Number of cycles: Read 1=50 nt targeted sequencing, Read 2=7 nt index sequencing; ii) Flowcell Version: HiSeq Flow Cell v3; iii) Flow cells were clustered using TruSeq SR Cluster Kit v3 - cBot – HS; iv) the sequencing chemistry used was TruSeq SBS Kit v3 - HS (50-cycles), with Illumina softwares RTA Version 1.13.48 and CASAVA Version 1.8.1”.

“Genotyping of the two nonsynonymous exonic SNPs, rs1048661 and rs3825942, was performed by digesting PCR generated amplicons (432 bp long) containing these SNPs with the enzymes Smal and HinfI respectively. In the case of the SNP rs1048661, PCR fragments with the risk allele G at codon 141 (141R allele) yielded three fragments of 201 bp, 146 bp and 85 bp long each, upon digestion with Smal. In contrast, fragments with the allele T at codon 141 (141L allele), lacked the Smal restriction site, yielding only 2 fragments of 347 bp and 85 bp long. However, PCR amplicons with the heterozygous genotype GT yielded all the 4 fragments (347 bp, 201 bp, 146 bp and 85 bp) upon digestion with Smal”.

“Genotyping of the SNP rs3825942 was carried out by generating amplicons of 432 bp long containing the SNP, and Hinf I restriction enzyme digestion. Amplicons with A at codon 153 (153D allele) were sensitive to Hinf I digestion resulting in 2 fragments of 322 bp and 110 bp, whereas amplicons with G at codon 153 (153G allele) lacked the Hinf I restriction site and therefore were resistant to digestion, resulting in an intact fragment of 432 bp. Amplicons with the heterozygous GA genotype yielded 3 DNA fragments (347 bp, 322 bp, and 110 bp) upon digestion with Hinf I”.

“Genotyping of intronic SNP rs2165241 was performed by digesting a 536 bp PCR amplicon containing this SNP with the enzyme SspI. The presence of the risk allele T resulted in two fragments of 331 bp and 205 bp, whereas the presence of the allele C caused resistance to SspI digestion. The presence of both alleles (TC) produced three fragments of 536 bp, 331 bp and 205 bp, respectively”.

“Amplicons harboring the SNP rs16958477 were genotyped using the restriction enzyme BauI. PCR-generated amplicons 416 bp long containing the risk allele C were sensitive to BauI digestion, creating two fragments of 246 bp and 170 bp fragments, whereas, amplicons with the allele A were resistant to BauI digestion. Amplicons containing the heterozygous AC produced three fragments (416 bp, 246 bp and 170 bp), upon digestion with BauI”.

“Finally, genotyping of the SNP rs3522 was carried out by digesting 309-bp PCR-generated amplicons containing the SNP with the enzyme BsaHI. The presence of the allele C in the SNP conferred sensitivity to BsaHI digestion, yielding two fragments of 204 bp and 105 bp. DNA amplicons containing the allele T at the same position resisted BsaHI digestion, resulting an intact undigested fragment of 309 bp. Digestion of amplicons containing the heterozygous CT genotype with BsaHI yielded three fragments (309 bp, 204 bp and 105 bp)”.

2. Reviewer’s comment: “Results obtained in this study on the recently described rs41435250 risk SNP and a brief Discussion on such results would be of interest for readers”.

Response to reviewer’s comment: As suggested by the Reviewer’s comment, on page 12, lines 4-8, the following paragraph has been added: “In contrast, the recently described coding variant rs41435250, located in exon 1 and identified as a risk variant in South Indian and Mexican subjects [32,37], has not been included in this analysis. This SNP did not associate with XFG in our pilot study (p=0.4), which is in agreement with the result obtained in other populations including South African subjects (p=0.49) [34] and a Saudi Arabian population, where it was found to be monoallelic [36]”.

3. Reviewer’s comment: “A sentence indicating if the geographic origin of cases and controls is similar needs to be added in Material and Methods section”.

Response to reviewer’s comment: As suggested by the Reviewer’s comment, in Methods section, page 5, lines 10 and 11, the following sentence has been added: “Most subjects involved in the study (75% and 66% of XFG and controls, respectively) originated in the Northernwestern regions of Spain including Galicia, Asturias and Cantabria”
4. **Reviewer’s comment:** Introduction, page 4, line 15, "The former study suggested the possibility of a LOXL1 intragenic epistatic effect.", must be changed to "The latter study.."

**Response to reviewer’s comment:** Sorry for the error. As suggested, on page 4, line 15, of the introduction section, the words “The former study…” have been replaced by “The latter study…”

5. **Reviewer’s comment:** “Were controls checked for the absence of exfoliation material? This should be stated in Methods section”.

**Response to reviewer’s comment:** As suggested by the Reviewer’s comment, in Methods section, page 5, lines 17 and 18, the sentence “Control subjects were selected from patients undergoing cataract surgery who did not present with glaucoma” has been changed by the following sentence: “Control subjects were selected from patients undergoing cataract surgery who did not present signs of glaucoma or exfoliative material”.

6. **Reviewer’s comment:** Discussion, page 14, line 35, the authors stated that "... 75% and 66% of XFG and control participants from the Northwest regions of Spain, where the prevalence of XFG is higher than in other regions of Spain. Differences between Spanish regions could explain the discrepancies between our results and those reported recently in different Spanish populations". It is not clear why differences in XFG prevalence among Spain regions could explain conflicting results as the lack of relationship between XFG and the G allele of rs1048661 in a previous Spanish population (de Juan-Marcos et al. 2014). Please explain more in detail.

**Response to reviewer’s comment:** On page 13, lines 17-24, the paragraph: “The Spanish population studied here comprised of approximately 75% and 66% of XFG and control participants from the Northwest regions of Spain, where the prevalence of XFG is higher than in other regions of Spain. Differences between Spanish regions could explain the discrepancies between our results and those reported recently in a different Spanish population [35]” has been replaced by the following: "The Spanish population studied here comprised of approximately 75% and 66% of XFG and control participants from the Northwestern regions of Spain, where the prevalence of XFG is higher than in other regions of Spain, which could reflect a different genetic background of subjects from these regions. Thus, differences in the geographic origin of the Spanish populations could explain the discrepancies between our results and those reported recently in a different Spanish population [35]. It is well known that the frequencies of risk alleles and their association with XFG vary not only among different ethnic groups, but also among populations of the same country, as described in studies with different populations from Greek, China or India (see Table 1)".

7. **Reviewer’s comment:** “A brief comment on the possibility that the use of CEU population controls for excluding novel LOXL1 SNPs as risk variants (Suppl table 1) could lead to false negative results, should be added”.

**Response to reviewer’s comment:** In agreement with Reviewer’s suggestion, on page 11, lines 21 and 22, the following sentence has been added: …“we can not rule out any false negative result”.

8. **Reviewer’s comment:** “Please correct spelling mistakes through the manuscript”.

**Response to reviewer’s comment:** Language of the manuscript has been thoroughly revised, and we hope the new version meets with the Reviewer’s satisfaction as well as that of the editorial board members.

Responses to Reviewer #2:

1. **Reviewer’s comment:** Page 14, line 14. Do the authors mean... ‘conferring or not a risk for XFG’
instead of at-risk?

Response to reviewer’s comment: Sorry for the error. On page 13, line 7, the sentence: “… conferring or not at-risk…” has been changed by the following “… conferring or not a risk…”

2.- Reviewer’s comment: Page 16, line 1. Correctly ‘Lox11 null mice’ instead of LOXL1 that is for the human gene.

Response to reviewer’s comment: As suggested by the reviewer’s comment, on page 14, line 24 the words “LOXL1 null mice” have been replaced by the following: “Lox11 null mice”

3.- Reviewer’s comment: Page 15, lines 26, 27 to page 16, lines 1, 2. There is indeed abundant evidence to show that, beyond the LOXL1 gene, there are other genetic and/or environmental factors involved in the development of XFS and XFG. However, the lack of either ocular or systemic XFS features in Lox11 null mice does nor provide support for this argument. There appears to be no evidence to indicate that any of the risk-associated allelic variants of LOXL1 would represent a null condition, or that there would be an absence or absent function of the LOXL1 protein in either XFS or XFG.

Response to reviewer’s comment: We thank the reviewer for her constructive comment. We agree that instead of the absence of the LOXL1 protein, an alteration in its function and/or structure may be related to the deposition of exfoliative material. Following reviewer’s suggestion, on page 14, lines 23-27, the sentence “In agreement with this view, Wiggs et al., recently showed that LOXL1 null mice did not exhibit ocular nor systemic features of XFS and the loss of LOXL1 did not result in deposition of exfoliative material or glaucoma ” has been changed to the following: “Recently, Wiggs et al., showed that Lox11 null mice did not exhibit ocular nor systemic features of XFS and the absence of LOXL1 protein did not result in deposition of exfoliative material or glaucoma. An alteration in the structure and/or function of the LOXL1 protein, rather than its lack, could be related to the deposition of exfoliative material”.

List of additional changes to the manuscript

1.- As a result of the inclusion of a new additional file in the revised version of the manuscript, the old “Additional file 1” has been labeled “Additional file 2”.

2.- We have detected and corrected some mistakes in the numbering of some of the references listed in Table 1.