Analysis of LOXL1 polymorphisms in a Saudi Arabian population with pseudoexfoliation glaucoma

Khaled K. Abu-Amero,1 Essam A. Osman,2 Ahmed S. Dewedar,2 Silke Schmidt,3 R. Rand Allingham,3,4 Saleh A. Al-Obeidan2

1Ocular Genetics Laboratory, College of Medicine, King Saud University, Riyadh, Saudi Arabia; 2Department of Ophthalmology, College of Medicine, King Saud University, Riyadh, Saudi Arabia; 3Center for Human Genetics, Duke University Medical Center, Durham, NC; 4Department of Ophthalmology, Duke University Eye Center, Durham, NC

Purpose: To investigate whether single nucleotide polymorphisms (SNPs) in the lysyl oxidase-like 1 (LOXL1) gene are associated with pseudoexfoliation glaucoma (PEG) in the Saudi Arabian population.

Methods: The coding regions of LOXL1 were fully sequenced in 93 clinically diagnosed PEG patients and 101 healthy controls. Both groups were Saudi Arabs. Previously reported and newly identified SNPs were evaluated for possible association with PEG and their pathological consequences on the gene were assessed.

Results: The “G” allele frequencies of both rs1048661 and rs3825942 SNPs differed between PEG patients and control subjects from Saudi Arabia (p=0.0056 and p=0.000005, respectively). This significance remained after applying the Bonferroni correction. Two non-synonymous novel SNPs in LOXL1 were detected in the PEG patients and not in the controls. One of these SNPs was in exon 4 (g.25722 C>G; codon change D484E) of LOXL1 and was predicted to be non-pathological; the other was in exon 6 of LOXL1 (g.28084 T>G; codon change Y559D) and was predicted to be probably damaging. All alleles of SNPs (rs28706550, rs35203737, rs12906373, rs41435250, and rs13329473) were monoallelic in this population. No allele frequency difference for rs8818 and rs3522 SNP between patients and controls (p values were 0.126 and 0.994 respectively).

Conclusions: Similar to almost all non-African populations tested thus far, the “G” allele of both rs1048661 and rs3825942 SNPs were associated with the risk of PEG in the Saudi Arab population.

Pseudoexfoliation syndrome (XFS) is characterized by deposits of grayish-white material seen primarily in the anterior segment of the eye. The deposits are primarily found along the pupillary border and often produce a characteristic pattern on the anterior lens surface [1]. XFS is frequently associated with pseudoexfoliation glaucoma (PEG), which often has a more aggressive clinical course and worse prognosis than the more common primary open angle glaucoma (POAG) [2]. The incidence of PEG in XFS patients varies and seems highest among individuals with Scandinavian and Northern European ancestry and lower among African Americans and in West Africa [3-5]. The prevalence of PEG in Saudi Arabia is unknown. The Glaucoma unit at King Abdulaziz University Hospital (where most PEG patients were recruited for this study) sees around 600 new glaucoma patients every year, and about 10% of those are PEG.

Thorleifsson and colleagues [6] have reported a genome-wide association study that identified a strong association between three single nucleotide polymorphisms (SNPs) in the lysyl oxidase-like 1 (LOXL1) gene. They identified one intronic SNP (rs2165241) and two non-synonymous coding SNPs (rs1048661 and rs3825942) with significant disease association in Icelandic and Swedish subjects. LOXL1 belongs to the “LOX” family of extracellular enzymes that have multiple functions including the cross-linking of collagen and elastin by oxidatively deaminating lysine residues. Since XFS deposits are associated with the extracellular and basement membrane regions, the LOX genes are legitimate functional candidates to be involved with PEG pathogenesis [7].

The association of LOXL1 SNPs (in particular rs1048661 and rs3825942) with XFS/PEG has now been studied in Caucasian populations in the USA [8], Australia [9], Austria [10], Germany [11], Italy [12], and Finland and in other ethnic groups, including Japanese [13], Indian [14], Chinese [15], and recently black South Africans [16]. The “G” allele of SNP rs3825942 is significantly associated with XFS/PEG in all populations tested to date [17] with the exception of black South Africans where the “A” allele is the risk allele [16].

The “G” allele of SNP rs1048661 is associated with XFS/PEG in all populations except in the Indian [14] and Chinese [15] populations. In other studies, the opposite “T” allele of SNP rs1048661 was shown to be the risk allele for PEG in the Chinese [18] and Japanese [19] populations.
This study was conducted to examine the frequency of various polymorphisms of \textit{LOXL1} in the Saudi Arabian population with PEG and to evaluate whether SNPs in the \textit{LOXL1} gene associated with the risk of PEG in this population.

**METHODS**

**Study population:** The study adheres to the tenets of the Declaration of Helsinki, and all participants signed an informed consent. The study was approved by College of Medicine ethical committee (proposal number # 08–657). All study subjects were self identified as Saudi Arabian ethnicity. Family names were all present in the database of Arab families of Saudi Arabian origin. Additionally, these names indicated that all five major Saudi Arabian provinces were represented in the study population. Expatriates were excluded from this study and all patients and controls were Saudi Arabs. Subjects with clinically diagnosed PEG and healthy controls were recruited into the study at King Abdulaziz University Hospital in Riyadh, Saudi Arabia. All participants underwent a standardized detailed ophthalmic examination, which included measurement of intraocular pressure (IOP) by Goldmann applanation tonometry, slit lamp biomicroscopy, gonioscopy, and dilated examination of the lens and fundus. Subjects with PEG were defined as those with clinical evidence of exfoliation material on the pupil margin or anterior lens surface and the presence of glaucomatous optic neuropathy with associated visual field loss in one or both eyes and documented IOP ≥22 mmHg in either eye. Saudi Arab subjects with normal anterior segment and optic nerve examination, IOP <18 mmHg, and no clinical signs of exfoliation were recruited as control subjects.

**DNA analysis:** Five ml of peripheral blood were collected in EDTA tubes from all participating individuals. DNA was extracted using the illustra blood genomicPrep Mini Spin Kit from GE Healthcare (Buckinghamshire, UK), and stored at −20 °C in aliquots until required. PCR amplifications of the 7 exons and the promoter region of \textit{LOXL1} were performed using the primers listed in Table 1. Successfully amplified fragments were sequenced in both directions using the M13 forward and reverse primers and the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster city, CA). Fragments were then run on the 3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturer protocol. All the sequenced fragments were then analyzed using SeqScape software v2.6 (Applied Biosystems). Allele frequencies for SNP rs1048661 and rs3825942 were confirmed by repeating the sequencing in both the forward and reverse directions. Table 1 details the sequence of the primers used, the PCR annealing temperature, and the expected amplicon size.

| Exon | Primer sequence | Annealing temperature (°C) | Amplicon size (bp) |
|------|-----------------|---------------------------|------------------|
| Promoter-F | TGTTAAGACGCGCCAGT | 60 | 465 |
| Promoter-R | CAGGAAACAGCTAGACC | 60 | 789 |
| 1A-F | TGTTAAGACGCGCCAGT | 60 | 810 |
| 1A-R | CAGGAAACAGCTAGACC | 57 | 553 |
| 1B-F | TGTTAAGACGCGCCAGT | 60 | 209 |
| 1B-R | CAGGAAACAGCTAGACC | 60 | 232 |
| 1C-F | TGTTAAGACGCGCCAGT | 58 | 265 |
| 1C-R | CAGGAAACAGCTAGACC | 58 | 210 |
| 2-F | TGTTAAGACGCGCCAGT | 60 | 234 |
| 2-R | CAGGAAACAGCTAGACC | 60 | 104 |
| 3-F | TGTTAAGACGCGCCAGT | 58 | 553 |
| 3-R | CAGGAAACAGCTAGACC | 57 | 810 |
| 4-F | TGTTAAGACGCGCCAGT | 57 | 465 |
| 4-R | CAGGAAACAGCTAGACC | 57 | 465 |
| 5-F | TGTTAAGACGCGCCAGT | 55 | 210 |
| 5-R | CAGGAAACAGCTAGACC | 55 | 210 |
| 6-F | TGTTAAGACGCGCCAGT | 60 | 234 |
| 6-R | CAGGAAACAGCTAGACC | 60 | 234 |
| 7-F | TGTTAAGACGCGCCAGT | 60 | 234 |
| 7-R | CAGGAAACAGCTAGACC | 60 | 234 |

In the table, F: Forward; R: Reverse; *SNPs rs1048661 and rs3825942 were amplified with this primer set. Bold and underlined sequences are those of M13.
polymorphisms of \textit{LOXL1} were subjected to Bonferroni correction for multiple testing.

\textbf{RESULTS}

Ninety three PEG patients and 101 controls were recruited into this study. Of the 93 PEG patients there were 61 males and 32 females with a mean age of 72.3 (SD 12.02). Of the 101 controls there were 64 males and 37 females with a mean age of 69.3 (SD 12.4). The full coding region, exon-intron boundaries, the promoter region (470 bases before the transcriptional start site), and the 5′UTR and 3′UTR of the \textit{LOXL1} gene was sequenced in all subjects.

The “G” allele frequencies of both rs1048661 (0.876) and rs3825942 (0.968) SNPs differed significantly between PEG patients and controls subjects (p=0.0056 and 0.000005, respectively). If we consider the 4 common SNPs (rs1048661, rs3825942, rs28706550, and rs35203737), then the Bonferroni corrected p-value should be 0.0125. After applying the Bonferroni correction, SNPs rs1048661 and rs3825942 remained significant. There was no statistically significant difference in genotype frequencies between patients and controls for SNP rs1048661 (p values were 0.409 and 1.000 for genotypes G/G and G/T respectively). As for SNP rs3825942, there was a statistically significant difference between patients and controls for the G/G genotype (p=0.049), but that significance disappeared after applying Bonferroni correction. As for the G/A genotype of SNP rs3825942, there was no difference in genotype frequency between patients and controls (p=1.000).

Genotypes of SNPs rs28706550 and rs35203737 were monogeneic in all patients and controls (Table 2).

There was no significant difference in allele frequencies between cases and controls for the rs8818 and rs3522 SNPs (p values were 0.126 and 0.994, respectively; Table 3). Genotypes at SNPs rs1048661, rs3825942, rs8818, and rs3522 were in HWE for both patients and controls (p ≥0.05).

\begin{table}[h]
\centering
\caption{Genotype frequencies of the four most common LOXL1 SNPs.}
\begin{tabular}{|l|l|l|c|c|}
\hline
SNP I.D. & Nucleotide change & Genotype & XFG patients (n=93) & Controls (n=101) & p value \\
\hline
rs1048661 & g.5758 G>T & G/G & 72 (77.4%) & 57 (56.4%) & 0.409 \\
& & G/T & 19 (20.4%) & 40 (39.6%) & 1.000 \\
& & T/T & 2 (2.2%) & 4 (4%) & reference \\
rS3825942 & g.5794 G>A & G/G & 88 (94.6%) & 70 (69.3%) & 0.049 \\
& & G/A & 4 (4.3%) & 25 (24.7%) & 1.000 \\
& & A/A & 1 (1.1%) & 6 (6%) & reference \\
rS28706550 & g.25067 A>C & A/A & 93 (100%) & 101 (100%) & - \\
& & A/C & 0 (0) & 0 (0) & - \\
& & C/C & 0 (0) & 0 (0) & reference \\
rS35203737 & g.28103 C>A & C/C & 93 (100%) & 101 (100%) & - \\
& & C/A & 0 (0) & 0 (0) & - \\
& & A/A & 0 (0) & 0 (0) & reference \\
\hline
\end{tabular}
\end{table}

LOXL1 indicates lysyl oxidase-like 1; PEG indicates exfoliation glaucoma. Alleles in bold were considered the risk alleles in calculating p value. Nucleotide are numbered as in GenBank accession number NG_011466. Numbers in parenthesis represent the allele frequency. Novel indicates not previously reported. N/A indicates not applicable.

\begin{table}[h]
\centering
\caption{Allele frequencies of various LOXL1-SNPs in PEG patients and controls.}
\begin{tabular}{|l|l|l|l|l|}
\hline
SNP I.D. & Nucleotide change & Amino acid change & Allele & PEG patients (n=93) & Controls (n=101) & p value \\
\hline
rs1048661 & g.5758 G>T & R141L & G & 163 (0.876)* & 154 (0.762) & 0.0056 \\
rs3825942 & g.5794 G>A & G153D & G & 180 (0.968) & 165 (0.817) & 0.000005 \\
rS28706550 & g.25067 A>C & N437H & A & 186 (1) & 202 (1) & N/A \\
rS35203737 & g.28103 C>A & S565Y & C & 186 (1) & 202 (1) & N/A \\
Novel & g.25722 C>G & D484E & C & 184 (0.99) & 202 (1) & N/A \\
Novel & g.28084 T>G & Y559D & T & 185 (0.995) & 202 (1) & N/A \\
rS8818 & g.30490 C>G & - & G & 141 (0.758) & 138 (0.683) & 0.126 \\
rS3522 & g.30556 C>T & - & C & 116 (0.624) & 125 (0.619) & 0.994 \\
rS41429348 & g.6212 C>T & - & C & 186 (1) & 202 (1) & N/A \\
rS12906373 & g.6272 C>T & - & C & 186 (1) & 202 (1) & N/A \\
rS41435250 & g.6296 G>T & - & G & 186 (1) & 202 (1) & N/A \\
rS13329473 & g.25737 C>T & - & C & 186 (1) & 202 (1) & N/A \\
\hline
\end{tabular}
\end{table}
Amino acid change
Location
Interspecies conservation
PolyPhen prediction

Non-synonymous indicates sequence change which results in an amino acid change. PolyPhen=Polymorphism Phenotyping is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. “Probably damaging” constitutes high confidence of affecting protein function. “Possibly damaging” reflects a likelihood of affecting protein function or structure, while “Benign” changes most likely lack phenotypic effect.

In summary we showed that the “G” allele of SNP rs1048661 in cases versus controls (p=0.000005). Despite our in silico analysis using PolyPhen which predicted that this SNP was possibly damaging and that the amino acid glycine was highly conserved at codon 153 in many species, some doubts about the pathological role of this SNP in PEG were raised. This stems from previous reports that this SNP does not appear to affect LOXL1 gene expression levels in blood or ocular tissues [6,22]. Additionally, the recent study in a South African population [16], where the “G” allele of SNP rs3825942 was protective and the opposite allele “A” was the risk allele for PEG, raises further uncertainties about the pathological role of this SNP.

Sequencing the coding region of the LOXL1 gene revealed two previously unreported SNPs (g.25722 C>G and g.28084 T>G) detected in PEG patients and not in the controls. In silico analysis using PolyPhen predicted that g.25722 C>G is benign and that g.28084 T>G was probably damaging. We cannot be certain what role these two non-synonymous SNPs play in the development of PEG, although it is possible that either one of these two SNPs can be used for PEG risk assessment.

In summary we showed that the “G” allele of LOXL1 SNPs rs1048661 and rs3825942 are associated with PEG in
the Saudi populations in a fashion similar to other non-African populations.

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