The LOXL1 Gene Variations Are Not Associated with Primary Open-Angle and Primary Angle-Closure Glaucomas

Subhabrata Chakrabarti, Kollu Nageswara Rao, Inderjeet Kaur, Rajul S. Parikh, Anil K. Mandal, Garudadri Chandrasekhar, and Ravi Thomas

PURPOSE. Glaucoma is a complex disease involving multiple genetic factors. Recently, single nucleotide polymorphisms (SNPs) in the LOXL1 gene have been implicated in exfoliation syndrome (XFS) and exfoliation glaucoma (XFG) but not in the primary glaucomas. This study was conducted to determine the possible involvement of these SNPs in cases of primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG).

METHODS. The three associated SNPs of LOXL1 (rs1048661, rs3825942, and rs2165241) were screened in 208 unrelated and clinically well-characterized glaucoma cases comprising patients with POAG (n = 112) or PACG (n = 96) along with 105 ethnically matched normal control subjects from Indian populations. Subjects with exfoliative material on the lens and radial pigmentation in the periphery of the lens that could be earlier signs of XFS were excluded. These SNPs were screened by resequencing and further confirmed by PCR-based restriction digestion. Haplotypes were generated with the three SNPs in cases and control subjects, and linkage disequilibrium (LD) and haplotype analysis were performed with the Haploview software, which uses the EM (expectation-maximization) algorithm.

RESULTS. The SNPs of LOXL1 did not exhibit any significant association with POAG or PACG, unlike previous studies from Icelandic, Swedish, U.S., and Australian populations with XFS/XFG. Haplotypes generated with these intragenic SNPs did not indicate any significant risk with POAG or PACG phenotypes. The risk haplotype G-G in XFS/XFG in other populations was present in 46% of the normal control subjects in the present cohort.

CONCLUSIONS. The results from the present study do not indicate the involvement of the LOXL1 SNPs in POAG and PACG. (Invest Ophthalmol Vis Sci. 2008;49:2343–2347) DOI:10.1167/iovs.07-1557

Globally, glaucoma is considered to be the second leading cause of irreversible blindness,1 and it is estimated that it will affect ~80 million people by the year 2020 worldwide.2 It is a group of clinically and genetically heterogeneous optic neuropathies characterized by a gradual and progressive loss of vision.3-5 Gonioscopically, primary glaucomas are classified as primary open-angle glaucoma (POAG; OMIM 137750; Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/Omim/) or high-risk (G-A).23 The significantly strong association of XFS with weak zonules that may cause anterior movement of the lens, contributing to angle closure and glaucoma.12,26 Because glaucoma is a complex disease attributed to multiple gene variants with various magnitudes of effect,27 we wondered whether the LOXL1 SNPs causing XFS...
and XFG may also be associated with primary glaucomas, which may vary between populations. The \textit{LOXL1} SNP (rs2165241) showed a weak association with POAG in the Icelandic population.\textsuperscript{25} However, to the best of our knowledge this has not been studied in the primary glaucomas in other populations. The present study was undertaken to determine the involvement of these XFS and XFG-associated SNPs of \textit{LOXL1} in a cohort of POAG and PACG patients in an ethnically different (Indian) population.

**METHODS**

**Clinical Details of the Subjects**

The study protocol adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board. The cohort comprised unrelated, consecutive patients with POAG (\(n = 112\)) and PACG (\(n = 96\)), and 105 normal control subjects, seen at the L. V. Prasad Eye Institute, Hyderabad, India, between January 2002 and March 2007. The diagnoses of POAG and PACG were independently confirmed by two surgeons based on the following inclusion and exclusion criteria mentioned in our preceding publication.\textsuperscript{28} In addition, we looked for exfoliative material and also for radial pigmentation in the periphery of the lens (that could be an earlier sign of XFS) on a dilated slit lamp examination.

Ocular hypertension, normal-tension glaucoma, lens-induced glaucoma, neovascular and XFG, and secondary-open angle glaucoma were excluded. Other ocular diseases that can lead to secondary glaucoma, neovascular and XFG, and secondary-open angle glaucoma were excluded. Other ocular diseases that can lead to secondary glaucoma were also excluded.

Normal adult individuals without any signs or symptoms of glaucoma and other systemic diseases served as control subjects. Their visual acuity ranged from 20/20 to 20/40, and IOP was measured with a Goldmann applanation tonometer. Peripheral blood samples (5–10 mL) were collected from each subject by standard protocols.\textsuperscript{29} The three \textit{LOXL1} SNPs from exon 1 (rs1048661 and rs3825942) and intron 1 (rs2165241) were amplified with these three predesigned primers (Table 1) in a thermal cycler (model 9700; Applied Biosystems, Inc. [ABI], Foster City, CA) at an annealing temperature of 60°C. The amplicons were purified with spin columns (Sigma-Aldrich, St. Louis, MO) and screened by resequencing (BigDye chemistry, ver. 3.1; model 3100 DNA Analyzer; ABI), according to the manufacturer’s protocol. Sequencing analysis software was used to read the individual sequences. Subsets of the patient and control samples were further confirmed by restriction digestion of the amplicons at 37°C overnight with appropriate restriction enzymes (Table 1) according to the manufacturer’s guidelines (New England Biologicals, Beverly, MA). The digested amplicons were electrophoresed on 8% nondenaturing polyacrylamide gels, along with an undigested amplicon that served as an internal control. The band patterns were read on the gel documentation system (model 9700; Applied Biosystems, Inc., Foster City, CA) at an exposure time of 1 h. The restriction enzymes were used to read the individual sequences.

**Molecular Analysis**

Peripheral blood samples (5–10 mL) were collected from each subject by venipuncture, with prior informed consent, and DNA was extracted by standard protocols.\textsuperscript{29} The three \textit{LOXL1} SNPs from exon 1 (rs1048661 and rs3825942) and intron 1 (rs2165241) were amplified with these three predesigned primers (Table 1) in a thermal cycler (model 9700; Applied Biosystems, Inc. [ABI], Foster City, CA) at an annealing temperature of 60°C. The amplicons were purified with spin columns (Sigma-Aldrich, St. Louis, MO) and screened by resequencing (BigDye chemistry, ver. 3.1; model 3100 DNA Analyzer; ABI), according to the manufacturer’s protocol. Sequencing analysis software was used to read the individual sequences. Subsets of the patient and control samples were further confirmed by restriction digestion of the amplicons at 37°C overnight with appropriate restriction enzymes (Table 1) according to the manufacturer’s guidelines (New England Biologicals, Beverly, MA). The digested amplicons were electrophoresed on 8% nondenaturing polyacrylamide gels, along with an undigested amplicon that served as an internal control. The band patterns were read on the gel documentation system (model 9700; Applied Biosystems, Inc., Foster City, CA) at an exposure time of 1 h. The restriction enzymes were used to read the individual sequences.

**Statistical Analysis**

Haploview software that incorporates the EM (expectation-maximization) algorithm was used to determine the maximum-likelihood estimates of allele frequencies, Hardy-Weinberg equilibrium, and haplotype frequencies from the genotype data at the three SNP loci.\textsuperscript{30} Pair-wise linkage disequilibrium (LD) between the individual SNPs was calculated with the LD plot function of the software. The \(\chi^2\) analysis was used to test the significance between the minor and major genotype frequencies. The odds ratios were calculated to assess the risk of the individual alleles and genotypes of the three SNPs.

**RESULTS**

**Distribution of the \textit{LOXL1} SNPs in POAG and PACG**

The study cohort conformed to Hardy-Weinberg equilibrium. The distributions of the allele frequencies for the three SNPs and their corresponding odds ratios are provided in Table 2. As is evident from the table, the frequencies of the XFS/XFG-associated SNPs across POAG and PACG cases were different (Indian) population.

**TABLE 1. Details of the Primers Sequences and Restriction Enzymes Used to Screen the Three \textit{LOXL1} SNPs**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>SNPs Screened</th>
<th>Primer Sequences</th>
<th>Fragment Size (bp)</th>
<th>Restriction Enzymes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOXL1_1</td>
<td>rs1048661</td>
<td>GCGAGCTGTTACAGCTGGTCTCA</td>
<td>464</td>
<td>Smal (–)</td>
</tr>
<tr>
<td>LOXL1_1</td>
<td>rs3825942</td>
<td>GCGAGCTGTTACAGCTGGTCTCA</td>
<td>464</td>
<td>HinI (+)</td>
</tr>
<tr>
<td>LOXL1_2</td>
<td>rs2165241</td>
<td>TAGGGCCCGTGGAGAATAG</td>
<td>264</td>
<td>SspI (+)</td>
</tr>
</tbody>
</table>

* (–) indicates abolition and (+) indicates the creation of restriction sites for the respective variants.

**TABLE 2. Distribution of Allele Frequencies and Their Odds Ratios for the Three \textit{LOXL1} SNPs across POAG and PACG Cases in the Present Cohort and POAG Cases in Other Populations**

<table>
<thead>
<tr>
<th>Populations (Phenotype)</th>
<th>\textit{rs1048661 (G)}</th>
<th>\textit{rs3825942 (G)}</th>
<th>\textit{rs2165241 (T)}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freq.</td>
<td>OR (95% CI)</td>
<td>(P)</td>
</tr>
<tr>
<td>Iceland (POAG) [(n = 90)]\textsuperscript{25}</td>
<td>0.711</td>
<td>1.32 (0.96-1.82)</td>
<td>0.085</td>
</tr>
<tr>
<td>Sweden (POAG) [(n = 200)]\textsuperscript{25}</td>
<td>0.638</td>
<td>0.82 (0.61-1.10)</td>
<td>0.19</td>
</tr>
<tr>
<td>Present Study (POAG) [(n = 112)]</td>
<td>0.616</td>
<td>0.70 (0.40-1.24)</td>
<td>0.112</td>
</tr>
<tr>
<td>Present Study (PACG) [(n = 96)]</td>
<td>0.667</td>
<td>0.88 (0.49-1.59)</td>
<td>0.332</td>
</tr>
</tbody>
</table>
associated alleles were not significantly different between the POAG and PACG cohorts and control subjects. The genotype frequencies of these alleles also did not exhibit any significant difference across the three LOXL1 SNPs in the POAG and PACG cohorts (Table 3).

**LD and Haplotype Analysis at the LOXL1 Locus**

The three intragenic SNPs were typed at the LOXL1 locus to generate haplotypes among the cases and control subjects. Pair-wise LD analysis indicated a strong LD (i.e., D' = 1, rs1048661 and rs3825942, and D' = 0.93, between rs3825942 and rs2165241; data not shown).

Four different haplotypes (with frequency >5%) were generated with these three SNPs among POAG and PACG cases and control subjects. There were no significant differences in the haplotype frequencies between the POAG and PACG cases compared with those in the control subjects (Table 4). The results were consistent even after reanalysis of the haplotype data with the two XFS/XFG-associated LOXL1 SNPs (rs1048661 and rs3825942).

**DISCUSSION**

The exfoliation syndrome is an age-related condition characterized clinically by the progressive deposition of fibrillar material throughout the anterior segment. Glaucoma occurs more commonly in eyes associated with XFS; such patients are also predisposed to PACG. The LOXL1, which belongs to the family of lysyl oxidase proteins, performs multiple functions in different tissues and is involved in a variety of disorders. It has been suggested that the formation of the extracellular matrix (ECM) of the eye is based on the expression of LOXL1 in the ocular tissues that may be involved in the ECM formation. It has been speculated that the chronic accumulation of the abnormal fibrillar material in the trabecular meshwork can lead to an increase in IOP that would eventually predispose to glaucoma. Recently, the expression of LOXL1 in the anterior segment of the eye has been convincingly demonstrated. However, the proposed functions of the two conserved nonsynonymous coding variants rs1048661 (R141L) and rs3825942 (G153D) in XFS based on their reduced expressions in the adipose tissue is still very speculative.

In summary, we tried to determine the involvement of the XFS/XFG-associated LOXL1 SNPs, in glaucoma pathogenesis based on possible commonalities in the pathophysiology. Morphometric and ultrastructural evidence suggest that the deposition of the exfoliation material in the juxtacanalicular area may lead to the development of glaucoma. Several in vitro studies have demonstrated the differential expression of various genes at different stages of development in the anterior segment of the eye. Although the significant involvement of the LOXL1 SNPs with XFS and XFG knockout mice have shown abnormalities in other tissues, but their role in ocular tissues leading to disease pathogenesis is yet to be determined.

To the best of our knowledge, other than the Icelandic and Swedish study, this is the first report to screen for the LOXL1 SNPs in POAG; we also screened for their involvement in PACG. The data from the present study indicated that the three XFS/XFG-associated SNPs were not involved with POAG or PACG. Whereas there was a very mild association of the intronic SNP (rs2165241) with POAG (P = 0.04) in the homogeneous Icelandic population, the association was not observed in the relatively heterogeneous POAG (P = 0.426) and PACG (P = 0.262) populations from India. Overall, the results obtained in the present study were similar to those observed among the patients with POAG from Iceland and Sweden (Table 2). Neither the LOXL1 genotype (Table 3) nor haplotype (Table 4) frequencies exhibited any significant association to POAG or PACG.

The risk haplotype with the rs1048661 and rs3825942 SNPs (G-G) in XFS in other studies was observed in equal frequencies among POAG, PACG, and control subjects in the present study (Table 4). But unlike previous studies, the proportion of T-G haplotype was higher among POAG and PACG cases than among the control subjects (Table 5). It was shown that relative to the low risk G-A haplotype, the G-G and T-G haplotypes conferred substantial risk in XFS and XFG, but the same was not observed in the present cohort (Table 5). Intriguingly, the haplotype supposed to have the lowest risk (T-A) was not observed in the present cohort, similar to all the previous studies. It was also noted that the risk haplotype G-G had a very high frequency in the normal population (~46%) similar to that observed in the general population elsewhere (Table 5).

In summary, we tried to determine the involvement of the XFS/XFG-associated LOXL1 SNPs, in glaucoma pathogenesis based on possible commonalities in the pathophysiology. Recently, the expression of LOXL1 in the anterior segment of the eye has been convincingly demonstrated. The LOXL1 gene is known to be involved in the formation of the extracellular matrix (ECM) and is expressed in various tissues. It has been suggested that the chronic accumulation of the abnormal fibrillar material in the trabecular meshwork can lead to an increase in IOP that would eventually predispose to glaucoma.

### Table 3. Distribution of Genotype Frequencies and Their Odds Ratios for the Three LOXL1 SNPs in POAG and PACG

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Genotypes</th>
<th>POAG (n = 112)</th>
<th>PACG (n = 96)</th>
<th>Controls (n = 105)</th>
<th>OR (95% CI) [POAG vs. Controls]</th>
<th>OR (95% CI) [PACG vs. Controls]</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1048661</td>
<td>GG</td>
<td>0.37</td>
<td>0.40</td>
<td>0.49</td>
<td>0.56 (0.23–1.40)</td>
<td>0.106</td>
<td>1.09 (0.36–3.27)</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>0.50</td>
<td>0.52</td>
<td>0.41</td>
<td>0.93 (0.38–2.30)</td>
<td>0.437</td>
<td>1.70 (0.57–5.10)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>0.13</td>
<td>0.08</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3825942</td>
<td>GG</td>
<td>0.71</td>
<td>0.61</td>
<td>0.60</td>
<td>2.12 (0.73–6.14)</td>
<td>0.080</td>
<td>1.00 (0.38–2.65)</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>0.23</td>
<td>0.30</td>
<td>0.30</td>
<td>1.35 (0.43–4.22)</td>
<td>0.300</td>
<td>0.97 (0.35–2.73)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>0.06</td>
<td>0.09</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2165241</td>
<td>CC</td>
<td>0.47</td>
<td>0.49</td>
<td>0.44</td>
<td>1.25 (0.49–3.20)</td>
<td>0.318</td>
<td>0.83 (0.28–2.43)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>0.41</td>
<td>0.42</td>
<td>0.48</td>
<td>0.80 (0.45–1.40)</td>
<td>0.216</td>
<td>0.79 (0.44–1.45)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>0.12</td>
<td>0.09</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 4. Estimated LOXL1 Haplotype Frequencies across POAG and PACG Cohorts

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>%POAG</th>
<th>%Controls</th>
<th>P</th>
<th>%PACG</th>
<th>%Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-G-C</td>
<td>37.9</td>
<td>29.8</td>
<td>0.079</td>
<td>30.8</td>
<td>29.5</td>
<td>0.781</td>
</tr>
<tr>
<td>G-G-T</td>
<td>32.1</td>
<td>31.9</td>
<td>0.965</td>
<td>25.7</td>
<td>31.8</td>
<td>0.183</td>
</tr>
<tr>
<td>G-A-C</td>
<td>16.4</td>
<td>22.3</td>
<td>0.123</td>
<td>23.0</td>
<td>22.4</td>
<td>0.886</td>
</tr>
<tr>
<td>G-G-C</td>
<td>13.0</td>
<td>14.3</td>
<td>0.707</td>
<td>16.8</td>
<td>14.2</td>
<td>0.475</td>
</tr>
</tbody>
</table>
Iowan (88%) and Icelandic (99%) cohorts strongly suggest that these variants are pathogeneses of which remain to be elucidated. Field with XFG, they are indeed more complex disorders, the SNPs in our cohort supports this notion. Although LOXL1 functions are yet uncharacterized, the population-attributable risks for the high-risk haplotype in the Nordic (99%) and Iowan (88%) cohorts strongly suggest that these variants are exclusive of XFG and XFG. The lack of association with LOXL1 SNPs in our cohort supports this notion. Although POAG and PACG share clinical features in the disc and visual field with XFG, they are indeed more complex disorders, the pathogeneses of which remain to be elucidated.

Acknowledgments

The authors thank all the patients and normal volunteers for participating in the study and especially Sreelatha Komatireddy and Koilkonda R. Devi for collecting the POAG and PACG samples for haplotyping.

References