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

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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 digestions. 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.[2,3] 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/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), which is more common in the Western world[2,4,5] and primary angle closure glaucoma (PACG), which is more common among the Asian populations.[6,7] Both these conditions are characterized by optic nerve head changes, degeneration of retinal ganglion cells, and visual field loss[2,3] and may be associated with an elevated intraocular pressure (IOP).[8]

Exfoliation syndrome (XFS; OMIM 177650) is an age-related systemic condition with clinically detectable accumulation of microfibrillar deposits in the lens and anterior segment.[9,10] The deposition of such material in the trabecular meshwork leads to secondary glaucoma.[11,12] The prevalence of XFS increases with age, and it may be associated with other vascular conditions.[9,13,14] The overall prevalence of XFS in population-based studies has been reported from south Indian populations, which varies from 3.0% to 6.0% among subjects over 40 years of age.[15–17]

POAG exhibits extensive genetic heterogeneity, and 11 chromosomal loci (*GLC1A-GLC1K*) have been mapped[18,19] and three genes: myocilin (*MYOC*; OMIM 601652),[20] optineurin (*OPTN*; OMIM 602432),[21] and *WDR36* (OMIM 609669)[22] have been characterized. Approximately 15 candidate genes have been identified based on case–control association studies but most of these have not been replicated in other populations.[18]

Recently, it has been shown by genome-wide association studies that single nucleotide polymorphisms (SNPs) in the *LOXL1* gene (OMIM 153456) at 15q24.1 are involved in XFS and XFG.[23] An extensive screening using a gene microarray (Hap300 Beadchip; Illumina, San Diego, CA) showed that two nonsynonymous SNPs in exon I of *LOXL1* (rs1048661 [R141L] and rs3825942 [G135D]) exhibited a strong association with XFS and XFG in two different populations. The initial study conducted on the Icelandic population was later replicated in a Swedish population. Jointly, the two SNPs in exon 1 accounted for >99% of all cases of XFG. It was also shown that an individual with the homozygous risk haplotype (G-G) had a 700 times greater chance of having XFG than those with the low-risk haplotype (G-A).[23] The significantly strong association of the coding SNPs (rs1048661 and rs3825942) has been independently replicated in two diverse cohorts of Caucasian XFS patients from Australia[24] and the Midwestern United States.[25]

XFS has been identified as the most common cause of open-angle glaucoma.[11] There is also a biological explanation for the 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

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

Primer Name	SNPs Screened	Primer Sequences		Fragment Size (bp)	Restriction Enzymes*
		Sense Strand (5′–3′)	Antisense Strand (5′–3′)		
LOXL1_1	rs1048661	GCAGGTGTACAGCTTGCTCA	ACACGAAACCCTGGTCGTAG	464	*Sma*I (–)
LOXL1_1	rs3825942	GCAGGTGTACAGCTTGCTCA	ACACGAAACCCTGGTCGTAG	464	*Hin*fI (+)
LOXL1_2	rs2165241	TAGGGCCCCTTGGAGAATAG	GTCCCATTCCCCTCTCAATC	264	*SSP*I (+)

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

and XFG may also be associated with primary glaucomas, which may vary between populations. The *LOXL1* SNP (rs2165241) showed a weak association with POAG in the Icelandic population.[23] 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 *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.[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 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 <21 mm Hg. Clinical examination on stereo biomicroscopy did not reveal any changes in the optic disc suggestive of glaucoma. This diagnosis was essentially one of exclusion: normal pattern of neuroretinal rim and absence of notching or thinning of the rim, disc hemorrhage, or nerve fiber layer defects. Cup-to-disc ratio suitable for the disc size, asymmetry of cup-to-disc ratio ≤ 0.2:1 (corrected for size) and absence of beta zone peripapillary atrophy were "soft" signs. The patients and control subjects were matched in ethnicity and geographical region of habitat.

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.[29] The three *LOXL1* SNPs from exon I (rs1048661 and rs3825942) and intron I (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 based on the abolition or creation of restriction sites for the three variants (Table 1) were generated, and sizes of the corresponding fragments were visualized with the help of a 100-bp DNA ladder (Fermentas, Hanover, MD). The genotypes were directly scored from the gels and correlated with the sequencing data. Each experiment was repeated independently by two investigators who were masked to the phenotypes.

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.[30] Pair-wise linkage disequilibrium (LD) between the individual SNPs was calculated with the LD plot function of the software. The χ^2 analysis was used to assess the test of significance between the allele and 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 *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-

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

Populations (Phenotype) [n]	rs1048661 (G)			rs3825942 (G)			rs2165241 (T)		
	Freq.	OR (95% CI)	P	Freq.	OR (95% CI)	P	Freq.	OR (95% CI)	P
Iceland (POAG) [n = 90][23]	0.711	1.32 (0.96–1.82)	0.085	0.872	1.25 (0.81–1.91)	0.32	0.550	1.36 (1.01–1.83)	0.04
Sweden (POAG) [n = 200][23]	0.638	0.82 (0.61–1.10)	0.19	0.863	0.87 (0.57–1.31)	0.49	0.488	0.83 (0.63–1.09)	0.18
Present Study (POAG) [n = 112]	0.616	0.70 (0.40–1.24)	0.112	0.830	1.53 (0.78–2.98)	0.105	0.321	0.95 (0.54–1.67)	0.426
Present Study (PACG) [n = 96]	0.667	0.88 (0.49–1.59)	0.332	0.755	0.94 (0.49–1.79)	0.456	0.296	0.82 (0.45–1.50)	0.262

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

SNPs	Genotypes	POAG (n = 112)	PACG (n = 96)	Controls (n = 105)	OR (95% CI) [POAG vs. Controls]	P	OR (95% CI) [PACG vs. Controls]	P
rs1048661	GG	0.37	0.40	0.49	0.56 (0.23–1.40)	0.106	1.09 (0.36–3.27)	0.439
rs1048661	GT	0.50	0.52	0.41	0.93 (0.38–2.30)	0.437	1.70 (0.57–5.10)	0.168
rs1048661	TT	0.13	0.08	0.10				
rs3825942	GG	0.71	0.61	0.60	2.12 (0.73–6.14)	0.080	1.00 (0.38–2.65)	0.496
rs3825942	GA	0.23	0.30	0.30	1.35 (0.43–4.22)	0.300	0.97 (0.35–2.73)	0.478
rs3825942	AA	0.06	0.09	0.10				
rs2165241	CC	0.47	0.49	0.44	1.25 (0.49–3.20)	0.318	0.83 (0.28–2.43)	0.368
rs2165241	CT	0.41	0.42	0.48	0.80 (0.45–1.40)	0.216	0.79 (0.44–1.43)	0.193
rs2165241	TT	0.12	0.09	0.08				

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., $D' = 1$, between 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.[31] Glaucoma occurs more commonly in eyes associated with XFS; such patients are also predisposed to PACG.[9]

The LOXL1, which belongs to the family of lysyl oxidase proteins, performs multiple functions in different tissues[23,32] and is involved in a variety of disorders.[33–35] 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.[36–38] 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.[39] Recently, the expression of LOXL1 in the anterior segment of the eye has been convincingly demonstrated.[24] 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.[23] Similarly, LOXL1 knockout mice have shown abnormalities in other tissues,[40] 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,[23] 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,[23] 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[23–25] was observed in equal frequencies among POAG, PACG, and control subjects in the present study (Table 4). But unlike previous studies,[23–25] 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,[23–25] 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.[23–25] 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 pathophysiologies.[9,11,12,26] Morphometric and ultrastructural evidence suggest that the deposition of the exfoliation material in the juxtacanalicular area may lead to the development of glaucoma.[41] Several in vitro studies have demonstrated the differential expression of various genes at different stages of development in the anterior segment of the eye.[42,43] Although the significant involvement of the *LOXL1* SNPs with XFS and XFG

TABLE 4. Estimated *LOXL1* Haplotype Frequencies across POAG and PACG Cohorts

Haplotypes	%POAG	%Controls	P	%PACG	%Controls	P
T-G-C	37.9	29.8	0.079	30.8	29.5	0.781
G-G-T	32.1	31.9	0.963	25.7	31.8	0.183
G-A-C	16.4	22.3	0.123	23.0	22.4	0.886
G-G-C	13.0	14.3	0.707	16.8	14.2	0.475

TABLE 5. Distribution of Estimated Haplotype Frequencies and Their Odds Ratios for the Two SNPs (rs1048661 and rs3825942) across POAG and PACG in the Present Cohort and Other Populations

Populations, [*n* (Cases, Controls)]	Phenotype	G-G Haplotype				T-G Haplotype			
		%Cases	%Controls	OR (95% CI)*	*P*	%Cases	%Controls	OR (95% CI)*	*P*
Sweden [399, 198][23]	XFG	83.3	56.1	35.72†	2.2×10^{-16}	16.2	31.8	12.36†	1.6×10^{-6}
Iceland [195, 14474][23]	XFG	81.4	49.8	18.94†	3.3×10^{-12}	17.3	34.9	5.74†	0.0027
Iowa (USA) [72, 75][25]	XFS	80.6	48.0	14.50†	2.7×10^{-5}	18.1	40.0	3.90†	0.12
Australia [86, 2422][24]	XFS	74.0	51.0	2.71 (1.91–3.92)	3.8×10^{-9}	22.0	34.0	0.54 (0.36–0.78)	7.8×10^{-4}
India [112, 105] (Present study)	POAG	45.2	46.1	1.45 (0.70–2.98)	0.158	37.8	29.9	1.88 (0.87–4.03)	0.052
India [96, 105] (Present study)	PACG	42.1	46.0	0.87 (0.43–1.75)	0.346	33.4	30.0	1.07 (0.51–2.27)	0.424

* The odds ratios were calculated with respect to the G-A haplotype.
† 95% CIs were not reported in the studies.

highlights their potential role in the disease pathogenesis, their functions are yet uncharacterized. The population-attributable risks for the high-risk haplotype in the Nordic (99%)[23] and Iowan (88%)[25] cohorts strongly suggest that these variants are exclusive of XFS and XFG.[23–25] 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.[23]

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