LOXL1 Promoter Haplotypes Are Associated with Exfoliation Syndrome in a U.S. Caucasian Population

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Purpose. *LOXL1* is a major genetic risk factor for exfoliation syndrome (ES) and exfoliation glaucoma (EG). Recent evidence documenting reversal of risk alleles for the disease-associated missense variants R141L and G153D suggests that these variants are not causative and that they may be proxies for other unknown functional *LOXL1* variants. The purpose of this study was to investigate the disease association of *LOXL1* variants spanning the gene region, including the 5′ and 3′ regulatory regions, in a U.S. Caucasian case–control sample.

Methods. Twenty-five *LOXL1* single-nucleotide polymorphisms (SNPs), distributed throughout the gene, were genotyped in 196 Caucasian patients with ES/EG and 201 matched controls. Genotype data were analyzed for single SNP associations, SNP interactions, and haplotype associations.

Results. Promoter region haplotypes that included the risk alleles for rs12914489, a SNP located in the distal promoter region and independently associated with ES, and rs16958477, a SNP previously shown to affect gene transcription, were associated with increased disease risk ($P = 0.0008$; odds ratio [OR], 2.34; 95% confidence interval [CI], 1.42–3.85) and with protective effects ($P = 2.3 \times 10^{-6}$; OR, 0.38; 95% CI, 0.25–0.57). Haplotypes containing rs12914489 and rs16958477 risk and protective alleles also significantly influenced the disease risk associated with missense alleles R141L and G153D.

Conclusions. *LOXL1* promoter haplotypes were identified that are significantly associated with ES/EG in a U.S. Caucasian population. These results suggest that promoter region SNPs can influence *LOXL1* gene expression, potentially causing a reduction of enzyme activity that may predispose to disease. (*Invest Ophthalmol Vis Sci.* 2011;52:2372–2378) DOI: 10.1167/iovs.10-6268

Glaucoma is a term used to describe a group of disorders that have in common a distinct type of optic nerve damage causing irreversible defects in the visual field. Collectively, glaucoma is a leading cause of blindness throughout the world and is estimated to affect more than 60 million people by 2010.[1] Exfoliation syndrome (ES), characterized by the deposition of microfibrillar material throughout the anterior segment of the eye, is a significant risk factor for glaucoma, with intraocular pressure and optic nerve degeneration developing in more than 50% of affected individuals by the age of 70.[2]

The genetics of ES and the related exfoliation glaucoma (EG) are complex. A genome-wide association study in cases and controls from Iceland initially identified lysyl oxidase-like 1 (*LOXL1*), an enzyme necessary for elastogenesis and elastin homeostasis, as a major genetic risk factor for the condition, with a population-attributable risk of more than 99%.[3] Subsequently, this association has been replicated in populations throughout the world.[4–22] Recent studies suggest that loss of LOXL1 function or reduced *LOXL1* expression is the genetic mechanism that contributes to the disease (Wiggs JL, et al., manuscript submitted).[23] *LOXL1* risk alleles are also very prevalent in control individuals arguing that *LOXL1* is necessary but not sufficient for disease and that other factors, both genetic and environmental, may contribute to disease development.

Missense changes in exon 1 (rs1048661, R141L, and rs3825942, G153D) are significantly associated with exfoliation syndrome in populations worldwide; however, the frequency of the risk allele for both variants varies among different ethnic populations (Fig. 1). In Caucasians the arginine allele at position 141 is associated with disease risk, while in the Japanese, leucine is the risk allele.[15–20] Recent studies of cases of exfoliation in South African blacks show that the aspartate at position 153 is the risk allele, whereas in all other populations studied, glycine is associated with exfoliation.[22] These findings suggest that these missense changes are not biologically causative and that other *LOXL1* variants may predispose to exfoliation syndrome by affecting gene expression or protein function. The purpose of this study was to investigate the disease association of other *LOXL1* gene variants by evaluating polymorphisms across the whole gene region of *LOXL1* and the corresponding promoter region in a U.S. Caucasian case–control sample.

Methods

Patients and Control Subjects

One hundred ninety-six unrelated patients with ES were recruited from the Glaucoma Consultation Service at the Massachusetts Eye and Ear Infirmary. Patients with ES were identified by the presence of the characteristic fibrillar material on the lens capsule or pupillary margin. Patients with iris transillumination defects without the presence of the fibrillar material were not identified as cases or controls. Of the 196 patients with ES, 145 had glaucoma and 51 did not. Glaucoma was defined as evidence of optic nerve damage in

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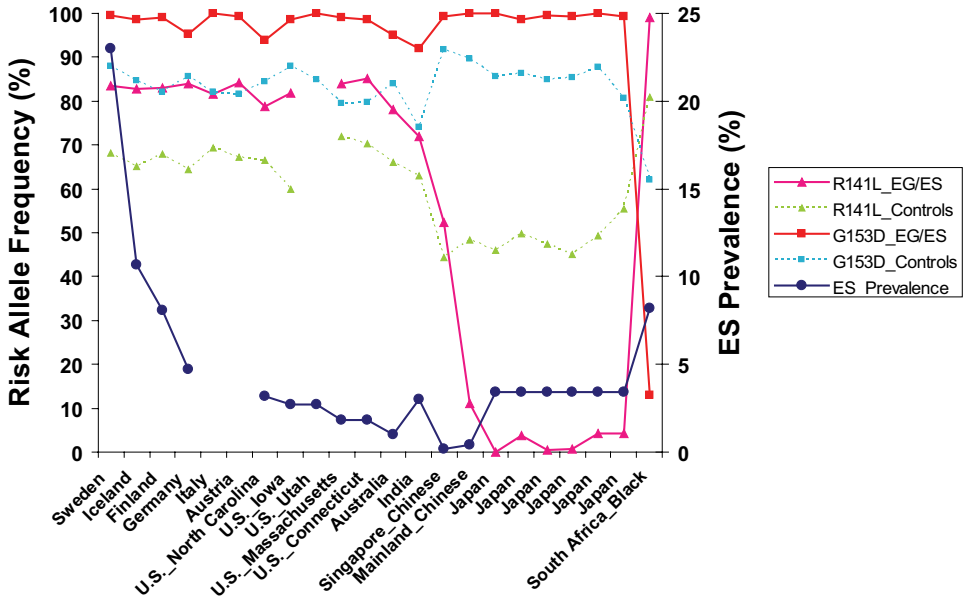

FIGURE 1. Prevalence of ES and risk allele frequencies of *LOXL1* coding variants (R141L and G153D; risk allele G) in EG or ES and controls across different populations .[4–34]

both eyes, visual field defects consistent with optic nerve damage and characteristic of glaucoma in at least one eye, intraocular pressure >22 mm Hg in both eyes on two occasions, or intraocular pressure >19 mm Hg in both eyes on treatment with two or more glaucoma medications. For the analyses reported in this study, all ES patients (with or without glaucoma) were included in the case

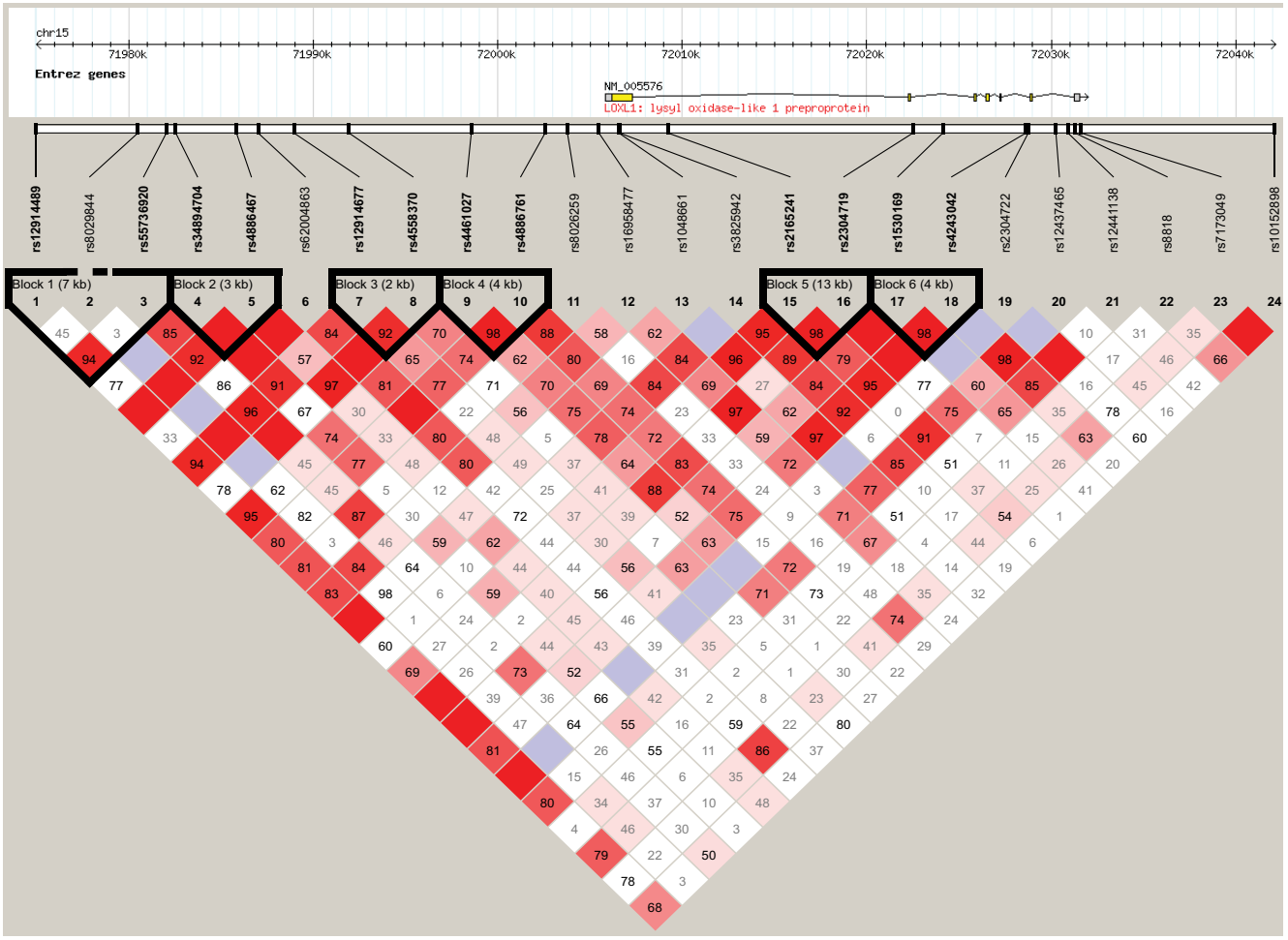

FIGURE 2. *LOXL1* linkage disequilibrium (LD) plot. LD plot of 24 SNPs in *LOXL1*. The numbers in the diamonds refer to D′. The LD block was defined according to the standard confidence intervals.

group. Two hundred one unrelated control subjects were recruited from the Comprehensive Ophthalmology Service at the Massachusetts Eye and Ear Infirmary. Control subjects had no evidence of exfoliation or glaucoma after clinical examination. The average age of the ES patients was 75. Because of the age-dependence of the ES, only controls older than age 60 were used for this study with an average age of 71. This study population (cases and controls) included only Caucasian patients of European ancestry. Of the patients with ES, 61% were women and 39% were men, and of the control subjects, 50% were women and 50% were men. This study adhered to the Tenets of the Declaration of Helsinki and has been reviewed and approved by the Institutional Review Board of the Massachusetts Eye and Ear Infirmary. Informed consent was obtained from all patients and controls.

Polymorphisms and Genotyping

A total of 25 SNPs (16 tag SNPs, 6 promoter region SNPs and the 3 SNPs initially associated with the condition in the Icelandic population: rs1048661, rs3825942, and rs2165241) distributed throughout the *LOXL1* gene were selected to capture 100% of alleles at r^2 greater than 0.8, including all exons, introns, 5′UTR and 3′UTR, and the proximal promoter region (Fig. 2). Tag SNPs were selected according to the HapMap CEU data (release 22) with Haploview (version 4.1).[35] The minimum minor allele frequency for checking markers was set to 0.01. Genotyping was performed by real time PCR assays (TaqMan assays; Applied Biosystems, Inc. [ABI], Foster City, CA). Oligonucleotide primers were ordered from ABI (Assay by Demand) and performed according to the manufacturer's instructions. The genotypes of rs1048661, rs3825942, rs8818, and rs3522 were further confirmed by direct sequencing. Products from PCR amplification were purified and sequenced by using dye termination chemistry (BigDye; ABI) and an automated genetic analyzer (model 3100; ABI). Sequence data were then analyzed (Vector NTI Suite, ver. 8; Invitrogen, Carlsbad, CA).

Statistical Analysis

Association analysis was performed with PLINK (version 1.07).[36] Hardy-Weinberg equilibrium was assessed by χ^2 test. The linkage disequilibrium (LD) plot was generated with Haploview (ver. 4.1).[34] Individual SNP associations were analyzed with the χ^2 test. To determine which SNPs had an independent effect on ES, we evaluated the association of each SNP by logistic regression after adjustment for age and all other SNPs. The association of each *LOXL1* SNP with ES was further evaluated by logistic regression after adjustment for age, rs1048661 (R141L) and rs3825942 (G153D). An additive effects model was applied to analysis of allele dosage in which the genotypes AA, AB, and BB were coded as 0, 1, and 2, respectively, where A represents the minor allele and B represents the common allele. The interaction effect between rs12914489, rs16958477 and G153D was analyzed by including an interaction term in the logistic regression model. Haplotype frequencies were estimated by using the standard E–M algorithm and tested with the χ^2 test. The omnibus *P* value was obtained from the omnibus test. Specific *P* values were obtained from the haplotype-specific test. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for each of individual haplotypes compared to all the other haplotypes. Conditional haplotype analysis was performed for rs12914489 and rs16958477 to evaluate whether there was a significant independent effect of these SNPs on ES after adjustment for age and the missense variants R141L and G153D. Multiple comparisons were corrected by the Bonferroni method.

Table 1. Association of 24 SNPs of *LOXL1* in Caucasian Patients with ES and Control Subjects

SNP	Chromosomal Position*	Minor Allele	Minor Allele Frequency						
			ES	EG	Controls	*P*†	$P_{adjusted}$‡	$P_{adjusted}$§	OR (95% CI)
rs12914489	**71974990**	**A**	**0.147**	**0.145**	**0.071**	**0.0006**	**0.017**	**0.04**	**2.27 (1.41–3.66)**
rs8029844	71980525	T	0.031	0.035	0.028	0.76			1.14 (0.50–2.62)
rs55736920	71982067	T	0.173	0.167	0.136	0.15			1.33 (0.90–1.97)
rs34894704	71982575	A	0.133	0.130	0.202	0.0097			0.61 (0.41–0.89)
rs4886467	**71985866**	**T**	**0.415**	**0.404**	**0.535**	**0.0008**	**0.22**	**0.96**	**0.62 (0.46–0.82)**
rs62004863	71987041	C	0.111	0.120	0.068	0.034			1.71 (1.04–2.83)
rs12914677	71988982	G	0.368	0.367	0.460	0.009			0.68 (0.51–0.91)
rs4558370	**71991963**	**T**	**0.080**	**0.074**	**0.162**	**0.0005**	**0.87**	**0.34**	**0.45 (0.28–0.71)**
rs4461027	**71998601**	**C**	**0.604**	**0.596**	**0.426**	**8.2×10^{-7}**	**0.17**	**0.72**	**2.04 (1.54–2.78)**
rs4886761	**72002604**	**T**	**0.521**	**0.511**	**0.391**	**0.0002**	**0.25**	**0.72**	**1.70 (1.28–2.25)**
rs8026259	72003812	C	0.247	0.269	0.196	0.082			1.35 (0.96–1.89)
rs16958477	**72005519**	**A**	**0.580**	**0.573**	**0.403**	**6.4×10^{-7}**	**0.60**	**0.47**	**2.05 (1.54–2.72)**
rs1048661‖	**72006599**	**T**	**0.160**	**0.153**	**0.271**	**0.0003**	**0.99**	**N/A**	**0.51 (0.35–0.74)**
rs3825942‖	**72006635**	**A**	**0.010**	**0.007**	**0.189**	**5.6×10^{-17}**	**0.018**	**N/A**	**0.04 (0.02–0.12)**
rs2165241‖	**72009255**	**T**	**0.785**	**0.783**	**0.469**	**9.4×10^{-20}**	**0.25**	**0.05**	**4.17 (3.03–5.56)**
rs2304719	**72022553**	**T**	**0.065**	**0.071**	**0.290**	**4.2×10^{-16}**	**0.43**	**0.31**	**0.17 (0.11–0.27)**
rs1530169	72024173	T	0.180	0.158	0.237	0.051			0.71 (0.50–1.00)
rs4243042	**72028677**	**T**	**0.253**	**0.241**	**0.538**	**6.2×10^{-16}**	**0.94**	**0.13**	**0.29 (0.21–0.39)**
rs2304722	72028802	C	0.008	0.007	0.003	0.30			3.11 (0.32–30.04)
rs12437465	**72030299**	**T**	**0.174**	**0.172**	**0.437**	**1.3×10^{-15}**	**0.83**	**0.32**	**0.27 (0.20–0.38)**
rs12441138	72030950	A	0.039	0.032	0.043	0.81			0.92 (0.45–1.86)
rs8818	72031331	C	0.171	0.167	0.236	0.026			0.67 (0.47–0.95)
rs7173049	**72031663**	**G**	**0.130**	**0.123**	**0.231**	**0.0002**	**0.80**	**0.65**	**0.50 (0.34–0.72)**
rs10152898	72042174	T	0.206	0.200	0.135	0.0099			1.66 (1.13–2.43)

* Base pair position in NCBI build 36.3 (National Center for Biotechnology Information, Bethesda, MD).

† χ^2 test. The Bonferroni corrected significance level was 0.0021 (0.05/24). Significant SNPs are highlighted in bold.

‡ Logistic regression after adjustment for age and all the other 12 significant SNPs. The Bonferroni corrected significance level was 0.0038 (0.05/13).

§ Logistic regression after adjusting for age, rs1048661 and rs3825942. The Bonferroni-corrected significance level was 0.0038 (0.05/13). N/A, not applicable.

‖ Significant association with ES has been reported in our previous study of a subgroup of samples.[4]

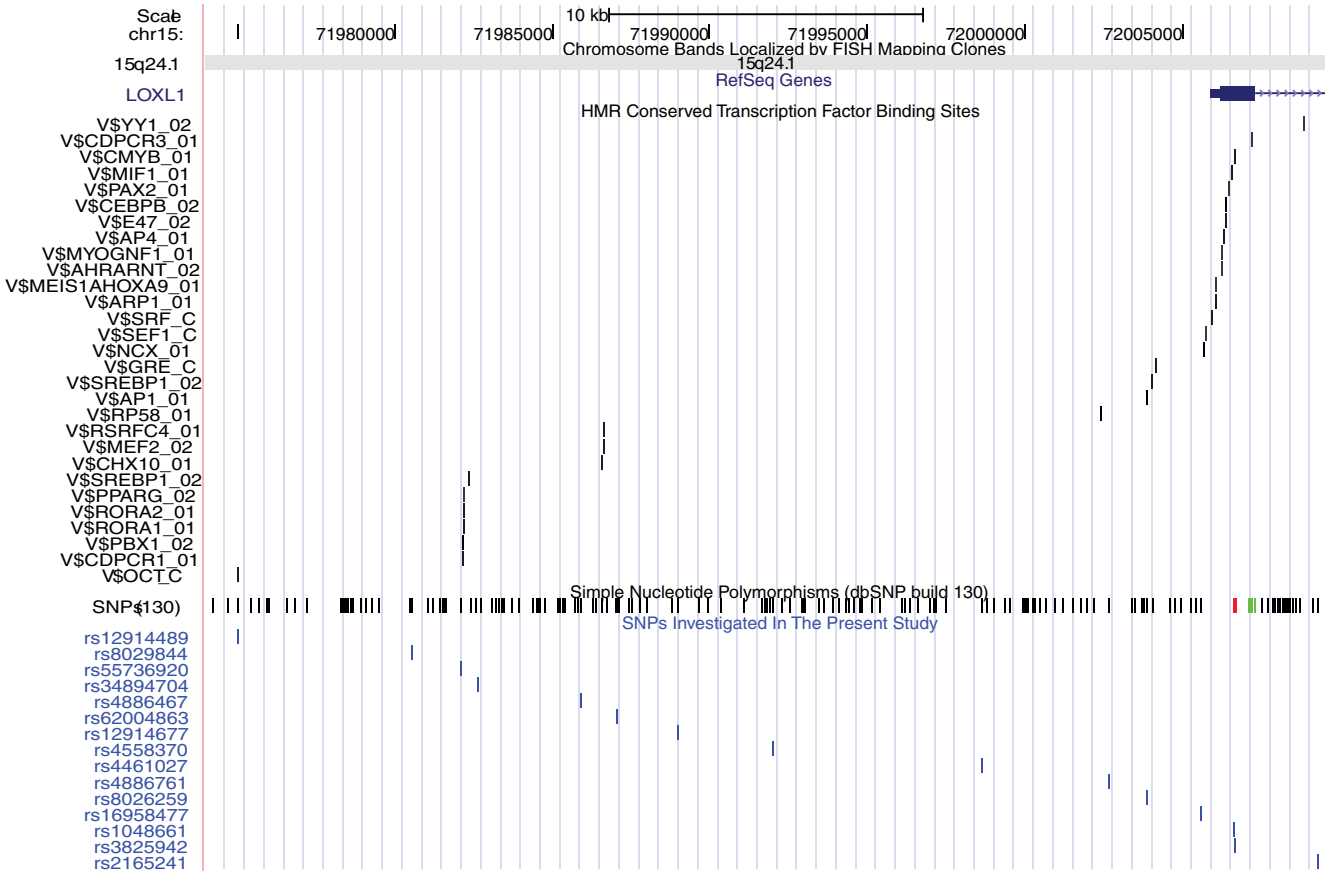

Figure 3. Transcription factor binding sites in the *LOXL1* promoter region and the SNPs investigated in the present study.

Results

Hardy-Weinberg Equilibrium

Except for SNP rs3522, the observed genotypes of all the other SNPs did not significantly deviate from Hardy-Weinberg equilibrium in both the ES patients and the controls ($P > 0.01$). The SNP rs3522 significantly deviated from Hardy-Weinberg equilibrium in the controls ($P = 0.00002$) and trended toward deviation in the ES patients ($P = 0.09$). Resequencing of the region did not identify genotyping errors, but suggested that rs3522 could be a pseudo-SNP due to repetitive sequences in this genomic region. Of interest, this SNP also tends to deviate from Hardy-Weinberg equilibrium in the HapMap European Caucasian population ($P = 0.05$). SNP rs3522 was thus excluded from further analysis.

Association between Single SNPs and Exfoliation Syndrome

Among the remaining 24 SNPs included in our analysis, 13 demonstrated a significant association with ES after the Bonferroni correction (nominal $P < 0.0021$), including the 3 SNPs (rs1048661, rs3825942, and rs2165241) initially reported in the Icelandic study,[3] 6 SNPs in the promoter region (rs12914489, rs4886467, rs4558370 rs4461027, rs4886761,

Table 2. Haplotype Analysis of Six Significant SNPs in the Promoter Region of *LOXL1*

	Estimated Haplotype Frequency				
Haplotype*	**ES**	**Controls**	**Combined**	***P*†**	**OR (95% CI)†**
GGGCTA	**0.337**	**0.237**	**0.286**	**0.002**	**1.64 (1.20–2.24)**
GGGTCC	0.172	0.162	0.167	0.69	1.08 (0.74–1.58)
GTGTCC	**0.098**	**0.222**	**0.161**	**2.3×10^{-6}**	**0.38 (0.25–0.57)**
ATGCTA	**0.135**	**0.063**	**0.098**	**0.0008**	**2.34 (1.42–3.85)**
GTTTCC	**0.049**	**0.119**	**0.085**	**0.0005**	**0.39 (0.22–0.68)**
Total	0.791	0.803	0.797	3.7×10^{-9}‡	

* The six SNPs included in the haplotype analysis are rs12914489, rs4886467, rs4558370, rs4461027, rs4886761, and rs16958477. Only haplotypes with a frequency higher than 5% in the combined samples were shown.

† Obtained from the haplotype-specific test using Haploview. The OR was calculated for each of individual haplotypes compared with all the other haplotypes. The Bonferroni-corrected significance level was 0.01 (0.05/5). Significant SNPs are highlighted in bold.

‡ Omnibus test with PLINK.

TABLE 3. Haplotype Analysis of Promoter SNPs rs12914489 and rs16958477 with Missense Variants R141L and G153D

	Estimated Haplotype Frequency				
Haplotype*	**ES**	**Controls**	**Combined**	***P*†**	**OR (95% CI)†**
GAGG	**0.400**	**0.282**	**0.340**	**0.0005**	**1.70 (1.26–2.29)**
GCGG	0.284	0.209	0.246	0.014	1.51 (1.09–2.09)
GCTG	**0.120**	**0.215**	**0.169**	**0.0004**	**0.50 (0.34–0.73)**
AAGG	**0.140**	**0.059**	**0.098**	**0.0001**	**2.56 (1.55–4.24)**
GCGA	**0.010**	**0.163**	**0.088**	**3.9×10^{-14}**	**0.05 (0.02–0.15)**
Total	0.954	0.928	0.941	1.7×10^{-17}‡	

* The four SNPs included in the haplotype analysis are rs12914489, rs16958477, rs1048661 (R141L) and rs3825942 (G153D). Only haplotypes with a frequency over 5% in the combined samples were shown.

† Obtained from the haplotype-specific test using Haploview. The OR was calculated for each of the haplotypes compared with all the other haplotypes. The Bonferroni corrected significance level was 0.01 (0.05/5). Significant SNPs are highlighted in bold.

‡ Obtained from the omnibus test with PLINK.

and rs16958477) and 4 SNPs in the gene region (rs2304719, rs4243042, rs12437465, and rs7173049; Table 1). SNP rs16958477 is located near the transcription start site (Fig. 3), and has been shown to influence transcriptional activity, the C allele being associated with increased transcription.[37] Among the 13 significant SNPs, only two SNPs, rs12914489 and rs3825942 (G153D), showed an independent effect on ES (P = 0.017 and 0.018, respectively) after adjustment for age and all the other SNPs. After further analysis adjustment for age, and the effects of SNPs rs1048661 and rs3825942, only rs12914489, located in the distal promoter region, remained significantly associated with ES (P = 0.04; Table 1). However, this adjusted P value did not reach the significance level after Bonferroni correction ($P_{adjusted} > 0.0038$).

Association between Promoter Haplotypes and ES

Since an SNP in the promoter region had evidence suggestive of independent association with ES, we investigated further the potential association of promoter region SNPs by analyzing the haplotypes constructed from six SNPs distributed throughout the promoter region with significant associations after the initial analyses: rs12914489, rs4886467, rs4558370, rs4461027, rs4886761, and rs16958477 (Table 2). These promoter region SNPs were chosen because of their proximity to transcription factor binding sites (Fig. 3). Of the five haplotypes with frequencies over 5%, haplotype ATGCTA, containing alleles A for rs12914489 and A for rs16958477 was most significantly associated with disease risk (P = 0.0008; OR, 2.34; 95% CI, 1.42–3.85); while haplotype GTGTCC, containing the opposite alleles for rs12914489 and rs16958477 was significantly protective ($P = 2.3 \times 10^{-6}$; OR, 0.38; 95% CI, 0.25–0.57). Interestingly, haplotype GTTTCC, also containing the C allele at rs16958477, was significantly protective (P = 0.0005; OR, 0.39; 95% CI, 0.22–0.68), which argues that the rs16958477 C allele associated with increased transcriptional activity has significant disease relevance. Haplotype GGGCTA containing the A allele for rs16958477 and the G allele at rs12914489 was associated with disease risk, but not as significantly as the haplotype containing both A alleles, suggesting that rs12914489 also contributes to the disease risk. Contributions for SNPs rs4886467, rs4558370, rs4461027, and rs4886761 to disease risk were not as apparent from these analyses.

To assess the impact of the promoter SNPs rs12914489 and rs16958477 on the disease risk associated with the *LOXL1* missense variants R141L and G153D, we analyzed the haplotypes containing alleles from these four SNPs (Table 3). Haplotype AAGG containing the risk alleles for all four SNPs has the highest risk for disease (P = 0.0001; OR, 2.56; 95% CI, 1.55–4.24). The two haplotypes containing protective alleles for rs12914489 and rs16958477 and with one missense risk allele (GCTG and GCGA) are both protective, with GCGA showing the strongest overall effect ($P = 3.9 \times 10^{-14}$; OR, 0.05; 95% CI, 0.02–0.15). Conditional haplotype analysis showed that rs12914489 had a significant independent effect on ES after adjustment for age and the missense variants R141L and G153D ($P_{adjusted}$ = 0.029), whereas an independent effect of rs16958477 on ES could not be demonstrated after adjustment for age, R141L and G153D ($P_{adjusted}$ = 0.51).

Joint Effects between Promoter SNPs, Missense Variants and ES Risk

Logistic regression modeling was used to investigate the joint effects between rs12914489, rs16958477, and G153D (the

TABLE 4. Genotype Frequencies of rs12914489, rs16958477, and G153D in Joint Analysis

	ES rs12914489					Controls rs12914489				
	GG rs16958477		GA+AA rs16958477			GG rs16958477		GA+AA rs16958477		
G153D	**CC**	**CA+AA**	**CC**	**CA+AA**	**Total**	**CC**	**CA+AA**	**CC**	**CA+AA**	**Total**
AA	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	1	8 (4.1)	2 (1.0)	1 (0.5)	1 (0.5)	12
AG+GG	30 (15.9)	107 (56.6)	0 (0.0)	51 (27.0)	188	61 (3.1)	97 (49.7)	2 (1.0)	23 (11.8)	183
Total	31	107	0	51	189	69	99	3	24	195

Data are presented as genotype count (%).

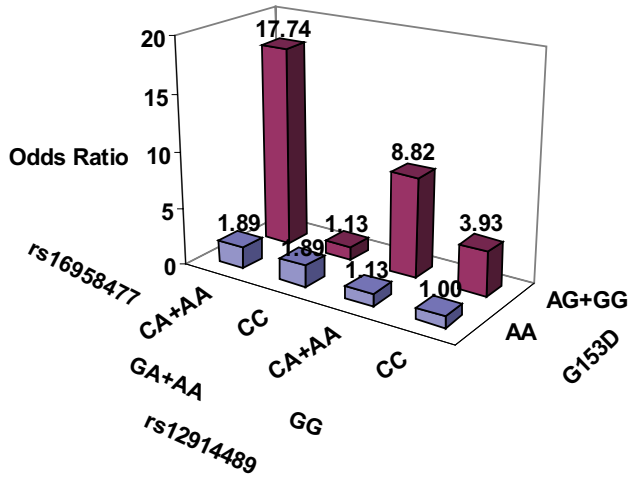

FIGURE 4. Joint effects among rs12914489, rs16958477, and G153D. The OR is plotted as a function of the genotypes of three SNPs associated with ES. Logistic regression suggests an additive effect. Since rs16958477 and G153D correlated highly in our population, the additive effect is primarily between rs12914489 and rs16958477.

missense variant with the strongest disease association in our population; Table 4). These analyses showed that the joint effects between these SNPs are additive but not interactive ($P_{\text{interaction}} > 0.05$). For G153D non–risk allele carriers (genotype AA), carrying rs16958477 risk allele A or rs12914489 risk allele A did not significantly change the risk of disease (OR, 1.13–1.89). However, for G153D risk allele carriers (genotypes AG or GG), carrying the rs16958477 risk allele A and rs12914489 risk allele A significantly increased the risk of disease (OR, 17.74; Fig. 4). Since rs16958477 correlates highly with G153D in our population, the additive effect is primarily between rs12914489 and rs16958477.

DISCUSSION

LOXL1 is a major risk factor for exfoliation syndrome in populations worldwide. The protein is one member of a protein family necessary for the formation of cross-links in collagen and elastin. LOXL1 initiates the cross-linking in elastin as a first step in the formation of elastin polymers.[38] The N-terminal of the LOXL1 protein contains the catalytic domain that is necessary for enzyme activation, substrate recognition and binding. Missense variants R141L and G153D are located within this domain, and as such, the initial discovery of their association with the disease suggested that these variants could affect enzymatic activity. Subsequent studies demonstrating reversal of the risk alleles argue that these missense changes are not biologically causal and that their significant disease association is due to other *LOXL1* gene variants that may be in linkage disequilibrium with R141L and G153D.

In this study, we have identified an *LOXL1* promoter region haplotype that is significantly associated with the disease in a U.S. Caucasian population. The risk haplotype includes the A allele of rs16958477 previously shown to cause a reduction in active gene transcription. A second SNP, rs12914489 in the distal promoter region is independently associated with the disease and contributes to the overall risk of this promoter haplotype. Although rs12914489 is approximately 30 kb from the transcription start site, it is located near a transcription factor binding site that may influence the transcriptional activity of the gene. Haplotypes containing the opposite alleles for SNPs rs16958477 (allele C) and rs12914489 (allele G) are both protective providing additional evidence to support a biological role for these SNPs. Collectively, these results suggest that the promoter region SNPs influence expression of the *LOXL1* gene and that the risk haplotype is associated with reduced gene expression and loss of active enzyme. A reduction of enzyme activity may cause impaired elastin formation and/or elastin maintenance, with subsequent compromise of elastin integrity predisposing to disease development. Further study of *LOXL1* promoter activity will be necessary to confirm this hypothesis.

The previously identified risk alleles R141L and G153D are significantly correlated with the transcriptionally relevant SNP rs16958477 in our population (Fig. 2), suggesting that these missense changes are associated with the disease risk because of linkage disequilibrium with the rs16958477 risk allele. The reversed risk allele at R141L and G153D in some populations is consistent with this hypothesis as the rs16958477 risk allele could be on an opposite haplotype in other populations. Other gene variants influencing gene expression could also be in linkage disequilibrium with these SNPs. Evaluation of these promoter SNPs in other populations and deep resequencing of the LOXL1 promoter region are important steps toward establishing the causality of these gene variants.

LOXL1 is clearly a major genetic risk factor for ES, and a reduction in *LOXL1* gene expression and enzyme activity is likely to be the genetic mechanism responsible for the associated disease risk. Although *LOXL1* is a necessary genetic risk factor, it is not sufficient to cause the disease. The high frequency of the associated variants in control populations as well as the reduced penetrance of the disease in some populations despite high frequencies of risk alleles suggest that other genetic and/or environmental factors must also play a role in disease development. Additional genetic and population studies are needed to fully elucidate the genetic architecture of this common blinding disease.

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