LOXL1 Gene Sequence Variants and Vascular Disease in Exfoliation Syndrome and Exfoliative Glaucoma

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Purpose: To investigate whether the single nucleotide polymorphisms (SNPs) of the LOXL1 gene associated with exfoliation syndrome (XFS) and exfoliative glaucoma (XFG) are different in XFS/XFG patients with and without cardiovascular disease (CVD); and to compare the allele frequencies in XFS/XFG with those in ischemic cerebrovascular disease (stroke), in the Hungarian population.

Methods: G153D and R141L allele frequencies were determined for 56 XFS/XFG patients (10 patients with and 45 without CVD, 1 patient unclassified), and for 189 patients with stroke.

Results: For G153D the frequencies of guanine (G) and adenine (A) alleles were 71.4% and 28.6% in the ischemic stroke group, and 58.0% and 42.0% in XFS/XFG (χ^2 test, $P = 0.008$). The corresponding figures in XFS/XFG without CVD were 56.7% and 43.3%, and 60.0% and 40.0% in XFS/XFG with CVD ($P = 0.785$). For R141L the frequencies of G and timidine (T) alleles were 68.2% and 31.7% in stroke patients, and 82.1% and 17.9% in XFS/XFG ($P = 0.004$). No difference was seen for allele frequency distribution between XFS/XFG patients without and with CVD (84.4% and 15.6%; 80.0% and 20.0%, respectively, $P = 0.738$).

Conclusions: In Hungarians, the frequency of G (risk) allele of G153D SNP was low in XFS/XFG. The frequency of G allele in R141L and G153D SNPs of the LOXL1 gene did not differ between XFS/XFG patients with and without CVD, but its frequency was different in XFS/XFG and ischemic stroke. These results suggest that the G allele in these SNPs has no direct role in the development of vascular diseases associated with XFS/XFG.

Key Words: exfoliation syndrome, exfoliative glaucoma, LOXL1 gene polymorphism, cardiovascular disease, cerebrovascular disease

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Exfoliation syndrome (XFS) is an elastosis-like systemic condition characterized by excessive ocular and extraocular synthesis and progressive accumulation of a fibrillar extracellular material called exfoliation material.[1–4] The progressive accumulation of the extracellular material in the eye frequently leads to the development of secondary open-angle glaucoma (exfoliative glaucoma, XFG), which is 1 of the leading causes of glaucoma-related visual impairment.[5,6] Recently 2 nonsynonymous single nucleotide polymorphisms (SNPs; rs3825942, G153D and rs1048661, R141L) of the lysyl oxydase-like 1 gene (LOXL1) located on 15Q were found to be strongly associated with the presence of XFS and XFG in Icelanders, and in the Swedish, German, and Italian populations, and in that of the United States.[7–13] In the Japanese population, however, these associations were not fully reproduced.[14,15] As the LOXL1 gene encodes an amine oxydase protein that catalyses the first step of cross-linking in collagen and elastin synthesis,[16] the potential role of its alterations in the development of elastosis-like diseases, such as XFS and XFG, seems possible, even though the exact mechanism by which the risk allele (guanidine, G allele) of the G153D and R141L SNPs leads to the production of exfoliation material has not yet been identified.

In XFS/XFG, in addition to the ocular presence of exfoliation material and its accumulation in the iris vessels,[17–20] exfoliation material has consistently been detected systemically at numerous sites, including in the vessel wall, in the myocardium, in smooth and striated muscle cells, skin, and in visceral organs.[3–6,21] In XFS/XFG the plasma total homocysteine concentration is elevated[22–25]; and this elevation may have a role in the development of a variety of vascular diseases including stroke, myocardial infarction, and venous occlusions.[26–28] Recently, large-artery and capillary dysfunction, and impaired baroreflex and heart-rate regulation have been identified in XFS/XFG.[29–33] Despite these suggestive items of evidence, however, the clinical significance of the XFS-related systemic vascular dysfunction remains controversial, because published results in this field are inconsistent. Association between XFS and cardiovascular and cerebrovascular morbidity has indeed been detected in some studies[32–36]; but in other studies such an association was not found.[37–40] An association between XFS and aorta aneurysm, which was reported in 1 study[41] was not confirmed by the results of another similar study,[42] despite the fact that both aorta aneurysm and XFS can be explained by elastosis.[1,2,42] These apparent contradictions can only partially be explained by the differences of clinical presentation of XFS/XFG in different European populations.[43] Another potential explanation is disturbed extracellular material metabolism in the vessel wall. As LOX knockout mice have decreased elastin synthesis that leads to cardiovascular dysfunction and aortic dissection,[44] the

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evaluation of the potential role of LOXL1 gene polymorphism in the development of systemic vascular alterations connected to human XFS/XFG may have clinical importance.

In this study, we wished to investigate the potential role of the recently discovered nonsynonymous SNPs of the LOXL1 gene in the association of XFS/XFG with severe systemic vascular disease. To do this, we compared the corresponding allele frequencies between patients with ischemic cerebrovascular disease (stroke) and those with XFS/XFG; and, within the XFS/XFG group, between the patients with and without cardiovascular disease (CVD).

METHODS

Patients

The research protocol was approved by the Central Ethics Committee of the Hungarian Ministry of Health, and informed consent was obtained from all participants before DNA sampling and storage. Fifty-six XFS/XFG patients were enrolled in the investigation between August and October 2008 (These were patients attending for normal follow-up examinations in the Glaucoma Unit of the Department of Ophthalmology of Semmelweis University, Budapest). Before enrollment, for each patient a detailed ophthalmologic and general medical history was recorded by 1 of the investigators (PK). The XFS/XFG patients were classified into those with and without CVD. Ongoing or earlier severe CVD was defined as systemic or ocular venous occlusion, ischemic cardiac disease, and myocardial or cerebral infarction or ischemic event. Owing to its very high frequency in the Hungarian general population, medically controlled systemic hypertension was not considered as a disease indicating severe CVD. Each XFS/XFG potential participant further underwent a detailed ophthalmologic examination with pharmacologically dilated pupil; for inclusion in the study, verification of exfoliation material in at least 1 eye was necessary.

DNA samples of 189 patients with ischemic cerebrovascular disease (provided by the Biobank of the Clinical and Research Centre for Molecular Neurology of Semmelweis University) were also analyzed. The cause of ischemic stroke was classified according to the TOAST criteria (Trial of ORG 10172 in Acute Stroke Treatment) into 1 of 5 ischemic stroke subtypes, as follows: large-artery atherosclerosis, cardioembolism, small-artery occlusion, stroke of other determined etiology, and stroke of undetermined etiology. These stroke patients were not investigated ophthalmologically, and were not classified as to their XFS/XFG status. Demographic and medical data of the participants are shown in Table 1.

Molecular Genetic Investigation

DNA was extracted from blood using an ABI PRISM 6100 PrepStation nucleic acid isolation system (Applied Biosystems, Foster City, CA), operated according to the manufacturer's instructions.

A 244 base pair fragment of the mtDNA (mRNA position nt. 674-917) was amplified by polymerase chain reaction from 100 ng of DNA. In the reaction 200 nmol/L each of the sense and the antisense primers was used. The sequence of the sense primer used was 5′-GCCA GGCGCGGCACCCAT -3′ and that of the reverse primer was 5′-GCGGGGTCGTAGTTCTCGTA-3′. The PCR processing schedule was as follows: 94°C/5 minutes (1 cycle); followed by 94°C/30 seconds, 68°C/30 seconds, 72°C/30 seconds (35 cycles); followed by 72°C/7 minutes (1 cycle). The G153D (rs3825942) and R141L (rs1048661) mutations were further investigated by PCR-RFLP analysis. The PCR product was digested with Ban II (G153D) and Ava I (R141L) restriction enzymes (Promega Corporation, Madison, WI), used according to the manufacturer's instructions. After digestion, the PCR fragments were transferred to 4% agarose gel and visualized by ethydium-bromide staining. Each genotype was validated with bidirectional direct sequencing (Figs. 1 and 2).

Statistical Analysis

χ^2 test was used to investigate deviation from the Hardy-Weinberg equilibrium. The corresponding allele frequencies were compared using the χ^2 test. A P-value < 0.05 was considered as statistically significant.

RESULTS

Of the XFS/XFG patients, 56 in all, 45 had no severe CVD, whereas10 of them did currently or earlier suffered from severe CVD. The 1 remaining XFG patient was impossible to classify, owing to unclear CVD history. Of the 189 patients with ischemic stroke, it was possible to analyze samples for all 189 for R141L; but for technical reasons only 187 samples were analyzed for G153 D. For the G153D SNP both the XFS/XFG group and the stroke

TABLE 1. Demographic and Medical Data of the Patients in the Study

	All XFS/XFG (n = 56)	XF/XFG Without CVD (n = 45)	XF/XFG With CVD (n = 10)	Stroke Patients (From the Register)
No. Caucasian, Hungarian Race/nationality	56 (100%)	45 (100%)	10 (100%)	189 (100%)
Age (mean ± SD, range, years)	73.9 ± 7.6 (range: 56-88)	73.02 ± 8.08 (range: 56-88)	77.40 ± 3.92 (range: 72-83)	73.5 ± 9.0 (range: 50-81)
Gender (male/female)	22/34	18/27	4/6	93/96
Classification for ischemic stroke type (n, %)	NA	NA	NA	
Large artery atherosclerosis				39 (20.9%)
Cardioembolism				55 (29.4%)
Small vessel occlusion				41 (21.9%)
Other determined etiology				12 (6.4%)
Etiology undetermined				40 (21.4%)

NA indicates not applicable.

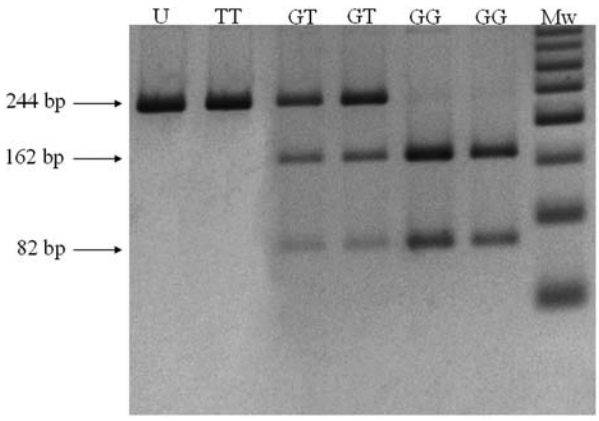

FIGURE 1. Genetic determination of the R141L (rs1048661) polymorphisms of the LOX1 gene was carried out with PCR-RFLP with Ava I digestion. Bp indicates base pair; G, guanine; MW, molecular weight marker (50 bp); T, timidine; U, uncut PCR product.

group deviated from the random mating model (χ^2 test, $P < 0.001$, and $P = 0.001$, respectively). For the R141L SNP no deviation from the random mating model was found ($P > 0.05\%$ for both groups). The distribution of the allele frequencies for both SNPs is shown in Table 2. For G153D the frequencies of guanine (G) and adenine (A) alleles were 71.4% and 28.6% in the stroke group, and 58.0% and 42.0% in XFS/XFG ($P = 0.008$, χ^2 test). The corresponding figures were 56.7% and 43.3% in XFS/XFG without CVD, and 60.0% and 40.0% in XFS/XFG with CVD ($P = 0.785$). For R141L the frequencies of G and timidine (T) alleles were 68.2% and 31.7% in the stroke group, and 82.1% and 17.9% in XFS/XFG, respectively ($P = 0.004$). No difference was seen for G and T allele distribution between XFS/XFG patients without and with CVD (84.4% and 15.6%; 80.0% and 20.0%, respectively, $P = 0.738$).

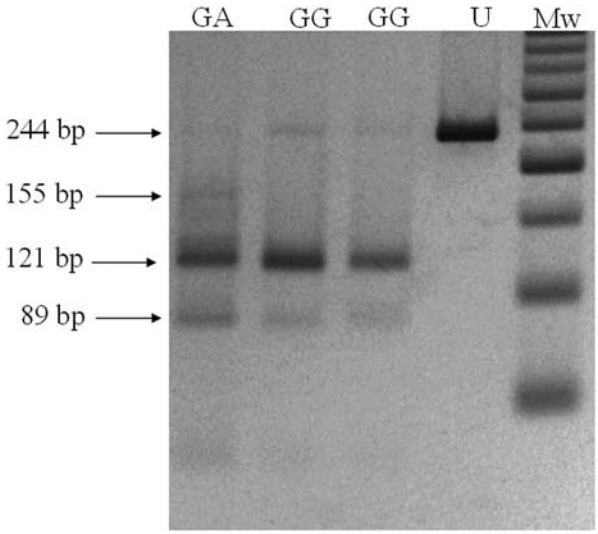

FIGURE 2. Genetic determination of the G153D (rs3825942) polymorphisms of the LOX1 gene was carried out with PCR-RFLP with Ban II digestion. A indicates adenine; bp, base pair; G, guanine; MW, molecular weight marker (50 bp); U, uncut PCR product.

DISCUSSION

Systemic vascular alterations in XFS/XFG have been studied using both epidemiologic[34–42] and pathophysiologic methods.[29–33] The epidemiologic reports suggest an association between XFS/XFG and cardiovascular and cerebrovascular diseases, but in the various studies the relationship has not been consistently found. This lack of consistency might partially be caused by the considerable geographic regional differences in clinical presentation of XFS and XFG.[43] Using various pathophysiologic methods and age-matched control groups, in XFS and XFG we consistently found pathologic regulation of capillary and large vessel function, and impaired baroreflex sensitivity and heart rate regulation, in Hungarian patients.[29–33] This suggests that, at least in the Hungarian population, XFS and XFG are indeed associated with pathologic vascular alterations. The consistency of the above-mentioned pathophysiologic changes provided the background for our current investigation, in which we determined the allele frequencies of 2 SNPs (rs3825942, G153D and rs1048661, R141L) of the LOXL1 gene in XFS/XFG patients and in patients with ischemic cerebrovascular disease. In earlier published studies, these SNPs have been found to be strongly associated with XFS/XFG, representing up to 99% of population attributed risk for these conditions.[7–13] As the LOXL1 gene encodes a protein with an important role in the crosslinking of collagen and elastin,[16,44] alterations of this gene may potentially influence the connective-tissue components of the blood vessels. XFS and XFG are considered elastosis-like diseases,[1–4] thus vascular diseases found in XFS/XFG patients may potentially be related to alteration of elastin function in the vessel wall. This hypothesis is supported by the decreased elastin synthesis, cardiovascular dysfunction, and aortic dissection shown in LOX-knockout mice.[44]

Similarly to several other Caucasian XFS/XFG populations[7–10,45] the high G allele (risk allele) frequency for R141L was present in our XFS/XFG patients (82.1%). But, interestingly, for G153D the frequency of G allele (58.0%) was considerably lower than that reported for the other Caucasian XFS/XFG populations.[7–13,45] As shown in Table 2, the allele distribution was similar for those XFS/XFG patients who had never had any serious CVD (systemic or ocular venous occlusion, ischemic cardiac disease, and myocardial or cerebral infarction or ischemic event) and for those XFS/XFG patients who earlier or at the time of the investigation did suffer from such diseases. Although the number of patients in the latter group was small, which is a limitation of our study, the similarity of the corresponding figures in the 2 XFS/XFG subgroups suggests that our findings are real and not just a statistical artifact.

The other goal of our investigation was to compare the allele frequencies in our total XFS/XFG group to those of the ischemic stroke patients. The DNA material of the ischemic cerebrovascular disease patients was obtained 4 years earlier the current investigation, and had then been stored for use in future investigations, of which this study is one. Thus, unfortunately, no information on the presence of exfoliation material in these patients was available. The stroke patients had suffered from severe ischemic cerebrovascular diseases at the time of DNA sampling during their hospitalization in a stroke center; those selected for this study were matched for age with the XFS/XFG patients. Our XFS/XFG patients showed a

TABLE 2. Distribution of LOXL1 Alleles in Patients With XFS/XFG, Stroke, and XFS/XFG Without and With Cardiovascular Disease

Allele	All XFS/XFG Patients (n = 56) (1)	Stroke Patients (n = 187 for G153D n = 189 for R141L) (2)	XFS/XFG Patients Without CVD (n = 45) (3)	XFS/XFG Patients With CVD (n = 10) (4)	*P** (1) vs. (2)	*P** (3) vs. (4)
G153D (rs3825942)					0.008	0.785
G	65 (58.0%)	267 (71.4%)	51 (56.7%)	12 (60.0%)		
A	47 (42.0%)	107 (28.6%)	39 (43.3%)	8 (40.0%)		
R141L (rs1048661)					0.004	0.738
G	92 (82.1%)	258 (68.2%)	76 (84.4%)	16 (80.0%)		
T	20 (17.9%)	120 (31.7%)	14 (15.6%)	4 (20.0%)		

*χ^2 test.
A indicates adenine; CVD, cardiovascular disease; G, guanine; T, timidine; XFS/XFG, exfoliation syndrome/exfoliative glaucoma.

significant difference in both allele distributions compared with the stroke patients. For G153D, the frequency of G allele was significantly lower in XFS/XFG than in the stroke group (58.0% vs. 71.4%). In contrast, for R141L the frequency of G allele was significantly higher in XFS/XFG (82.1%) than in ischemic cerebrovascular disease (68.2%). Although the distribution of the alleles investigated by us has not been evaluated for the healthy aging population of Hungary, which limits the conclusion of this investigation, the above findings suggest that the frequencies of the tested SNPs are different in ischemic stroke and XFS/XFG patients, in the Hungarian population.

All these findings suggest that vascular diseases associated XFS/XFG cannot be directly related to G allele (the risk allele for XFS and XFG) in G153D and R141L SNPs. This result, in fact, is not unexpected because the molecular background of the extracellular material diseases is complex, involves comodulating factors regulated by several different genes, and is also influenced by environmental factors.[46]

In conclusion, although this study is limited by the relatively small number of XFX/XFG patients, our findings suggest that the vascular pathophysiologic alterations that have been consistently reported for XFS and XFG in the Hungarian population[29–31,33] are not directly connected with the G153D and R141L SNPs of the LOXL1 gene.

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