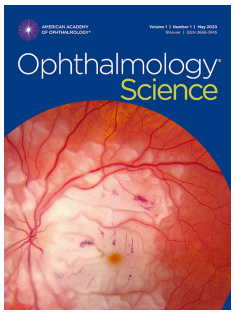

Association of two Lysyl oxidase (LOX) Gene single nucleotide polymorphism with Keratoconus; a nationwide registration study

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Association of two Lysyl oxidase (LOX) Gene single nucleotide polymorphism with Keratoconus; a nationwide registration study

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Abbreviations and Acronyms

C (Cytosine)

ECM (Extracellular matrix)

G (Guanine)

GWAS (Genome-Wide Association Study)

GLM (General Linear Model)

FN1 (Glycoprotein fibronectin)

HWE (Hardy-Weinberg equilibrium)

KC (Keratoconus)

LOX (Lysyl oxidase)

MMP9 (Metalloproteinase 9)

T (Thymine)

T-ARMS PCR (Tetra-primer amplification-refractory mutation system-polymerase chain reaction)

TIMP1, TIMP2 (Tissue inhibitor of metalloproteinase 1 and 2)

THBS1 (Thrombospondin1)

TGFBI (Transforming growth factor beta-induced gene)

SNPs (Single nucleotide polymorphisms)

Abstract

Purpose: Keratoconus (KC) is the most common primary ectatic corneal disease, characterized by progressive thinning of the cornea, affecting its shape and structure and leading to visual loss. Lysyl oxidase (*LOX*) is an important component of the extracellular matrix (ECM) and contributes to the homeostasis of corneal stromal ECM via enzymatic reaction. This nationwide registration study aims to examine the association of KC with two known single nucleotide polymorphisms (SNPs), rs2956540 and rs10519694, in a population of Iranian descent.

Design: Case-control

Participants: One hundred seventy-eight subjects with KC and one hundred eighty clinically healthy subjects participated in the study.

Methods: Genomic DNA was extracted from peripheral blood samples, and their genotypes were determined using tetra-primer amplification-refractory mutation system-polymerase chain reaction (T-ARMS PCR).

Main outcome measured: Allele frequency for rs2956540 and rs10519694

Results: Genotypic frequency was significantly different between cases and controls for rs2956540 (p-value = 0.019). The rs2956540 C allele carriers were significantly more frequent among KC cases than healthy controls (p-value chi-square = 0.015, p-value Fisher's Exact = 0.010). There was a significant difference in genotype frequency between groups for rs10519694 (p-value = 0.001). T allele carriers were significantly more frequent among KC patients (p-value chi-square = 0.002, p-value Fisher's Exact = 0.001). Sex stratification revealed no significant differences in genotype frequency between males and females in cases and controls. Fitting the general linear model (GLM) showed that rs10519694 could be considered a predictor for developing of KC (p-value = 0.001); however, this was not observed for rs2956540 (p-value = 0.323) .

Conclusions: rs2956540 and rs10519694 are associated with KC in a population of Iranian descent. rs10519694 could potentially be used for KC risk prediction.

Keratoconus (KC), or conical cornea, is characterized by bilateral, progressive, non-inflammatory thinning and protrusion of the cornea, which usually leads to visual impairment from myopia and irregular astigmatism[1]. KC is the most common corneal ectatic disorder[2] and one of the most common reasons for corneal transplantation, especially in younger patients[3-5]. Its estimated prevalence is 0.17 to 47.90 per 1000 in different populations[6-8]. Both genders are equally affected, and the disease is more prevalent among Asians than Caucasians[8,9].

KC is a multifactorial disease with a single etiology remaining unknown. Family-based and twin studies have demonstrated both genetic and environmental factors impact pathogenesis[10-12].

Histochemical analysis shows disturbances in the homeostasis of the extracellular matrix (ECM) of the keratoconic cornea[13,14]. Lysyl oxidase (*LOX*)[15], metalloproteinase 9 (*MMP9*)[16,17], tissue inhibitor of metalloproteinase 1 and 2 (*TIMP1*, *TIMP2*)[16,17], transforming growth factor beta-induced gene (*TGFBI*)[16,18,19], glycoprotein fibronectin (*FN1*)[16], integrin[16], and thrombospondin1 (*THBS1*)[16,20] are some examples of ECM-related genes involved in the development of KC.

Lysyl oxidase is a copper-dependent enzyme encoded by the *LOX* gene (Location: 5q23.2) that plays an important role in maintaining the ECM by forming elastin and collagen cross-links using oxidative deamination catalysis of lysine and hydroxylysine residues[21]. Connections between *LOX* chromosomal location in familial KC and altered *LOX* activity in individuals with KC support a promising role of *LOX* in the incidence of KC[22-24]. A case-control Genome-Wide Association Study (GWAS) in American Caucasians revealed the association of KC with single nucleotide polymorphisms (SNPs) lies within *LOX*[15]. Several studies have already highlighted the role of *LOX* variants, especially SNPs, in the risk of KC[15,23,25-27]. A study in an Iranian population demonstrated that rs1800449 is significantly associated with KC and increasing KC risk, whereas a similar effect was not observed in rs2288393[21]. Another study revealed that both rs1800449 and rs2956540 are significantly associated with KC[15], as did a separate study of European descendants examining rs2956540 and rs3735520[28].

Among all SNP variants located in *LOX*, rs2956540 and rs10519694 are two intronic variants located in the fourth intron of *LOX*. Accordingly to the Genotype-Tissue Expression (GTEx) database (https://gtexportal.org/home/), rs2956540 and rs10519694 have been known as expression quantitative trait loci (eQTLs) in several tissues. Therefore, it is possible that rs2956540 and rs10519694 might be eQTLs in corneal tissue too, and alter the risk of KC. Also SNP rs2956540 may affect gene expression through transcriptional regulation, as predicted by Genomatrix (http://www.genomatix.de/) that it can alter the binding sites of several transcription factors like PTX1, CMYB, and ISM1[29].

Few studies have evaluated the association of these two variants with KC among different populations[29]. The latest meta-analysis in 2015 showed that rs2956540 and rs10519694 variants have a significant association with KC, whereas it failed to replicate this result in rs1800449 and rs2288393[29]. This study explores the association of rs10519694 and rs2956540 with KC in a population of Iranian descent.

Methods

The study was performed in Negah Eye Hospital, Tehran, Iran between January 2020 and January 2022. It was approved by the institutional review board and ethics committee of the Ophthalmic Research Center, Research Institute for Ophthalmology and Vision Science, Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.ORC.REC.1399.015) and met the Helsinki Declaration criteria. KC was primarily diagnosed based on clinical criteria including Vogt's striae, Fleischer's ring, corneal stromal thinning and protrusion, and video-keratography findings[30-32]. The Pentacam HR (Oculus, Wetzlar, Germany) and the Corvis ST (OCULUS Optikgeräte GmbH; Wetzlar, Germany) were done for all suspicious cases to detect mild forms of KC (Tables 1 and 2) [33-36]. All diagnoses were approved by two cornea specialists (FD, AB). Complete medical histories were obtained from all patients with KC. No other ocular disorders or risk factors (including contact lens use, eye rubbing, atopy, etc.) or systematic diseases (including connective

tissue disorders) were uncovered. All healthy controls had no personal or familial history of eye-related, metabolic, or immune system-related disease. All participants or their legal guardians signed consent forms.

Genotyping

Five ml of peripheral blood was collected from all participants and stored in an EDTA tube at -20 °C until DNA was extracted. Total genomic DNA was extracted using a salting-out protocol[37]. Agarose 1% gel electrophoresis and UV-spectroscopy were used to assess the quality and quantity of extracted DNA, respectively. rs2956540 and rs10519694 were genotyped using tetra-primer amplification-refractory mutation system-polymerase chain reaction (T-ARMS PCR). 3'allele-specific primers designed by primer1 online tool (http://primer1.soton.ac.uk/primer1.html) were used for amplification. The T-ARMS PCR was carried out in 20 µl mixtures containing 100 ng of genomic DNA, 10 µl of Taq DNA Pol 2X Master Mix Red (Amplicon, Denmark), 0.5 µl of two outer primers, 1 µl of two inner primers for each SNP, and the remainder came from double-distilled water. PCR reactions were performed using the Eppendorf thermocyclers (Eppendorf AG 22331 OMIM 148300) under the following conditions: 94 °C for 4 min, followed by 35 cycles including denaturing at 94 °C for 30 s, annealing for 30 s at 60 °C and 62 °C for rs2956540 and rs10519694, respectively, and extension at 72 °C for 30 s. A final extension was conducted at 72 °C for 5 min. Finally, the PCR product ran on 2% gel electrophoresis. Based on the sizes of the PCR products on the gel electrophoresis, the genotypes were determined (Table 3).

Statistical analysis

All statistical analyses were performed using SPSS Statistics for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA). Two-tailed Mann-Whitney and Pearson Chi-Square tests were used to compare age and gender. Pearson Chi-Square test was used to compare allele and genotype frequency between cases and controls and to assess divergence from Hardy-Weinberg equilibrium (HWE). The allele risk was evaluated using two-tailed Fisher's Exact Test and Odds Ratio (OR) with a 95% confidence interval. Logistic regression was used to fit the general linear model (GLM) to the data to predict KC risk using rs2956540 and rs10519694. Statistical significance was defined as a P value of 0.05.

Results

Demographic data

A total of 358 unrelated individuals (178 KC and 180 healthy controls) were enrolled in the study. Mean age of KC patients (92 males and 86 females) and healthy controls (97 males and 83 females) were 31.97 ± 9.37 years and 32.71 ± 6.88 years, respectively (p-value = 0.33). Gender was matched in both groups (p = 0.75) (Supplementary Table S4).

Hardy-Weinberg equilibrium (HWE)

In association studies, we typically hypothesize that healthy controls remain in HWE, whereas the subject groups deviate from HWE. This means one of the frequencies of the genotypes is higher or lower than what would be expected from mutation, natural selection, etc. Thus, in both rs2956540 and rs10519694, KC subjects departed from HWE (rs2956540: p-value = 0.038 / rs10519694: p-value = 0.048), whereas healthy controls remained in HWE (rs2956540: p-value = 0.93 / rs10519694: p-value = 0.090) (Supplementary Table S5).

Allele frequency

To calculate the frequency of each allele, the homozygotes of that allele were multiplied by two and added to the heterozygotes, and the result was divided by twice the population of the group. In this set of data, both rs2956540 and rs10519694 risk alleles showed higher frequencies compared to those displayed in the dbSNP database (rs2956540 C allele = 0.3, rs10519694 T allele = 0.2). Allele frequency did not significantly differ for rs2956540 (p-value = 0.2654) and rs10519694 (p-value = 0.08263) between cases and controls (Table 6).

Genotype frequency

To calculate the genotypic frequency in each group, the total number of each genotype was divided by the total population of that group. Genotypic frequency was significantly different between cases and controls for rs2956540 (p-value = 0.019). rs2956540 C allele carriers (CC & CG) were significantly more frequent among KC cases than healthy controls (p-value $_{\text{chi-square}}$ = 0.015, p-value $_{\text{Fisher's Exact}}$ = 0.010). There was a

significant difference in genotype frequency between groups for rs10519694 (p-value = 0.001). T allele carriers were significantly more frequent among KC patients (p-value $_{\text{chi-square}}$ = 0.002, p-value $_{\text{Fisher's Exact}}$ = 0.001) (Tables 7 and 8). Sex stratification revealed no significant differences in genotype frequency between males and females in cases and controls (Table 9). Fitting the general linear model (GLM) showed that rs10519694 could be considered a predictor for developing of KC (p-value = 0.001); however, this was not observed for rs2956540 (p-value = 0.323) (Table 10).

Discussion

KC is a complex, multifactorial, genetic condition that can manifest in isolation or in association with other systemic genetic disorders. Bilaterality, familial aggregation[38-40], concordant monozygotic twins[41], correlation with inflammatory bowel disease, Down syndrome[42], Leber congenital amaurosis[43], diabetes mellitus[26], and ethnic[44] differences in prevalence and incidence[45] all point to a genetic cause. Genetic relationships in KC will facilitate discovering biomarkers for early detection and monitoring progression. 14% of KC patients have a family history[40]. It remains uncertain how familial and sporadic KC differ genetically. Since family history does not alter genetic severity, genetic research can pool all cases[46]. Numerous studies have investigated associations between isolated KC and genetic factors[2,46-49]. Several have suggested *LOX* to be one of the most promising genes associated with KC[23]. Using an oxidative deamination catalytic reaction, *LOX* affects ECM homeostasis and maintenance in collagen and elastin-rich tissues, such as the cornea[50].

Thus far, numerous studies have examined the association of KC with a wide spectrum of *LOX* single nucleotide variants. Thr392Pro and Pro32Leu substitutions are examples of exonic, non-synonymous point mutations observed in two Brazilian and Chinese KC patients[8,51], whereas –116C > T and –58C > T are two *LOX* 5'UTR mutations that have been observed in two advanced KC patients[51]. rs1800449 and rs2288393 are two well-known intergenic SNPs whose relationships to KC have been studied extensively[15,21,52,53]. The

rs2956540 and rs10519694 are two well-known SNPs found in the fourth intron of the *LOX* gene and connected to KC in different populations[8,12,29,54].

Alterations in *LOX* corneal tissue expression in KC patients have been demonstrated. One study showed *LOX* to be significantly downregulated in KC patients compared to controls[55]. Dudakova et al. observed lower *LOX* activity in corneal fibroblast cultures from KC individuals[14].

In addition to a significant drop in *LOX* mRNA levels, Pahuja et al. showed a reduction in the ratio of *LOX* expression with increasing KC grades[56]. Collectively, the results of these studies suggest that alterations in *LOX* biological activity through changes in DNA sequences, particularly in non-coding intronic sequences, correlate with the risk of KC development.

We looked at the relationship between two known intronic SNPs in the *LOX* gene, rs2956540 and rs10519694, and KC in a population of Iranian descent. A recent study identified rs2956540-G as a risk allele for KC in a population of Europeans[28]. However, we found rs2956540-C allele highly prevalent in KC patients, whereas the G-allele was more common in the healthy population. Additionally, we noted no significant difference in rs10519694 allele frequency between cases and controls. Furthermore, we found that being a rs10519694-T carrier was more frequently and significantly observed among Iranian KC patients. While previous studies did not clearly define which rs10519694 allele is the risk allele, we found rs10519694-T to be a significant risk factor for KC. As in previous studies, we found no significant association between gender and rs2956540 and rs10519694 alleles in both KC subjects and healthy controls. In this study, we examined gene and allele frequencies in a referral center in a consecutive, non-random group of Iranian descent. Further studies on KC patients evaluating more SNPs with larger sample sizes in different populations are needed for definitive results.

Conclusion

We evaluated the association between two well-known *LOX* gene SNPs, rs2956540 and rs10519694, and KC in a population of Iranian descent for the first time. We found the rs10519694 variant is more prevalent in KC patients. Further studies should be conducted integrating other genetic information and

questionnaires to assess environmental factors and characterize the severity of allergies and frequency of eye rubbing.

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Table 1. Pentacam criteria for risk of keratectasia

Criteria	Normal	Suspect	Abnormal
Pentacam			
K max (D)	<47.2	47.2-49	>49
Against the rule astigmatism (D)	<1	1-2	>2
Corneal astigmatism (D)	<6	6-7	>7
Thinnest point (µm)	>500	470-500	<470
Difference between pachy apex and thinnest location (µm)	<10	10-20	>20
Difference in central thickness between two eyes (µm)	<10	10-30	>30
Displacement of the thinnest point from the center (mm)	<0.5	0.5-1	>1
Skewed Steepest Radial Axis (SRAX) (degrees)	<10	10-20	>21
IS value (Inferior-Superior difference at the 3 mm) (D)	<1.4	1.4-1.9	>1.9
IS value (Inferior-Superior difference at the 5 mm) (D)	<1.4	1.4-2.5	>2.5
Anterior elevation (µm)	<10	10-12	>12
Posterior elevation (µm)	<15	15-17	>17

Table 2. The Clinically Suggested Cut-off Values for Keratoconus Indices in Screening Clinical and Subclinical Cases

Parameter	Clinical Keratoconus	Subclinical Keratoconus
Tomographic		
CKI	**	**
KI	1.07	**
IHA	10.4	**
IHD	0.017	**
TKC	1.0	2.0
ISV	36.6	**
IVA	0.28	0.15
Rmin	7.04	**
PE	20.5	10.5
IS value	1.1	1.9
KISA	60%	100%
Pachymetric		
ART-Min	606	**
ART-Max	356	368
ART-Avg	444	490
BAD_D	2.02	1.31
CCT	515	518
PPI-Min	0.87	0.80
PPI-Max	1.53	1.40
PPI-Avg	1.18	1.08
TCT	506	502

CKI: central keratoconus index, KI: keratoconus index, IHA: index of height asymmetry, TKC: topographic keratoconus classification, ISV: index surface variance, IVA: index of vertical asymmetry, IHD: index of highest decentration, Rmin: minimum radius of curvature, PE: prediction error, IS value: the Inferior-Superior value, ART: Ambrosio's relational thickness indices, BAD_D: Belin/Ambrósio deviation, CCT: central corneal thickness, PPI: pachymetric progression indices, TCT: thinnest corneal thickness, KISA: The KISA index was derived from the following 4 indices: central K; I–S; the SRAX index, an expression of irregular astigmatism occurring in keratoconus; and the astigmatism index (AST), which quantifies the degree of the regular corneal astigmatism (simulated K1 − simulated K2)

Table 3. T-ARMS-PCR primer sequences, Melting temperatures (T_m) and the size of PCR product for every genotype.

SNP	Primer sequence	PCR product	Genotype (bp)
rs2956540	Forward inner primer (C allele): ACTTATTTTTCCTCCATTTGCTAAGCC	209	CC (417, 209)
	Reverse inner primer (G allele): GTTTTATGCTGAAAATAGAATAGTGGTAGC	265	GG (417, 265)
	Forward outer primer (5' - 3'): CTGACATAGATTTTAACTGACACGCATT	417	CG (417, 265, 209)
	Reverse outer primer (5' - 3'): CAGTCCACAATGAAGAACAAAAATTTAC		
rs10519694	Forward inner primer (C allele): AAATATTCACATCAATAAGTAAATGAAGGC	252	CC (402, 252)
	Reverse inner primer (T allele): TATTTTTCTCCTCCCAGCCTGTAGACGA	208	TT (402, 208)
	Forward outer primer (5' - 3'): TGGTTTTGAGTTTAGGTAATCAAGGTCC	402	CT (402, 252, 208)
	Reverse outer primer (5' - 3'): TGCTAGAATTGAATGGCAGTATTGAGTT		

SNP: single nucleotide polymorphisms, PCR: polymerase chain reaction, T-ARMS: tetra-primer amplification-refractory mutation system

Table 6. rs2956540 and rs10519694 allele frequency

	rs2956540		rs10519694	
	C	G	C	T
Case	174 (48%)	182 (52%)	210 (58%)	146 (42%)
Control	160 (44%)	200 (56%)	236 (65%)	124 (35%)
Pearson Chi-Square	1.2402		3.0124	
df	1		1	
p-value	0.2654		0.0826	

C: Cytosine, G: Guanine, T: Thymine

Table 7. rs2956540 and rs10519694 genotype frequency

	rs2956540			rs10519694		
	CC	CG	GG	CC	TC	TT
Case	34	106	38	54	102	22
Control	39	82	59	84	68	28
Pearson Chi-Square	7.94			14.03		
df	2			2		
p-value $_{\text{Chi-square}}$	0.019			0.001		

C: Cytosine, G: Guanine, T: Thymine

Table 8. Comparison of C allele carriers of rs2956540 and T allele carriers of rs10519694 between cases and controls

	rs2956540		rs10519694	
	CC + CG	GG	CC	TT + TC
Case	140	38	54	124
Control	121	59	84	96
Pearson Chi-square	5.91		10.08	
df	1		1	
p-value $_{\text{Chi-square}}$	0.017		0.002	
p-value $_{\text{Fisher's exact}}$	0.017		0.002	
OR (CI = 0.95)	1.79		2.01	

C: Cytosine, G: Guanine, T: Thymine, OR: odd ratio

Table 9. Gender-specific genotypic stratification

		rs2956540			rs10519694		
		CC	CG	GG	CC	TC	TT
Case	Female	17	53	16	22	49	15
	Male	17	53	22	32	53	7
Pearson Chi-square		0.746			4.721		
df		2			2		
P-value		0.689			0.094		
Control	Female	19	37	27	42	31	10
	Male	20	45	32	42	37	18
Pearson Chi-square		0.142			1.737		
df		2			2		
P-value		0.932			0.420		

C: Cytosine, G: Guanine, T: Thymine

Table 10. Fitting logistic regression model to genotype data

	Beta	Standard Error	p-value	OR
rs2956540	-0.265	0.269	0.323	0.767
rs10519694	-0.725	0.223	0.001	0.484
Intercept	0.670	0.290	0.021	1.954

Precis

This case-control study showed that rs10519694, known as single nucleotide polymorphisms (SNPs), could be considered a predictor for the development of keratoconus by registration of Iranian descent.; however, this was not observed for rs2956540.