

## *VSX1* gene variants are associated with keratoconus in unrelated Korean patients

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**Abstract** Keratoconus is a bilateral ectatic disorder characterized by the central thinning of corneal tissue leading to visual impairment. To investigate the possibility of visual system homeobox 1 (*VSX1*) as a candidate susceptibility gene for Korean patients with keratoconus, we performed a mutation screening of the *VSX1* gene in 249 unrelated patients with keratoconus and 208 control subjects without the ocular disorder. We found two heterozygous novel missense mutations in exon 2: N151S and G160V. The G160V mutation was identified in 13 keratoconus patients (5.3%), and the N151S mutation was found in only one keratoconus patient (0.4%). We also detected three synonymous polymorphisms and four intragenic polymorphisms. The *IVS1-11\*a* allele was associated with a significantly increased risk of keratoconus in Korean patients [3.6 vs. 0.5%,  $p = 0.001$ , odds ratio (OR) = 7.76, 95% confidence interval (CI) 1.989–30.241]. Other polymorphisms did not show an association with keratoconus risk. Our data is the first reported *VSX1*

mutation screening in Korean keratoconus patients. We detected two novel missense mutations and one intragenic polymorphism in the *VSX1* gene, which show a strong statistical association with unrelated keratoconus patients. Consequently, our study suggests that *VSX1* gene variants seem to be significant genetic variants for keratoconus predisposition in unrelated Korean patients.

**Keywords** Korean · Keratoconus · *VSX1* · SNP · G160V mutation

### Introduction

Keratoconus (KTCN; OMIM 148300) is a noninflammatory, bilateral ectatic disorder characterized by central thinning of corneal tissue with myopia and astigmatism that leads to visual impairment (Krachmer et al. 1984). Histologically, thinning and scarring of corneal stroma, iron deposition in the epithelial basement membrane, disruption of Bowman's layer, and eventual protrusion of the cornea can be observed (Nakamura et al. 2005; Sherwin and Brookes 2004). The prevalence of keratoconus is estimated to be 1 per 2,000 in the general population. The onset of keratoconus is in puberty, and the progression of the disease occurs through the third or fourth decade of life, when it usually stops (Karseras and Ruben 1976; Rabinowitz 1998).

The exact cause of keratoconus is unclear, although its pathogenesis may involve genetic, environmental, and behavioral factors. It may be associated with various factors, including atopy, eye rubbing, hard contact lenses, and numerous multisystemic genetic diseases, including Down syndrome, Leber's congenital amaurosis, and Ehlers-Danlos syndrome (Bawazeer et al. 2000; Chwa et al. 2006;

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Elder 1994; Flanders et al. 1984; Kenney and Brown 2003; Kuming and Joffe 1977; Rabinowitz 1998; Shapiro and France 1985). Family-based and twin studies have shown that genetic factors in particular play a significant role in the development of keratoconus and that there is a high concordance rate for keratoconus in monozygotic twins (Edwards et al. 2001; McMahon et al. 1999; Parker et al. 1996; Wang et al. 2000). A familial inheritance has been reported in 6–23% of patients with keratoconus, which reveals a genetic heterogeneity from autosomal dominant, with incomplete penetrance to autosomal recessive, but most reported keratoconus cases are sporadic (Bawazeer et al. 2000; Rabinowitz 1998; Wang et al. 2000; Wilson et al. 1992).

Six keratoconus susceptibility genomic loci, 20p11.21 (KTCN 1, MIM 148300), 16q22.3-q23.1 (KTCN 2, MIM 608932), 3p14-q13 (KTCN 3, MIM 608586), 2p24 (KTCN 4, MIM 609271), 5q14.3-q21.1, and 15q22.23-q24, have been previously identified through linkage studies of multigenerational families with keratoconus (Brancati et al. 2004; Dash et al. 2006; Heon et al. 2002; Hughes et al. 2003; Hutchings et al. 2005; Tang et al. 2005; Tynismaa et al. 2002). Based on studies reporting significant linkage of keratoconus, several pathogenic mutations in *VSX1* (MIM 605020; visual system homeobox gene 1, zebrafish, homolog of), located on 20p11.21, have been detected in multigenerational pedigrees of keratoconus, with a strong association and genetic variants of *VSX1* that has been validated by multiple studies (Aldave et al. 2006; Bawazeer et al. 2000; Bisceglia et al. 2005; Chwa et al. 2006; Heon et al. 2002; Kenney and Brown 2003; Kuming and Joffe 1977; Liskova et al. 2007; Tang et al. 2008). Although several variants of *VSX1*, e.g., L17P, L159 M, and R166 W, have been reported to be pathogenic in multigenerational pedigrees of keratoconus with various associations, the identified pathogenic mutations has shown various associations in familial keratoconus patients with different ethnicities (Aldave et al. 2006; Bisceglia et al. 2005; Heon et al. 2002; Liskova et al. 2007; Tang et al. 2008). The association between variations in the *VSX1* gene and keratoconus in Korean patients, however, has yet to be investigated. Therefore, it is of importance to identify the genetic factors that determine the susceptibility to keratoconus in Korean patients to gain insight into the pathogenesis of keratoconus. In particular, *VSX1* is expressed in vitro and in vivo during corneal wound healing and in the corneal stroma, which may shed light on the pathophysiological origin of keratoconus (Barbaco et al. 2006). In this study, we assessed the influence of *VSX1* variants on the incidence of sporadic keratoconus in Korean patients by screening the *VSX1* gene.

## Materials and methods

### Subjects

This study consisted of 249 unrelated keratoconus patients and 208 controls who were all of Korean ancestry. All keratoconus patients, and controls lacking corneal disease, were identified from the Korea Eye Tissue and Gene Bank Related to Blindness, Department of Ophthalmology, the Catholic University of Korea. Appropriate informed consent was obtained from each subject, and all studies were performed according to the tenets of the Declaration of Helsinki. The patients were diagnosed with keratoconus based on the following criteria: (1) symptoms of keratoconus (Munson sign, protrusion, Vogt's striae, corneal thickness, scarring, Fleishcher ring, photokeratotomy signs, video keratography signs, and refractive errors) and (2) medical histories (age, gender, contact lense wearing, eye rubbing, systemic disease, atopy, and connective tissue disease (McMahon et al. 2006; Rabinowitz 1998). Control individuals were selected from the general population without keratoconus.

### Mutation screening of the *VSX1* gene

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA blood kit (QIAGEN, Valencia, USA). All polymerase chain reaction (PCR) studies were carried out with 25 ng of genomic DNA as a template in a mixture of PCR buffer, 2.5 mM magnesium chloride (MgCl<sub>2</sub>), 200 nM deoxynucleotide triphosphate (dNTP), 0.4 pmol of each primer, and 0.75 U of h-Taq polymerase (Solgent, Daejeon, Korea). To search mutations in this gene, we designed seven sets of primers that were used to amplify the PCR products of the entire coding region as well as exon–intron junctions of the gene (Table 1) (Bisceglia et al. 2005). The genotypes of single nucleotide polymorphisms (SNPs) spanning the coding region of the *VSX1* gene were determined by single-strand conformational polymorphism (SSCP) analysis and direct sequencing. For SSCP, the amplified products were mixed with loading buffer with the addition of formamide, denatured at 95°C for 5 min, cooled in ice, and separated on a nondenaturing polyacrylamide gel. After electrophoresis, DNA bands were visualized by the silver staining method, and then those showing variation were directly sequenced. For DNA sequencing, amplified DNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, USA) and sequenced directly according to the protocols accompanying the BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The novel mutation G160V was confirmed by analysis of the *Bst*II restriction fragment length

**Table 1** Primer sequences for *VSX1* screening

Exon	Primer ID	Primers	bp
1	VSX1-EX1F	CAGCTGATTGGAGCCCTTC	599
	VSX1-EX1R	CTCAGAGCCTAGGGGACAGG	
2	VSX1-EX2F	GCACTAAAAATGCTGGCTCA	393
	VSX1-EX2R	GCCTCCTAGGAACTGCAGAA	
3	VSX1-EX3F	CATTAGAGGTGGGGTGTT	419
	VSX1-EX3R	TCTTGTGGTGCCTTCAGCTA	
4	VSX1-EX4F	GATCATGCTCGGGAGAGAAG	394
	VSX1-EX4R	CGTTGCTTTGCTTTGAAAAT	
5	VSX1-EX5F1a	AGATAGGCACTGACAAGGAC	304
	VSX1-EX5R1a	AGCTGGAGAGGTCAATAGC	
	VSX1-EX5F1b	CTTCAAAGAAGGTTCTAGCC	
	VSX1-EX5R1b	GGCATTGCATTTTATCTTGAC	

polymorphism (RFLP). Nucleotide sequences were compared with the published *VSX1* sequences (GenBank accession number NM\_014588).

### Statistics

To determine statistically significant differences between the groups by genotyping of SNPs and haplotypes, we used the  $\chi^2$  test and  $2 \times 2$  and  $2 \times m$  Fisher's exact test for the contingency table file. The  $2 \times 2$  contingency tables for each individual allele and the  $2 \times m$  contingency tables for each locus were used, where  $m$  refers to the number of marker alleles detected in the population. The Hardy–Weinberg equilibrium (HWE) of SNPs in patients and controls was calculated using the GenePop program. Values of  $p < 0.05$  were considered statistically significant. The strength of the association was estimated by odds ratio (OR) of risk and 95% confidence intervals (CI) (JavaStat, <http://members.aol.com/johnp71/ctab2x2.html>). Haplotype frequencies and linkage disequilibrium measures were estimated using the Haploview package (Barrett et al. 2005).

### Results

The 249 unrelated keratoconus patients and 208 controls recruited for this study had their entire *VSX1* gene coding region and the exon–intron junctions sequenced by PCR–direct sequencing. Screening in the coding region of the *VSX1* gene identified two heterozygous nonsynonymous mutations in exon 2 N151S (EX2 + 28A > G) and G160V (EX2 + 55G > T), and three heterozygous synonymous substitution, L176L (EX3 + 25G > A), A182A (rs12480307, EX3 + 43G > A) in exon 3 and G239G (EX4 + 90G > A) in exon 4 (Fig. 1). Exons 1 and 5 did not contain any mutations.

The allele and genotype distribution and OR of these variants are given in Tables 2 and 3. In exon 2, the heterozygous transversion of EX2 + 55G > T (G160V) lies near the positively charged *VSX1* nuclear localization signal and was present in 5.3% of unrelated keratoconus patients. Another nonsynonymous mutation, EX2 + 28A > G (N151S), was found in only one keratoconus patient (0.4%). Two nonsynonymous mutations, N151S and G160V, were not found in unaffected individuals from the control group.

Although the heterozygous mutations of EX3 + 25G > A (L176L) and EX3 + 43G > A (A182A) in the homeobox domain and EX4 + 90G > A (G239G) in the CVC domain were identified in affected individuals, these mutations were polymorphisms, the frequency of which did not differ significantly between cases and controls (Table 2).

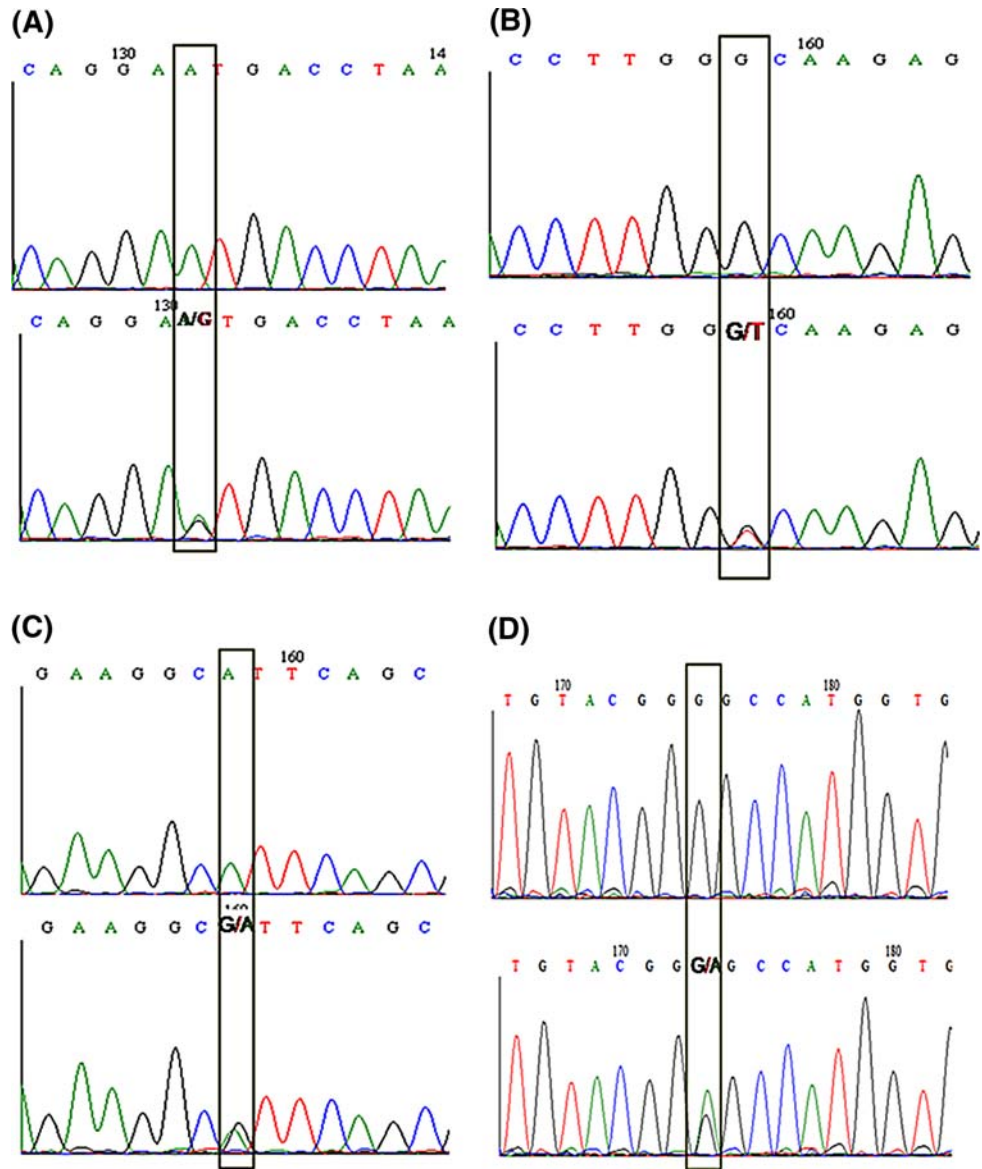
In this study, we also found four intronic nucleotide changes, IVS1-11 t > a, IVS3 + 22 c > t, IVS3-23 g > a (rs6138482), and IVS3 + 84 t > a (rs62217198) (Fig. 2, Table 3). The *IVS1-11\*t/a* genotype was significantly higher in keratoconus patients than in the control group (7.2 vs. 1.0%,  $p = 0.001$ , OR = 8.03, 95% CI: 2.042 << 31.432), and the *IVS1-11\*a* allele frequency was higher in keratoconus patients compared with controls (3.6 vs. 0.5%,  $p = 0.001$ , OR = 7.76 95% CI: 1.989–30.241). There were no significant genotype or allele frequency differences at IVS3 + 22 c > t, IVS3 + 23 g > a, and IVS3 + 84 t > a between keratoconus patients and control subjects. The genotype distributions of all variants of *VSX1* among the control subjects and the affected individuals were in Hardy–Weinberg equilibrium.

In haplotype analysis, we identified two haplotype block sets: IVS1-11t > a, N151S (EX2 + 28A > G), G160V (EX2 + 55G > T), L176L (EX3 + 25G > A) for block A and A182A (EX3 + 43G > A), IVS3 + 22c > t, IVS3 + 23 g > a, IVS3 + 84t > a for block B. The AAGG of block A was more prevalent in keratoconus patients than in control subjects and with higher risk to keratoconus ( $p = 0.004$ , OR = 5.85, 95% CI 1.475 << 23.151). The TATG of block A was present in only keratoconus patients ( $p < 0.001$ ), whereas AAGG ( $p = 0.001$ ) of block A and ACGT ( $p = 0.021$ ) of block B were less frequent in the keratoconus compared with the control individuals (Table 4).

### Discussion

The *VSX1* gene, also named the retinal inner nuclear layer homeobox gene, is part of a paired-like subfamily of homeodomain transcription factors and contains a CVC domain (Hayashi et al. 2000; Passini et al. 1998; Semina

**Fig. 1** Electropherograms showing mutations by direct sequencing of the *VSX1* gene exonic region: **a** N151S mutation, **b** G160 V mutation, **c** A182A polymorphism, **d** G239G polymorphism



et al. 2000). *VSX1* is expressed in the embryonic cranio-facial area and is restricted to cone bipolar cells of the inner nuclear layer, where it maintains function in the adult retina (Hayashi et al. 2005; Mintz-Hittner et al. 2004). Recently, Hosseini et al. (2008) reported the assessment of *VSX1* expression in neonatal human cornea during perinatal endothelial differentiation. Barbaro et al. (2006) suggested that *VSX1* is expressed in vitro and in vivo during human corneal wound healing, a process in which differentiation of corneal keratocytes into myofibroblasts occurs, but not in freshly obtained normal corneas. These results suggest a strong correlation between *VSX1* expressions and wound-healing responses.

The *VSX1* gene located within 20p11.21 of the candidate gene region for keratoconus and several pathogenic mutations in *VSX1* gene were also found in various corneal dystrophies such as keratoconus and posterior polymorphous

corneal dystrophy (PPCD, MIM122000) (Aldave 2005; Aldave et al. 2005; Heon et al. 2002). Heon et al. (2002) identified mutations in the *VSX1* homeobox gene in patients with either keratoconus or PPCD. The R166W mutation responsible for keratoconus altered the homeodomain and impaired DNA binding. This decreased DNA binding was demonstrated by an electrophoretic mobility shift assay. Two mutations, L159M and G160D, near the nuclear localization signal of the *VSX1* gene were associated with keratoconus and PPCD, respectively. Bisceglia et al. (2005) evaluated the role of the *VSX1* gene in a series of 80 keratoconus-affected Italian subjects. Three described missense mutations, D144E, G160D, and P247R, were identified, and the novel mutation L17P was found in 8.7% of unrelated patients and three families. This mutation is transmitted as an autosomal dominant trait with variable expressivity and incomplete penetrance. Although Aldave et al. (2006)

**Table 2** Genetic variations in the exonic region of the *VSX1* gene in Korean keratoconus patients

Nucleotide change	Codon	Minor allele	No. of carriers (%)			
			Keratoconus		Controls	
			Het.	Hom.	Het.	Hom.
EX2 + 28A > G	N151S	G	1 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)
EX2 + 55G > T	G160 V	T	13 (5.3)	0 (0.0)	0 (0.0)	0 (0.0)
EX3 + 25G > A	L176L	A	1 (0.4)	0 (0.0)	4 (2.0)	0 (0.0)
EX3 + 43G > A	A182A	G	7 (2.9)	1 (0.4)	2 (1.9)	0 (0.0)
EX4 + 90G > A	G239G	A	4 (1.7)	0 (0.0)	2 (1.0)	0 (0.0)

*Het* heterozygous genotype,  
*Hom* homozygous genotype

**Table 3** Allele and genotype frequencies in intragenic region of *VSX1* gene polymorphisms in the Korean keratoconus patients

Nucleotide change	Case		Control		<i>P</i> value	OR	95% CI
	<i>n</i>	%	<i>n</i>	%			
IVS1-11t > a							
tt	231	92.8	206	99.0	0.001	8.03	2.042 << 31.432
ta	18	7.2	2	1.0	0.001		
aa	0	0.0	0	0.0	0.984		
t	472	0.964	414	0.995	0.001	7.76	1.989 << 30.241
a	18	0.036	2	0.005	0.001		
IVS3 + 22c > t							
cc	243	99.2	197	100.0	0.502		
ct	2	0.8	0	0.0	0.052		
tt	0	0.0	0	0.0	0.998		
c	488	0.996	394	1.000	1.000		
t	2	0.004	0	0.000	1.000		
IVS3 + 23 g > a							
gg	43	17.5	49	24.9	0.077		
ga	119	48.8	91	46.2	0.633		
aa	83	33.9	57	28.9	0.304		
g	205	0.582	189	0.480	0.077		
a	285	0.418	205	0.520	0.077		
IVS3 + 84t > a							
tt	238	97.1	195	99.0	0.310		
ta	7	2.9	2	1.0	0.310		
aa	0	0.0	0	0.0	0.999		
t	483	0.986	392	0.995	0.312		
a	7	0.014	2	0.005	0.312		

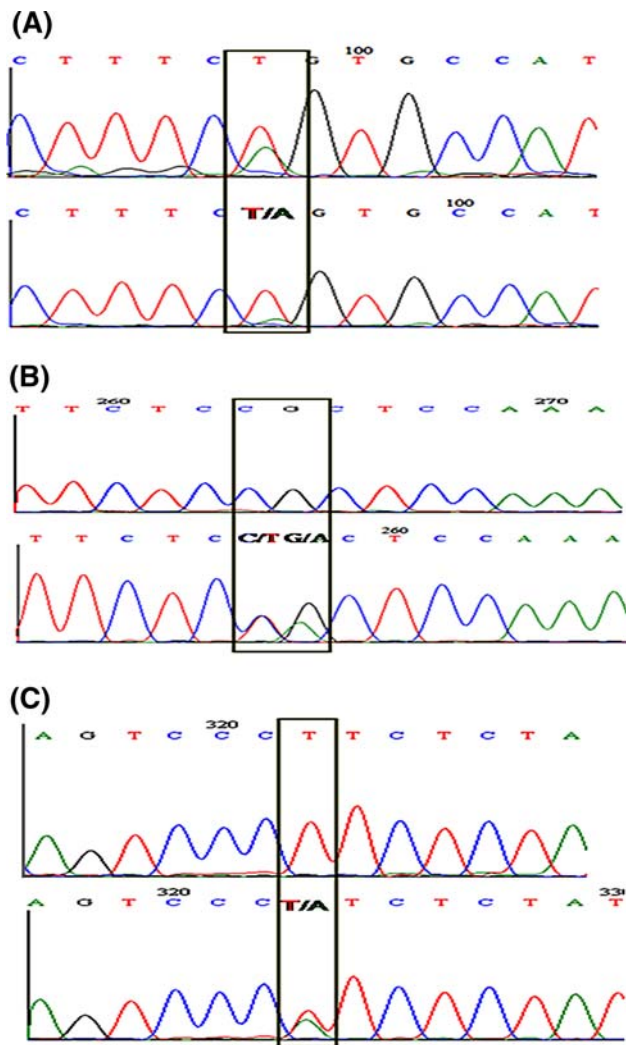
*OR* odds ratio, *CI* confidence interval

identified only one mutation, D144E, in a single affected keratoconus patient, D144E is a non-disease-causing polymorphism. Liskova et al. (2007) reported the D144E mutation and two novel intronic SNPs in a white British family, but these variants did not cosegregate with the disease phenotype. Tang et al. (2008) could not confirm the association of the three polymorphisms L159 M, R166 W, and H244R in the *VSX1* gene in a larger pedigree with keratoconus because the distribution of the three polymorphisms was not significant enough to support a pathogenetic role in keratoconus. Aldave et al. (2006) and Tang et al. (2008) reported that the previously identified pathogenic

mutations did not observe the association with sporadic keratoconus patients. Therefore, it is important to identify the genetic factors that determine susceptibility to keratoconus to gain insight into the pathogenesis of keratoconus in Korean patients because the susceptibility of mutations in *VSX1* can vary in different ethnicities.

In this study, we report the results of a mutation screening of the *VSX1* gene in unrelated Korean patients with keratoconus. Mutational analysis of the *VSX1* gene revealed six novel mutations, EX2 + 28A > G (N151S), EX2 + 55G > T (G160V), EX3 + 25G > A (L176L), EX4 + 90G > A (G239G), IVS1-11t > a, and IVS3

+ 22c > t, and three previously described changes, EX3 + 43G > A (A182A, rs12480307), IVS3 + 23G > A (rs6138482), and IVS3 + 84 t > a (rs622177198). Among



**Fig. 2** Electropherograms showing mutations by direct sequencing of the *VSX1* gene intronic region: **a** IVS1-11 t > a polymorphism, **b** IVS3 + 22 c > t and IVS3 + 23 g > a polymorphisms, **c** IVS3 + 84 t > a polymorphism

these mutations, the heterozygous two-base-pair transition of EX2 + 55G > T near the positively charged *VSX1* nuclear localization signal of exon 2 results in the substitution of glycine by valine (G160V). G160V was present in 5.3% of unrelated keratoconus patients, whereas it was not found in unaffected individuals from the control group or from any patients with other corneal diseases. The N151S mutation was present in 0.4% of unrelated keratoconus patients. In intragenic variants, *IVS1-11t/\*a* genotype ( $p = 0.001$ , OR = 8.03, 95% CI: 2.042 << 31.432) and *IVS1-11\*a* allele ( $p = 0.001$ , OR = 7.76, 95% CI: 1.989–30.241) frequencies were significantly higher in keratoconus patients than in the control group. With the haplotype analysis, the risk haplotypes, carrying *IVS1-11\*a* and EX2 + 55\*T, were identified in our Korean study population. The AAGG and TATG haplotypes of IVS1-11t > a, EX2 + 28A > G (N151S), EX2 + 55G > T (G160V), and EX3 + 25G > A (L176L) combination were significantly associated with keratoconus. However, the common TAGG haplotype was found to be significantly decreased in keratoconus. These results suggest a role of *VSX1* variants in the risk of unrelated Korean patients developing keratoconus and identify IVS1-11t > a and G160V variants as putative susceptibility variants. Both individual genotype data and corresponding haplotype data are consistent with a contribution of the IVS1-11t > a and G160V variants to keratoconus risk. Although in this study none of the mutations previously described as being mutations associated with keratoconus were identified in any of the affected patients, we found new mutations of the *VSX1* gene in unrelated Korean keratoconus patients were considerably different from other ethnic groups.

Our data is the first report of *VSX1* mutation screening in Korean keratoconus patients and detected novel missense mutation G160V and intragenic polymorphism IVS1-11t > a in the *VSX1* gene. These show strong statistical association with unrelated keratoconus patients. Consequently, the study suggests that two novel variants, IVS1-11t > a and G160V, in the *VSX1* gene seem to be possible

**Table 4** Haplotype combination frequencies by SNPs in *VSX1* gene

Haplotypes				Case	Control	$\chi^2$	P value
Block A							
IVS1-11t > a	N151S	G160 V	L176L				
T	A	G	G	0.940	0.985	11.24	0.001
A	A	G	G	0.029	0.005	6.93	0.004
T	A	T	G	0.021	0.000	8.29	<0.001
Block B							
A182A	IVS3 + 22c > t	IVS3 + 23 g > a	IVS3 + 84t > a				
A	C	A	T	0.578	0.519	3.04	0.080
A	C	G	T	0.400	0.475	4.95	0.029
G	C	G	A	0.014	0.005	1.84	0.312

candidate variants for keratoconus predisposition in unrelated Korean patients. However, it is not known how SNPs in the *VSX1* gene might control progression and onset of keratoconus, and they clearly require further analysis to elucidate their relevance to this impairment.

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