Original Article

The association between *VSX1* exon3 gene variants and keratoconus in Malaysian patients

Jenny P Deva, Yun F Ngeow, Thaw Zin

Purpose: This case-control study aims to examine possible associations of *VSX1* exon3 gene variants with the development of keratoconus (KC) in Malaysian patients. **Methods:** A case-control study was done on 42 keratoconus cases, 127 family member controls, and 96 normal controls. **Results:** Three gene variants, p.A182A, p.P237P, and p.R217H showed significant associations with keratoconus ($P < 0.05$). While p.A182A and p.P227P were more prevalent than in the family and normal controls (OR 3.14–4.05), the reverse was observed with p.R217H (OR 0.086–1.59). With Haploview analysis, p.A182A and p.P237P were shown to be in linkage disequilibrium (LD) (LOD (logarithm of the odds score) score of 2.0, r2 of 0.957, and 95% confidence interval (CI) of 0.96–1.00). **Conclusion:** The study results suggest that the p.A182A and p.P237P variants could have contributed to the development of keratoconus in some Malaysians and that these two variants are likely to be co-inherited. In contrast, the p.R217H variant appeared to confer some protection against the development of keratoconus.

Key words: Gene variants, keratoconus, keratoconus genetic risk factor, VSX1

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Keratoconus (KC) has been a well-known corneal ectatic degeneration for more than 150 years.[1] Worldwide, the incidence of this disease has been reported to be around 1:2000 and the prevalence is as high as 54.5 per 100,000 population.[2] A recent meta-analysis which included 50 million individuals from 50 countries, estimated the global prevalence to be 138 per 100,000 population indicating an increase in incidence or improved disease detection.[3]

Keratoconus is characterized by a progressive thinning of the cornea resulting in high myopic astigmatism and reduced visual acuity, typically starting in early adolescence and progressing until the mid-thirties in age.[4,5] Researchers have repeatedly claimed a multifactorial etiology involving genetics as well as cultural, environmental, and other factors but to date, the etiopathogenesis of keratoconus remains an enigma.[5,6]

In the "polygenic" theory hypothesized for keratoconus, different candidate genes and variants were identified as possible risk factors. *VSX1* is one of the most frequently researched candidate genes.[2,7] It regulates the functions of cone opsin genes, controls activation of the red-green visual pigment in the locus control region, and is involved in retinal and anterior eye development.[8] It is also expressed in corneal tissues and is thought to play a role in corneal wound healing.[9] Heon *et al*.[10] were the first to identify *VSX1* mutations in patients with keratoconus and posterior polymorphous corneal dystrophy (PPCD). Their observations led to the postulation that mutations in *VSX1* were causative factors of this disease. Subsequently, both exogenic and intragenic polymorphisms were found to be associated with keratoconus, and studies in different ethnic groups showed the presence of *VSX1* variants in patients from different populations.[11-13] Tanwar *et al*.[14] identified four *VSX1* variants, p.A182A (c.546A>G), p.P237P (c.627+84T>A), p.R217H (c.627+23G>A), and c504-24C>T. In Tanwar's study, while two of the variants p.A182A and p.P237P were present in both keratoconus cases and controls, the other two, p.R217H and c.504-24 C>T were present only in patients, implying a pathogenic role for the latter two variants. Shetty *et al*.[15] identified two novel missense substitutions p.R166W and p.L159M. Regrettably, despite a lot of research work done on *VSX1* variants, there is still a lack of consistent association confirming the relationship between gene variants and pathogenicity in keratoconus. This could be due to the small number of cases analyzed in most studies conducted so far.

In Malaysia, few genetic studies on keratoconus have been reported as yet. Only two different eye disease studies have alluded to the incidence of this disease in the Malaysian population. Reddy *et al*.[16] reported only four (0.3%) keratoconus patients among 1169 eye cases screened. Another study by Mohd-Ali *et al*.[17] identified only 159 cases after screening 13,000 patients (1.2%). In the only molecular characterization study, Ng studied the association of *VSX1* exon3 variants with clinical presentations of keratoconus, to better understand their molecular characterization and role in

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the pathogenesis of the KC disease.[18] He found p.A182A and p.P237P to be closely linked to each other, in a single haplotype block. Similar findings were also mentioned in a Korean study by Mok *et al*.[11]

Methods

Ethical approval

This study obeyed the tenets of the Declaration of Helsinki and was approved by the Institutional Scientific and Research Review Board (UTAR IRB FMHS Oct. 22, 2013) of Universiti Tunku Abdul Rahman, Sungai Long Campus, Malaysia.

Study populations

For this case-control study, 42 patients who were diagnosed with keratoconus for the first time, 126 unaffected family controls, and 97 other normal controls were recruited. Patients were diagnosed based on clinical features such as the presence or absence of corneal thinning, Vogt's striae, Fleischer's ring, Munson's sign, topographical details, and refractive findings. Family member controls were those who were screened for keratoconus because they were blood relatives of keratoconus cases. Normal controls were college students who volunteered to be screened for eye refractive disorders and were not found not to have keratoconus.

Recruitment of participants

Keratoconus patients were recruited after their diagnosis was confirmed in an ophthalmology clinic. Their family members were invited to participate in the study when they visited the same clinic for eye screening. Similarly, college students were recruited when they turned up at a community eye screening event. After obtaining informed consent, each participant was asked to answer a questionnaire, which included questions on age, gender, family history, medical history including atopy, and the participant's habit of eye rubbing.

Blood collection and DNA analysis

From each participant, finger prick blood was collected onto an FTA card (Whatman, Classic) for transportation to the laboratory where genomic DNA was extracted, purified, and amplified in a polymerase chain reaction (PCR) assay using primer pairs specific for the *VSX1* exon 3 (Forward primer 5′-3′CATTCAGAGGTGGGGTGTT; Reverse primer 5′-3′ TCTTGTGGTGCCTTCAGCTA) and carried out on an Eppendorf mastercycler (Westbury, NY). PCR products were sequenced on an ABI-3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing data were analyzed using BLAST software and compared against reference sequences for *VSX1* (Gen Bank accession number: NM_014588) using an alignment software (Sequencher® ver. 5.1.0, Gene Codes Corporation, Ann Arbor, MI, USA).[19] The variations identified were evaluated using Alamut software version 2.1e (Interactive Biosoftware, Rouen, France). The nomenclature, location, and classification of variations were done based on Alamut output (Apical Science Outsource Company input).

Statistical analysis

The SPSS Statistic software (IBM, United States) and Microsoft Excel software (Microsoft Corporation, United States) were used for statistical analyses. The three genetic models (dominant, recessive, and additive) were used in SNPSTATs, to assess the association of single nucleotide polymorphisms (SNPs) with the risk of keratoconus. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by unconditional logistic regression analyses, and adjusted for age and sex. All SNPs were examined for genetic variation patterns, using the haplotype association test.

Results

A total of 42 keratoconus cases, 127 family member controls, and 96 normal controls were recruited. The DNA sequencing analysis of blood samples from all participants revealed only four nucleotide changes [Table 1] which have been previously reported by other researchers.[14,15] The distribution of these variants found in this study is summarized in Table 2.

As the variant c.504-24C>T was rarely seen among both cases and controls, it was dropped from further discussion. With reference to Table 2, the other three variants p.A182A, p.P237P, and p.R217H were examined for their role as risk factors for keratoconus development. All three variants conformed to Hardy–Weinberg equilibrium (HWE) $P > 0.05$. Both the p.A182A and p.P237P variants were seen in about a third (33.3%) of keratoconus patients compared to 12–15% in the controls. The p.R217H on the other hand was more in controls (59.72%) than in patients (42.83%). The Odds Ratios

Table 1: Sequence variants observed in *VSX1* exon

SNP ID	VSX transcript ID	c.DNA change	VSX1 protein ID	Amino acid change
rs12480307	NM_014588	c.546A>G	NP_055403	p.A182A
rs56157240	NM_014588	c.627+84T>A	NP_055403	p.P347P
rs6138482	NM_014588	c.627+23G>A	NP_055403	p.R217H
(IVS3-24C)	NM_014588	c.504-24C>T	NP_055403	-

Table 2: The frequencies of *VSX1* gene variants in keratoconus cases and controls

Study groups (*n*)	p.A182A	p.P237P	p.R217H	IVS3-24C
	c.546A>G	c.627+84T>A	c.627+23G>A	c.504-24C>T
	(*n*, %)	(*n*, %)	(*n*, %)	(*n*, %)
Keratoconus (42)	14, 33.3	14, 33.3	18, 42.83	3, 7.14
Family Control (126)	20, 15.74	20, 15.74	13, 10.24	1, 0.78
Normal Control (97)	12, 12.37	12, 12.37	48, 49.48	6, 6.18

calculated [Table 3] indicated 3–4 times increased risk of keratoconus for both p.A182A and p.P237P when compared to family and normal controls. The reverse was seen with p.R217H (OR 0.082, *P* value < 0.0001 against normal controls), which implied a possible "protective" role for this variant against the development of keratoconus.

An interesting finding was the constant pairing of the two SNPs, rs 12480307 in variant p.A182A and rs 56157240 in p.P237P. This pairing was further studied using the Haploview software, which is a bioinformatics software, designed to estimate haplotype frequencies and analyze linkage disequilibrium (LD) in genetic data.[20] With this software, the likelihood that the chromosomal sites of rs12480307 and rs56157240 were near enough to each other, for them to be inherited together was assessed. For this assessment, the LOD (logarithm of the odds score), confidence interval of D' values, and MAF (minor allele frequencies) were calculated. A LOD score of 3 or higher indicates that there is at least a 1000:1 likelihood that the two SNPs are linked and therefore inherited together.

The haplotype block 1 [Fig. 1] included four SNPs, of which, only rs12480307 (SNP2) and rs56157240 (SNP3) were shown to be in LD in Block 1. The Haploview analysis revealed three haplotype matchups based on the alleles from these two SNPs. The three matchups were AT (89.9%), GA (9.7%), and AA (0.4%). For the AA and GA haplotypes, there was no difference between patients and controls ($P > 0.05$). However, the AT haplotype showed a difference in distribution between patients and controls ($P = 0.0354$). The results from χ^2 and allelic analysis for all *VSX1* SNPs using Haploview were similar to the results from the χ^2 analysis from SPSS.

Clinical topographic findings and features in the relatives of keratoconus patients

It was very interesting too, to have observed a few special clinical characteristics of family members, like myopia, which included, moderate myopia with moderate myopic astigmatism, which was "regular," meaning mostly 90° or 180° axis, unlike keratoconus astigmatism, which was typically skewed or irregular. Also in KC the degree of myopia and astigmatism was higher, with keratometry ranging from -45.00 to -70.00 diopters and high astigmatism , between -3.00 to -10.00 diopters or more. Family Control keratometry range however, was average >45.00 to -47.00 diopters. Astigmatism was also lower by-1.00 to -3.00diopters. In addition, KC patients showed lower pachymetry readings than controls, ranging from 350μ-450μ, as expected.[21]

In addition, vision could easily be corrected, with spectacles or soft lenses for family and relatives. Whereas, KC cases needed the extra advantage of the smooth anterior spherical surface of hard or rigid gas permeable contact lenses, to overcome the irregular keratoconic anterior cornea, to even attain a good vision of 6/6.

Family pachymetry was normal range 450–550 range, unlike thin keratoconic cornea.

Perhaps this study is too small, for us to adequately and properly correlate and study to explore whether the absence or presence of KC findings was influenced by the type of mutation found in the VSX gene in KC patients. Future studies will definitely help to clarify all these doubts and enable keratoconus to become even more treatable, through gene therapy.

Discussion

Mutations in *VSX1* have been described as pathogenic (contributing to the keratoconus phenotype) in some populations but non-pathogenic in other populations.[12,15,22-24]

In this study, all four *VSX1* variants were present in keratoconus patients and control groups but at different frequencies. Two of them, p.A182A and p.237p, appeared to be pathogenic (OR of 3.14 and 3.61, respectively) whereas p.R217H was not (OR of 0.082). This finding contradicted the

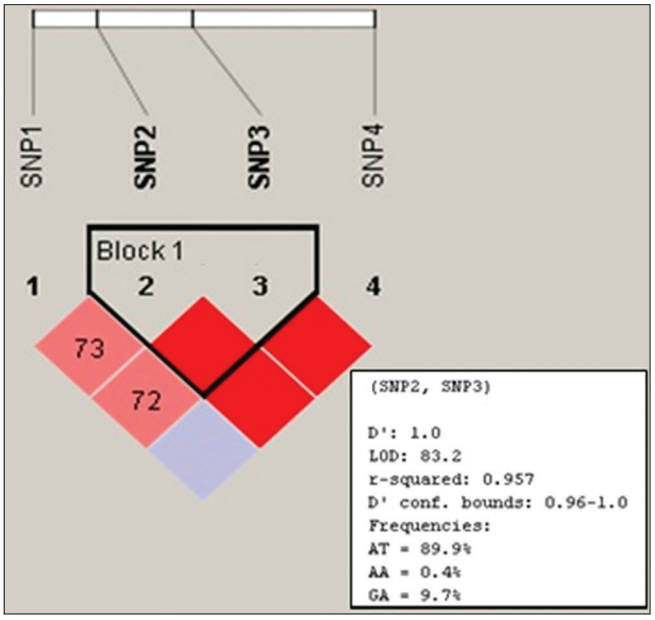

Figure 1: Haplotype block (Block 1) generated with Haploview. LOD score >2.0; r2 = 0.95; MAF>0.05; and D'= 1.0. SNP1 (c.504-24C>T),; SNP2, rs12480307; SNP3, rs56157240; SNP4, rs6138482

Table 3: Strength of association between *VSX1* exon3 SNPs and keratoconus

Comparison Group	*VSX1* exon3 SNP	Position	Minor Allele	Dominant Allele	*P*	OR	L95	U95
Normal Control	rs12480307	25059546	G	A	0.026	3.14	1.14	8.6
	rs56157240	25059381	T	A	0.012	3.61	1.13	9.94
	rs 6138482	25059442	C	T	0.04	0.082	0.031	0.237
Family Control	rs12480307	25059546	G	A	0.029	3.78	1.11	12.79
	rs56157240	25059381	T	A	0.02	4.05	1.21	13.56
	rs6138482	25059442	C	T	0.33	1.59	0.62	4.67

results of Tanwar *et al*.[14] which showed p.R217H as occurring more frequently in keratoconus cases. However, many observations were consistent with those reported by Wang *et al*.[2] for the Han population in China, although the Malaysian participants were from different ethnic groups comprising Indians, Malays, and Chinese. In their study of 97 cases and 107 normal controls, Wang *et al*.[2] showed a positive association of four SNPs with keratoconus, two of which were rs12480307 and rs56157240 which were found in this study. Likewise, their data also implied the pairing of these two SNPs, and a "possible protective effect" with rs6138482 (p.R217H). However, instead of the "AT" genotype in rs12480307 and rs56157240 found to be possibly pathogenic in this study, they showed the "AA" genotype of rs2071376 to be at risk for keratoconus.

The Haploview findings are similar to those from a Korean study in which the authors also reported the association of the three SNPs (rs12480307, rs56157240, and rs6138482) in a haplotype block.[11] Additionally, the sequence variants corresponding to these three SNPs (c.546A>G, c.627+84T>A, and c.627+23G>A) were also among the seven variants in the haplotype block described by Ng *et al*.[24]

The main limitation of this study is the small sample size of keratoconus cases. No separate analyses were done for ethnicity and gender as the small number of cases would not allow for significant differences. However, some similarities could be seen comparing the research work done by others.[25-27] The association with mutations in other candidate genes for keratoconus such as the COL4A3 and SOD1 genes was not analyzed because previous studies had shown negligible numbers of variants in these two genes among Malaysian patients and the general public.[28]

Conclusion

The etiopathogenesis of keratoconus is still inadequately investigated. Although genetic studies have identified various possible pathogenic gene variants, the significance of these findings is still largely controversial. In this study on *VSX1* exon 3, we corroborated the findings of other researchers that a pair of SNPs (rs12480307 and rs56137240) might be important for the development of keratoconus while other SNPs such as rs 6138482 might be protective against keratoconus.

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Conflicts of interest

There are no conflicts of interest.

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