Visual System Homeobox 1 (VSX1) Gene Analysis in Keratoconus: Design of Specific Primers and DNA Amplification Protocols for Accurate Molecular Characterization

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SUMMARY

Background: Keratoconus is an ocular degeneration characterized by the thinning of corneal stroma that may lead to varying degrees of myopia and visual impairment. Genetic factors have been reported in the pathology of keratoconus where Asians have a higher incidence, earlier onset, and undergo earlier corneal grafts compared to Caucasians. The visual system homeobox 1 (VSX1) gene forms part of a paired-like homeodomain transcription factor which is responsible for ocular development. The gene was marked as a candidate in genetic studies of keratoconus in various populations. Single nucleotide polymorphisms (SNPs) in the VSX1 gene have been reported to be associated with keratoconus. The detection of the SNPs involves DNA amplification of the VSX1 gene followed by genomic sequencing. Thus, the objective of this study aims to establish sensitive and accurate screening protocols for the molecular characterization of VSX1 polymorphisms.

Methods: Keratoconic (n = 74) and control subjects (n = 96) were recruited based on clinical diagnostic tests and selection criteria. DNA extracted from the blood samples was used to genotype VSX1 polymorphisms. In-house designed primers and optimization of PCR conditions were carried out to amplify exons 1 and 3 of the VSX1 gene. PCR conditions including percentage GC content, melting temperatures, and differences in melting temperatures of primers were evaluated to produce sensitive and specific DNA amplifications.

Results: Genotyping was successfully carried out in 4 exons of the VSX1 gene. Primer annealing temperatures were observed to be crucial in enhancing PCR sensitivity and specificity. Annealing temperatures were carefully evaluated to produce increased specificity, yet not allowing sensitivity to be compromised. In addition, exon 1 of the VSX1 gene was amplified using 2 different sets of primers to produce 2 smaller amplified products with absence of non-specific bands. DNA amplification of exons 1 and 3 consistently showed single band products which were successfully sequenced to yield reproducible data.

Conclusions: The use of in-house designed primers and optimized PCR conditions allowed sensitive and specific DNA amplifications that produced distinct single bands. The in-house designed primers and DNA amplification protocols established in this study provide an addition to the current repertoire of primers for accurate molecular characterization of VSX1 gene polymorphisms in keratoconus research.

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VSX1, keratoconus, Malaysia, polymorphism, primer design

INTRODUCTION
Keratoconus (OMIM: 148300), a progressive non-inflammatory eye disorder, is characterized by the conical shape of the patient's cornea as a result of eye protrusion due to thinning of the stromal layer. This disorder may lead to varying degrees of myopia and visual impairment [1]. Affecting either one or both eyes, the severity of the disease and clinical signs presented can vary depending on its progression level. Keratoconus is also associated with several ocular and systemic diseases, such as Down's syndrome, posterior polymorphic dystrophy, Leber's congenital amaurosis, and connective tissue disorders [2-4]. The estimated prevalence of keratoconus worldwide is 50 - 230 per 100,000 in the population, with a fourfold higher rate in Asians [5]. In a limited study in Malaysia, an estimate of one in a hundred patients referred for eye-related problems were confirmed to have keratoconus [6].

Most cases of keratoconus are sporadic. However, cases which involve genetic inheritance and ethnicity have been reported [1,7]. Generally, Asians have a higher incidence, earlier onset, and undergo earlier corneal grafts compared to Caucasians [5,7]. In addition, family history and consanguinity, gender, ultraviolet (UV) exposure, and atopy are potential factors that can affect prevalence and incidence rates [5,6,8-12].

A number of genetic studies have reported an association between genetic factors and keratoconus. The visual system homeobox 1 (VSX1) gene, a paired-like homeodomain transcription factor, is located on chromosome 20 [13]. The VSX1 gene comprises five main exons spanning about 6.6 kilobases (kb). The nuclear localization signal homeodomain and CVC domains are features in the VSX1 gene coding region involved in the development of retina and anterior segment of the eye and play a role in activation of the red-green visual pigment of the eyes [14,15]. The VSX1 gene has alternate splicing sites that produce different transcripts [14] and single nucleotide polymorphisms (SNPs) have been reported within the five exons, introns, and untranscribed regions. SNPs in the VSX1 gene were implicated in keratoconus cases in Italian, Canadian, and Korean populations [4,8,16]. However, SNP studies by other investigators showed the mutations to be non-pathogenic [9,10,17,18]. Thus, the VSX1 gene is an interesting gene complex to study because of reported SNPs that have been associated with predisposition and progression of keratoconus in specific populations.

In Malaysia, the focus on keratoconus is on the clinical aspects whereby higher numbers of Malays and Indians were observed to be affected compared to Chinese, and with a higher prevalence in males [6]. Most of the Malaysian keratoconus patients exhibit asthma, which is one of the common atopy factors associated with keratoconus [5,6,19]. Thus, although the clinical signs are understood, the genetic basis of this disease has not been fully elucidated in Malaysia. The objective of this study aims to establish a sensitive and accurate screening platform for the molecular characterization of VSX1 polymorphisms.

MATERIALS AND METHODS
Subjects
A total of 74 confirmed unrelated keratoconus patients who attended the Ophthalmology Clinic, University Malaya Medical Centre (UMMMC) and the Ophir Eye Clinic and Surgery, Malaysia were recruited for this study. In addition, 96 control subjects without keratoconus were also recruited during eye-screening drives. Demographic characteristics of study subjects were recorded. A series of clinical eye tests was performed in order to classify subjects as keratoconic or non-keratoconic. The clinical tests included a visual acuity test using Snellen’s chart, a test for refractive power using Zeiss ATLAS model 900 (Carl Zeiss, California, USA), corneal topography using Zeiss Visante OCT model 1000 (Carl Zeiss, California, USA), and a biomicroscopy test using models BP900® LED powered and AT900® (Haag-Streit AG, Koeniz, Switzerland). Participation was voluntary with written and informed consent obtained from all patients/guardians before blood collection. This study was approved by the Medical Ethics Committee, UMMC (MEC reference number: 775.8) in accordance with the Declaration of Helsinki.

Inclusion and exclusion criteria
A subject was categorized as keratoconic when a minimum K-reading cutoff value of 45 D and maximum pachymetry reading cutoff value of 450 µ was observed. A subject was a suspected keratoconic when the pachymetry reading was between 450 - 500 µ. Normal individuals possessed K-readings of ≤ 44 D and pachymetry readings of > 500 µ. Patients with ocular diseases such as cataract and glaucoma were excluded. Patients having systemic diseases such as diabetes and heart diseases were also excluded.

DNA extraction and purification
Blood was collected in EDTA tubes. The blood samples were immediately stored in ice in cooler boxes until arrival in the laboratory. Blood tubes were then transferred to a -20°C freezer and DNA extraction was carried out the following day. Blood tubes were thawed at room temperature and DNA was extracted using the conventional phenol-chloroform method. Purified DNA was solubilized in double distilled water and aliquots were kept at -80°C for storage and 4°C for molecular characterization. The concentration and purity of extracted DNA were measured using a spectrophotometer at
Table 1. Primer sequences for DNA amplification of the VSX1 gene, DNA amplified product size and PCR conditions.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence 5’ → 3’</th>
<th>PCR product (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 *</td>
<td>VSX1-1aF: 5’-ACTTTTTCAGGGA CAGGCAG-3’</td>
<td>320</td>
<td>Denaturation: 95°C; 1 minute Annealing: 61°C; 30 seconds Extension: 72°C; 1 minute Final extension: 72°C; 7 minutes</td>
</tr>
<tr>
<td></td>
<td>VSX1-1aR: 5’-CCTCTCTGTAGGAC GTTTGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VSX1-1bF: 5’-TCCGCAACGGGTC CAGAG-3’</td>
<td>492</td>
<td>Denaturation: 94°C; 1 minute Annealing: 60°C; 30 seconds Extension: 72°C; 1 minute Final extension: 72°C; 7 minutes</td>
</tr>
<tr>
<td></td>
<td>VSX1-1bR: 5’-GAAGCGCGGCGCT GATTA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 #</td>
<td>VSX1-2F: 5’-GCACTAAAAATGCTG GCTA-3’</td>
<td>393</td>
<td>Denaturation: 95°C; 1 minute Annealing: 59°C; 30 seconds Extension: 72°C; 1 minute Final extension: 72°C; 7 minutes</td>
</tr>
<tr>
<td></td>
<td>VSX1-2R: 5’-GCCTCCTTAGAACGT CAGAA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 *</td>
<td>VSX1-3F: 5’-TTCCAGAGGTGGGGTG TTCCA-3’</td>
<td>417</td>
<td>Denaturation: 93°C; 1 minute Annealing: 60°C; 30 seconds Extension: 72°C; 1 minute Final extension: 72°C; 7 minutes</td>
</tr>
<tr>
<td></td>
<td>VSX1-3R: 5’-TCTTGTGTGGCTTCTT AGCTA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 #</td>
<td>VSX1-4F: 5’-GATCATGCTCGGGA GAGAAG-3’</td>
<td>394</td>
<td>Denaturation: 95°C; 1 minute Annealing: 61°C; 30 seconds Extension: 72°C; 1 minute Final extension: 72°C; 7 minutes</td>
</tr>
<tr>
<td></td>
<td>VSX1-4R: 5’-CGTTGCTTTGCTTTGG AAAT-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* - In-house designed primers, # Published primer sequences and PCR products from Owens H et al. [20].

Molecular genotyping
Genotyping was carried out to detect DNA amplification products in the coding region including four exon-intron junctions of the VSX1 gene. DNA amplification was carried out using four previously reported primer pair sequences [20]. DNA amplification was successful in exons 2 and 4 while inconsistent results were obtained in exons 1 and 3. Thus, three novel sets of primers for VSX1 polymorphisms were designed - 2 sets for exon 1 and one set for exon 3. In addition, in-house PCR protocols to enhance sensitivity and specificity were also developed for DNA amplification using the new primers. These primers were designed using the PrimerBasic Local Alignment Search Tool (Primer-BLAST), an online web tool on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [21]. The primer sequences used in this study, PCR product size, and PCR conditions are listed in Table 1. DNA amplification was carried out using a thermal cycler (Veriti, Applied Biosystems, Foster City, CA, USA). The PCR reaction mixture contained 200 ng of DNA template, 0.5 μM of each primer, 1.5 mM MgCl₂, 200 μM of each dNTP, and 1 unit of Taq DNA polymerase in a final reaction volume of 25 μL (Promega GoTaq® Green Master Mix (Promega, Madison, WI, USA). DNA amplified products were observed by electrophoresis using 2% w/v pre-stained ethidium bromide agarose gels. All PCR products were purified and then subjected to genomic sequencing for detection of SNPs using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing data was compared against a reference sequence for VSX1 (GenBank: NG_008101.1) using an alignment software (Sequencher® ver.5.1.0, Gene Codes Corporation, Ann Arbor, MI, USA).

RESULTS
Confirmation of keratoconus was carried out by ophthalmologists using clinical tests and suspected keratoconus patients were excluded from this study. DNA from a total of 74 confirmed keratoconus patients and 96 non-keratoconus individuals were used for optimization studies for VSX1 gene analysis. DNA was amplified using published and in-house designed primer sequences followed by genomic sequencing to detect VSX1 polymorphisms.

DNA amplification using published primer sequences and PCR conditions for exons 2 and 4 produced distinct PCR products without any need for optimization. However, published primer sequences and PCR conditions for exons 1 and 3 produced inconsistent results showing no amplification or weak PCR products. Thus, new primer sequences were designed for DNA amplification for exons 1 and 3 together with further optimization of

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Table 2. Single nucleotide polymorphisms (SNPs) reported from various populations and SNPs confirmed in this study.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Published SNPs * (in both patients and controls)</th>
<th>Patient DNA (n = 74)</th>
<th>Control DNA (n = 96)</th>
</tr>
</thead>
</table>

* - References: [4,8,16,22,24-26,28,29].

Table 3. Comparison of parameters used in primer design for amplification of exons 1 and 4 in the VSX1 gene.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Published study *</th>
<th>Current study</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSX1 exon 1</td>
<td>VSX1-EX1</td>
<td>VSX1-1a</td>
</tr>
<tr>
<td>1 PCR product size</td>
<td>599 bp</td>
<td>320 bp</td>
</tr>
<tr>
<td>2 Forward primer: GC content (%)</td>
<td>57.89</td>
<td>55.00</td>
</tr>
<tr>
<td>3 Melting temperature</td>
<td>58.21°C</td>
<td>59.96°C</td>
</tr>
<tr>
<td>4 Reverse primer: GC content (%)</td>
<td>65.00</td>
<td>60.00</td>
</tr>
<tr>
<td>5 Melting temperature</td>
<td>60.76°C</td>
<td>60.46°C</td>
</tr>
<tr>
<td>6 Difference in melting temperature</td>
<td>2.55°C</td>
<td>0.50°C</td>
</tr>
</tbody>
</table>

| VSX1 exon 3                   | VSX1-EX3          | VSX1-3        |
| 1 PCR product size            | 419 bp            | 417bp         |
| 2 Forward primer: GC content (%) | 52.63            | 55.00         |
| 3 Melting temperature         | 56.94°C           | 60.70°C       |
| 4 Reverse primer: GC content (%) | 50.00            | 50.00         |
| 5 Melting temperature, °C     | 58.94°C           | 58.94°C       |
| 6 Difference in melting temperature | 2.00°C        | 1.76°C        |

* - Data for published study was obtained from Owens H et al. [20].

annealing temperatures to increase specificity. Using the in-house designed primers and optimized PCR conditions, DNA amplification was successfully carried out for exon 1 producing 2 PCR products of 320 bp (Figure 1A lanes 2, 7, 8, and 9) and 492 bp (Figure 1B, lanes 2 - 8), and exon 3 where a 417 bp product was amplified (Figure 1C, lanes 2 - 9). Initial annealing temperatures at 59°C (Figure 1A, lanes 5 and 6) and 60°C (Figure 1A, lanes 3 and 4) to increase sensitivity produced non-specific PCR products in exon 1, thus an optimal annealing temperature of 61°C was selected which produced reproducible and specific distinct PCR products. Amplified products from the 4 exons in the 74 confirmed keratoconus patients and 96 control subjects were purified and sequenced. Table 2 shows the 25 reported SNPs in the VSX1 gene and the 7 SNPs identified in this study. The electropherograms for p.L17V in exon 1 and p.A182A in exon 3 are shown respectively in Figures 2A and 2B. The seven SNPs detected in this study were p.L17V (exon 1), p.G160V (exon 2), p.G239R (exon 4) and 4 SNPs: p.A182A, c.627+84T>A, c.627+23G>A and c.504-24C>T in exon 3.
Molecular Technique for Characterization of VSX1 in Keratoconus

Figure 1A. Gel electrophoresis of PCR products amplified from exon 1 in the VSX1 gene using primer set 1A.

Lanes 1 and 11 - 100 bp DNA marker, lane 2 - 320 bp amplified product from normal control DNA (annealing temperature - 61°C), lanes 3 and 4 - 320 bp amplified products with non-specific amplified bands (annealing temperature - 60°C), lanes 5 and 6 - 320 bp amplified products with non-specific amplified bands (annealing temperature - 59°C), lanes 7 - 9 - 320 bp products amplified with DNA from keratoconus patients, lane 10 - PCR control where no DNA was added.

Figure 1B. Gel electrophoresis of PCR products amplified from exon 1 in the VSX1 gene using primer set 1B.

Lanes 1 and 10 - 100 bp DNA marker, lanes 2 - 4 - 492 bp amplified products from normal control DNA, lanes 5 - 8 - 492 bp products amplified with DNA from keratoconus patients, lane 9 - PCR control where no DNA was added.

Figure 1C. Gel electrophoresis of PCR products amplified from exon 3 in the VSX1 gene using primer set 3.

Lanes 1 and 11 - 100 bp DNA marker, lanes 2 - 4 - 417 bp amplified products from normal control DNA, lanes 5 - 10 - 417 bp products amplified with DNA from keratoconus patients, lane 9 - PCR control where no DNA was added.
DISCUSSION

The protein encoded by the VSX1 gene contains a paired-like homeodomain and binds to the core of the locus control region of the red/green visual pigment gene cluster. The encoded protein may regulate expression of the cone opsin genes early in eye development. Polymorphisms in this gene have been implicated in two corneal disorders, namely posterior polymorphous corneal dystrophy and keratoconus [4]. Genetic studies of polymorphisms in the VSX1 gene are controversial due to equivocal findings [4,8,16,18,20,22-29].

The main objective of designing in-house primer sequences for exons 1 and 3 was to enhance sensitivity and specificity for DNA amplification. Primer sequences which were effective in reported studies [8,16,18,20] were not successful for DNA amplification for exons 1 and 3 in this study. PCR efficiency is dependent on various parameters which include primer design, PCR inhibitors, number of PCR cycles, and even the type of thermal cycler used [30,31]. In this study, two PCR reactions were carried out for exon 1 instead of one reaction [20]. Primer design was aimed at amplifying the VSX1 exon 1 to produce two shorter PCR products in order to increase sensitivity and specificity, thus leading to enhanced DNA amplification. For VSX1 exon 3, the primer specificity was increased by redesigning the forward primer, whereas the reverse primer was maintained as the same sequence used in previously published studies [20].

Several other parameters in primer design, GC content, melting temperature, and differences in melting temperature between forward and reverse primers, were also taken into account during primer design and optimization of PCR conditions (Table 3). The GC content of the exon 1 reverse primer was 65% in previous studies [8,16,18,20], and excess GC content has been reported to influence the annealing temperature. The optimum GC content should be between 40 - 60% as GC content also influences primer specificity [32]. The GC content of the reverse primer in exon 1 was reduced to 60%. Other favorable factors in primer design include similar melting temperatures between forward and reverse primers. In this study, the GC content of in-house designed primers in exon 3 was between 50 - 60% and the difference in melting temperatures between the forward and reverse primers was between 0.50 - 1.76°C.

CONCLUSION

The in-house designed primers and optimized PCR conditions were more specific and yielded reproducible distinct DNA amplifications. This produced good quality sequencing data that allowed optimal detection of SNPs necessary for accurate molecular characterization of VSX1 gene polymorphisms in keratoconus research.

Authors’ Contribution:

JBN and AYLL performed the molecular analysis. JBN wrote the paper for this study. RYYP and JAMA supervised the project and revised the article. KRL, VS, and JPD performed the clinical tests to confirm keratoconus. All authors have read and approved the final version of this manuscript.
Molecular Technique for Characterization of VSX1 in Keratoconus

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Declaration of Interest:
The authors declare that they have no competing interests.

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