

No *VSX1* Gene Mutations Associated with Keratoconus

Anthony J. Aldave,¹ Vivek S. Yellore,¹ Andrew K. Salem,¹ Gina L. Yoo,¹ Sylvia A. Rayner,¹ Huiying Yang,² George Y. Tang,² Yoana Piconell,² and Yaron S. Rabinowitz²

PURPOSE. To determine whether mutations of the *VSX1* gene play a pathogenetic role in the development of keratoconus (KTCN).

METHODS. DNA extraction, PCR amplification, and direct sequencing of the *VSX1* gene were performed in 100 unrelated patients with diagnoses of clinical and topographic features of KTCN.

RESULTS. Of the four previously identified presumed pathogenic mutations in the *VSX1* gene (Leu17Pro, Asp144Glu, Leu159Met, and Arg166Trp), only Asp144Glu was identified in a single affected patient. Two novel single nucleotide polymorphisms (SNPs), both resulting in synonymous substitutions, were identified: c.53G>T (Ser6Ser) in four affected patients and c.209G>T (Pro58Pro) in two affected patients. Two previously reported SNPs were also identified: c.426C>A (Arg131Ser) in one affected patient and c.581A>G (Ala182Ala) in 51 of the 100 affected patients.

CONCLUSIONS. Only one of the presumed pathogenic mutations in the *VSX1* gene, Asp144Glu, was identified in a single member of the cohort of affected patients. However, as previously demonstrated, Asp144Glu is a non-disease-causing polymorphism. The absence of pathogenic mutations in the *VSX1* gene in a large number of unrelated KTCN patients indicates that other genetic factors are involved in the development of this disorder. (*Invest Ophthalmol Vis Sci.* 2006;47:2820-2822) DOI:10.1167/iovs.05-1530

Keratoconus (KTCN; Online Mendelian Inheritance in Man [OMIM], 148300) is a noninflammatory type of corneal ectasia characterized by thinning of the corneal stroma and progressive conical corneal protrusion, each of which results in impaired vision.¹ Although patients less severely affected may use eyeglasses and contact lenses for vision correction, those more severely affected often require corneal transplantation for visual rehabilitation. Keratoconus is the most common of the noninflammatory ectatic disorders of the cornea, with an estimated incidence between 1 in 500 and 1 in 2000 persons.² Given the prevalence of this disorder, it is not surprising that keratoconus is one of the most common indications for corneal transplantation.^{3,4} Although most cases of

keratoconus are sporadic, 5% to 10% of patients have a positive family history.^{2,5} In such cases, both autosomal dominant and recessive patterns of inheritance have been described.⁶⁻⁸

Genomewide linkage analysis of affected pedigrees has demonstrated evidence of linkage to several different chromosomal loci, including 5q14.3-q21.1,⁹ 16q22.3-q23.1 (designated keratoconus 2),⁷ 3p14-q13 (keratoconus 3),¹⁰ and 2p24 (keratoconus 4).¹¹ However, the genetic locus designated keratoconus 1 on chromosome 20p11.2 was not identified by linkage analysis. Instead, the visual system homeobox 1 gene (*VSX1*), located within the chromosome 20p11-q11 candidate gene region for posterior polymorphous corneal dystrophy (PPCD; MIM, 122000), was initially selected for screening in patients with PPCD.¹² The *VSX1* gene belongs to a family of homeodomain transcription factors that are thought to control cell differentiation in craniofacial and ocular development, making it a good functional candidate gene for PPCD.^{13,14} Although neither Fuchs endothelial corneal dystrophy (FECD) nor keratoconus has been linked to this region, screening of the *VSX1* gene was performed in patients with FECD because PPCD and FECD are corneal endothelial dystrophies and may share common clinical features.¹² Previous reports linking PPCD and keratoconus¹⁵⁻¹⁹ led the authors to speculate regarding a common genetic etiology for the two disorders; thus, they also performed mutation screening of the *VSX1* gene in patients with keratoconus (these patients did not have PPCD). The authors did acknowledge, however, that the two disorders demonstrate distinct clinical features and that the major pathologic defects in keratoconus lie in the anterior cornea, whereas PPCD is limited to the posterior cornea.¹²

The identification of sequence variants in the *VSX1* gene in patients with keratoconus and PPCD has led to the assumption in the ophthalmic research community that mutations in the *VSX1* gene are responsible for PPCD and keratoconus. Several research articles have been published purporting to identify pathogenic mutations in patients with keratoconus and other ocular disorders.^{20,21} We suspected different pathogenetic mechanisms for these two disparate corneal disorders and have demonstrated that the *VSX1* gene sequence variants identified in PPCD patients are most likely only polymorphisms.²²

In this study, we report the results of mutation analysis of the *VSX1* gene in 100 unrelated patients with keratoconus. Because only a single patient was found to harbor one of the previously identified presumed pathogenic mutations, which may be considered a polymorphism because it has been identified in persons without keratoconus, we conclude that the *VSX1* gene has not been demonstrated to play a role in keratoconus.

MATERIALS AND METHODS

This study was conducted in accordance with the tenets of the Declaration of Helsinki.

Patient Identification

After institutional review board (IRB) approval was granted (UCLA IRB 94-07-243-22B; Cedars-Sinai IRB 939), informed consent was obtained from patients diagnosed with KTCN in the clinical practices of AJA and YSR. The diagnosis of KTCN was based on the presence of character-

From the ¹Cornea Service, The Jules Stein Eye Institute, David Geffen School of Medicine at UCLA, Los Angeles, California; and the ²Cornea Genetic Eye Institute, Cedars-Sinai Medical Center, Los Angeles, California.

Supported by the Center for Keratoconus (AJA, YSR), the Emily Plumb Estate and Trust (AJA), and the Skirball Program in Molecular Ophthalmology and National Eye Institute Grant 09052 (YSR).

Submitted for publication December 1, 2005; revised February 11, 2006; accepted May 17, 2006.

Disclosure: **A.J. Aldave**, None; **V.S. Yellore**, None; **A.K. Salem**, None; **G.L. Yoo**, None; **S.A. Rayner**, None; **H. Yang**, None; **G.Y. Tang**, None; **Y. Piconell**, None; **Y.S. Rabinowitz**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Anthony J. Aldave, Cornea Service, The Jules Stein Eye Institute, David Geffen School of Medicine at UCLA, 100 Stein Plaza, Los Angeles, CA 90095; aldave@jsei.ucla.edu.

istic topographic features of keratoconus (inferior or central corneal steepening or asymmetric bow tie pattern with skewing of the radial axes) and the presence of one or more of the following characteristic clinical features in one or both eyes: conical corneal deformation, corneal stromal thinning, Fleischer ring, or Vogt striae.

DNA Collection and PCR Amplification

Informed consent was obtained from each subject after an explanation of the nature and possible consequences of study participation. Genomic DNA was isolated from peripheral blood leukocytes or buccal epithelial cells (QIAamp DNA Mini Kit spin protocol; Qiagen, Valencia, CA).

All five exons and intron/exon boundaries of the *VSX1* gene were amplified with the use of custom-designed oligonucleotide primers (Table 1). Each reaction was carried out in a 25- μ L mixture containing 12.5 μ L premix (Failsafe PCR 2 \times PreMix D; Epicenter, Madison, WI), 0.2 μ M each primer, 0.5 U genomic DNA polymerase (RedTaq; Sigma-Aldrich Corp., St. Louis, MO), 2.5 μ L 10 \times PCR buffer (RedTaq; Sigma-Aldrich Corp.) with 2.5 mM MgCl₂, and approximately 100 ng genomic DNA. Thermal cycling was performed in a thermal cycler (iCycler; Bio-Rad, Hercules, CA), under the following conditions: initial denaturation for 3 minutes at 95°C; 35 cycles of 94°C for 30 seconds, 58°C (exons 1-4) or 55°C (exon 5) for 35 seconds, 72°C for 60 seconds; and a final extension for 10 minutes at 72°C.

DNA Sequencing

Sequencing reactions were performed with BigDye Terminator Mix version 3.1 (Applied Biosystems [ABI], Foster City, CA). Samples were denatured at 96°C for 2 minutes, then cycled 25 times at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Unincorporated nucleotides were removed using the CleanSeq reagent and a SPRI plate (Agencourt Bioscience Corp., Beverly, MA) according to the manufacturer's instructions and then were analyzed on an ABI-3100 Genetic Analyzer (ABI) after resuspension in 0.1 mM EDTA. Nucleotide sequences were compared with the published *VSX1* cDNA sequence (GenBank accession number NM_014588).

RESULTS

The 100 unrelated patients with keratoconus recruited into the study ranged in age from 18 to 79 years. Two of the patients had a first- or second-degree relative with keratoconus, and one patient also had PPCD. Two of the patients had undergone unilateral corneal transplantation before presentation; thus, the diagnosis of KTCN was based on clinical and topographic findings in the unoperated eye.

Screening of the *VSX1* gene demonstrated that of the four mutations previously associated with keratoconus (Leu17Pro, Asp144Glu, Leu159Met, and Arg166Trp), only Asp144Glu was identified in a single affected patient. This patient, who did not have a personal or family history of glaucoma, was diagnosed with bilateral keratoconus at age 16, and at age 17 underwent penetrating keratoplasty in her left eye 7 months after the development of hydrops. Additionally, none of the three mutations considered possibly associated with keratoconus (Gly160Asp, His244Arg, Pro247Arg) were identified in any of

the affected patients. Two novel SNPs were identified, both resulting in synonymous substitutions—c.53G>T (Ser6Ser) in four patients and c.209G>T (Pro58Pro) in two patients. Two previously reported SNPs were also identified: c.426C>A (Arg131Ser; refSNP ID, rs6050307) in one affected patient and c.581A>G (Ala182Ala; refSNP ID, rs12480307) in 51 of the 100 affected patients.

DISCUSSION

Although the *VSX1* gene is expressed in the retina, where it is thought to play a role in the development of retinal bipolar interneurons,^{23,24} no expression has been detected in the mouse cornea^{25,26} or in one of two studies using RT-PCR performed on RNA isolated from adult human cornea.^{12,13} As expected, *VSX1* mutant mice do not demonstrate a mature cone bipolar cell phenotype.²⁴ However, the discovery that no corneal abnormalities were observed through gross examination or with light and electron microscopic examination in *VSX1*^{+/-} and *VSX1*^{-/-} mice provides functional evidence suggesting the loss of *VSX1* gene function may not result in the keratoconus or PPCD disease phenotypes.

Heon et al.¹² selected the *VSX1* gene for screening as a positional candidate gene for PPCD (MIM, 122,000) because it is positioned within the chromosome 20p11-q11 PPCD1 candidate region. Although the PPCD candidate gene region is not one to which keratoconus has been linked previously, the investigators chose to screen the *VSX1* gene in keratoconus patients because of previous reports of the coexistence of the two corneal disorders.¹⁵⁻¹⁹ Sequence variants were identified in four patients; two mutations (Leu159Met and Arg166Trp) were not identified in control subjects and were considered pathogenic, whereas the other two mutations (Asp144Glu and His244Arg) were also identified in subjects without keratoconus and thus were considered only possibly pathogenic. However, insufficient evidence exists to consider either the Leu159Met or the Arg166Trp mutation as causing disease because neither was demonstrated to sort with the disease phenotype: the Leu159Met mutation was identified in one pedigree (three affected siblings and one affected parent), but no unaffected family members were examined. In addition, the Arg166Trp mutation was identified in a single sporadic patient with keratoconus; *VSX1* screening was not performed in any family members.

Bisceglia et al.²¹ identified four missense mutations in 7 of 80 patients with keratoconus. One of the two mutations considered pathogenic, Leu17Pro, was not identified in the cohort screened by Heon et al.,¹² whereas the other, Asp144Glu, was reported by Heon et al.¹² in a patient without keratoconus and has been reported by the authors in an unaffected control subject.²² The other two missense mutations identified, Gly160Asp and Pro247Arg, were considered possibly pathogenic, though these mutations were previously associated with PPCD, not keratoconus,^{12,22} and the Gly160Asp mutation was not found to segregate with the keratoconus phenotype in an affected pedigree.²¹

TABLE 1. Primer Sequences Used for *VSX1* Amplification

Exon	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)	Coding Region (bp)
1	GCAGCCCAATCCTATAAAGCTTCC	GTGGGGCGATGGTCTGTGAC	590	424
2	CTTAAGTACCCAAGAGGTTTCATAACT	GAAACCACTGGGCCTGCTATCAT	297	79
3	AAGCAGGCACGGTGGTCTCTTA	AAGGGACTGCTGATTGGCTCACT	294	124
4	GCTCGGGAGAGAAGATCC	TCAGTAACTGACGTTGCTTTG	400	181
5	GGAAATTTACTTCATTGCTGAATTT	TGGCATTGCATTTTATCTTGACA	496	290

From these two reports, four mutations in the *VSX1* gene—Leu17Pro, Asp144Glu, Leu159Met, and Arg166Trp—purportedly cause keratoconus. We identified only one of these previously reported mutations, Asp144Glu, in a single affected patient. Given that the Asp144Glu mutation has already been demonstrated in two reports in unaffected control subjects, it may be considered a polymorphism. The other three mutations have been identified in only one (Leu159Met and Arg166Trp) or three (Leu17Pro) affected probands from a total of almost 250 unrelated patients with keratoconus in whom *VSX1* gene screening has been reported (including this report).^{12,21} Thus, the lack of evidence to support the previous conclusions that these mutations are pathogenic, the failure to demonstrate corneal expression of the *VSX1* gene, the absence of any corneal abnormality in the *VSX1* knockout mouse model, and the identification of presumed pathogenic mutations in the *VSX1* gene in less than 2% of keratoconus patients provides strong evidence that the *VSX1* gene does not play a major role in the pathogenesis of keratoconus.

References

- Krachmer JH, Feder RS, Belin MW. Keratoconus and related non-inflammatory corneal thinning disorders. *Surv Ophthalmol*. 1984;28:293-322.
- Rabinowitz YS. Keratoconus. *Surv Ophthalmol*. 1998;42:297-319.
- Kang PC, Klintworth GK, Kim T, et al. Trends in the indications for penetrating keratoplasty, 1980-2001. *Cornea*. 2005;24:801-803.
- Cosar CB, Sridhar MS, Cohen EJ, et al. Indications for penetrating keratoplasty and associated procedures, 1996-2000. *Cornea*. 2002;21:148-151.
- Kennedy RH, Bourne WM, Dyer JA. A 48-year clinical and epidemiologic study of keratoconus. *Am J Ophthalmol*. 1986;101:267-73.
- Hughes AE, Dash DP, Jackson AJ, Frazer DG, Silvestri G. Familial keratoconus with cataract: linkage to the long arm of chromosome 15 and exclusion of candidate genes. *Invest Ophthalmol Vis Sci*. 2003;44:5063-5066.
- Tyynismaa H, Sistonen P, Tuupainen S, et al. A locus for autosomal dominant keratoconus: linkage to 16q22.3-q23.1 in Finnish families. *Invest Ophthalmol Vis Sci*. 2002;43:3160-3164.
- Wang Y, Rabinowitz YS, Rotter JI, Yang H. Genetic epidemiological study of keratoconus: evidence for major gene determination. *Am J Med Genet*. 2000;93:403-409.
- Tang YG, Rabinowitz YS, Taylor KD, et al. Genomewide linkage scan in a multigeneration Caucasian pedigree identifies a novel locus for keratoconus on chromosome 5q14.3-q21.1. *Genet Med*. 2005;7:397-405.
- Brancati F, Valente EM, Sarkozy A, et al. A locus for autosomal dominant keratoconus maps to human chromosome 3p14-q13. *J Med Genet*. 2004;41:188-192.
- Hutchings H, Ginisty H, Le Gallo M, et al. Identification of a new locus for isolated familial keratoconus at 2p24. *J Med Genet*. 2005;42:88-94.
- Heon E, Greenberg A, Kopp KK, et al. *VSX1*: a gene for posterior polymorphous dystrophy and keratoconus. *Hum Mol Genet*. 2002;11:1029-1036.
- Semina EV, Mintz-Hittner HA, Murray JC. Isolation and characterization of a novel human paired-like homeodomain-containing transcription factor gene, *VSX1*, expressed in ocular tissues. *Genomics*. 2000;63:289-293.
- Hayashi T, Huang J, Deeb SS. RINX(*VSX1*), a novel homeobox gene expressed in the inner nuclear layer of the adult retina. *Genomics*. 2000;67:128-139.
- Blair SD, Seabrooks D, Shields WJ, Pillai S, Cavanagh HD. Bilateral progressive essential iris atrophy and keratoconus with coincident features of posterior polymorphous dystrophy: a case report and proposed pathogenesis. *Cornea*. 1992;11:255-261.
- Bechara SJ, Grossniklaus HE, Waring GO 3rd, Wells JA 3rd. Keratoconus associated with posterior polymorphous dystrophy. *Am J Ophthalmol*. 1991;112:729-731.
- Driver PJ, Reed JW, Davis RM. Familial cases of keratoconus associated with posterior polymorphous dystrophy. *Am J Ophthalmol*. 1994;118:256-257.
- Gasset AR, Zimmerman TJ. Posterior polymorphous dystrophy associated with keratoconus. *Am J Ophthalmol*. 1974;78:535-537.
- Weissman BA, Ehrlich M, Levenson JE, Pettit TH. Four cases of keratoconus and posterior polymorphous corneal dystrophy. *Optom Vis Sci*. 1989;66:243-246.
- Mintz-Hittner HA, Semina EV, Frishman LJ, Prager TC, Murray JC. *VSX1* (RINX) mutation with craniofacial anomalies, empty sella, corneal endothelial changes, and abnormal retinal and auditory bipolar cells. *Ophthalmology*. 2004;111:828-836.
- Bisceglia L, Ciaschetti M, De Bonis P, et al. *VSX1* mutational analysis in a series of Italian patients affected by keratoconus: detection of a novel mutation. *Invest Ophthalmol Vis Sci*. 2005;46:39-45.
- Aldave AJ, Yellore VS, Principe AH, et al. Candidate gene screening for posterior polymorphous dystrophy. *Cornea*. 2005;24:151-155.
- Hayashi T, Huang J, Deeb SS. Expression of *rinx/vsx1* during postnatal eye development in cone-bipolar, differentiating ganglion, and lens fiber cells. *Jpn J Ophthalmol*. 2005;49:93-105.
- Ohtoshi A, Wang SW, Maeda H, et al. Regulation of retinal cone bipolar cell differentiation and photopic vision by the CVC homeobox gene *Vsx1*. *Curr Biol*. 2004;14:530-536.
- Chow RL, Volgyi B, Szilard RK, et al. Control of late off-center cone bipolar cell differentiation and visual signaling by the homeobox gene *Vsx1*. *Proc Natl Acad Sci USA*. 2004;101:1754-1759.
- Chow RL, Snow B, Novak J, et al. *Vsx1*, a rapidly evolving paired-like homeobox gene expressed in cone bipolar cells. *Mech Dev*. 2001;109:315-322.