

No Pathogenic Mutations Identified in the *COL8A2* Gene or Four Positional Candidate Genes in Patients with Posterior Polymorphous Corneal Dystrophy

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PURPOSE. To identify the genetic basis of posterior polymorphous corneal dystrophy (PPCD) through screening of four positional candidate genes and the *COL8A2* gene, in which a presumed pathogenic mutation has previously been identified in affected patients.

METHODS. DNA extraction, PCR amplification, and direct sequencing of the *COL8A2*, *BFSPI*, *CST3*, *MMP9*, and *SLPI* genes were performed in 14 unrelated, affected patients and in unaffected family members.

RESULTS. In the *COL8A2* gene, the previously identified, presumed pathogenic mutation (Gln455Lys) was not discovered in any of the affected patients. A missense mutation, Thr502Met, was identified in 2 of the 14 affected probands, although it was not considered to be pathogenic, as it has been identified in unaffected individuals. Although several novel and previously identified single nucleotide polymorphisms producing synonymous and missense amino acid substitutions were identified in the *COL8A2*, *BFSPI*, *CST3*, *MMP9*, and *SLPI* genes, no presumed pathogenic sequence variants were found.

CONCLUSIONS. No pathogenic mutations were identified in the *COL8A2* gene or in several positional candidate genes in a series of patients with PPCD, indicating that other genetic factors are involved in the development of this autosomal dominant corneal dystrophy. (*Invest Ophthalmol Vis Sci*. 2005;46:1599–1603) DOI:10.1167/iovs.04-1321

Posterior polymorphous corneal dystrophy (PPCD; MIM 122000; Mendelian Inheritance in Man; <http://www.ncbi.nlm.nih.gov/Omim/searchmorbid/> provided in the public domain by the National Center for Biotechnology Information [NCBI], Bethesda, MD) is a dominantly inherited corneal disorder that may show significant phenotypic variability in affected patients who may be asymptomatic or have significant visual impairment from associated corneal edema or glaucoma.

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Efforts to identify the genetic basis of PPCD began a decade ago, when Heon et al.¹ performed a genome-wide linkage analysis in a large American family with PPCD, localizing the PPCD locus to a 30-cM region on the long arm of chromosome 20 at region 11. Several years ago, Heon et al.² reported identifying two mutations (Asp144Glu and Gly160Asp) in the visual system homeobox gene 1 (*VSX1*; MIM 605020) in 2 of 22 affected patients with PPCD. This gene, positioned within the previously identified candidate gene region on chromosome 20, was an attractive positional candidate gene, as it is one of the homeodomain-containing transcription factor genes that regulate the expression of cellular differentiation during development in various tissues, including ocular tissues.³ We have screened the *VSX1* gene in 14 patients (12 families) affected with PPCD, as well as in unaffected family members and healthy control subjects.⁴ The previously identified Gly160Asp missense change was not present in any of our 12 probands, and the Asp144Glu mutation was identified in one affected patient as well as one unaffected control individual. Thus, the absence of the Gly160Asp missense change in any of the 14 affected patients that we examined, as well as its presence in only 1 of the 22 patients with PPCD reported by Heon et al.² suggests that this is either an unusual cause of PPCD, or more likely, a rare polymorphism. In addition, our discovery of the Asp144Glu missense change in a single affected patient as well as a healthy control patient who did not display any clinical features of PPCD argues against this change being mutagenic and leads to the conclusion that it too most likely represents a rare polymorphism.

We sought to identify the genetic basis of PPCD by haplotype and crossover analysis in large families, to refine the candidate gene interval, and to prioritize and screen positional candidate genes based on the known or predicted function of the encoded proteins. As the histopathologic and ultrastructural examination of affected corneas demonstrates abnormal development, proliferation, and migration of the endothelial cells, genes encoding regulatory proteins that control cell differentiation, division, or growth were considered likely candidate genes. In accordance with these criteria, four genes mapped to the long arm of chromosome 20, region 11, were selected for screening: matrix metalloproteinase 9 (*MMP9*; MIM 120361), cystatin 3 (*CST3*; MIM 604312), secretory leukocyte protease inhibitor (*SLPI*; MIM 107285), and beaded filament structural protein 1 (*BFSPI*; MIM 603307).

MMP-9 (gelatinase B) has been implicated in a wide variety of corneal disorders, including recurrent corneal erosions,⁵ ocular rosacea,⁶ vernal keratoconjunctivitis,⁷ and subepithelial scarring after photorefractive keratectomy.⁸ In addition, matrix metalloproteinases have been identified in the corneal endothelium in normal and keratoconus corneas,^{9,10} and both *MMP-2* (gelatinase A) and *MMP-9* have been implicated in the pathogenesis of keratoconus, prompting the screening of *MMP-9* as a candidate gene in patients with keratoconus.^{11–13} As PPCD has been associated with keratoconus in several

published reports¹⁴⁻¹⁸ and the two conditions have been reported to share a common genetic origin,² we also chose to screen the MMP-9 gene in our cohort of patients with PPCD.

Cystatin 3 (cystatin C) is the most abundant extracellular inhibitor of cysteine proteases, expressed in virtually all tissues and fluids, including the eye and tears.¹⁹⁻²¹ Like matrix metalloproteases, cysteine proteases have been implicated in several pathologic corneal processes, such as corneal angiogenesis,²² and altered levels have been noted in the tears of patients with a variety of corneal disorders, including corneal dystrophies.²³ Corneal endothelial migration has been demonstrated to be dependent on protease function, as protease inhibitors have been shown in a wound repair model to inhibit significantly the movement of corneal endothelial cells along Descemet's membrane.²⁴ Thus, as protease inhibitors such as cystatin 3 have been associated with corneal dystrophies and demonstrated to regulate corneal endothelial cell migration, the *CST3* gene was selected for screening in patients affected with PPCD.

SLPI is the principal serine protease inhibitor (serpin) measured in human reflex tear fluid²⁵ and thus has properties similar to those of *CST3*. Given this, and the demonstration that *SLPI* is able to mediate the proliferation of human epithelial cells by regulation of growth-associated genes,²⁶ we also chose to screen the *SLPI* gene as a functional candidate gene for PPCD.

BFSP1 (filensin) encodes the protein filensin, an intermediate filament that is an essential component of the crystalline lens, contributing to lens fiber cell differentiation and maintenance of cell conformation and transparency.²⁷ Although filensin is considered a lens-specific intermediate filament, inherited disorders of the cornea, such as Meesmann's corneal dystrophy, have been associated with mutations in genes that encode intermediate filaments.²⁸ Given the role of *BFSP1* in lenticular cell differentiation and structure, as well as the common origin of the lens and cornea from surface ectoderm, we chose to screen the *BFSP1* gene as well in patients with PPCD.

Although not mapped to 20q11, the collagen, type VIII, α -2 gene (*COL8A2*; MIM 120252) on chromosome 1 was also selected for screening as a missense mutation has been reported in two related patients (15 patients screened) with histologically verified PPCD.²⁹

MATERIALS AND METHODS

The researchers followed the tenets of the Declaration of Helsinki in the treatment of the subjects reported herein. Study approval was obtained from the institutional review board at each participating center: The Jules Stein Eye Institute, The University of California, Los Angeles (UCLA IRB 02-10-092-01); The University of Vermont (IRB 04-119); Emory University (IRB ID 617-2003); and Wills Eye Hospital, Thomas Jefferson University (IRB 03-583E).

Candidate Gene Selection

The NCBI Map Viewer function was selected for viewing all the genes localized to human chromosome 20 (<http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?taxid=9606&chr=20>). The markers previously identified as defining the interval to which PPCD has been linked¹ were entered to define the region displayed, allowing identification of the candidate genes in this region. The function of each gene within and immediately adjacent to the candidate region was then evaluated, with prioritization given to those genes that were known to play a role in disorders of the cornea or that encoded regulatory proteins controlling cell differentiation, proliferation, or growth.

Patient Identification and DNA Collection and Preparation

All patients affected with PPCD who were identified by one of the investigators (AJA, LE-B, GCT, IR, SBH, RDS) were offered enrollment in the study. The diagnosis of PPCD was based on characteristic corneal endothelial changes, including clustered vesicles with a surrounding gray halo, bands with parallel borders, and geographic gray opacifications, and on histopathologic examination of excised corneal buttons, when available. After informed consent was obtained, affected patients, unaffected family members, and unrelated, unaffected, healthy control individuals were enrolled in the study, and a slit lamp examination was performed to confirm their affected or unaffected status. Genomic DNA was obtained from peripheral blood samples and/or from buccal epithelial cells by using a brush (CytoSoft CP-5B; Medical Packaging Corp., Camarillo, CA). Genomic DNA was prepared from the buccal epithelial cells and peripheral blood leukocytes (QIAamp DNA Mini Kit spin protocol; Qiagen, Valencia, CA).

PCR Amplification

The coding regions of the *MMP9*, *CST3*, *SLPI*, *BFSP1*, and *COL8A2* genes were amplified by polymerase chain reaction (PCR) with the custom-designed primers (Primer 3; http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi; provided in the public domain by Massachusetts Institute of Technology, Cambridge, MA) shown in Table 1. All primers were designed so that they would be positioned on intronic segments 60 to 80 nucleotides on either side of the intron-exon boundary to ensure complete reading of the exons.

Each reaction was performed in a 25- μ L mixture containing 12.5 μ L of a PCR premix (FailSafe PCR 2 \times PreMix D; Epicenter, Madison, WI), 0.15 μ L of each primer (10 pM/ μ L), 0.2 U of *Taq* DNA polymerase (New England Biolabs, Beverly, MA), and 60 ng of genomic DNA.

Thermal cycling was performed (iCycler; BioRad, Hercules, CA) under the following conditions: initial denaturation for 10 minutes at 95°C; 35 cycles of 94°C for 30 seconds, 58°C for 40 seconds, and 72°C for 80 seconds; and a final extension for 10 minutes at 72°C.

PCR Purification and DNA Sequencing

The PCR products were analyzed on an agarose gel to confirm the product size and ensure adequate purity and quantity of the amplicons for sequencing reactions. Enzymatic purification of amplified DNA was performed by incubating 15 to 30 ng DNA with 5 U exonuclease I and 0.5 U shrimp alkaline phosphatase (USB Corp., Cleveland, OH) for 15 minutes at 37°C. After inactivation of the nucleases (80°C/15 minutes), the sequencing reactions were set up by the addition of 2 μ L dye-terminator chemistry mix (BigDye Terminator Mix v3.1; ABI, Foster City, CA), 2 μ L of premixed sequencing dilution buffer (SeqSaver; Sigma-Aldrich, St. Louis, MO) and 0.2 μ L primer (10 pM/ μ L). 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 by using a dye terminator chemistry removal method (CleanSeq reagent and an SPRI plate [solid phase reversible immobilization]; Agencourt Bioscience Corp., Beverly, MA) according to the manufacturer's instructions and then analyzed on an DNA sequencer (model 3100; ABI) after resuspension in 0.1 mM EDTA. Nucleotide sequences, read manually as well as on computer (Mutation Surveyor, ver. 2.2; Softgenetics, State College, PA), were compared with the published GenBank cDNA sequence for each gene: *MMP9* (NM_004994), *CST3* (NM_000099), *SLPI* (NM_003064), *BFSP1* (NM_001195), and *COL8A2* (NM_005202) (<http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by NCBI, Bethesda, MD).

Identification of Reference Single-Nucleotide Polymorphism ID for Sequence Variants

The GenBank number for each gene was entered into the NCBI Entrez Nucleotide web page (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide>; NCBI). The selection of Graph as

TABLE 1. Primer Sequences Used for COL8A2, BFSP1, CST3, MMP9, and SLPI Gene Amplification

Gene	Exon	Forward Primer (5'–3')	Reverse Primer (5'–3')	Product Size (bp)	Exon Size (bp)
COL8A2	1	TGGGAATTGCTTCTCAGCCTTTT	AGGCCCTCTGGGGTATTAGGAAA	497	193
	2A*	CCATTCTTCTCCTCCCGTGTACC	cccagactcacccttgtctcctg	2311	1919
	2B	gcctcaaggggggataatggagt	agttggtccagggggtccat		
	2C	tggaggacccccaggagtg	cgctgtggccattgtagagagtc		
BFSP1	2D	ctgtctgcccacacacac	AGCAGAGGCCAGGACTCACAAAG		
	1	AACTGCTGATCGGCTCTGGAAC	GTGCCATCAAAAACAGAAGCCAGT	699	377
	2	GGCATCGTACCCAGGTGAATTT	AAAACCTGCACCTCCACCATTCC	231	61
	3	TCATATACCCGGCCAGCTTTACC	ACCCTCGGCTTACCTGTACAAAC	237	96
	4	CGGAAGTGTCCATTCCTGTTCTC	GGGAGTCTCCAGGTACAGCTTCC	246	93
	5	CTCTGCCCTTTTCTCTCTGCAT	CGGGTAACACCTCCATGAAACAT	229	108
	6	CAGGTTCTTTTCTGGTGAGGT	ATGCCACTGCCTATAACTGTGC	397	221
	7	AGAGTCGCTCTTGCCTCTGAC	TTCCAGATGGATCTGAAGCAAGC	249	86
	8A*	AGCGCTTTGTTCCGAGAGAGTT	cctttgcttatctgcccctccate	864	615
	8B	tgacacaagaaggggctccag	ctaaagggggcttgggtgactc		
CST3	8C	ctgtgctttatgacggccaggt	CAAGCACCTCAGCTCCATCCTG		
	1	GAAGGCCGGAAGGGGATAAAACC	GCCGGAGAGGAGCAGCAC	477	318
	2	AATGTGGGCAGCTCATGAAGGTA	TACACACACTCAGGCACATGCAC	364	114
MMP9	3	CCCAGTGTTCACCTCCATCCTT	CTTGCCTGATGGCCTTGCTGT	598	343
	1	CTTAAAGCCCCACAACAGCAG	ACACTCAACACCCTCACGGACAG	243	136
	2	GGGCAGAGAAAGGGGTCAGAGAT	CGTCCAAGACAGGACATGTTAC	373	232
	3–4†	GGACGGCAGAGCATTGTGTAT	CCCTCGAAGATGAAGGGGAAGT	834	149/129
	5	ATCACCCGCCGCCCTAACT	GCACACTGGCCAGAGCTGGAA	299	174
	6	CGCCTTCTCCCCCTTTCC	AGAGGGCGGGCTGAACCT	250	174
	7	TTGGACTCATCCATCTGGCTCAT	CACCCCACCACCACCACTG	280	177
	8	GTTCCCGGCAGTGGTGGT	CACAGCCACGCCCTTTCC	287	156
	9	AGGGGTACAGAGGTATGCAGGAA	GGCAGCCCTCCGCAAAG	388	280
	10	GTCCAAGGCTTAGAGCCCGTCTC	TCTTGAGCCTCCTGACTGATGG	244	140
	11	GAGTAGCTGCTCCCTCTGC	CGGAGGAGACAGGTGCTGA	287	151
	12	CAGGGGGAAGATGCTGCTGTT	GGAGCTTAGTGGTGTCTCACGA	288	104
	13	TGTTCTATTTCTGTATATGTGGGAGA	GGTTGGTCCAGTGGGGATTAC	236	119
SLPI	1	CTCCTTGGTGTCCAGGATTTCTGG	CTCAGGGAGGTCTCCGAAACTA	366	85
	2	GGAGGCATGATGGGTCTCAGTA	ATCACCCAGTTCGGTCCCTCT	291	159
	3	GGGATGGGAGGGGTTGTATAAAG	CATATGCCTGGGCCTCCAGA	289	150
	4	GGCAGGAGGAAGGATGTGGATA	CCAACGAATCACAGAAGCAGGAT	280	2

* COL8A1 exon 2 and BFSP1 exon 8 were amplified as a large fragment by flanking primers (upper case) and sequenced with additional internal primers (lower case) because of their large size.

† MMP9 exons 3 and 4 were amplified and sequenced as one amplicon, because of their proximity.

the display option provided the cDNA and amino acid sequences for each gene, as well as the location and refSNP ID number of previously identified single-nucleotide polymorphisms (SNPs).

RESULTS

Forty-three affected and 22 unaffected subjects from 28 families with PPCD were identified and enrolled in the study from the four participating centers. For the purpose of candidate gene screening, 14 affected patients and 5 unaffected relatives from 14 unrelated families were selected. At least 100 unaffected, unrelated control subjects were used to identify the frequency in the general population of each novel candidate gene sequence variant that was not also identified in an unaffected relative.

Screening of the COL8A2 gene revealed four synonymous amino acid substitutions, two of which have been reported previously²⁹ (Table 2). A single missense substitution, c.1512C→T (Thr502Met), was identified in two affected patients; this mutation has already been identified in unaffected control individuals.³⁰

Sequencing of the MMP9 gene revealed four previously reported SNPs: c.855G→A, Arg279Gln; c.1740C→G, Arg574Pro; c.1840A→C, Gly607Gly; and c.2022G→A, Arg668Gln (Table 2). A previously unidentified, synonymous substitution, Val694Val (c.2101G→A), was found in two affected patients as well as an unaffected relative.

Analysis of the CST3 gene revealed three previously reported SNPs: c.87C→T (Pro4Pro); c.148G→A (Ala25Thr); and c.180G→A (Leu35Leu) (Table 2).

Three previously reported SNPs and one previously unreported missense mutation (c.1125G→T; Glu374Asp) were identified in the BFSP1 gene (Table 2). The c.1125G→T missense change was also identified in 3 of 111 unaffected control individuals. In addition, each of the 14 probands and the control individuals tested were homozygous for the c.9G→C (Arg3Arg) sequence variant, suggesting that this "sequence variant" represents the wild-type nucleotide.

No coding region sequence changes were identified in the SLPI gene.

DISCUSSION

It is estimated that 95% of all coding exons and almost all genes on chromosome 20 have been identified.³¹ Therefore, although gene annotation may not be complete, one of the 275 positional candidate genes (defined as those between the markers D20S98 and D20S108; build 35.1) on 20q11 is likely to be involved in the pathogenesis of PPCD. As the number of positional candidate genes precludes brute-force screening of each, we have chosen to prioritize screening of the candidate genes based on the known or predicted function of the encoded protein. No pathogenic mutations were identified in any of the

TABLE 2. Sequence Variants in the *COL8A2*, *BFSP1*, *CST3*, *MMP9*, and *SLPI* Genes in Patients with PPCD

Gene	Nucleotide Change	Amino Acid Change	Affected Patients (n = 14)	refSNP ID (dbSNP build 121)	Control Individuals (n ≥ 100)
<i>COL8A2</i>	c.112G→A	Ala35Ala*	5 (4 heterozygous)	None	
	c.598G→T	Gly197Gly	1 (heterozygous)	None	1/114
	c.1012C→G	Leu335Leu	2 (heterozygous)	None	1/116
	c.1492G→A	Gly495Gly*	5 (1 heterozygous)	None	
<i>MMP9</i>	c.1512C→T	Thr502Met†	2 (heterozygous)	None	
	c.855G→A	Arg279Gln	10 (4 heterozygous)	rs17576	
	c.1740C→G	Arg574Pro	4 (heterozygous)	rs2250889	
	c.1840A→C	Gly607Gly	10 (8 heterozygous)	rs13969	
	c.2022G→A	Arg668Gln	2 (heterozygous)	rs17577	
	c.2101G→A	Val694Val	2 (heterozygous)	None	
<i>CST3</i>	c.87C→T	Pro4Pro	1 (heterozygous)	rs1055084	
	c.148G→A	Ala25Thr	2 (heterozygous)	rs1064039	
<i>BFSP1</i>	c.180G→A	Leu35Leu	1 (heterozygous)	rs6138024	
	c.1125G→T	Glu374Asp	1 (heterozygous)	None	3/111
	c.1500G→A	Ala500Ala	6 (5 heterozygous)	rs6136118	
	c.1749A→G	Pro583Pro	11 (5 heterozygous)	rs6080718	
<i>SLPI</i>	c.1926C→T	Thr642Thr	5 (heterozygous)	rs6080717	
	None				

* Reported by Biswas et al.²⁹

† Reported by Kobayashi et al.³⁰

four positional candidate genes (*MMP9*, *CST3*, *SLPI*, or *BFSP1*) that were selected for screening.

Examination of the *COL8A2* gene did not reveal the previously identified, presumed pathogenic Gly455Lys mutation or a novel gene mutation in any of the patients affected with PPCD. Molecular genetic evidence indicating that mutations in the *COL8A2* gene play a role in PPCD was provided by the performance of a genome-wide linkage analysis in a three-generation family with early-onset Fuchs corneal dystrophy.²⁹ Significant linkage was demonstrated to a 6- to 7-cM region on chromosome 1, within which the *COL8A2* gene is positioned. As this gene encodes the $\alpha 2$ chain of type VIII collagen, which is a component of endothelial basement membranes and is highly expressed in human corneal Descemet's membrane, it was considered an excellent functional and positional candidate gene for both Fuchs corneal dystrophy and PPCD. However, the fact that the Gly455Lys mutation, previously identified in only two affected individuals from one family (no unaffected family members screened), was not present in any of the 13 other affected patients that these investigators screened, in any of the 5 unrelated Japanese patients with PPCD reported by Kobayashi et al.³⁰ or in any of the 14 affected probands that we examined, indicates that it either represents an unusual cause of PPCD, or more likely, a rare polymorphism.

A third locus for PPCD has recently been mapped to an 8.55-cM region on the short arm of chromosome 10, indicating that locus heterogeneity exists for this autosomal dominant disorder.³² The existence of another genetic locus for PPCD had been suggested previously by investigations that excluded the PPCD locus on 20q11 and the *COL8A2* gene on 1p in two large families with PPCD.^{33,34} Twenty-six known genes and hundreds of predicted genes have been localized to the recently identified chromosome 10 locus.³² Therefore, as genetic heterogeneity has been demonstrated for PPCD, evaluation of linkage to the previously identified PPCD loci on chromosomes 1, 10, and 20 must be performed before further candidate gene screening commences. Because most of the affected probands reported in this study did not have a large number of affected and unaffected family members available for examination and specimen collection, linkage analysis in these families would not be informative in confirming or excluding linkage to the chromosome 1, 10, or 20 loci for PPCD. However, our labora-

tory has recently completed initial fine mapping of the PPCD locus on the long arm of chromosome 20, region 11, with the suggestion of linkage in four families with PPCD to this region (maximum single-point LOD scores of 2.25 and 2.16 for markers *D20S484* and *D20S195* [both at $\theta = 0.0005$], respectively; data not shown). Evaluation of the candidate gene region with more densely spaced markers will allow further refinement of the candidate interval, thus reducing the number of positional candidate genes. To permit the efficient evaluation of these remaining positional candidate genes, avoiding duplication of efforts by independent investigators, we encourage the publication of future candidate gene screening studies investigating the genetic basis of PPCD.

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