A Novel Variant of Granular Corneal Dystrophy Caused by Association of 2 Mutations in the TGFBI Gene—R124L and ΔT125-ΔE126

Paul Dighiero, MD; Séverine Drunat, MD; François D’Hermies, MD; Gilles Renard, MD; Marc Delpech, MD, PhD; Sophie Valleix, MD, PhD

Objective: To characterize the molecular defect in the TGFBI gene in a French family affected with an atypical granular corneal dystrophy.

Patients: This family comprises 9 affected individuals across 3 generations without consanguineous marriage.

Methods: Light and electron microscopy were used to examine corneal buttons from patients. Exons of the TGFBI gene were amplified by polymerase chain reaction and sequenced directly using an automated method. Restriction digestion analysis and heteroduplex screening were performed to confirm that the mutations identified were not polymorphisms.

Results: Round or snowflakes-like deposits that stained red with Masson trichrome and appeared as dense, rod-shaped structures were observed in the most anterior layers of the central stroma. All patients were heterozygous for the R124L mutation and a novel mutation predicting the deletion of 2 amino acid residues—threonine (T) and glutamic acid (E)—at codons 125 and 126.

Conclusions: This French family is affected with a novel variant of granular dystrophy that is caused by a molecular defect in the TGFBI gene, reported here for the first time.

Clinical Relevance: These 2 mutations cause a novel variant of granular dystrophy that is intermediate in severity between the classical and superficial variant forms.


IX AUTOSOMAL dominent corneal dystrophies—granular Groenouw corneal dystrophy type I, superficial variant of granular corneal dystrophy (SVGCD), lattice corneal dystrophy type I, lattice corneal dystrophy type IIIA, Avellino corneal dystrophy, and Reis-Bücklers corneal dystrophy—have all been shown to result from specific point mutations in the TGFBI gene.1-4 This gene maps to 5q31 and encodes a 683–amino acid protein, denoted as keratoepithelin. It contains a secretory peptide signal and an arg-gly-asp motif, usually found in cell adhesion molecules.5,6

The 2 corneal dystrophies of granular type (GCD), included in the previous paragraph, are characterized by the deposition of grayish white round or snowflakes-like opacities in the anterior central stroma. These deposits stain red with Masson trichrome and appear as dense, rod-shaped bodies by electron microscopy.7-9 Visual impairment usually occurs in the fifth decade, when the intervening corneal stroma between the deposits develops a diffuse, ground-glass appearance. Two clinical variants of GCD, the classical and the superficial forms, have been described previously.7,10,11 Superficial variant of GCD, the severe form, begins early in childhood with frequent recurrent erosive attacks and rapid visual loss. Confluent subepithelial deposits are found by histological examination, and this form is associated with the Arg124Leu mutation of keratoepithelin. The classical variant is a milder, late-onset form with few corneal erosions and is characterized by multiple snowflakes-like opacities that lead to visual impairment only in adulthood. This form is caused by the Arg555Trp mutation of keratoepithelin.12

In addition to these 2 well-characterized subtypes of granular dystrophy, other rare clinical variants exist. Their diagnosis remains confused, however, because they have not yet been defined at the molecular level. We report the clinical, histological, ultrastructural, and molecular study of a French family with 9 members through 3 generations who were affected with a rare variant subtype of GCD with autosomal dominant inheritance.

From the Department of Ophthalmology, Hôtel-Dieu Hospital (Drs Dighiero, D'Hermies, and Renard), and the Laboratoire de Biochimie et Génétique Moléculaire (Drs Drunat, Delpech, and Valleix), Paris, France.
PATIENTS AND METHODS

PATIENTS

The Table summarizes the clinical data from 9 patients with atypical GCD examined in the Department of Ophthalmology, Hôtel-Dieu Hospital, Paris, France. They were examined with a slitlamp biomicroscope (Hagg-Streit 900; Koeniz, Switzerland), and corneal photographs were taken with a slitlamp camera (model SI-6E; Topcon, Tokyo, Japan). None of the patients had any other ocular disease that might have decreased their best-corrected visual acuity.

The first symptoms usually occurred during the first decade of life and included severe recurrent erosions (7 of 8 patients) or decreased best-corrected visual acuity (5 of 8 patients). Bilateral corneal opacities always occurred before age 10 years, recurrent attacks of pain from corneal epithelial erosions appeared in infancy or early childhood, and decrease of visual acuity was present before age 30 years. The corneas of young, untreated patients had numerous opacities, which could be round or resemble snowflakes, that involved predominantly the superficial stroma and subepithelial layers (Figure 1, A). Initially the stroma between the opacities was clear (before age 15 years), but later (at 20-25 years old) the intervening corneal stroma developed a ground-glass appearance, which differs, however, from that seen in SVGCD. Figure 2, A, shows the pedigree of this family, which is consistent with an autosomal dominant inheritance pattern. No consanguineous marriage was noted.

METHODS

Light and electron microscopy (transmission electron microscope, CM 10; Philips, Eindhoven, the Netherlands) were used to examine corneal buttons obtained from 4 patients after penetrating keratoplasty. Each corneal button was bisected. Half was fixed in Bouin solution and prepared for histological processing. Corneal sections were stained with hematoxylin-eosin, periodic acid–Schiff, Alcian blue, Masson trichrome, and Congo red. The other half of each button was immersed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, embedded in epoxy resin, and sectioned for transmission electron microscopy. Semithin sections were stained with toluidine blue.

Genomic DNAs were extracted by standard protocols from peripheral blood leukocyte samples collected from the 9 patients, their healthy family members (members II:3, III:4, III:6, and III:8), and 95 unrelated control subjects after informed consent was obtained. Exons of the TGFBI gene were amplified from genomic DNA of each participant by polymerase chain reaction (PCR) using the appropriate forward and reverse primers previously reported. Approximately 300 ng of DNA was used in a 50-µL PCR amplification mixture containing 10-mmol/L Tris-hydrochloride (pH 8.3), 50-mmol/L potassium chloride, 0.2-mmol/L deoxynucleoside triphosphate (Pharmacia, St Quentin Yvelines, France), 15 mmol/L of magnesium chloride, 250 mmol/L of each primer, and 0.5 U of Taq polymerase (Boehringer Mannheim, Meylan, France). The PCR conditions were 3 minutes at 94°C followed by 30 cycles of 94°C for 40 seconds, 55°C for 40 seconds, and 72°C for 40 seconds, with a terminal extension step at 72°C for 3 minutes. Each PCR product was purified and sequenced on both strands using a sequencing kit (Big Dye Terminator; Applied Biosystems, Warrington, England) according to the manufacturer’s recommendations. The products were then resolved on an automatic fluorometric DNA sequencer (model ABI Prism 377; Applied Biosystems). Nucleotide sequences were compared with the nucleotide sequence of the TGFBI human complementary DNA reported by Skonier et al. Restriction enzyme digestion analysis with RsII (Boehringer Mannheim) was used to screen for the R124L mutation in the healthy family members and 95 unrelated controls.

For heteroduplex analysis, PCR products of exon 4 were heated at 95°C for 3 minutes and then slowly cooled to room temperature for 45 minutes. The annealed reaction products were electrophoresed through a nondenaturing 8% polyacrylamide gel (16 × 18 cm) at 8 mA over night. Gel preparation and electrophoresis were done according to standard procedures. After electrophoresis, the gel was stained in ethidium bromide and examined with an ultraviolet transilluminator to visualize bands.

Electron microscopy of 3 corneas revealed the characteristic features of GCD with electron-dense, rod-shaped structures. The deposits were irregular in shape and could appear as a single small rod or as a large mass, which may be the result of fusion of smaller elongated deposits in the extracellular spaces (Figure 1, B). No curly fibers were observed.

MOLECULAR GENETIC ANALYSIS

Sequence analysis of PCR products from exon 4 of the TGFBI gene first revealed a heterozygous single base pair (bp) transversion from G to T of the second nucleotide position of codon 124 (CGC/CTC). This would cause the replacement of arginine by leucine at codon 124 of keratoepithelin (R124L). This nucleotide substitution removed a restriction site for the RsII restriction enzyme that allowed for rapid PCR-based testing from all the family members. None of the healthy family members and 95 control
subjects tested were positive for this mutation (data not shown). Immediately after this G to T transversion, we observed a shift of the sequence of exon 4 in the affected patients, which might correspond to a short deletion. To confirm this observation, we analyzed PCR products of exon 4 by polyacrylamide gel electrophoresis to test for heteroduplex formation.14 Using this technique, heterozygous patients with small deletion or insertion mutations can be easily identified on the basis of the presence of specific DNA heteroduplexes formed during PCR amplification and seen in nondenaturing polyacrylamide gels. In theory, heterozygotes should generate 4 heteroduplex bands that reassociate to form 2 DNA heteroduplexes and 2 DNA homoduplexes. Each DNA heteroduplex differs in conformation and thus migrates with distinctive electrophoretic mobility. We therefore displayed the PCR products of exon 4 from 2 affected patients (patients II:5 and III:9) and 1 healthy family member (member II:3) on an 8% nondenaturing polyacrylamide gel. As shown in Figure 2, B, affected individuals have an altered band pattern compared with the

Figure 1. Clinical, microscopic, and ultrastructural photographs. A, Slitlamp photograph of the cornea from the proband (patient III:3) at age 23 years showing the corneal opacities. B, Electron micrograph of the cornea from patient III:1 at age 31 years showing typical rod-shaped bodies (d); c indicates collagen fibers (original magnification ×22600). Scale bar indicates 1 mm. C, Line of continuous deposits (arrows) underlying corneal epithelium in a zone of central cornea stained with Masson trichrome (patient III:3, 29 years old) (original magnification ×260). D, Discontinuous trapezoidal-shaped deposits (arrows) in peripheral cornea stained with toluidine blue (patient III:3, 29 years old) (original magnification ×260).

Clinical Characteristics of the 9 Patients*

<table>
<thead>
<tr>
<th>Patient No./Sex/Age, y</th>
<th>Age at Onset, y</th>
<th>BCVA</th>
<th>Surgical Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCVA</td>
<td>OD</td>
<td>OS</td>
<td>Right Eye</td>
</tr>
<tr>
<td>I:1/M/dead at 68, 10</td>
<td>20/100</td>
<td>20/100</td>
<td>2 PK</td>
</tr>
<tr>
<td>II:2/F/62, 10</td>
<td>20/50</td>
<td>20/50</td>
<td>2 PK</td>
</tr>
<tr>
<td>II:3/M/60, 7</td>
<td>20/30</td>
<td>20/100</td>
<td>1 PK, 1 PTK</td>
</tr>
<tr>
<td>III:5/F/27, 6</td>
<td>20/25</td>
<td>20/30</td>
<td>1 PTK</td>
</tr>
<tr>
<td>III:7/F/25, 6</td>
<td>20/100</td>
<td>20/30</td>
<td>0</td>
</tr>
<tr>
<td>III:9/M/29, 5</td>
<td>20/40</td>
<td>20/25</td>
<td>1 PK</td>
</tr>
<tr>
<td>IV:1/M/6, 5</td>
<td>20/20</td>
<td>20/15</td>
<td>0</td>
</tr>
</tbody>
</table>

*BCVA indicates best-corrected visual acuity; PK, penetrating keratoplasty; PTK, phototherapeutic keratectomy; and ellipses, data not available.
healthy family member. As expected, in addition to a homoduplex band of normal size, a mutant homoduplex band of smaller size and 2 heteroduplex bands of slower mobility are present. To characterize each allele of exon 4 from the patients, the 4 bands were eluted from the gel and sequenced directly on both strands. We thus identified a 6-bp deletion that included codons 125 and 126 predicting the deletion of threonine (T) and glutamic acid (E) residues. Sequencing analysis of each allele demonstrated that the 6-bp deletion, involving codons 125 and 126, and the G to T transversion at codon 124 were associated on the same chromosome. These 2 mutations were transmitted in all 9 affected family members with complete penetrance. None of the unaffected family members or the 95 unrelated individuals tested possessed this 6-bp deletion in exon 4, suggesting that it is not a polymorphism. We also screened for all the previously reported mutations of the TGFBI gene by sequencing exons 11 and 12, and none were present in these patients.1,2,15

We investigated patients from a French family affected with a dominant GCD that seemed to show substantial phenotypic variation compared with the 2 granular subtypes previously recognized. All the affected family members fulfilled the diagnostic criteria stated previously for granular dystrophy.7-9 All of them displayed the same phenotype, and no clinical variations were observed among patients. The clinical features consisted of round or snowflake-like opacities in the subepithelial and the most anterior layers of the central stroma. Thus, this dystrophy could easily be confused with the classic GCD form, but it differs by the prominent disposition of the deposits in the superficial stroma, its early onset, and the constant presence of recurrent painful corneal erosions during childhood. Rapid spreading of the deposits and an early ground-glass appearance of the intervening stroma are features that

Figure 2. A, Pedigree of the studied family affected with a novel variant of granular corneal dystrophy. Solid symbols indicate affected individuals; open symbols, unaffected family members. B, Heteroduplexes and homoduplexes analyzed on an 8% nondenaturing polyacrylamide gel. Healthy family member II:3 presented a unique expected band of approximately 220 base pair (bp), and patients II:5 and III:9, heterozygous for one 6-bp deletion mutation in exon 4, showed a pattern of 4 bands. Lane M represents the HaeIII marker 5 (MBI Fermentas) as size marker. C, Electropherograms of the wild (left) and mutant (right) alleles from patient II:5. Asterisks indicate the G to T transversion; black bar, the site of the 6-bp deletion in exon 4. Partial sequences of the normal and mutant alleles are shown at the bottom of the figure. Stars indicate the deleted nucleotides.
led to early loss of visual acuity (<20/40 in all patients at 25 years old) in patients affected with this GCD. Thus, this atypical form of GCD is reminiscent of SVGCD and classical GCD and is intermediate in severity.

Results of molecular investigations showed that this phenotype segregated in the 9 patients with 2 distinct mutations in exon 4 and involved 3 consecutive codons of the TGFBI gene. In addition, we demonstrated by sequencing of each allele in patients that these 2 mutations were located on the same chromosome and that patients were thus heterozygous for each of these mutations. These data were also in concordance with familial analysis segregation. The 6-bp inframe deletion, ΔACGGAG, in exon 4 is expected to produce a final shorter peptide, and that is likely to be one of the disease-causing mutations. The R124L mutation has been previously reported in one Japanese family and in a Japanese patient with sporadic SVGCD. The 6-bp deletion (ΔT125-ΔE126) has never before been reported in any of the six 5q31-linked autosomal dominant corneal dystrophies. Other atypical granule dystrophies have been described already, but the cases reported thus far have been shown to result from a homozygous mutational status of the TGFBI gene in patients.16-18

In conclusion, this corneal dystrophy seems to be a rare, novel clinical variant of the granular type. The molecular defect that we characterized is intriguing because the presence of the R124L mutation alone is responsible for an SVGCD, whereas its association with deletion of codons 125 and 126 produces significant phenotypic variations of the granular corneal disease. Thus, these data indicate that even the least variation in phenotype might reflect an underlying variation in genotype.

Accepted for publication December 18, 1999.

This work was supported by grants from La Fédération des Aveugles et des Handicapés Visuels de France, Paris.

We thank Michele Savoldelli for her help with light and transmission electron microscopy.

REFERENCES


©2000 American Medical Association. All rights reserved.