

BIGH3 Exon 14 Mutations Lead to Intermediate Type I/IIIA of Lattice Corneal Dystrophies

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PURPOSE. To screen the *BIGH3* gene in three unrelated families with lattice corneal dystrophy (LCD), two of which disclosed a particular phenotype.

METHODS. Genomic DNA was extracted from peripheral leukocytes of the affected patients and their family members. The entire coding sequence of the *BIGH3* gene was screened for mutations by means of transcript analysis on total RNA isolated from peripheral leukocytes by reverse transcription-polymerase chain reaction performed with primers designed for this study. Each mutation was confirmed at the genomic level, by using published primers.

RESULTS. One family that had a typical form of LCD, had the described R124C mutation in the *BIGH3* gene. Two families with atypical forms of LCD were negative for the previously known mutations of the gene. Direct sequencing of the *BIGH3* mRNA in the latter two families allowed us to identify two mutations located in exon 14. They consist of a 9-bp insertion at position 1885^1886 and one missense mutation at position 1877 of the *BIGH3* gene. Three new polymorphisms were also observed.

CONCLUSIONS. Two mutations different from those linked to LCD have been found in clinically distinguishable forms of this disease, intermediate between LCDs types I and IIIA. The DNA segment comprising both alterations normally encodes for a highly conserved region of the fourth internal domain of the β ig-h3 protein, suggesting that this region may be of functional and/or structural importance. The identification of new mutations by screening of the complete *BIGH3* gene and the comparative analysis of the induced modifications in β ig-h3 protein should shed light in the understanding of the molecular mechanisms underlying LCDs resulting from mutations in the *BIGH3* gene, and may help to explain their phenotypic heterogeneity. (*Invest Ophthalmol Vis Sci.* 2000;41:1302-1308)

Different blinding corneal dystrophies have been related to recurrent specific mutations in the *BIGH3* gene (transforming growth factor β -induced gene) on chromosome 5q31. These include lattice corneal dystrophy (LCD) types I and IIIA, deep stromal LCD reported by Fujiki et al.,¹ granular corneal dystrophy type I, granular corneal dystrophy type II (a mixed type of lattice and granular dystrophy previously named Avellino dystrophy), and Reis-Bückler corneal dystrophy. These conditions are defined by their clinical manifestations and the histologic characteristics of their corneal deposits.^{2,3} The deposits observed in lattice dystrophies fulfill the criteria of amyloidosis (Congo red-positive staining with apple green birefringence under polarized light and fibrillar

appearance in electron microscopy). With time, these deposits often induce severe impairment of vision and require surgical procedures to restore visual acuity.

Amyloid corneal dystrophy has been divided into five forms. LCD type I (122200 at Online Mendelian Inheritance in Man OMIM), (<http://www.ncbi.nlm.gov/Omim>), is characterized by thin grayish, linear, branching deposits of amyloid material that progressively accumulate in the subepithelial and stromal layers of the cornea. LCD type III (OMIM 204870) has a late clinical adult onset (70-90 years), a presumably autosomal recessive mode of inheritance and affects Japanese individuals.⁴ LCD type IIIA differs from LCD type III in that it has autosomal dominant trait of inheritance, affects white individuals, and frequently associates superficial corneal erosions.⁵ LCD type II (OMIM 105120), the Finnish type,⁶ is associated with familial systemic amyloidosis caused by mutations in the gelsolin gene.⁷ Gelatinous droplike corneal dystrophy (MIM 204870) is a nonlattice primary amyloidosis that has been reported in Japan and is not one of the *BIGH3*-mutation corneal dystrophies.⁸

The *BIGH3* gene product (β ig-h3) is an extracellular matrix adhesion protein inducible by transforming growth factor (TGF)- β , first isolated by Skonier et al.⁹ It is a prominent protein in the cornea, skin, and matrix of many connective tissues.

Each form of 5q31-linked autosomal dominant corneal dystrophy has been associated with a different mutation: LCD

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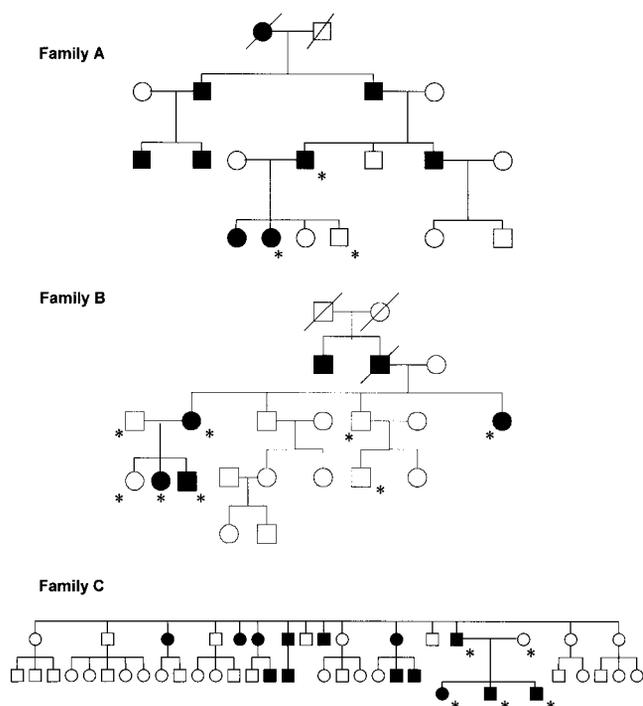


FIGURE 1. Pedigrees of families with LCD. *Individuals in whom molecular investigation of *BIGH3* mRNA and DNA was performed.

type I with R124C, granular corneal dystrophy type I with R555W, granular corneal dystrophy type II with R124H, Reis-Bückler corneal dystrophy with R555Q.¹⁰ Recently, a single deletion of codon Phe540 (Δ F540)¹¹ has been linked with a phenotypic variant of Reis-Bückler corneal dystrophy and two missense mutations, R124L¹² and R124S,¹³ with phenotypic variants of granular corneal dystrophy. Three novel missense mutations in the *BIGH3* gene responsible for LCD have also been reported: P501T¹⁴ for LCD type IIIA, L527R for an autosomal dominant form of LCD with deep stromal amyloid deposits,¹ and L518P¹⁵ for an early onset form of LCD classified as type I. Current molecular biologic knowledge of LCD suggests the existence of a mutation-phenotype correspondence, because each phenotype of *BIGH3* LCD seems to be linked to a specific mutation.

We present two families with a type of LCD clinically distinct from the previously identified LCDs (intermediate between LCD types I and IIIA). The first one was negative for all

the mutations previously described. The second one presented the H626R mutation that we and others have recently reported.^{16,17} Finally, we have included a third family with the classic LCD type I whose affected members shared the specific 370C>T mutation. These two newly described DNA modifications result in an alteration of the same region of the protein, the fourth internal domain, which is a highly conserved amino acid sequence.

METHODS

Patients

Three families with LCD were included in this study (Fig. 1) after obtaining informed consent for clinical and molecular investigations (according to the World Medical Association Declaration of Helsinki). The disease showed an autosomal dominant inheritance pattern in all of them. All the individuals included in the study underwent clinical examination before molecular investigation. No clinical signs suggestive of systemic amyloidosis were observed in any of the individuals.

DNA Extraction

Blood samples were obtained from 16 individuals (10 affected, 4 nonaffected, and 2 spouses), and genomic DNA was extracted from peripheral leukocytes of the LCD patients and their family members, by standard methods.

DNA Sequencing

We first screened exon 4 and exon 12, where hot spot sites for mutations in the *BIGH3* gene have been reported.¹⁸ Exons 4 and exon12 of the *BIGH3* gene were amplified by polymerase chain reaction (PCR) in a total volume of 50 μ l. The primers used were those published by Munier et al.¹⁰ The PCR products were purified (QIAquick PCR Purification Kit; Qiagen, Chatsworth, CA) and directly sequenced (Prism AmpliTaq FS dichloroRhodamine Dye Terminator kit; Perkin-Elmer, Applied Biosystems, Foster City, CA). The samples were resolved on a sequencer (model 377; Applied Biosystems). All sequence alterations identified initially in the *BIGH3* cDNA were confirmed at the genomic level by exon-specific PCR using primers published elsewhere.¹⁰ Twenty independent *BIGH3* genes from apparently unrelated individuals were sequenced in the region of each mutation to search for reproducible PCR-induced errors and polymorphisms. Sequence analysis was performed by manual DNA sequencing (Sequenase ver. 2.0 se-

TABLE 1. RT-PCR Primer Sequences

Fragments	Primers	Nucleotides
a	PCR1Fa: 5'-CCG TCG GTC GCT AGC TCG CT-3' PCR1Ra: 5'-ACA TCA GAC TCT GCA GCC AAT T-3'	-41 to 1163
b	PCR1Fb: 5'-ATC ATC TCC AAT AAA GAC ATC CTA G-3' PCR1Rb: 5'-AAC ATT CAA ACA GTC TCT GTG GAA A-3'	1051 to 2133
a1	PCR2Fa1: 5'-TGC GCG TCG TCC CGC TCC AT-3' PCR2Ra1: 5'-TCA TCA GTC AGG ACT CGC CT-3'	-18 to 551
a2	PCR2Fa2: 5'-TTG AGC TGC TCA ATG CCC T-3' PCR2Ra2: 5'-GTC TTG GCT GAG TCT GGG AT-3'	493 to 1133
b1	PCR2Fb1: 5'-AAC GGG GTG ATC CAC TAC AT-3' PCR2Rb1: 5'-TCA GTC CTG CAG ACT GGA T-3'	1081 to 1582
b2	PCR2Fb2: 5'-ATC GCT TTA GCA TGC TGG TA-3' PCR2Rb2: 5'-ATC TGA GAG AAA TTG GCG GAG A-3'	1538 to 2109

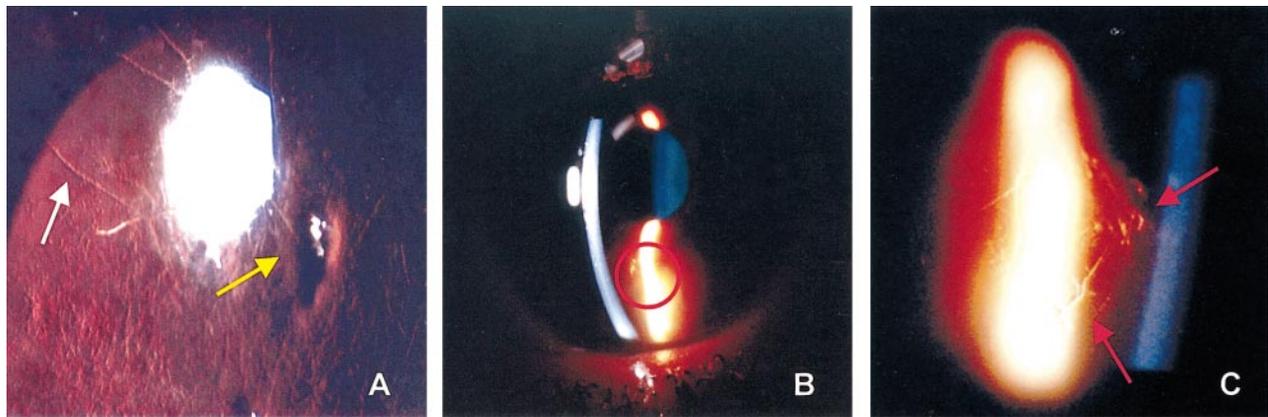


FIGURE 2. Family B. Thick lattice lines (*white arrow*) and subepithelial clump (*yellow arrow*) resulting in reduced visual acuity as low as 20/400 in a 58-year-old woman (A). Subepithelial stellate lattice deposits initiating in the lower center of one cornea of her 28-year-old son (B, *inside red circle*). Higher magnification of these initiating deposits (C, *red arrows*).

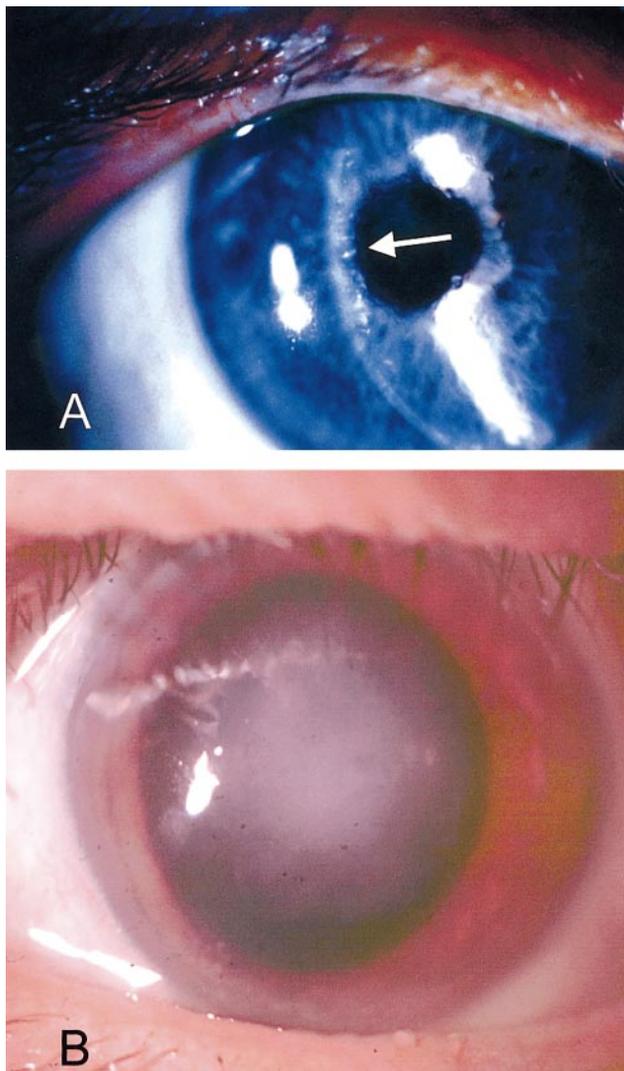


FIGURE 3. Family C. LCD type I. (A) Thin lattice corneal deposits in a 15-year-old boy with epithelial erosion. Subepithelial and stromal lattice lines (*arrow*) result in a light haze. (B) Strong corneal haze made of subepithelial and stromal lattice deposits resulting in severe loss of vision in his 55-year-old father.

quencing kit; USB, Cleveland, OH) and ^{35}S -deoxyadenosine triphosphate (ATP).

BIGH3 mRNA Screening for Mutations

The entire coding sequence of the *BIGH3* gene (2052 bp) was screened for mutations by means of transcript analysis. Total RNA was isolated from peripheral leukocytes using an isolation system (SV Total RNA ; Promega, Madison, WI), and reverse transcription (RT)-PCRs were performed as described.¹⁹ Primers (Table 1) were designed to amplify the total *BIGH3* gene cDNA in two partially overlapping fragments a and b. A 2- μl aliquot of the initial PCR-product (PCR1) was used as a template in a second, nested PCR (PCR2) reaction, to amplify 4 overlapping fragments of 600 bp each (fragments a1, a2, b1, and b2). PCR reactions were performed at 60°C in a total volume of 50 μl . The PCR products were purified (QIAquick PCR Purification Kit; Qiagen) and manually sequenced as described above.

Haplotype

Haplotype analysis of affected and unaffected individuals from family B was performed from PCR-amplified lymphocyte DNA by use of four microsatellite markers (D5S393, D5S399, D5S500, D5S399) within 1 cM of the disease locus.²⁰ All microsatellites have been obtained from Généthon.²¹ Fluorescence PCR assays were carried out in a total volume of 50 μl (model 2400; Perkin-Elmer, Applied Biosystems). The reaction mixes and the PCR conditions were those described for the specific primers. Fluorescence PCR products were separated using a standard semiautomated method on a sequencer (model 377; Applied Biosystems).

RESULTS

Phenotypes

Two families had features that were intermediate between LCD type I and LCD type IIIA. Affected individuals of family A had corneal lattice deposits thinner than those of family B but thicker than those of family C, and subepithelial haze with occurrence of corneal erosions. Disease onset was during the second decade of life. Penetrating keratoplasty was necessary

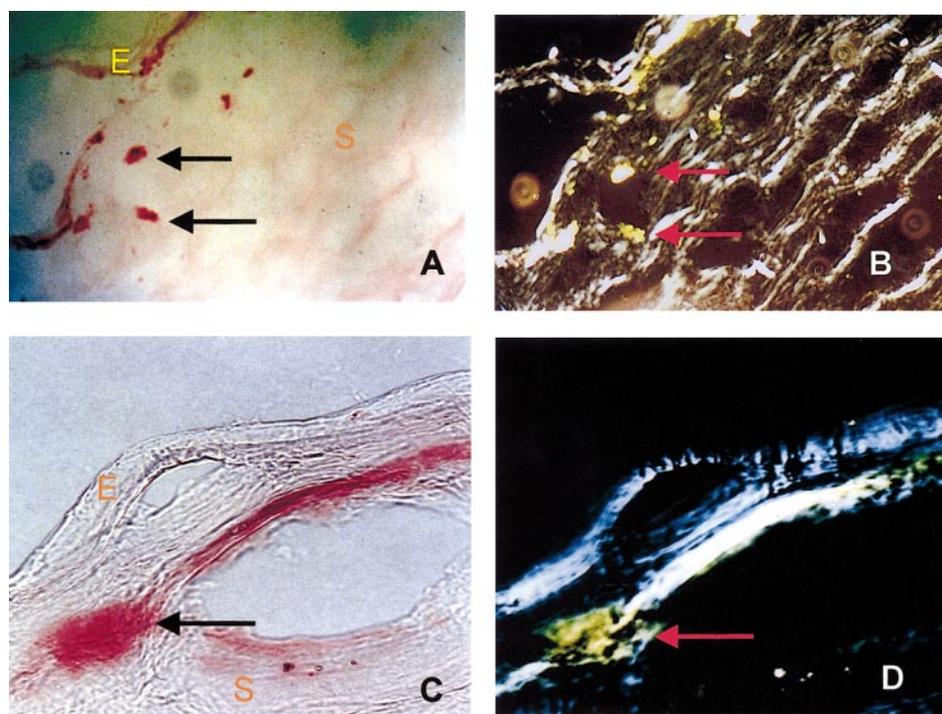


FIGURE 4. Congo red staining on one corneal button from a 55-year-old family C member (A, B) and one corneal button from a 58-year-old family B member (C, D) showing red-apple green dichroism of thin amyloid deposits in family C and thick amyloid deposits in family B. These findings confirmed the amyloid nature of the deposits displayed clinically by each of these families. E, corneal epithelium; S, corneal stroma. Arrows indicate amyloid deposits. Magnification, $\times 25$.

in this family in the fourth decade. The affected individuals of family B displayed thick lattice deposits in the stromal layer and subepithelial clumps with no occurrence of superficial corneal erosions (Fig. 2A). The onset of the disease was in adulthood, approximately 27 years of age. The disease was characterized at its onset, by asymmetrical stellate deposits located in the lower center of the cornea (Figs. 2B, 2C). Penetrating keratoplasty was necessary in affected members of this family in the fifth decade of life for severely reduced visual acuity.

The classic phenotype of LCD type I is represented in our study by family C. The affected members of this family disclosed thin linear branching deposits in the subepithelial and stromal layers resulting in corneal haze (Fig. 3). The deposits initiated during childhood, visual impairment was obvious in the second decade of age, and it was associated with frequent recurrent corneal erosions.

The amyloid origin of the deposits in families B and C was confirmed on corneal buttons by Congo red staining. They displayed a typical red-apple green dichroism under polarized light (Fig. 4). They were thicker in family B than in family C.

Because β ig-h3 protein is highly expressed in the skin,⁹ we screened, after obtaining informed consent, one 55-year-old affected individual of family C for the presence of dermoepidermal amyloidosis and compared the results with a normal control clinically negative for amyloidosis. Interestingly, Congo red staining was negative and electron microscopy did not show fibrillar deposits, demonstrating the absence of amyloid deposits in skin. These histologic features were similar on both the patient with LCD type I and the normal control (data not shown).

Mutation Screening

Family A. All the affected and unaffected members of family A were negative for the mutations previously reported in association with the LCDs (R124C, P501T, L518P) as well as

for those associated with other *BIGH3* corneal dystrophies (R124H, R124S, L527R, R555W, R555Q, Δ F540). Complete *BIGH3* mRNA sequencing analysis was performed to search for mutations in these patients. It demonstrated a shift in exon 14. Electrophoresis on a 10% polyacrylamide gel of PCR products of exon 14 amplified from genomic DNA demonstrated two amplicons separated by approximately 10 bp in affected individuals and a single amplicon in unaffected individuals as well as in control individuals. Genomic DNA sequencing of exon 14 in this family found a heterozygous 9-bp insertion at position 1885[^]1886 of the *BIGH3* gene [1885[^]1886ins9(CCAATGTTC)] (Fig. 5) leading to an in-frame insertion of three amino acids Asn-Val-Pro between codons 629 and 630 of the β ig-h3 protein (NVP629-630ins). This insertion consisted of a duplication of a 9-bp (1886-CCAATGTTC-1894) sequence of exon 14. Sequence analysis of the whole *BIGH3* cDNA in this family did not reveal any other putative disease-causing mutation.

Family B. Haplotype analysis showed that one *BIGH3* allele cosegregated with the disease and that the clinically unaffected 22-year-old individual (II-1 in Fig. 6) inherited this allele as had individuals with the clinical manifestations of LCD.

Confirming the haplotype analysis, a missense mutation in affected individuals was detected at codon H626 in exon 14 (Fig. 6). Genomic DNA sequencing of exon 14 confirmed the heterozygous 1-bp transition 1877A>G (CAT \rightarrow CGT) of codon 626 leading to a substitution of histidine for arginine: H626R. These findings were confirmed by using both sense and anti-sense primers. This mutation was found in all the affected members of the family as well as in the 22-year-old individual who was younger than the expected age of onset of the disease. None of the previously reported LCD-associated *BIGH3* mutations was detected in this family.

Family C. Direct sequencing analysis of exon 4 amplified from genomic DNA demonstrated that one allele of all the affected individuals from family C had the C>T transition

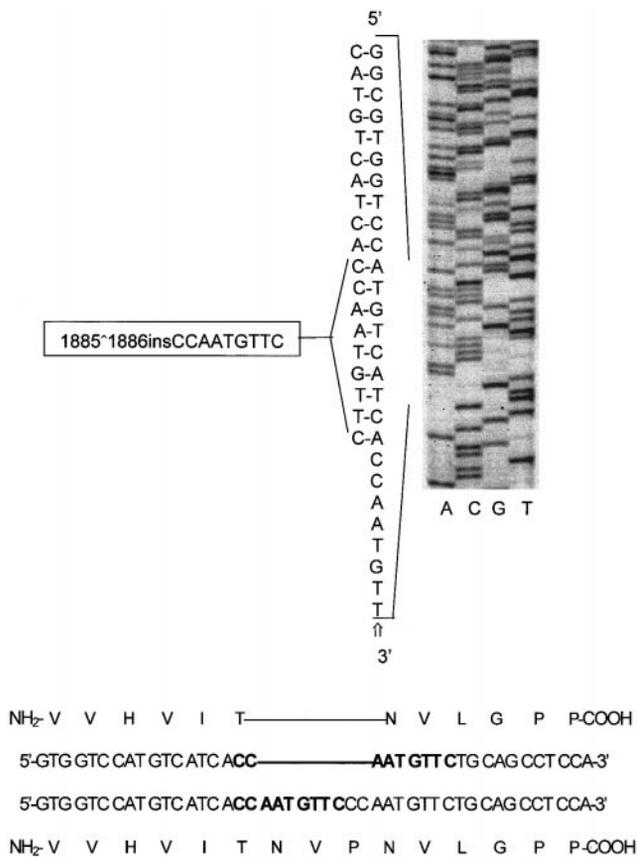


FIGURE 5. Direct sequencing of the *BIGH3* gene exon 14 in one affected individual of family A showing a 9-bp insertion and duplication (1885^1886insCCAATGTTT) leading to the insertion of an in-frame 3 amino acids Asn-Val-Pro at position 629–630 of the protein (NVP629–630ins). (↑) Normal sequence. The sequence context of the duplication (**bold type**) is presented.

TABLE 2. Polymorphisms Detected in the *BIGH3* Gene

Exon/Intron	Nucleotide	Codon
Exon 6	651 C/G	Leu217
Exon 8	981 A/G	Val327
Exon 11	1416 C/T	Leu472
Exon 12	1620 T/C	Phe540
Exon 12	1632 T/C	Asn544
Intron 5	IVS5+7insA	

(CGC→TGC) at position 370 that caused an arginine-to-cysteine substitution (R124C). Thus, family C expressed the typical phenotype of LCD type I, and presented the known mutation in the β ig-h3 protein (R124C).

Polymorphisms

We identified five nucleotide substitutions that did not change the amino acid sequence of the encoded protein, did not cosegregate with the disease, or were present in normal control individuals (Table 2). Three of these polymorphisms were identical with those that other investigators have reported.^{18,22}

The IVS5+7insA variation detected in family B created an *Hin*I digestion site and suppressed a *Nde*II digestion site. Digestion of PCR products from exon 5 amplification revealed that this insertion in intron 5 did not cosegregate with the disease. Transcript analysis demonstrated that this sequence variation, located in the vicinity of the donor splice site of intron 5 does not alter mRNA processing. cDNA products were of the expected size with no evidence of exon 5 skipping or intronic insertion. Sequencing of the RT-PCR products did not exhibit any sequence alteration.

DISCUSSION

In the present study, we report a novel 9-bp insertion/duplication of the *BIGH3* gene and one missense mutation in two apparently unrelated families with a type of LCD clinically

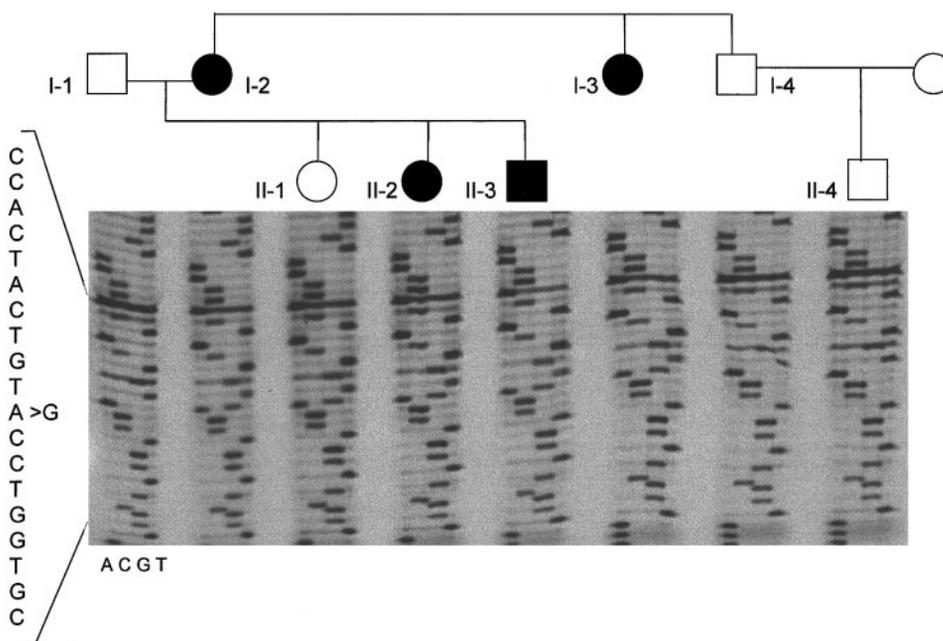


FIGURE 6. Direct sequencing of exon 14 of the *BIGH3* gene in family B. Sequencing of the normal and mutant alleles demonstrated a A>G transition at position 1877 (CAT→CGT) resulting in a histidine-to-arginine substitution (H626R) in the protein. Individual II-1, a 22-year-old and younger than the age of onset of the disease in this family inherited the mutant allele as shown by the sequencing of exon 14. Open symbols represent clinically unaffected individuals. Filled symbols represent clinically affected individuals.

distinct from the previously described types of LCD. Indeed, both phenotypes presented by these families clearly diverge from LCD type I displayed by family C, in which we identified the classic R124C mutation. They also differ from LCD type IIIA linked to the P501T mutation. Differences on LCDs type I and IIIA were also evident in several clinical respects: age of onset of the disease, age of severely reduced visual acuity, morphology of the subepithelial and stromal deposits, and occurrence of corneal erosions.

The *BIGH3* gene product β ig-h3 is a secreted protein of 683 amino acids, which contains an aminoterminal secretory sequence, a carboxyl-terminal RGD (Arg-Gly-Asp) sequence located from codon 642 to codon 644, and four homologous domains of 140 amino acids. The RGD sequence is known to act as a ligand-recognition site for integrins in several proteins. However, the integrins that specifically bind to corneal β ig-h3 protein await identification. It is thought that the four domains of the protein can be folded into a potential bivalent tetrameric structure that may act as a bridge between cells expressing the appropriate ligand.⁹ Thus, β ig-h3, similarly to other homologous cell adhesion proteins (human osteoblast specific factor 2: OSF-2; drosophila fasciclin-I: DrF-1; and *Mycobacterium bovis* MPB70)²³ may interfere in cell adhesion.

The 9-bp insertion at position 1885^1886 (1885^1886ins9) is the first insertion type DNA modification described in the *BIGH3* gene associated with LCD. It is located in the region encoding for the last portion of the protein, close to the 642-RGD-644 sequence of the protein. It is the most distal DNA alteration reported in this gene. The duplication occurred in a sequence context containing several palindromic or quasipalindromic sequences, direct and inverted repeats and a polypurimidine run of 5 bp. Such elements are known to contribute to the deletional or insertional mutagenesis and to promote mispairing in secondary DNA structures during DNA replication. This duplication results in the addition of three amino acid residues at position 629-630 of the protein: NVP629-630ins. Proline, frequently found in the bends of folded protein chains, has the recognized capability of disrupting the backbone structure of polypeptides and causes a sharp transition in the direction of the chain. These properties are of particular interest for LCD, because the cases studied by us were characterized by amyloid deposition of the β ig-h3 protein, and the β -pleated structure is one of the specific traits of amyloidosis.^{24,25} Valine is a hydrophobic amino acid. Again, it is noteworthy that hydrophobic residues are frequently observed in amyloid proteins and are thought to participate in their precipitation.²⁶ Finally, the location of the NVP insertion, close to the RGD sequence, may impair its binding to its natural ligands and alter its metabolism, favoring its accumulation.

The second new DNA modification found in the families reported in this study is the H626R mutation. Our preliminary report¹⁶ documenting the H626R mutation in LCD has been confirmed recently by Stewart et al.¹⁷ who observed clinical heterogeneity in age of onset in the patients with H626R mutation, the presence of epithelial erosions and the timing of bilateral involvement.¹⁷ In contrast to the family studied by Stewart et al.¹⁷ all affected members in one of our families (family B) had almost identical clinical manifestations. The phenotypic differences within and between families sharing the same mutation suggest that other factors participate in the manifestations of this disorder. Repairing of the corneal layers is associated with an increased expression of β ig-h3 pro-

tein.^{27,28} Therefore, the increase in the synthesis of the amyloid precursor protein in repetitive corneal lesions such as epithelial erosions may be one of the factors participating in the expression of the disease.

Both NVP629-630ins and H626R are located in the fourth internal domain of the β ig-h3 protein. This particular region has a highly conserved amino acid sequence between the four internal domains. H626 is conserved in domains 1, 2, and 4 of the human protein and in homologous proteins: *Drosophila* fasciclin 3 (DrF-3), grasshopper fasciclin 3 (GrF-3), and porcine and chick collagen fiber-associated protein (RGD-CAP). The constant presence of histidine residue in all these regions of the protein and the fact that it is observed across species suggests that H626 is of functional and/or structural importance. Therefore, a mutation in this region may have important consequences for the structure and/or the function of the protein. It is tempting to speculate that the new mutations presented here facilitate the β -pleated sheet structure favoring the accumulation of insoluble protein material and amyloid fibril formation.

Analysis of the predictive secondary structure of the native and mutated β ig-h3 proteins (<http://pbil.ibcp.fr/NPSA>) did not reveal any particular structure or site of processing suggestive of amyloid predisposition. There was no particular shared modification of the protein induced by the mutations in codons 124, 501, 518, 527, 540, 555, 626, 629-630 that would enable us to differentiate amyloid from nonamyloid-inducing mutations. These results also suggest that the mutation of *BIGH3* is not the only factor responsible for its precipitation in insoluble amyloid fibrils. The factors that have been proposed as participants in the formation of amyloid fibrils are misfolding of the protein, incomplete proteolysis, increased hydrophobicity, and ionic interactions.^{24,26,29,30}

Because the R124C mutated β ig-h3 protein was present, both in skin and in corneal tissues, the absence of amyloid deposits in the skin of the 55-year-old individual of family C strongly suggests that other factors, probably locally determined, are required for the mutated β ig-h3 protein to precipitate into amyloid fibrils. Whether, these factors include the classically evoked limited proteolysis, the interaction with other proteins with amyloid enhancing activity, or other specific corneal characteristics with unidentified facilitating capacity for the genesis of amyloidosis remains to be elucidated.

Autosomal dominant corneal dystrophies have been associated with a variety of mutations in the *BIGH3* gene.^{1,10-15} Among these dystrophies three clinically well-differentiated forms of *BIGH3*-linked LCD (type I, type IIIA, and the type described by Fujiki et al.¹) are the consequence of amyloid fibril deposition. The previous reports of mutations in the *BIGH3* gene suggest that each of the different mutations identified corresponds to a specific type of 5q31-linked LCD. This may also be the case for the mutations described in this study. Indeed, each of these two mutations (NVP629-630ins and H626R) in the *BIGH3* gene is associated with a phenotype that differs from the previously described *BIGH3*-linked LCD.^{5,15,31-33} The pathogenic mechanisms by which amyloidosis appears in LCD are not known, and the pathogenesis of amyloidosis in general is not fully understood. Five of the previously known mutations of the *BIGH3* gene, resulting in the correspondent protein mutations, have been recognized as inducing amyloidosis: R124C, R124H, P501T, L518P, and L527R. The comparative analysis of these mutations within

LCD, as well as the search for common points with other amyloidoses such as that occurring in Alzheimer's disease, hereditary neuropathy in Portuguese families, or others, should help in understanding the molecular mechanisms of the occurrence of LCD.

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