Molecular Vision 2007; 13:1976-83 <http://www.molvis.org/molvis/v13/a223/>
Received 11 July 2007 | Accepted 15 October 2007 | Published 18 October 2007

TGFBI (BIGH3) gene mutations in Hungary - report of the novel F547S mutation associated with polymorphic corneal amyloidosis

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Purpose: To identify mutations in the Transforming Growth Factor Beta Induced (*TGFBI*) gene in Hungarian patients with corneal dystrophy and to characterize histological features of their corneal buttons excised during penetrating keratoplasty.
Methods: Exons of *TGFBI* were sequenced in 38 members of 15 unrelated families with corneal dystrophy and exon 12 was also sequenced in 100 healthy controls from the same population. Immunohistological analysis of available corneal buttons excised during penetrating keratoplasty was also performed.
Results: Molecular genetic analysis revealed a heterozygous R124C mutation in 18 patients with lattice type I dystrophy. A R555W heterozygous mutation was detected in five patients with granular Groenouw type I corneal dystrophy and a R555Q heterozygous mutation was found in four patients clinically diagnosed with Reis-Bücklers (one patient) and Thiel-Behnke (three patients) dystrophy. Three patients with "atypical granular" dystrophy later diagnosed as Avellino dystrophy were heterozygous for the R124H mutation. A novel heterozygous mutation (T1640C) causing a F547S amino acid exchange was detected in a patient with polymorphic corneal amyloidosis. Immunohistochemistry showed the presence of BIGH3 protein deposits in all examined corneal buttons. Electron microscopy confirmed the presence of amyloid fibrils in the case of the novel mutation.
Conclusions: Our results indicate that molecular genetic analysis is required to confirm the diagnosis of corneal dystrophies. We report the first cases of Avellino dystrophy from Central-Eastern Europe. We conclude that the novel F547S mutation causes polymorphic corneal amyloidosis since no other mutations were detected in the *TGFBI* gene of this patient and the novel mutation could not be found in healthy controls.

5q31 linked corneal dystrophies are a clinically and histologically heterogeneous group of autosomal dominantly inherited corneal disorders. All these dystrophies result from mutations in the Transforming Growth Factor Beta Induced (*TGFBI*; BIGH3) gene, encoding for the BIGH3 protein, also called keratoepithelin [1]. Corneal dystrophies caused by specific mutations of *TGFBI* include lattice corneal dystrophies type I (LCDI), type IIIA (LCDIIIA), type I/IIIA (LCDI/IIIA), and type IV(LCDIV), granular Groenouw type I corneal dystrophy (GCDI), Avellino corneal dystrophy (ACD), Thiel-Behnke dystrophy or corneal dystrophy of Bowman layer type II (CDB2), and Reis-Bücklers' dystrophy (RBCD). The most frequent types of these dystrophies result from mutations of two mutational hot spots in exons 4 and 12 of the *TGFBI* gene, representing codons 124 (LCDI, ACD, and RBCD) and 555 (GCD and CDB2), respectively. Mutations responsible for rare lattice dystrophies (LCD types III and IV) localize to other sites within exons 12, 13, and 14, encoding for the fourth fasciclin1 domain of the BIGH3 protein [2,3]. Histological examination of corneal specimens show amyloid deposits in lattice dystrophies and Avellino dystrophy, hyaline accumulation in granular dystrophy, and the presence of subepithelial fibrous material in Reis-Bücklers and Thiel-Behnke dystrophy [4]. The two latter dystrophies, being relatively rare and somewhat similar in clinical appearance, were not always clearly distinguished in earlier studies [5,6]. However, the recent literature revealed distinct genetic, morphological, and clinical characteristics of these diseases. In Reis-Bücklers dystrophy, caused by the R124L mutation, subepithelial geographic corneal deposits can be seen on clinical examination, and rod shaped bodies can be observed by electron microscopy. Thiel-Behnke dystrophy, caused by the R555Q mutation, is characterized by honeycomb-shaped subepithelial deposits, which are composed of curly filaments as shown by electron microscopy [7,8].

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In several corneal dystrophies, the deposits contain the BIGH3 protein as demonstrated by immunohistological examination [9,10].

In this study, we present the results of a molecular genetic analysis of 15 families referred to our department between 1997 and 2006 because of presumed BIGH3-related corneal dystrophies. Immunohistochemical studies of available dystrophic corneal buttons excised during penetrating keratoplasty were performed using a polyclonal antibody detecting the BIGH3 protein. The clinical and histological characteristics of a corneal dystrophy similar to polymorphic corneal amyloidosis, caused by the novel F547S mutation, are described.

METHODS

Patients: Thirty-eight members from 15 families that were referred to the participating ophthalmology services between 1997 and 2006 were examined. One hundred unrelated, healthy volunteers from the same population who showed no sign of corneal disease on clinical examination were also recruited. All examinations were performed according to the tenets of the Declaration of Helsinki. All patients and controls were enrolled in the study after informed consent.

Clinical examinations included assessment of best-corrected visual acuity (BCVA), slit lamp examination, and slit lamp photography. On clinical examination, 18 patients showed the characteristics of lattice corneal dystrophy type I, five patients had granular dystrophy Groenouw type I, one patient was diagnosed with Reis-Bücklers, and three with Thiel-Behnke dystrophy. Three patients had "atypical granular" dystrophy and one patient was diagnosed with "atypical lattice" corneal dystrophy. Seven family members did not show any clinical sign of corneal dystrophy.

Table 1. Primers used for sequencing exons of *TGFBI*

Exon	primer	Sequence	Annealing temperature (°C)	product length (bp)
1	forward:	5-CCCTCCCGCTCGCAGCTTAC-3	55	424
	reverse:	5-CCCTCTCTCCTTCCACCGCG-3		
2	forward:	5-GACCCAAGGAGGCAAACACG-3	55	232
	reverse:	5-CCAGCGTGCATACAGCTTGG-3		
3	forward:	5-CTCTTCCTTGGCTGTGGAGG-3	55	228
	reverse:	5-TCCTCTCTCCCACCATTCCC-3		
4	forward:	5-CCCCAGAGGCCATCCCTCCT-3	58	226
	reverse:	5-CCGGGCAGACGGAGGTCATC-3		
5	forward:	5-TCACGAGGGCTGAGAACATG-3	55	341
	reverse:	5-CAGAAACCAGCCCACACATG-3		
6	forward:	5-GCTTTGGGACTATGCCTCTG-3	58	260
	reverse:	5-CTCTTGGGAGGCAATGTGTC-3		
7	forward:	5-GAGCTTGGGTTTGGCTTCTG-3	55	350
	reverse:	5-AGCAGGTCTTGGGCTCAATC-3		
8	forward:	5-GCTGCAAGTGGTCCCTGAGG-3	60	407
	reverse:	5-AGCTGTGAGCTGGGTCCAGG-3		
9	forward:	5-TGCTGATGTGTGTCATGCCC-3	55	426
	reverse:	5-CCCAAAGGCATTCTCTGCAC-3		
10	forward:	5-CCAAACTCAAGGAGGGATGG-3	58	373
	reverse:	5-CCTTCCTTGTCAGCAACCAG-3		
11	forward:	5-CTGAACAAATCAGGAGGCCC-3	55	288
	reverse:	5-CTTGACAAATAGCTGGGGCC-3		
12	forward:	5-TGTGCATTCCAGTGGCCTGG-3	55	339
	reverse:	5-GCCAACTGTTTGCTGCTGGG-3		
13	forward:	5-GTGCTTATTCCCTGGGCAGG-3	55	317
	reverse:	5-ATATGTCCTGGAGCCCTGCC-3		
14	forward:	5-TCGAGATCATGCCACTGCAC-3	55	344
	reverse:	5-TCTCCACCAACTGCCACATG-3		
15	forward:	5-AGCCAGAAAGCCCACCAGTG-3	58	275
	reverse:	5-AGCAGCCAAGGAAGACAGGG-3		
16	forward:	5-TCTGCCAAGGCCACCTGAGG-3	58	278
	reverse:	5-CAGCAGATGGCAGGCTTGGG-3		
17/A	forward:	5-AGAGCATGGCAGAAGGAGGC-3	55	306
	reverse:	5-CCCACATGCACAAGGCTCAC-3		
17/B	forward:	5-TACAGGAGGAATGCACCACG-3	55	376
	reverse:	5-AAACTGTGCCAGGACTTCCC-3		
17/C	forward:	5-GACTGCCTATGCCAAGTCCC-3	58	364
	reverse:	5-AGGTGTGGAGGAGCATGACG-3		

Primers have been optimized for PCR amplification of the 17 exons of *TGFBI* from genomic DNA. In addition to entire exons, short segments of their flanking introns were also covered. Exon 17 was sequenced in three overlapping segments.

Molecular genetic analysis: Peripheral blood (9 ml) was taken from patients, unaffected family members, and 100 healthy volunteers. Genomic DNA was isolated from buffy coat using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Exons of *TGFBI* were amplified using polymerase chain reaction (PCR) primers encompassing entire exons and short segments of flanking introns. Exon 17 was sequenced in three overlapping segments. Primers that we have optimized for amplification are listed in Table 1. For exon 4, they are identical to those previously published [1]. Primer annealing temperature was adjusted separately for each PCR reaction and ranged from 50 °C-60 °C. An initial step of denaturation at 95 °C was followed by 35-45 cycles of annealing, elongation at 72 °C, and denaturation at 95 °C. PCR products purified by ultrafiltration were sequenced with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Histology and immunohistochemistry: Corneal buttons of 15 patients affected by lattice corneal dystrophy type I (LCDI; 10 cases from five families), corneal dystrophy of Bowman layer type II (CDB2; two cases, one case initially diagnosed as Reis-Bücklers dystrophy), Groenouw corneal dystrophy type I (GCDI; two cases), and polymorphic corneal amyloidosis (one case) were either fixed in 98% ethanol-2% acetic acid solution and embedded in paraffin or frozen in TissueTek OCT (Bayer, Pittsburgh, PA) and stored in liquid nitrogen until use. One of the two CDB2 and GCDI corneal buttons were recurrences 15 and 11 years after the first transplantations, respectively. The patients with Avellino dystrophy had best corrected visual acuities between 20/30 and 20/20, therefore keratoplasty was not performed and corneal buttons with Avellino dystrophy could not be subjected to histological analysis. Tissue sections were stained with hematoxylin-eosin, congo red, and Masson's trichrome in each case. Congo red stained sections were examined under polarized light as well. Polyclonal anti-BIGH3 antibody against an 18 amino-acid peptide identical with the NH_2-terminal portion of the protein was prepared in chickens as described [11]. Sections of corneal buttons were placed on β-methacrylopropyltrimetoxy-silane-coated glass slides (Sigma, Schnelldorf, Germany), blocked with 5% horse serum, and then incubated with 1:100 diluted polyclonal antisera. Slides were developed with Sigma Fast Red tablets (Sigma) according to the manufacturer's instructions. Slides were examined with a Nikon Eclipse TS100 microscope (Nikon Instruments Europe B.V., Badhoevedorp, Netherlands) with 20X and 40X objectives.

Electron microscopy: One-quarter of the corneal button of the patient having the F547S mutation was fixed in 1% glutaraldehyde, dehydrated in methanol, and embedded in

Epon. Ultrathin sections were placed on coated 300 mesh copper grids and post-contrasted in uranyl acetate-lead citrate. Sections were examined with a Jeol 1010 transmission electron microscope at 80 kV.

RESULTS

Results of genetic and immunohistochemical examinations are summarized in Table 2. In all patients diagnosed with LCDI dystrophy, the *TGFBI* R124C mutation was found. No mutation in *TGFBI* was detected in seven clinically unaffected LCDI family members. In five patients showing the clinical signs of GCDI, the heterozygous R555W mutation was present. In one patient initially diagnosed as having Reis-Bücklers dystrophy and in three patients with CDB2 (Thiel-Behnke) dystrophy, the heterozygous R555Q mutation was detected. Thus, the diagnosis of Reis-Bücklers dystrophy was modified to CDB2 (Thiel-Behnke) dystrophy based on the results of the molecular genetic analysis.

Immunohistological examination of 10 LCDI corneal buttons with a polyclonal antibody against the NH_2-terminal portion of the BIGH3 protein revealed strong staining of the intraepithelial and subepithelial deposits and weaker staining around deep stromal deposits while no labeling within the deep deposits was observed (Figure 1A,B). Intra- or interfamilial differences were not noted among LCDI patients. In GCDI corneas, strong staining of the superficial and weaker staining of the deep granular deposits was observed (Figure 1C,D). In the CDB2 corneal buttons, strong labeling of the subepithelial fibrous deposit could be detected (Figure 1E,F).

In three members of a family, clinical examinations revealed breadcrumb-like deposits in the central anterior cornea, which had linear extensions toward the deeper stroma. Between the deposits, the cornea was clear. Based on the clinical appearance, the initial diagnosis was “atypical granular” dystrophy. Molecular genetic examination revealed the previously reported R124H mutation and established the diagnosis of Avellino dystrophy.

In both corneas of a 46-year-old patient, slit lamp examination revealed central, snowflake-like deposits mixed with fine linear and ice-chipped appearing deposits, which were more abundant in the central than in the peripheral cornea. Deposits were seen throughout the corneal stroma and numerous pre-Descemet deposits could be observed, however, no corneal guttae were present. A diffuse haze was seen between the central corneal deposits (Figure 2A,B). The visual acuity of the patient was 20/200 (right eye) and 30/200 (left eye). A novel heterozygous mutation, T1640C, causing the F547S amino acid exchange was detected in exon 12 of *TGFBI* in this patient (Figure 2F). Histological examination of her corneal button showed abundant congo red positive deposits mainly in the deep layers of the corneal stroma (Figure 2C), yielding green birefringence when examined under polarized light (Figure 2D). Immunohistochemistry showed strong staining of the deposits after labeling with a polyclonal anti-BIGH3 antibody (Figure 2E). Electron microscopic examination detected electron-dense deposits consisting of 8-10 nm thick, straight, nonbranching fibrils among collagen lamellae of the corneal stroma (Figure 3). All these findings indicate the pres-

Table 2. Results of genetic and histological examinations

Clinical diagnosis	Molecular genetic diagnosis	Number of patients	Mutation	Number of corneal buttons examined	Deposit type	Immunohistology (N-terminal BIGH3)
LCDI	LCDI	18	R124C heterozygous	10	Amyloid	Intra- and subepithelial deposits stained
GCDI	GCDI	5	R555W heterozygous	2	Hyaline	All deposits stained
CDB1 (Reis-Bücklers)	CDB2 (Thiel-Behnke)	1	R555Q Heterozygous	1	Subepithelial acidophilic fibers	All deposits stained
CDB2 (Thiel-Behnke)	CDB2 (Thiel-Behnke)	3	R555Q heterozygous	1	Subepithelial acidophilic fibers	All deposits stained
Atypical granular	Avellino	3	R124H heterozygous	-	-	-
Atypical lattice	Polymorphic corneal amyloidosis	1	F547S heterozygous	1	Amyloid	All deposits stained
Healthy family member	Healthy family member	7	None	-	-	-

The table summarizes clinical and genetic diagnoses as well as histological findings for 31 patients with dystrophic corneas and their 7 healthy family members. Previously reported genotype-phenotype correlations have been confirmed. Clinical diagnosis had to be amended based on genetic information in 4 cases (one Reis-Bücklers changed to Thiel-Behnke and 3 atypical granular to Avellino). A new mutation F547S was found in a case of polymorphic corneal amyloidosis. Deposits in all dystrophic corneal buttons contained the NH_3-terminal part of the BIGH3 protein.

ence of BIGH3 containing amyloid deposits in the cornea of this patient. The novel T1640C mutation was not detected in the 100 unrelated healthy controls from the same population. Therefore, we conclude that the T1640C mutation causing the F547S amino acid exchange in the BIGH3 protein is responsible for the corneal dystrophy of this patient.

DISCUSSION

In accordance with many previous reports [12-15], we have detected most of the mutations at codons 124 and 555 of *TGFBI*, indicating that these sites represent mutational hot spots in Hungarian corneal dystrophy patients as well. In four cases, the initial clinical diagnosis had to be corrected based

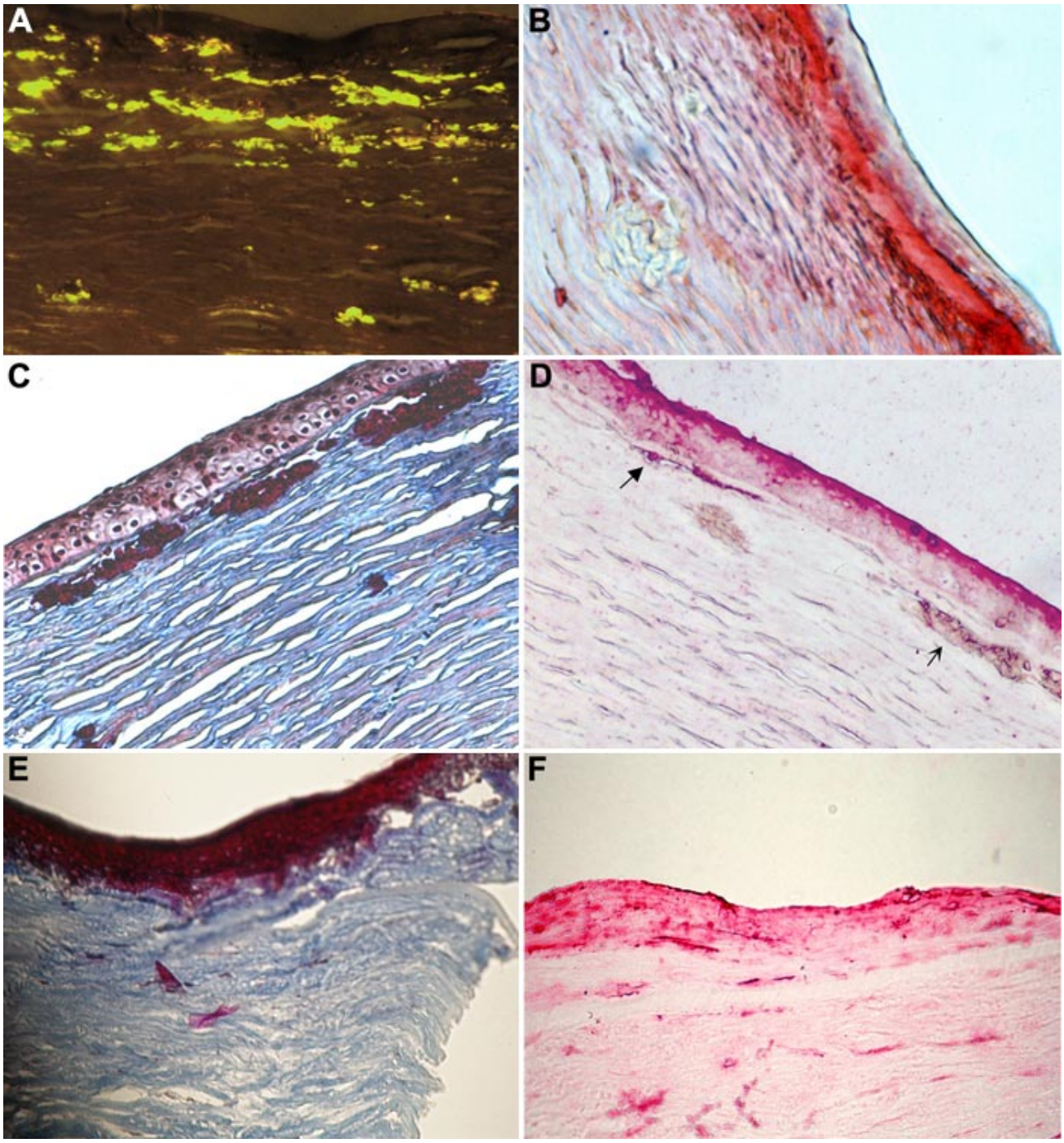

Figure 1. Histological examination of BIGH3-related corneal dystrophies. **A**: A congo red-stained LCDI cornea is shown under polarized light. Amyloid deposits are light green. **B**: Immuno-histological staining of an LCDI cornea with anti-BIGH3 antiserum is shown. Intra- and subepithelial deposits show red staining. **C**: In a GCDI cornea, deposits appear bright red with Masson's trichrome stain. **D**: Immuno-histological labeling of a cornea with recurrent GCDI shows the presence of BIGH3 protein in the deposits. **E** and **F**: Masson's trichrome and immuno-histological staining of a recurrent Thiel-Behnke dystrophy is illustrated. Subepithelially deposited fibrous material stains blue with Masson's trichrome and shows intensive immuno-labeling with the anti-BIGH3 antibody. Note that in **E** the epithelium is missing.

on the results of the molecular genetic examinations. In one case, initially diagnosed as Reis-Bücklers dystrophy, honeycomb-shaped subepithelial deposits as well as characteristic histological features of CDB2 were seen, thus the incorrect diagnosis could have resulted from confusing earlier literature data [1,5,6], rather than an unusual clinical picture.

Different staining patterns of normal corneas and BIGH3 containing deposits were published with various antibodies reacting with the NH_2-terminal, COOH-terminal, or middle portions of the BIGH3 protein [10,11,16]. Regarding LCDI

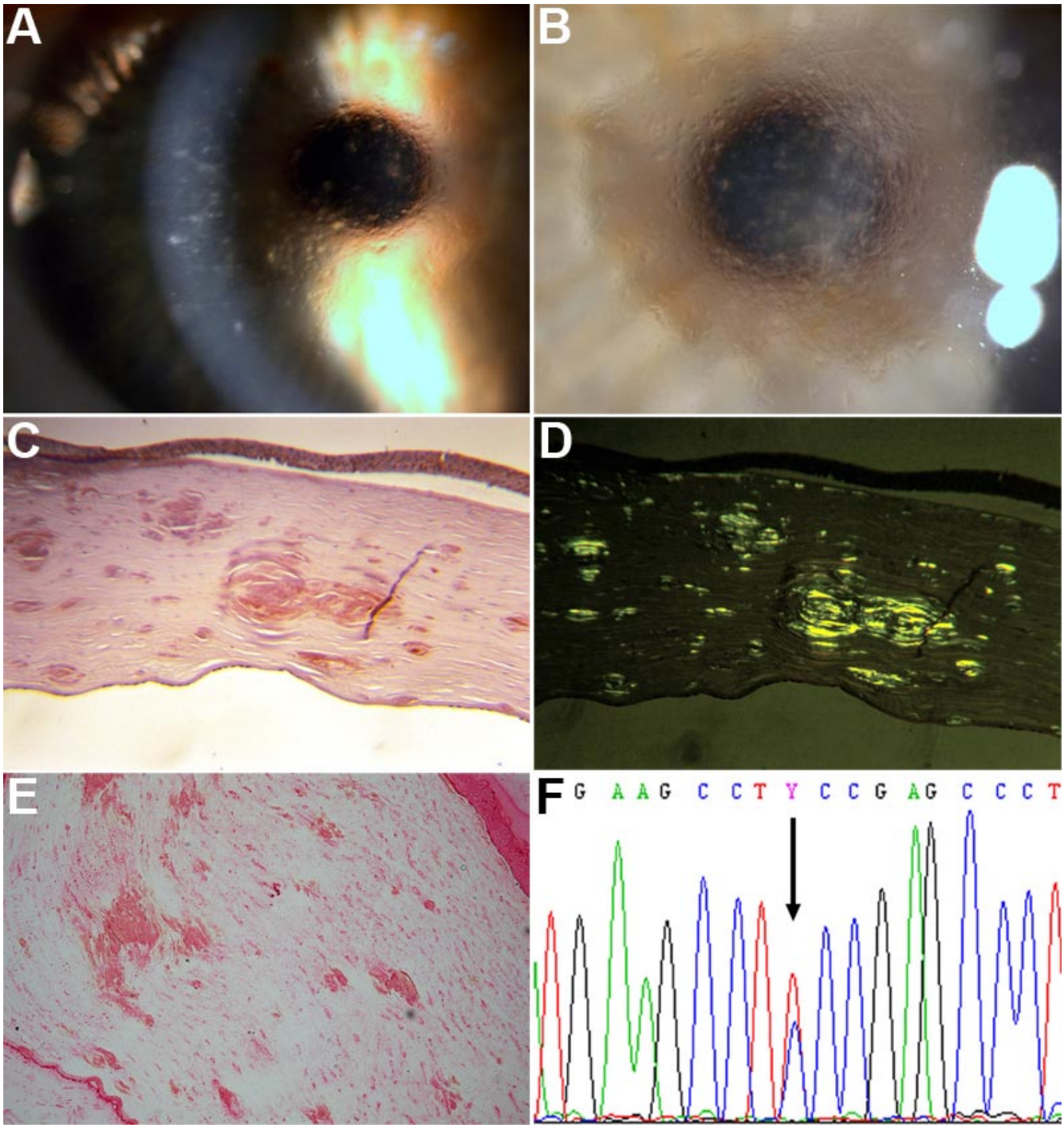

Figure 2. Slit lamp and histological images of the polymorph corneal amyloidosis caused by the F547S mutation. **A** and **B**: Clinical pictures of the polymorph corneal amyloidosis caused by the F547S mutation shows a snowflake-like central and fine linear corneal deposits. Deposits extend to the pre-Descemet level as shown in the photography with narrow slit (**A**). **C**: Congo red-stained cornea of the same patient shows large congophilic deposits in the corneal stroma, which exhibit green birefringence when viewed under polarized light (**D**). **E**: Cornea of the same patient, stained with anti-BIGH3 antibody, is shown in this section. All deposits are stained in red. **F**: The electropherogram of exon 12 of *TGFBI* of the same patient is shown. The arrow indicates the T>C heterozygous conversion at position 1640, causing the F547S amino acid exchange.

and GCDI, our findings corroborate earlier reports on the absence of NH_2-terminal immuno-reactive BIGH3 in intrastromal deposits. In CDB2, staining of the deposits has been reported with an antibody to the middle portion of BIGH3 [9]. Our results indicate that CDB2 deposits also contain the NH_2-terminal segment of BIGH3.

In three members of one family, molecular genetic examination revealed the R124H mutation and established the diagnosis of Avellino dystrophy, which has not been reported from Hungary or the Eastern-European region so far. This type of dystrophy was first described in an American-Italian family [17] but was since found in many populations and seem to be especially frequent in Asia [18-21]. Our study confirms that the R124H mutation may occur in any population.

In a patient initially diagnosed as "atypical lattice" corneal dystrophy, we detected the novel T1640C mutation causing the F547S amino acid exchange in BIGH3. No other mutation in *TGFBI* could be detected in this patient. The novel T1640C mutation was not found in 100 unrelated healthy subjects from the same population, indicating that the mutation does not represent a common polymorphism in Hungary. Histological and electronmicroscopic examination showed amyloid deposits in the corneal stroma, and immunohistochemistry showed the presence of BIGH3 in the patient's corneal deposits. These results indicate that T1640C is a novel mutation of *TGFBI* causing corneal amyloidosis. This mutation seems to be one of the rare mutations in the fourth fasciclin1 (FAS1) domain of BIGH3, which cause various forms of corneal amyloidosis with characteristic lattice lines described as LCDI, LCDI/IIIA, LCDIIIA, LCDIV, and polymorphic corneal amyloidosis [3,22-28]. In 2003, Clout and Hohenester [29] established a structural model of the fourth FAS1 domain

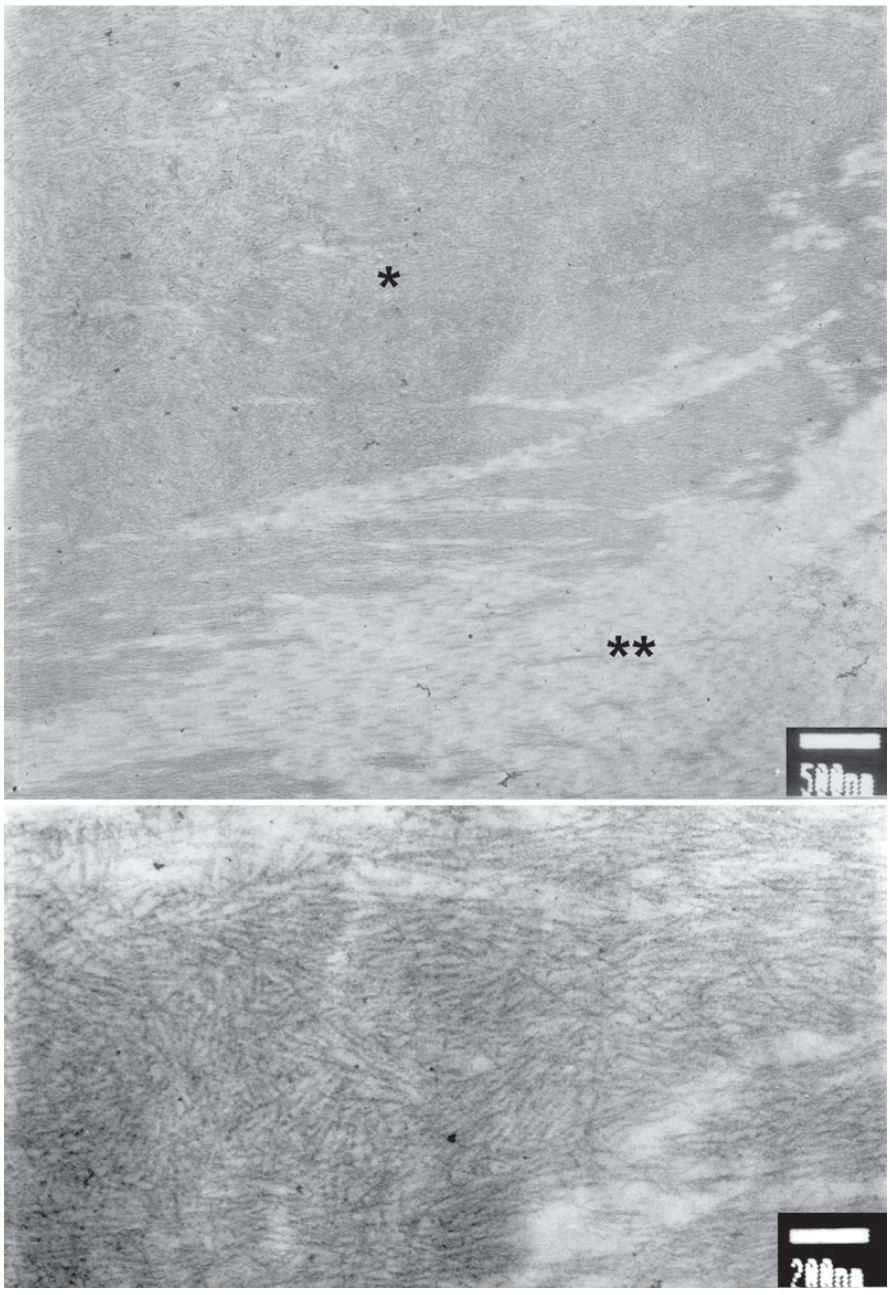

Figure 3. Electron micrograph of the cornea with the F547S mutation. Top: Electron micrographs of the cornea of the patient with the F547S mutation shows a large, electron-dense deposit (asterisk) among the collagen lamellae (double asterisk). Bottom: At higher magnification, nonbranching 8-10 nm fibrils are revealed, indicating the amyloid nature of the deposit.

of BIGH3, based on the crystal structure of drosophila fasciclin1. Using this model, they concluded that with the exception of R124 and R555, all amino acids implicated so far in dystrophy-related mutation sites in BIGH3 are highly conserved and probably have a role in protein folding and maintenance of protein structure. Phenylalanine 547 is located in a 10 amino acid long conserved region, which extends from Y537 to F547 [30]. According to the model, F547 would interact with L518 and other side chains in the formation of a buried hydrophobic protein core. The exchange of the hydrophobic phenylalanine in position 547 to serine, a hydrophilic and much smaller molecule, could disrupt this core and cause severe structural instability and protein misfolding. Interestingly, five mutations have been described in the conserved region between Y537 and F547, three of them (T538R, F540S, and A546T) causing LCDIIIA [3,31,32], one (N544S) causing LCDIV [33], and another (A546D) causing polymorphic corneal amyliodosis [34]. In the latter dystrophy, central deposits with chipped-ice appearance as well as fine, deep stromal linear deposits were described, and histology showed the presence of amyloid deposits in the corneal stroma. Because of its comparable clinical and histological characteristics, we have classified the dystrophy caused by the F457S mutation as polymorphic corneal amyloidosis.

Collectively, our results demonstrate that molecular genetic analysis is necessary to establish the appropriate diagnosis of corneal dystrophies. Detailed genetic and histological characterization helps our understanding of the pathomechanisms of *TGFBI*-related corneal dystrophies.

ACKNOWLEDGEMENTS

This work was supported by the Hungarian National Research Fund grants OTKA F046321 and K68616. G.V. is the recipient of an Öveges grant from the Hungarian National Office for Research and Technology.

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The print version of this article was created on 18 Oct 2007. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.