



Systemic investigation of keratoepithelin deposits in TGFBI/BIGH3-related corneal dystrophy

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Purpose: To investigate the location and tissue-specificity of the pathologic keratoepithelin (KE) deposition in a patient with a keratoepithelinopathy (KEP), *TGFBI/BIGH3*-related corneal dystrophy.

Methods: An autopsy was performed in a patient with lattice type I corneal dystrophy (LCDI) after authorization was obtained from the family. Mutation screening in *TGFBI/BIGH3* was done on the patient several years ago. Eighteen different tissues or organs, including brain, heart, lung, kidney, liver, lymph nodes, spleen, aorta, esophagus, bone marrow, urinary bladder (including a papillary urothelial carcinoma), samples of a metastatic squamous cell carcinoma, adrenal gland, parathyroid gland, muscle, prostate, and cornea were investigated, and sections from the tissues were labeled with KE2 rabbit TGFBI/BIGH3 antiserum.

Results: The patient, diagnosed with LCDI and Alzheimer's disease, died at 79 years of age from a complicated chronic obstructive lung disease. Mutation analysis showed the classical Arg124Cys mutation in exon 4 of *TGFBI/BIGH3*, associated with LCDI. Except for the cornea, immunostaining with KE2 antisera did not reveal any deposits in any of the 17 other organs analyzed.

Conclusions: Pathologic deposits caused by KE accumulation were only observed in the cornea and in no other tissue or organ in this patient. These results suggest a cornea-specific mechanism in the aggregation of KE. Further studies need to be done to investigate whether the degradation of mutated KE generates cornea-specific fragments that aggregate or whether the clearing of normal fragments is different in affected corneas, which then leads to aggregation.

Corneal dystrophies are inherited diseases of the cornea characterized by the progressive accumulation of deposits in the cornea [1]. Eight years ago, we reported that allelic mutations in one single gene, the *TGFBI/BIGH3* gene located on 5q31 and coding for a 683 amino acid secreted protein called keratoepithelin (KE), were responsible for many of these diseases [2]. KE is a member of the extracellular matrix and is ubiquitously found, but not in the brain [3]. We showed in mouse embryos that expression starts as early as 10.5 days after conception with high expression in all the mesoderms [4]. Until now, all the pathogenic mutations reported on the *TGFBI/BIGH3* gene were linked to corneal dystrophies and were not related to other diseases.

KE is expressed in various organs [5,6] and is also present in blood serum [7]. However, clinical examination of patients affected with 5q31-linked corneal dystrophies has never revealed KE deposits anywhere else than in the cornea. Schmitt-Bernard et al. [8] investigated the skin of a patient carrying LCDI. Although KE is abundantly expressed in the skin, they did not find amyloid deposits in this tissue, even after carefully checking with electron microscopy. Furthermore, Klintworth [9] has also added to this statement that despite the deposition of amyloid within the cornea in numerous in-

herited diseases, the corneal tissue is not affected in many genetically determined forms of amyloidosis that affect other tissues. Corneal deposits in TGFBI/BIGH3-related corneal dystrophies seem to be a tissue-specific local process. Although it has regularly been mentioned that deposits are only found in the corneas of affected patients [10-15], no systematic search for deposits elsewhere in the body has been performed and no autopsy of a patient with TGFBI/BIGH3-related corneal dystrophy has been reported.

The exact role of KE in the pathogenesis of the 5q31-linked corneal dystrophies is still unclear. Korvatska et al. [16] did present several hypotheses which could explain the origin of the corneal deposits in these inherited corneal dystrophies. One is the possible role of cornea-specific enzymes in the catabolism of normal and mutated KE. Alternatively, the local conditions observed in the cornea may favor the spontaneous aggregation of mutated KE peptides. Schmitt-Bernard et al. [8] have also presented a similar hypothesis of an alteration in the degradation of the mutated TGFBI/BIGH3 protein and a participation of local factors to explain KE amyloidogenesis.

It has already been shown that *TGFBI/BIGH3* transcripts and KE are intensively synthesized in the corneal epithelium of the adult eye and that primary corneal fibroblasts established from normal and 5q31-affected corneas secreted KE in the culture medium [17]. Recently, it was also shown that KE itself constituted most, if not all, of the content of the deposits, as opposed to a recruitment of other amyloidogenic peptides or proteins [16,17]. Finally, these authors showed that

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abnormal KE isoforms specifically observed in diseased corneas were not produced by cultured fibroblasts from these same corneas.

We present the first report of an autopsy of a patient affected with LCDI due to an Arg124Cys mutation. We did not observe deposits other than in the cornea. This suggests a corneal-specific protein processing that may explain why the cornea is the only site where pathologic deposits are found [10-15].

METHODS

An autopsy was performed after informed consent was obtained from the family. After careful macroscopic examination, the heart, lung, brain, and samples from liver, spleen, pancreas, kidney, esophagus, aorta, bone marrow, and lymph nodes were routinely processed and fixed in a 6% buffered formaldehyde solution. After dehydration and embedding in paraffin, 4 μ m sections were stained with hematoxylin and eosin (H&E) and Congo red.

KE2 antiserum and anti-amyloid P component (anti-P) were used as described previously in the literature [17]. Preimmune serum was also used to detect nonspecific staining and improve specificity. Samples of brain, heart, lung, kidney, liver, lymph nodes, spleen, aorta, oesophagus, bone marrow, urinary bladder (including a papillary urothelial carcinoma), adrenal gland, parathyroid gland, muscle, prostate, and samples of a metastatic squamous cell carcinoma were selected for immunohistochemical analysis. Samples of cornea obtained after keratoplasty of our patient were selected as a positive control.

All selected samples were immunostained using the peroxidase-anti-peroxidase (PAP) technique of Sternberger et al. [18] Tissue sections (4 μ m thick) were mounted on Superfrost® Plus slides (Milian SA, Geneva, Switzerland) deparaffinized in xylol, taken through to absolute alcohol, blocked for endogenous peroxidase with 0.1% hydrogen peroxidase in methanol for 45 min, and rehydrated through graded alcohols. Sections were incubated with KE2 antibody and preimmune serum. They were all first digested in 0.05% Pronase (Dako, Glostrup, Denmark) for 15 min at room temperature. To reduce nonspecific primary antibody binding, the slides were incubated for 10 min with normal goat serum (PelFreez Biologicals, Rogers, AR) diluted 1:30 in Tris-buffered saline (TBS). The primary antibody KE2 (1:50), preimmune serum and anti-P component (1:40; Dako), secondary antibodies, goat anti-rabbit immunoglobulins (1:100) and PAP complex (1:400; Sternberger Monoclonals, Baltimore, MD) were diluted in TBS containing 2 mg/ml skim milk. All incubations were for 30 min at room temperature, followed by washing in TBS.

RESULTS

The patient died at 79 years of age of bronchopneumonia and respiratory failure. He was suffering from mild Alzheimer's disease. An older sister has the disease as well [19]. Both individuals were part of a large Swiss family affected with LCDI. The patient was diagnosed when he was an adult (about 50

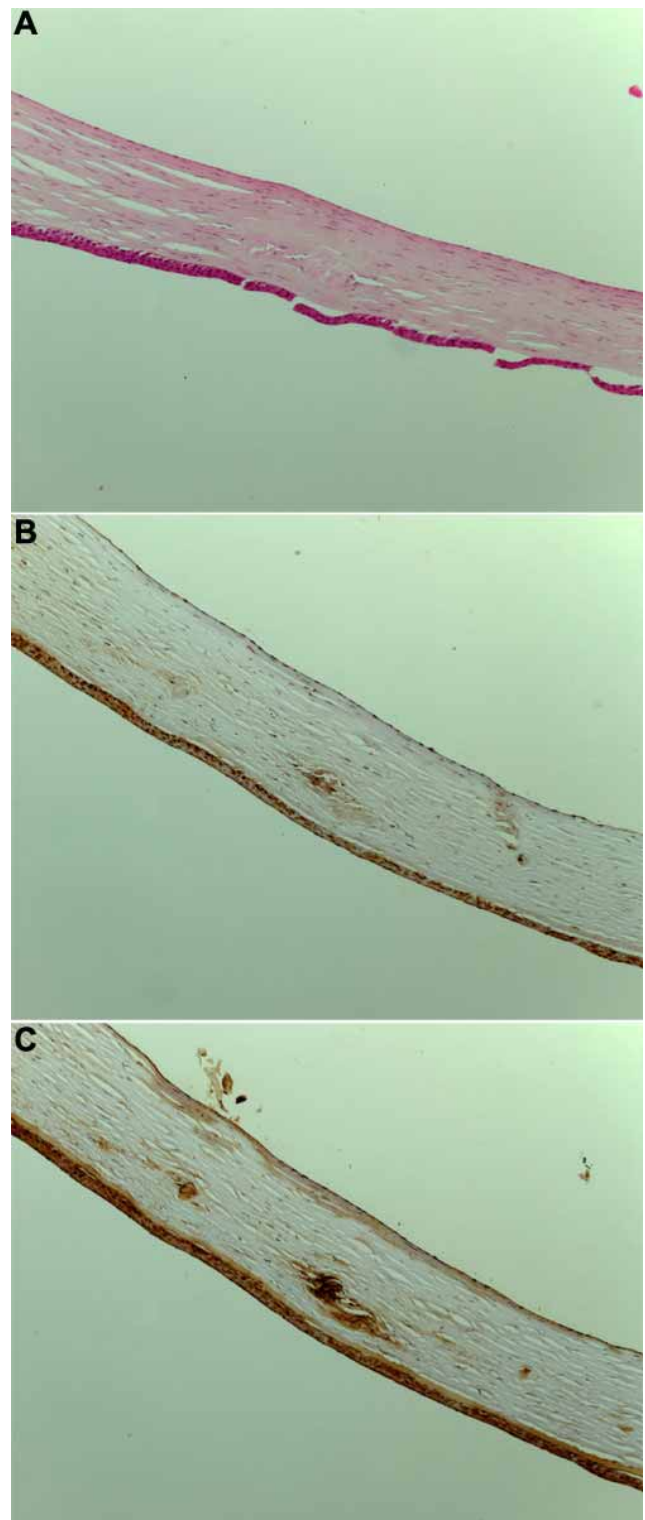


Figure 1. Keratoepithelin deposit in the patient's cornea (LCDI). **A:** Hematoxylin and eosin (H&E) staining of the cornea showed deposits that are disturbing the normal architecture of the corneal stroma. **B:** Control preimmune serum treatment on the cornea. **C:** Immunostaining with keratoepithelin antibody (KE2) revealed pathological intrastroma deposits of keratoepithelin. Corneal epithelium is uniformly stained with KE2 because it normally expresses keratoepithelin. In the images, KE refers to keratoepithelin and KE2 refers to antibody raised against KE.

years ago) and molecular analysis, performed in 1996, revealed an Arg124Cys mutation in exon 4 of the *TGFBI/BIGH3* gene [2]. His sister was not affected and did not carry the mutation.

Autopsy findings: A poorly differentiated squamous cell carcinoma of the inguinal fold with widespread metastases (inguinal, paraaortic, lung hilar lymph node, and multiple lung metastases) was diagnosed. Lung examination revealed also bronchopneumonia and emphysema with subsequent right

heart hypertrophy. A noninvasive papillary urothelial carcinoma was also diagnosed. Grading was not possible due to autolysis artefacts. For the purpose of this study, all H&E stained slides were carefully examined and no unusual eosinophil deposits were observed.

Immunohistochemistry: Corneal samples obtained after keratoplasty of our patient, known for hereditary corneal dystrophy (LCDI), several years before his death. These samples

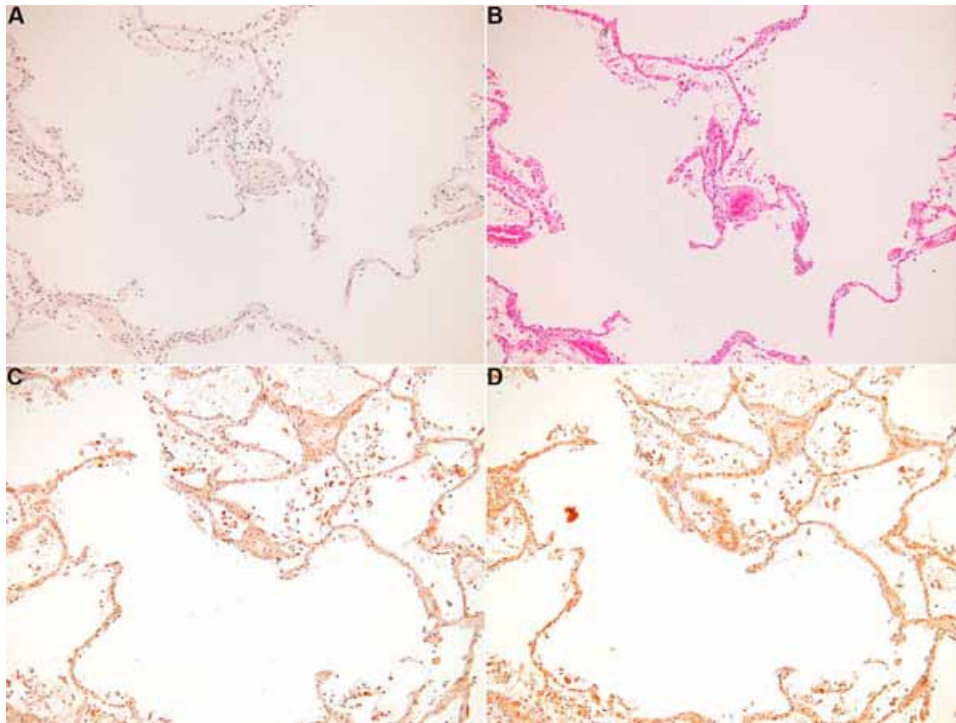


Figure 2. Absence of keratoepithelin deposits in the lung. **A:** Congo red staining showed no amyloid deposits. **B:** Hematoxylin and eosin (H&E) staining of lung tissue revealed no deposit. **C:** A control preimmune serum treatment on lung tissue. **D:** Immunostaining with KE2 is uniformly positive because the lung normally expresses keratoepithelin, but it is negative for pathological deposits of this protein that are characteristic of *TGFBI/BIGH3*-related corneal dystrophies.

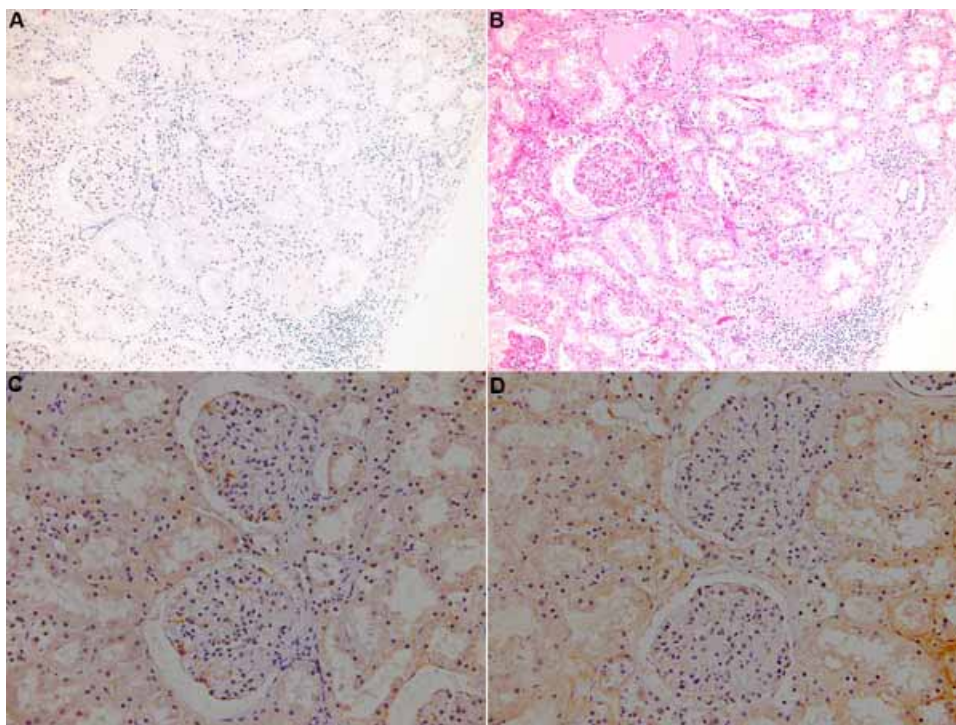


Figure 3. Absence of keratoepithelin deposits in the kidney. **A:** Congo red staining showed no amyloid deposits. **B:** Hematoxylin and eosin (H&E) staining of kidney tissue revealed no deposit. **C:** Control preimmune serum treatment on kidney tissue. **D:** Immunostaining with KE2 is uniformly positive because the kidney normally expresses keratoepithelin, but it is negative for pathological deposits of this protein that are characteristic of *TGFBI/BIGH3*-related corneal dystrophies. The brownish spots correspond to blood red cells.

were used as a positive control and showed eosinophil/amylophorous fusiform deposits upon H&E staining in the anterior part of the corneal stroma (Figure 1A). The amyloid nature of the deposits was confirmed by Congo red staining (Figure 1A) and immunostaining using nonspecific antisera to amyloid P component (not shown) which are nonspecific to disease type. The same deposits showed intense staining with the specific KE2 antibody (Figure 1D). Little nonspecific staining was present as shown in Figure 1C after incubation with preimmune serum. All selected and immunostained autopsy samples were carefully examined and no KE2-positive pathological deposits were present in any of the selected organs (Figure 2, Figure 3) or tumor samples (Figure 4). The staining of the samples incubated with either specific KE2 antibody or the preimmune serum is similar. Except for the cornea, immunostaining with KE2 antiserum and anti-P as well as staining with Congo red did not reveal any deposit in all the other tissues.

DISCUSSION

The expression of *TGFBI/BIGH3* has been demonstrated in different tissues by several groups. In the present study, deposition of KE reactive material was investigated in samples from 16 different organs and also from a squamous cell and a papillary urothelial carcinoma. Except for the cornea, immunostaining with KE2 antiserum did not reveal any deposit in all the other tissues. Samples were not available for transcript or protein quantification and we therefore cannot say whether the expression of *TGFBI/BIGH3* was normal or increased in these tissues. However, Billings et al. [20] have shown that immunohistochemical analysis of human lung localized Big-h3 to the vascular and airway extracellular matrix (ECM), and particularly to the septal tips of alveolar ducts

and alveoli, and that *TGFBI/BIGH3* protein is produced in lung by bronchial smooth muscle cells. They also mentioned that no abnormal findings relative to the lung have been reported in patients with *TGFBI/BIGH3* related corneal dystrophy. The same authors reported a positive immunostaining of the matrix and urothelium in intact bladder tissue with anti-*TGFBI/BIGH3* antibodies and high amounts of the *TGFBI/BIGH3* protein secreted by cells obtained from human bladder tissue. In addition, *TGFBI/BIGH3* has been found upregulated in many cancers such as colorectal tumors [21] and esophageal adenocarcinoma [22]. *TGFBI/BIGH3* mRNA expression was found to be 32.4 fold increased in pancreatic cancer in comparison to normal control tissues [23]. Finally, renal expression of *TGFBI/BIGH3* has also been shown in the rat [24].

Except in the cornea, no pathogenic mutation of *TGFBI/BIGH3* has been related to a disease in any other tissue. KE is a widely expressed protein in the ECM and may play an important role in organogenesis and in the maintenance of tissue architecture. Analysis of the cDNA deduced amino acid sequence of KE identified within the second and fourth internal fasciclin-like repeat domains shows two conserved sequences (Asn-Lys-Asp-Ile-Leu and Glu-Pro-Asp-Ile-Met, respectively) known to mediate cell adhesion [3]. In addition, the developmental expression pattern of *TGFBI/BIGH3* in the mouse embryo shows that *TGFBI/BIGH3* transcripts are abundantly expressed in the mesenchyme of most organs [4]. However, all of the mutations reported so far in *TGFBI/BIGH3* are exclusively related to different forms of inherited corneal dystrophy [9]. Our patient was suffering from LCDI, characterized by a progressive accumulation of amyloid corneal deposits.

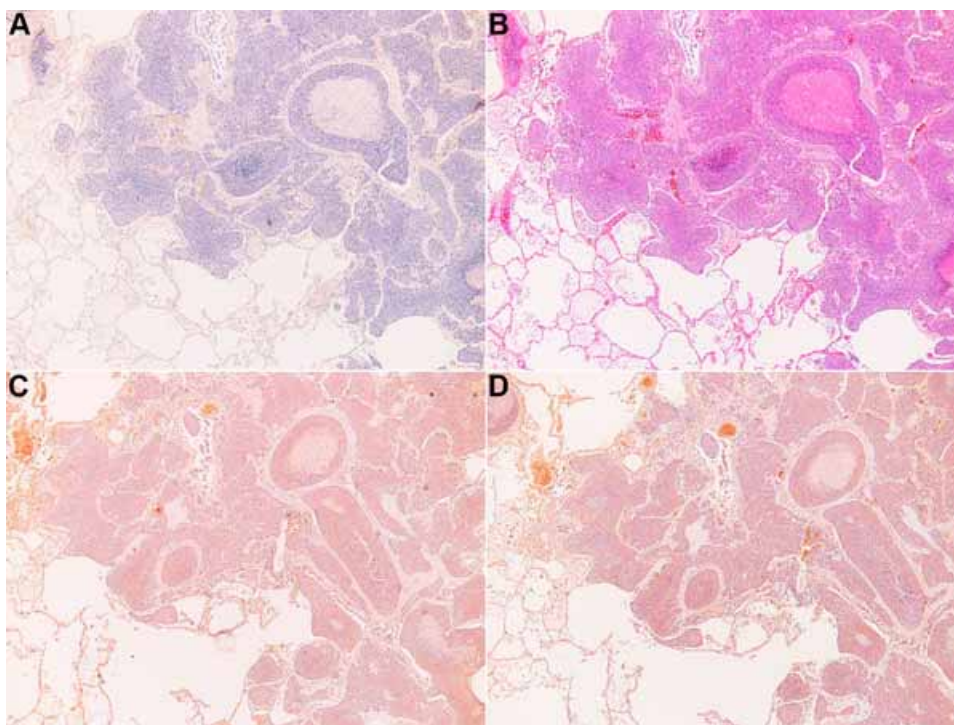


Figure 4. Absence of keratoepithelin deposits in the squamous cell carcinoma. **A:** Congo red staining showed no amyloid deposits. **B:** Hematoxylin and eosin (H&E) staining of squamous cell carcinoma tissue revealed no deposit. **C:** Control preimmune serum treatment on squamous cell carcinoma. **D:** Immunostaining with KE2 does not reveal any deposit in the tumoral pulmonary tissue. The brownish spots correspond to blood red cells.

Corneal amyloidogenesis in *TGFBI/BIGH3* related corneal dystrophy is likely a tissue and protein specific process [17]. Systemic amyloidoses are often the result of a chronic inflammatory process [25], while corneal amyloid deposits in *TGFBI/BIGH3* related corneal dystrophy precede the development of recurrent corneal erosions, rather than developing secondarily. This has been strongly suggested in a case reported by Aldave et al. [26], where a *TGFBI/BIGH3* related corneal dystrophy patient had subepithelial opacification in his left eye without any history of corneal erosion in this eye. Billings et al. [20] have reported that proinflammatory cytokine, interleukin 1 β , which upregulates a number of proinflammatory genes, had no effect on *TGFBI/BIGH3* expression levels. The mechanism leading to corneal amyloidosis is still unclear.

The analysis of KE structure may help in understanding this corneal amyloidogenesis process. It has been shown that corneal deposits are constituted by the mutant KE protein itself [16,17]. KE is an extracellular matrix protein containing an NH₂-terminal secretory signal peptide, four 140 amino acid repeats with internal homology, and an arg-gly-asp (RGD) motif at the COOH-terminus [2]. The RGD motif is found in many extracellular matrix proteins modulating cell adhesion and serves as a ligand recognition sequence for several integrins [27-29]. Most of the mutations in *TGFBI/BIGH3* occur at the two arginine codons Arg124 and Arg555 [2,30]. Schmitt-Bernard et al. [8] have assessed the influence of the structure of mutant *TGFBI/BIGH3* protein related peptides centered on codon 124 on amyloid formation. They have shown that the particular amino acid occupying position 124, the presence of valine-valine at positions 112-113, and the existence of disulfide and hydrogen bonding, are of utmost importance in determining the tendency of *TGFBI/BIGH3* protein related peptides to adopt a β -pleated sheet configuration and to form amyloid. They showed that of all the peptides analyzed, and on the contrary to the native Arg110-131 peptide (amino-acid sequence between codons 110 and 131), Cys110-131 is the peptide with the greatest tendency to form amyloid fibrils [8]. Since cysteine has the capacity to form disulfide bonds, it participates in the appearance of aggregates and predisposes to the formation of amyloid fibrils. Besides disulfide bonds, the same authors suggest that other factors participate to amyloidogenesis. Hydrophobicity is an important feature in the formation of β -pleated insoluble aggregates. The native Val112-Val113 hydrophobic sequence has been implicated in amyloid fibril formation from the *TGFBI/BIGH3* protein derived peptides mutated at codon 124 [8].

As suggested in a previous report [5], a corneal specific protein-protein interaction may explain the aggregation of abnormal KE isoforms that can also be produced by a corneal specific protease. Takacs et al. [31] showed that the 42 kDa protein variant found in LCDI corneas starts at residue 26 (Ala) whereas the NH₂-terminal amino acid of the non mutated KE is Gly24. An altered processing of the mutated KE protein could then explain the amyloid corneal deposition. It could be interesting to identify the KE isoforms that may be present in other organs. If these isoforms are different from those identi-

fied in the cornea, the investigations should be oriented toward a corneal specific protease as a likely causal element of 5q31 corneal dystrophies. Stix et al. [32] confirmed very recently that amyloid deposits in LCDI corneas contain proteolytic fragments of KE. They proposed the hypothesis of an unstable globular state of the mutated KE that leads to unfolded polypeptide chains favoring proteolytic cleavage and intermolecular association leading to amyloid formation. Proteolysis has also been presented as a mechanism for clearing the pathologic corneal protein aggregates. Whether secretion of the mutated protein or an inefficient clearing process is the causal mechanism of the corneal deposit is still unknown. As an ECM protein, KE interacts with other matrix proteins, such as collagen type I, II, IV, and VI [33-35], in order to maintain the corneal tissue architecture which is essential for corneal transparency. But the interaction between mutant KE and the other ECM proteins does not seem to be disrupted [36]. Aberrant interactions between mutant KE or its degradation products and ECM proteins or other corneal components may also explain KE related amyloidogenesis. Since deposits in *TGFBI/BIGH3* related corneal dystrophy are localized mainly in the center of the cornea as opposed to *BIGH3* being expressed in both the central and peripheral cornea, identification of a corneal component that is present mainly in the central cornea could help understanding KE aggregation.

In summary, our study confirms that deposits are only present in the cornea of patients affected with LCDI and probably in no other tissue, including tumoral tissues. This suggests that specific conditions may exist in the cornea. Such conditions could be responsible for the production of abnormal KE through a cornea specific catabolic pathway. Alternatively, cornea-specific physical or biochemical conditions may trigger the aggregation of abnormal KE products. Further investigations should focus on corneal specific components in order to identify the pathological mechanism of the 5q31 related corneal dystrophies.

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