Analysis of Filaggrin Mutations and Expression in Corneal Specimens from Patients with or without Atopic Dermatitis

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**Key Words**

Atopic dermatitis · Filaggrin · FLG gene · Keratitis · Cornea

**Abstract**

**Background:** Filaggrin is expressed in the epidermis and is essential for the maintenance of the epidermal barrier. Null mutations within the filaggrin gene (\textit{FLG}) lead to a disturbed epidermal barrier and are associated with a significantly increased risk of atopic dermatitis (AD). The association of AD with ocular surface disorders prompted us to speculate that common \textit{FLG} mutations may be particularly prevalent in AD patients with ocular comorbidities. **Methods:** Corneal buttons and biopsies from AD patients with ocular involvement (\textit{n} = 11) and from non-atopic patients (\textit{n} = 9) with a histological diagnosis of keratitis were included in the study. DNA samples obtained from paraffin-embedded corneal specimens were genotyped for the two most common \textit{FLG} mutations (R501X and 2282del4). Filaggrin protein expression was analysed by immunohistochemistry. **Results:** Normal skin and corneal specimens (\textit{n} = 6) were positive for filaggrin, which could be detected in the stratum corneum of the skin and in the basal epithelial layer of the cornea. Interestingly, all AD corneal specimens as well as the specimens from keratitis patients without AD were negative for filaggrin expression. Genotyping of the \textit{FLG} mutations R501X and 2282del4 revealed wild-type alleles in all analysed samples. **Conclusions:** The lack of filaggrin expression observed in the analysed corneal specimens from AD patients is not due to the two most common \textit{FLG} mutations (R501X, 2282del4) but is most likely secondary to inflammation, as all keratitis specimens of non-AD patients showed lack of filaggrin expression as well.

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**Introduction**

Filaggrin is expressed in the stratum corneum (SC) of healthy skin and is essential for the regulation of epidermal homeostasis. Derived from the large, complex, highly phosphorylated precursor profilaggrin, filaggrin monomers interact with keratin intermediate filaments to form macrofibrils. In the upper layers of the SC, filaggrin monomers are further degraded to free amino acids [1] that are important in maintaining the hydration of the SC. Up to 10% of the European population are affected by filaggrin deficiency due to inheritance of a single \textit{FLG} null mutation and suffer from dry and scaly skin and are at risk of developing atopic dermatitis (AD) [2–6]. Large-
scale meta-analyses showed that up to 25% of all and up to 50% of severe AD patients carry one FLG mutation and that these patients, in addition, have a greater risk of multiple allergies, asthma, rhinitis, and skin infections [3–6]. In Europeans, two recurrent mutations (R501X and 2282del4) account for 80% of the total risk alleles, while there are many low-frequency variants in Asian populations [7].

Many AD patients suffer from severe chronic ocular surface disorders, indicating that AD leads to a complex immunological disturbance of the ocular surface, often involving the lid margin, the conjunctiva and the cornea. These can result in any combination of blepharitis, conjunctivitis and/or keratitis. Bacterial and viral superinfections can worsen the clinical course [8], resulting in punctate epithelial keratopathy, persistent epithelial defects, shield ulcers, and corneal vascularization [9, 10]. Interestingly, apart from the ocular surface complications, AD is also associated with keratoconus [11].

The association of AD with ocular surface disorders prompted us to speculate that common FLG mutations may be particularly prevalent in AD patients with ocular comorbidities. Therefore, we looked for the most common FLG mutations R501X and 2282del4 in corneal specimens with AD-associated changes and compared the filaggrin expression in those specimens with corneal specimens from keratitis patients without AD.

### Material and Methods

#### Patients

Corneal buttons (n = 8) and biopsies (n = 3) from AD patients with ocular involvement and corneal buttons (n = 9) from non-atopic patients with a histological diagnosis of keratitis (5 cases of acute and 4 cases of chronic keratitis) were included in the study (table 1). Informed consent was received from all patients, and the study was approved by a local Research Ethics Committee. All specimens were collected in the Division of Ophthalmopathology.
of the University Eye Hospital Freiburg, Freiburg im Breisgau, Germany. The diagnosis of AD was based on the criteria of Hanifin and Rajka [12]; the ophthalmological diagnosis according to the patients’ files was confirmed histologically.

**Immunohistochemistry**

Paraffin-embedded samples were prepared as reported previously [13]. For immunohistochemistry (IHC) against filaggrin, a mouse anti-human monoclonal antibody (IgG1 kappa, clone 15C10; Vector Laboratories, Burlingame, Calif., USA), a biotinylated secondary goat anti-mouse antibody (LSAB kit; Dako, Carpinteria, Calif., USA), streptavidin conjugated with horseradish peroxidase, and the substrate 3-amino-9-ethyl carbazole were used. Sections were also counterstained with Mayer’s haematoxylin before analysis by light microscopy. Skin biopsies (n = 3) from patients without AD and normal postmortem corneoscleral specimens (n = 6) served as control samples. TrueBlue peroxidase substrate (KPL, Gaithersburg, Md., USA) was used in combination with mouse anti-human monoclonal anti-filaggrin antibodies (BioLogo, Kronshagen, Germany) to confirm the IHC results.

**Molecular Genetic Analysis**

DNA samples obtained from paraffin-embedded specimens were genotyped twice for R501X and 2282del4 using two different methods: the iPLEX™ method (Sequenom, San Diego, Calif., USA) by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and the TaqMan allelic discrimination method (Applied Biosystems, Foster City, Calif., USA), as described previously [14].

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**Fig. 1.** Filaggrin expression in normal skin and corneal samples. Filaggrin expression in the skin (a), a normal conjunctival and a normal corneal specimen (b, c) were detected by IHC. In skin specimens, the staining for filaggrin was detected in the SC and hair follicle (arrows; a, ×40). In ocular specimens, filaggrin was detected in the conjunctiva (b, ×40; c, ×100) and in the corneal epithelium (d, ×200; e, ×400). Higher resolution (e) of the corneal epithelium shows that filaggrin expression is more pronounced but not restricted to the nuclei of the basal cell layer. Filaggrin IHC was also performed in atopic (f, ×100) and non-atopic (g, ×100) keratitis samples. The stroma in a keratitis sample (f) shows an infiltration of chronic inflammatory cells; an additional retrocorneal membrane (arrowheads) can be detected. In the case of acute keratitis due to a *Pseudomonas* infection (g) in a patient without AD, a massive acute stromal inflammatory infiltration can be discovered. Neither corneal specimen (f, g) shows positive staining for filaggrin.
Results

Immunohistochemistry

As expected, the skin tissue displayed filaggrin expression in the SC (fig. 1a), whereas in the normal cornea, filaggrin expression was observed in the basal epithelial layer (fig. 1b–e) with rather pronounced nuclear staining (fig. 1e). In contrast, all other corneal specimens from AD patients did not display filaggrin staining (table 1; fig. 1f). Interestingly, the control specimens from keratitis patients without AD were also negative for filaggrin (fig. 1g).

Molecular Genetic Analysis

Genotyping of the FLG mutations R501X and 2282del4 revealed wild-type alleles in all samples from AD patients and in all non-atopic patients with keratitis.

Discussion

Filaggrin is a key epidermal protein important for the maintenance of skin barrier function. Interestingly, filaggrin is not only expressed in the skin but also in the corneal epithelium, but much less is known about its role here. As previously reported [15], we could confirm a positive expression of filaggrin in normal corneal epithelium, as well as in the SC of skin samples. Filaggrin was mainly expressed in the basal layers of corneal epithelium (fig. 1b–e) whereas it is expressed in the superficial layers of the epidermis (fig. 1a). This might already indicate a different function for filaggrin in the corneal epithelium than in the SC of the skin. Tong et al. [15] assumed that filaggrin plays a role in compensating ocular surface stress.

Since a common aetiology for ichthyosis vulgaris, AD and keratoconus is assumed, and filaggrin expression is altered in those diseases, we investigated its corneal expression in patients with AD and corneal diseases. We included a second group of corneal specimens from keratitis patients but without any clinical history of atopic diseases. None of the AD corneal or non-atopic keratitis samples showed any filaggrin expression. Therefore, we conclude that the lack of filaggrin expression in the ocular surface specimens from AD patients is most likely secondary, e.g. due to inflammation, no matter whether the inflammation is related to AD or a different aetiology. This assumption is supported by the fact that none of the AD patients were carriers of the common FLG mutations (R501X and 2282del4). Similar observations were also reported for clinically affected skin specimens from AD patients without any of the known FLG mutations. These samples revealed a reduced or even missing filaggrin expression, which was suggested to be related to inflammatory processes [16]. The reason for the absent filaggrin expression is yet unclear. Potential explanations include (1) a reduced expression related to a (low-grade) inflammatory status in cases of keratitis, or (2) an enhanced proteolytic processing of filaggrin by up-regulated or activated proteases/caspases that degrade filaggrin. Activation of proteolytic enzymes such as kallikreins, e.g. KLK-5 or KLK-7, or caspases, e.g. caspase-14 [1], could be induced by inflammation. Furthermore, other rare FLG mutations might lead to a reduced expression of filaggrin, which may have been missed. However, this seems unlikely since the analysed mutations account for 80% of the total risk alleles [2–6].

In conclusion, corneal specimens from all analysed AD patients showed a lack of filaggrin expression independently of the two most common FLG mutations (R501X and 2282del4). The reduced filaggrin expression in the corneal epithelium was most likely a consequence of an ongoing inflammatory process, since non-atopic inflammatory corneal disorders displayed the same phenotype.

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Disclosure Statement

The authors declare that they have no relevant conflicts of interest.

References


