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NIPAL4/Ichthyin Is Expressed in the Granular Layer of Human Epidermis and Mutated in Two Pakistani Families with Autosomal Recessive Ichthyosis

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Key Words
Ichthyosis · *NIPAL4* · Atopic dermatitis · Filaggrin · Autosomal recessive congenital ichthyosis

Abstract
Background: Autosomal recessive congenital ichthyosis (ARCI) can be divided into 3 types including lamellar ichthyosis (OMIM 242304), nonbullous congenital ichthyosiform erythroderma (OMIM 242100) and harlequin ichthyosis (OMIM 242500). The last type is uncommon since newborns with harlequin ichthyosis usually die shortly after birth. Several genes have been linked to ARCI, but these represent only 60% of the known genetic causes of this condition. ***Methods:*** After having performed a linkage analysis, we analyzed the DNA of 2 consanguineous Pakistani families with ARCI for *NIPAL4* mutations and performed in situ hybridization (ISH) for NIPAL4 mRNA in the epidermis. ***Results:*** The haplotype analysis revealed a linkage to chromosome 5, and we identified a recurrent missense mutation, p.A176D, in affected individuals from both families. We also determined by ISH that NIPAL4 mRNA is highly expressed in the granular cell layer of the epidermis, consistent with the ARCI phenotype. ***Conclusion:*** Our results expand the spectrum of the clinical manifestations of the *NIPAL4* gene and further extend our understanding of its molecular function.

Introduction

Autosomal recessive congenital ichthyosis (ARCI) can be clinically divided into 3 main categories. The first is lamellar ichthyosis (LI; OMIM 242304), the second nonbullous congenital ichthyosiform erythroderma (NBCIE; OMIM 242100) and the third harlequin ichthyosis (OMIM 242500), which is usually fatal shortly after birth. Because of this, several authors exclude harlequin ichthyosis from the classification and divide ARCI simply into 2 main subtypes.

In most cases of ARCI, infants are born with a collodion membrane. Clinically, LI is characterized by scales that are large, adherent, dark and pigmented, with an absence of skin erythema [1]. On the other hand, in NBCIE, the scales are fine and white on an erythematous background, although they are larger and grayish on the limbs [2, 3]. Despite this clinical distinction, many cases of ARCI do not fit this classification, and patients frequently have manifestations of both conditions in addition to other features.

At the molecular level, mutations have been detected in 60% of the cases [4]. To date, 6 different genes have been implicated in the pathogenesis of ARCI, and these include: transglutaminase 1 *(TGM1)*, *ABCA12*, 3-lipoxygenase *(ALOXE3)* and 12-lipoxygenase *(ALOX12B)*, NIPA-like domain containing 4 *(NIPAL4)* and *CYP4F22* [5].

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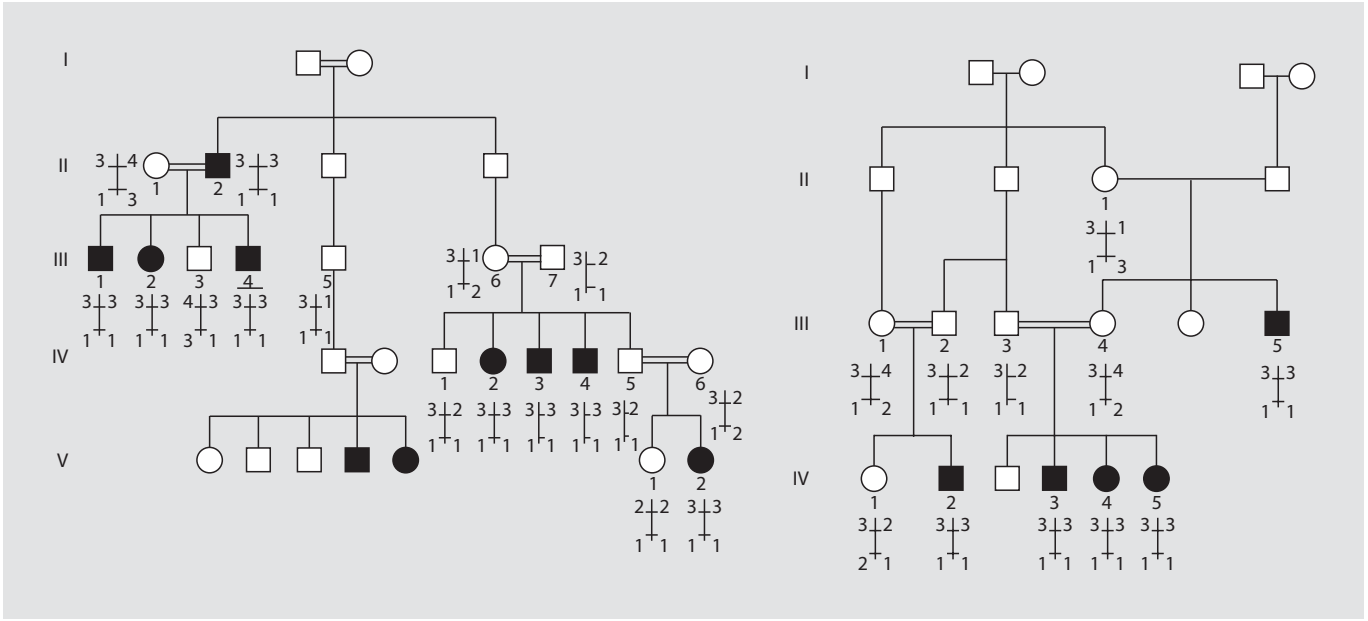

Fig. 1. Autosomal recessive inheritance is clearly seen in both pedigrees.

The pathophysiology of ARCI revolves around deficiencies in intercellular lipid [6]. The lipid that forms the 'cement' between corneocytes, and forms the epidermal barrier, originates from lamellar granules which are located in the granular cell layer [7, 8]. The lamellar granules contain polar lipids including cholesterol sulfate, phospholipids, sphingomyelin and glucosylceramides that are the precursors of the intercellular lipids of the stratum corneum. Generally, lamellar granules encapsulate lipids from the cytosol of the keratinocytes by specialized transporters such as ABCA12 and deliver them to the cell membrane via direct fusion [7]. In addition, lamellar granules also contain other enzymes and proteases that are required for the normal desquamation of the epidermis [7, 9]. Therefore, mutations in these proteins can cause a disturbance in barrier formation, leading to ARCI.

NIPAL4, also known as ichthyin, is composed of several transmembrane domains. Ichthyin-like proteins are localized on the plasma membrane, and share a homology to both transporters and G-protein-coupled receptors [2, 6]. Ichthyin is thought to be a membrane receptor for the trioxilins A3 and B3, which are components of the hepoxilin pathway, but its function is yet to be determined.

To date, only a limited number of mutations in the *NIPAL4* gene have been reported to be associated with ARCI. These include 6 missense mutations, 2 splice site mutations and 1 nonsense mutation. Here, we analyzed 2 Pakistani families with clinical features of ARCI for mutations in the *NIPAL4* gene. We also performed in situ hybridization (ISH) for NIPAL4 in the epidermis and localized it to the granular layer, consistent with the ARCI phenotype.

Patients and Methods

We analyzed 2 Pakistani families affected by an ichthyosiform disorder. After having obtained informed consent, we collected peripheral blood samples from members of these families in EDTA-containing tubes (under institutional approval and in adherence to the Declaration of Helsinki principles). Genomic DNA was isolated according to standard techniques. Both males and females were equally affected, suggestive of an autosomal recessive pattern of inheritance (fig. 1). On physical examination, dry skin was a common finding among all patients. Most young members in one family reported severe itching (fig. 2a), generalized multiple erythematous and nonerythematous crusted papules and hyperkeratotic plaques (fig. 2b), with fine whitish scales over the face (fig. 2c). Adult members of the same family had ectropion and severe whitish hyperkeratotic plaques over the face (fig. 2d), generalized semiadherent whitish-to-yellowish scales on an erythematous base (fig. 2e), joint contractures of the fingers and clubbing of the nails (fig. 2f). The second family also showed variation in the clinical presentation of its members with skin dryness and palmoplantar keratoderma (fig. 2g). Some patients showed

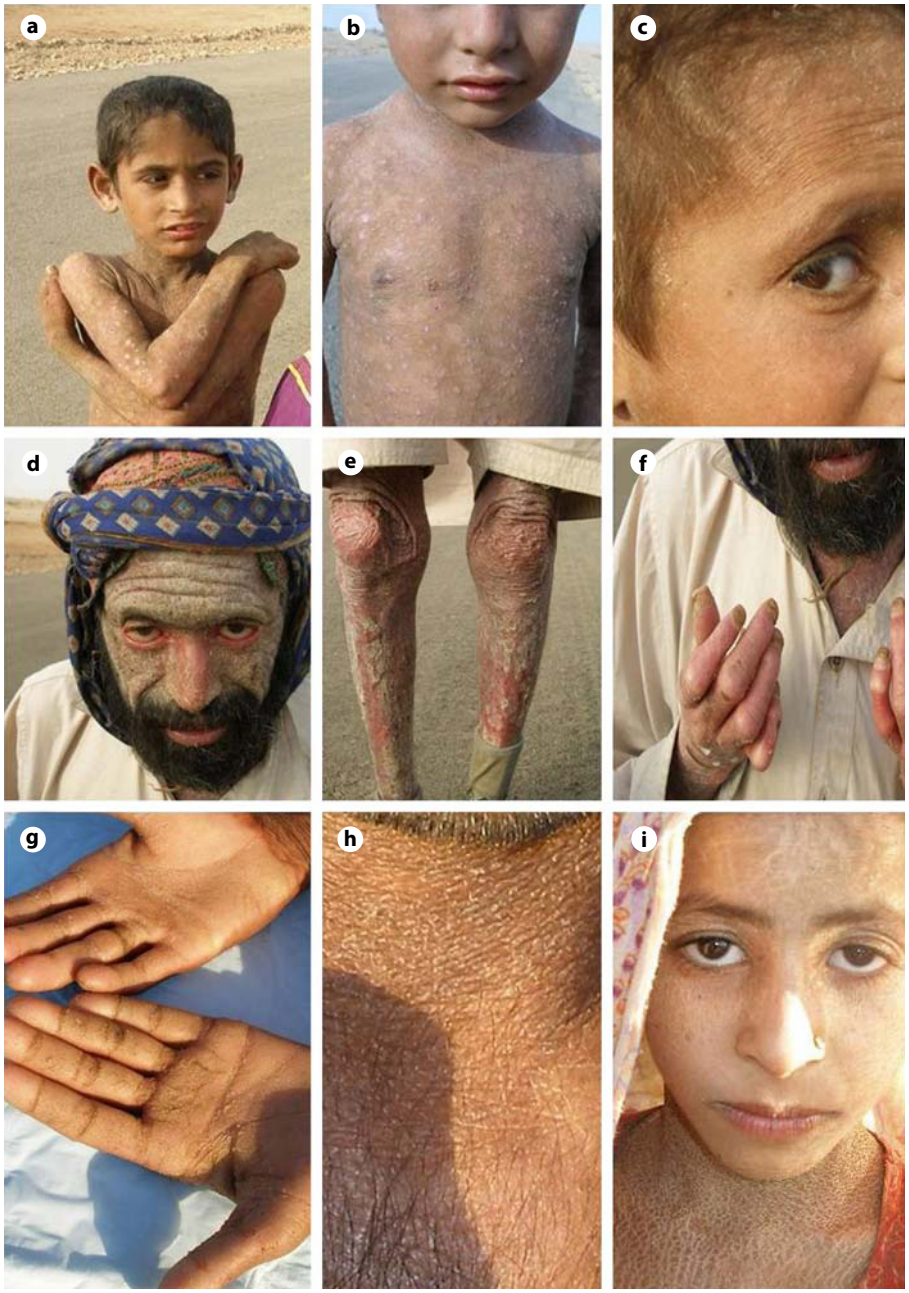

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Fig. 2. a Severe itching in an affected individual. **b** Patient presenting with atopic dermatitis. **c** Fine whitish scales over the face of an affected individual. **d** Facial hyperkeratosis with ectropion, resembling LI. **e** Semiadherent yellowish-to-whitish scales overlying an erythematous base. **f** Joint contractures of the fingers with nail clubbing. **g** Palmar hyperkeratosis. **h** Fine whitish scales overlying an erythematous base, involving the neck and chest. Typical findings in NBCIE. **i** Brown reticulated ichthyosis involving the chest and neck.

fine whitish scales involving the body and the face (fig. 2h), while other members had generalized reticulated brownish ichthyosis (fig. 2i) with an absence of the fine whitish scales over the face.

Haplotype Analysis

In order to determine if the phenotype was linked to any of the genes already known to cause ARCI, we performed a haplotype analysis using microsatellite markers around each of the known genes. PCR amplification was performed for both affected and unaffected individuals in the 2 families. The amplification conditions for each PCR were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 7 min. The PCR products were then run on 8% polyacrylamide gel. Genotypes were assigned by visual inspection.

Mutation Analysis

All exons of the *NIPAL4* gene and the filaggrin gene with adjacent sequences of exon-intron borders were amplified by PCR with primers and under conditions previously described [2, 10]. The amplified PCR products were directly sequenced in an ABI Prism 310 automated sequencer using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

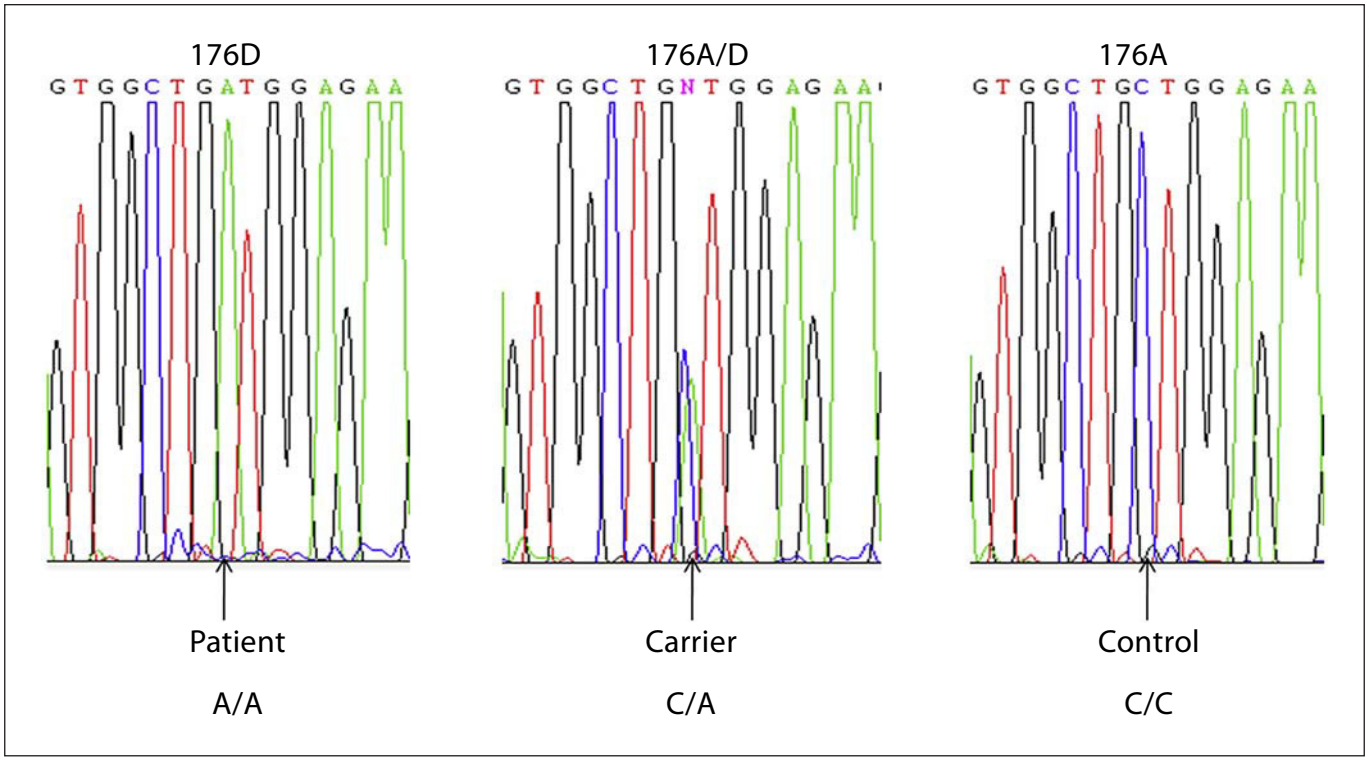

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Fig. 3. Homozygous mutation characterized by C → A transition leading to the substitution of aspartic acid (D) for alanine at codon 176 (A).

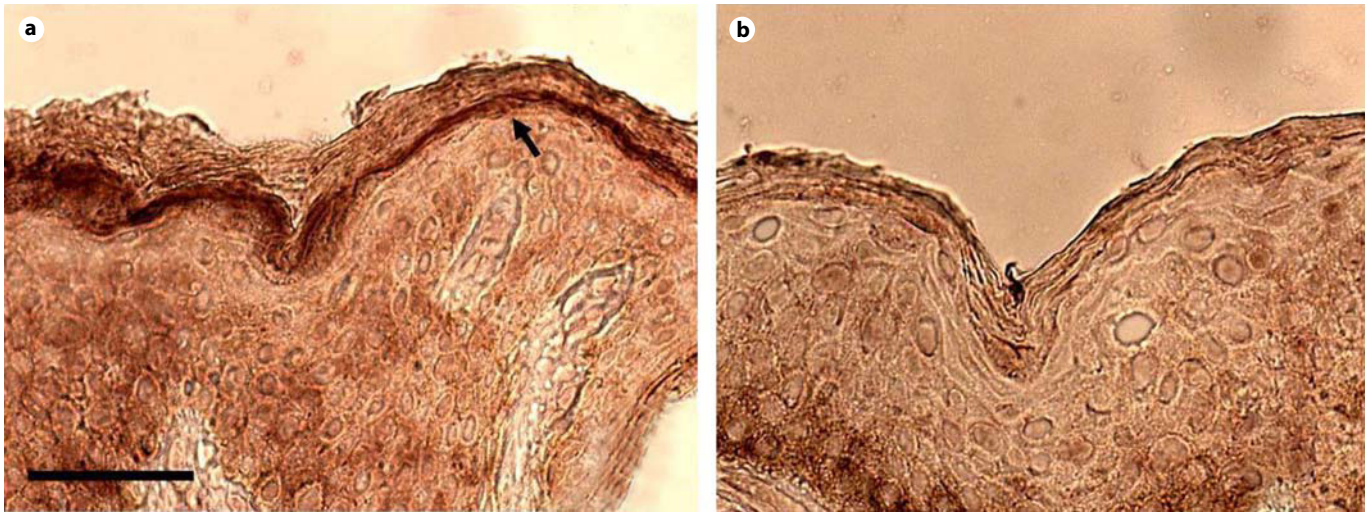

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Fig. 4. a ISH reveals the expression of NIPAL4 mRNA in the granular cell layer of the epidermis (arrow). **b** Negative control.

In situ Hybridization

A fragment of the coding region of the human NIPAL4 cDNA (GenBank accession No. NM_001099287: 437–935 nt) was cloned into the pCR®II-TOPO vector (Invitrogen). The antisense and sense digoxigenin-labeled cRNA probes were synthesized from the linearized vectors with T7 and SP6 RNA polymerases (Roche Applied Science), respectively. Skin tissue of a healthy control individual was fixed with 4% paraformaldehyde-PBS at 4°C overnight. After a dehydration step with 30% sucrose-PBS, the tissue was frozen in optimal cutting temperature compound and sectioned on glass slides at a thickness of 7 μm. ISH was performed following the methods previously described [11].

Results

Haplotype Analysis

The haplotype analysis showed that both families were linked to the region encompassing the *NIPAL4* gene on chromosome 5 (fig. 1), while neither family showed linkage to the other genes known to cause ARCI (data not shown).

Identification of Recurrent Mutation in NIPAL4 *Gene*

We performed a direct sequencing analysis for the *NIPAL4* gene in both families. Affected individuals from both families were homozygous for the mutation c.527C → A in exon 4, which leads to a substitution of the amino acid alanine by aspartic acid, designated A176D (fig. 3). The uninvolved members were either heterozygous for the mutation or had the wild-type sequence consistent with autosomal recessive inheritance. No mutation was found in the filaggrin gene.

NIPAL4 mRNA Expressed in Granular Cell Layer of Epidermis

In order to localize the expression of NIPAL4 in human skin, we performed ISH studies in skin sections of a healthy control individual. The results demonstrate that the human NIPAL4 mRNA is predominantly expressed in the granular cell layer of the epidermis (fig. 4). The sense probe did not show positive signals (fig. 4).

Discussion

NIPAL4 is one of 6 genes that have been implicated in ARCI, and only a few families have been reported with mutations in this gene. Patients with *NIPAL4* mutations resemble more the NBCIE subtype, despite the fact that some patients clinically look like LI patients. Clinically, they present with a collodion membrane, which is shed shortly after birth, although some may be born without this membrane [2]. They later develop generalized ichthyosis with erythema, fine whitish scales on the face and trunk, and larger brownish scales on the neck, buttocks and legs. In addition, patients develop palmoplantar keratoderma with fissures, sometimes associated with clubbing of the nails. Some reports describe ectropion, hypohidrosis, and patients with no or minimal erythema [12]. These features do not resemble the clinical picture of NBCIE, but are more similar to what is observed in LI. Therefore, the spectrum of clinical variability in patients with *NIPAL4* mutation is very wide, with no consistent clinical features.

Patients with *NIPAL4* mutations have been diagnosed from Mediterranean countries including Algeria, Turkey and Syria [2], and from Scandinavian countries including Sweden and Norway, in addition to a patient from the Faroe Islands and another from South America [12]. This report presents the first cases of *NIPAL4* mutations from Pakistan.

Consistent with what has already been reported, we detected clinical variability even among members of the same family. These clinical features included severe skin dryness, which was common among all patients. In one family, the younger patients had clinical symptoms consistent with atopic dermatitis, which has not been reported to be a major manifestation of *NIPAL4* mutations. This was associated with fine whitish scales over the face. The older affected member within the same family had ectropion, severe hyperkeratosis over the face and generalized ichthyosis with semiadherent whitish-to-yellowish scales on an erythematous base, associated with finger contractures and nail clubbing. These clinical manifestations represent a mixture of both LI and NBCIE phenotypes. In the second family, some members had a generalized reticulated brownish hyperkeratosis accentuated over the skin folds, associated with facial dyschromia, dryness and absence of the fine whitish scaling in addition to palmoplantar hyperkeratosis. On the other hand, some members of the same family did not have the reticulated brownish hyperkeratosis, but only had the fine whitish scales overlying an erythematous base over the body and face in addition to the palmoplantar hyperkeratosis. Despite having a common mutation, p.A176D, substantial clinical variation was evident. We therefore conclude that there is no genotype-phenotype correlation, and that environmental factors might play a major role in the different phenotypes.

Mutations in the *NIPAL4* gene should be differentiated from other genes causing ARCI, such as the *LOX*

and *CYP4F22* genes. In general, patients with *LOX* gene mutations and mainly those with *ALOX12B* gene mutations are born with a collodion membrane, but later on they improve markedly, most of them achieving complete healing of their skin and others having a mild NBCIE phenotype [13]. On the other hand, patients with *CYP4F22* are not born with collodion membranes, and this is very helpful to differentiate it from other genes causing ARCI, such as *NIPAL4* and *TGM1*, but later on the patients start developing the LI phenotype [14], and thus, at that stage, it would be hard to differentiate it from the other genes causing LI.

The recurrent mutation, A176D located in exon 4, is the most common mutation reported in patients with *NIPAL4* gene mutations. The fact that this mutation occurred in distinct populations suggests that it may be a hot spot for mutations at this site. In our case, both families showed the same disease-related haplotype, suggesting a common founder mutation in Pakistani families.

It has previously been found that levels of NIPAL4 cDNA are similar between affected patients and healthy controls [12], indicating that these mutations do not affect the stability of the transcript; therefore, the mechanism by which this mutation leads to the disease needs to be determined at the molecular level. It has been shown by electron microscopy that patients with the mutation A176D have abnormal lamellar bodies in the stratum granulosum and elongated perinuclear membranes [12]. Here, we showed by ISH that NIPAL4 is expressed in the granular layer of the epidermis, which is the site where the lamellar bodies are present. Our data provide evidence that NIPAL4 may be important for the formation of normal lamellar bodies, which are essential for the development of a normal skin barrier.

Although there were several individuals that had the clinical symptoms and features of atopic dermatitis, which were predominant over the ichthyosis, none of them had mutations in the *FLG* gene (data not shown), which is known to be involved in patients with atopic dermatitis [10]. Filaggrin is initially synthesized as profilaggrin, which is inactive. The subsequent processing of profilaggrin occurs in order to generate the active form, i.e. filaggrin monomers, which contribute to the skin barrier stability [15]. The disruption of profilaggrin processing by proteins from the lamellar bodies has previously been reported [16]. It has been shown in mice that matriptase, an enzyme found in lamellar bodies, is essential for the processing of profilaggrin, and that mutations in matriptase prevent the degradation of profilaggrin into its monomer form [16]. In humans, matriptase deficiency leads to ichthyosis, follicular atrophoderma and hypotrichosis (OMIM 602400). A second example is the Netherton syndrome (OMIM 256500), which clinically includes a combination of ichthyosis and atopic dermatitis in addition to other anomalies. This syndrome has a mutation in *SPINK5,* which codes for a protease inhibitor called LEKTI, located in the lamellar bodies [17]. Mutations in LEKTI have been shown to alter the processing of filaggrin [18]. The above evidence as well as the fact that syndromes that have alterations in filaggrin processing and filaggrin mutations, such as the Netherton syndrome and ichthyosis vulgaris (OMIM 146700), clinically develop ichthyosis and atopic dermatitis raise the possibility that the mechanism of action of NIPAL4 might be directly linked to filaggrin activity.

In conclusion, we detected a recurrent mutation in the *NIPAL4* gene in 2 Pakistani families with ARCI and extended the spectrum of clinical features. Additionally, we demonstrated the expression of NIPAL4 in the granular cell layer, emphasizing its importance for establishing a normal epidermal barrier formation and furthering our understanding of the molecular function of the *NIPAL4* gene.

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