nature genetics

Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris

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Ichthyosis vulgaris (OMIM 146700) is the most common inherited disorder of keratinization and one of the most frequent single-gene disorders in humans. The most widely cited incidence figure is 1 in 250 based on a survey of 6,051 healthy English schoolchildren[1]. We have identified homozygous or compound heterozygous mutations R501X and 2282del4 in the gene encoding filaggrin (*FLG*) as the cause of moderate or severe ichthyosis vulgaris in 15 kindreds. In addition, these mutations are semidominant; heterozygotes show a very mild phenotype with incomplete penetrance. The mutations show a combined allele frequency of ~4% in populations of European ancestry, explaining the high incidence of ichthyosis vulgaris. Profilaggrin is the major protein of keratohyalin granules in the epidermis. During terminal differentiation, it is cleaved into multiple filaggrin peptides that aggregate keratin filaments. The resultant matrix is cross-linked to form a major component of the cornified cell envelope. We find that loss or reduction of this major structural protein leads to varying degrees of impaired keratinization.

The phenotypic characteristics of ichthyosis vulgaris include palmar hyperlinearity, keratosis pilaris and a fine scale that is most prominent over the lower abdomen, arms and legs[2] (**Fig. 1**). We identified individuals affected by ichthyosis vulgaris of varying severity in 15 kindreds (**Fig. 2**). Marked presentation of ichthyosis vulgaris included prominent scaling (**Fig. 1**), whereas the very mild presentation consisted of palmar hyperlinearity, keratosis pilaris and, in some cases, fine scale. Filaggrin (filament aggregating protein) is important in the formation of the stratum corneum[3–5]. Keratohyalin granules in the granular layer of interfollicular epidermis are predominantly composed of the 400-kDa protein profilaggrin. Following a short, unique N-terminal domain, most of the profilaggrin molecule consists of 10–12 repeats of the 324-residue filaggrin sequence[6]. Upon terminal differentiation of granular cells, profilaggrin is proteolytically cleaved into ~37-kDa filaggrin peptides and the N-terminal domain containing an S100-like calcium binding domain. Filaggrin rapidly aggregates the keratin cytoskeleton, causing collapse of the granular cells into flattened anuclear squames. This condensed cytoskeleton is cross-linked by transglutaminases during formation of the cornified cell envelope (CCE). The CCE is the outermost barrier layer of the skin which not only prevents water loss but also impedes the entry of allergens and infectious agents[7]. Filaggrin is therefore a key protein in facilitating epidermal differentiation and maintaining barrier function.

Several lines of evidence point to a genetic defect in the gene encoding filaggrin (*FLG*) in ichthyosis vulgaris. Immunoblotting studies showed that filaggrin protein was absent or markedly reduced in the skin and/or keratinocytes[8–10] of individuals with ichthyosis vulgaris. In addition, decreased filaggrin mRNA has been demonstrated in some individuals with ichthyosis vulgaris[11]. A recessive mouse mutant, flaky tail (*ft*), bears the histological and ultrastructural hallmarks of human ichthyosis vulgaris[12], and strong genetic linkage has been obtained to the mouse filaggrin locus (*Flg*)[13,14]. Although biochemical analysis has shown defective profilaggrin processing in $ft^{-/-}$ mice[12], the genomic mutation has not yet been identified, owing to the considerable difficulties of analyzing the unusually large and repetitive gene encoding filaggrin, as discussed below.

Notably, we identified genetic linkage in an American family between ichthyosis vulgaris associated with a histologically absent granular layer and markers in the epidermal differentiation complex (EDC) on chromosome 1q21 (ref. 15). The EDC is a dense cluster of genes encoding scores of epidermal structural proteins including

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Received 1 November 2005; accepted 6 January 2006; published online 29 January 2006; doi:10.1038/ng1743

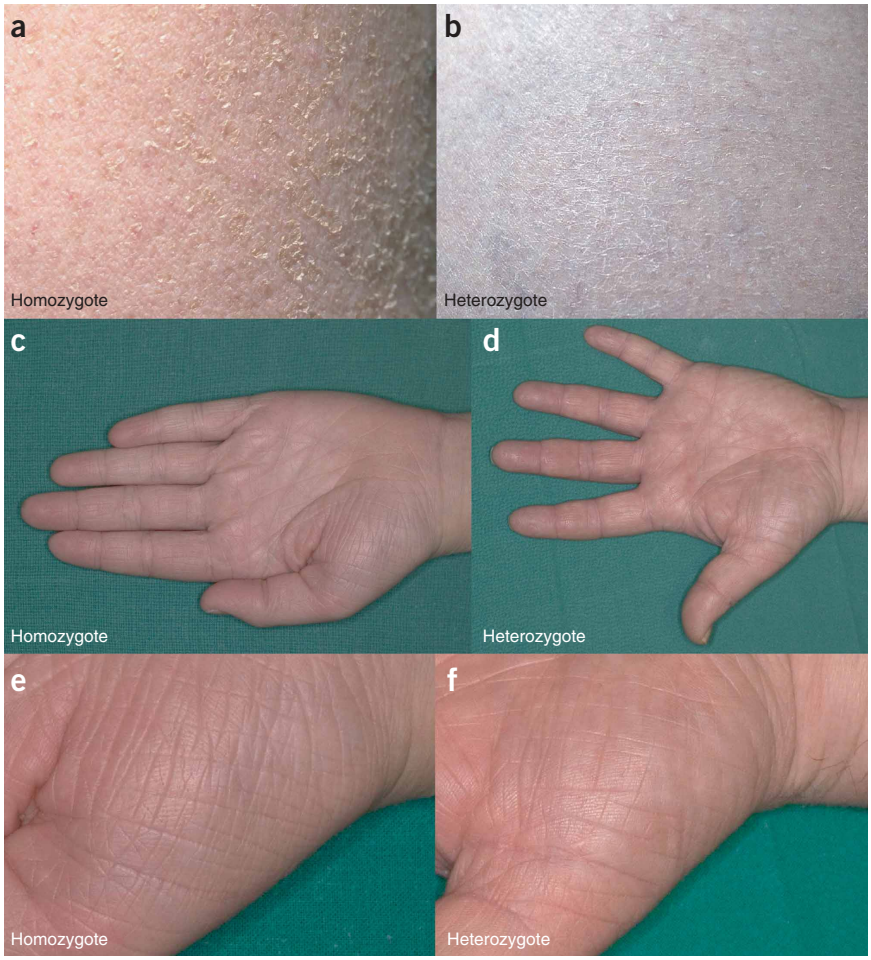

Figure 1 Clinical appearance of ichthyosis vulgaris. (**a**,**c**,**e**) Full presentation of ichthyosis vulgaris in *FLG*$^{-/-}$ individuals. (**b**,**d**,**f**) A milder but still readily recognizable phenotype in a *FLG* R501X heterozygote. Pedigrees are shown in **Figure 2**. (**a**) Diffuse, fine superficial scaling is seen here on the lower abdomen of this individual lacking filaggrin (R501X/2282del4 compound heterozygote, family 6, I:2). Scaling was widespread and obvious in this patient and other homozygous or compound heterozygous patients. (**b**) Fine scaling seen here in a close-up view of the calf of an R501X heterozygote (family 1, II:2). Scaling was less obvious and tended to have a less widespread distribution overall in heterozygotes. (**c**) Marked palmar hyperlinearity seen in an R501X homozygous patient (family 1, III:2). This phenotype becomes more marked with age. The patient shown here is 14 years of age, but note that the hands appear prematurely aged compared with his heterozygous father (**d**). (**d**) Palmar hyperlinearity is less marked in this R501X heterozygote (family 1, II:2) but is still readily discernable, particularly on the palmar surface of the proximal phalanges. (**e**) Enlargement of **c** showing the marked palmar hyperlinearity on the thenar eminence of this R501X homozygote, compared with his heterozygous father (**f**). (**f**) Enlargement of **d** showing the less pronounced palmar linearity on the thenar eminence of this R501X heterozygote.

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filaggrin, loricrin, involucrin, trichohyalin, small proline-rich proteins, S100 proteins and others[16]. We obtained a statistically significant lod score with genetic markers close to the *FLG* locus when we analyzed linkage data using either dominant ($Z_{max} = 3.6$) or recessive ($Z_{max} = 3.4$) modes of inheritance. These data established linkage of ichthyosis vulgaris to the EDC, but the inheritance pattern was unclear. Another report has also suggested mapping of ichthyosis vulgaris to 1q21-q22 in two Chinese families, consistent with a possible *FLG* mutation, although such a defect was not identified in that study[17]. This body of evidence led us to undertake molecular analysis of the gene encoding filaggrin in seven unrelated ichthyosis vulgaris families and eight additional 'sporadic' cases from Ireland, Scotland and the USA.

FLG consists of three exons[18,19]. Exon 1 (15 bp) consists only of 5′ UTR sequences, and exon 2 (159 bp) contains the initiation codon. Exon 3 is unusually large (12,753 bp) and encodes most of the N-terminal domain and all filaggrin repeats (**Fig. 3a**). The number of filaggrin repeats varies from 10 to 12 in the population[6]. The homology between the repeats at the DNA level is almost 100%, making conventional PCR-based sequencing for the internal regions of this exon almost impossible. We did not find any sequence changes in exons 1 or 2 in five ichthyosis vulgaris families. We developed long-range PCR conditions to amplify a 12-kb genomic fragment covering exon 3 and, therefore, all the repeat domains (**Fig. 3b**). Although we were unable to sequence this fragment fully, we detected a homozygous nonsense mutation, R501X, near the start of repeat 1 in three affected individuals from family 1 (**Fig. 3c–e**). Using a smaller PCR fragment, we confirmed segregation of R501X in family 1 and, in addition, we identified this mutation in the other 14 ichthyosis vulgaris kindreds studied. The mutation creates a new *Nla*III restriction enzyme site; we used this to confirm the mutation and screen populations (**Fig. 3f**). By this means, we found the mutation to be present at relatively high allele frequencies in Irish, Scottish and European American populations (combined frequency, 0.027; **Table 1**).

In three families, ichthyosis vulgaris patients with a very pronounced phenotype (**Fig. 1**) were homozygous for R501X (**Fig. 2**). In other families and isolated cases, we found individuals with the marked ichthyosis vulgaris phenotype to be heterozygous for R501X. Further sequencing in these cases showed the existence of a second mutation, 2282del4, in exon 3 (**Fig. 3g–i**). This leads to a premature termination codon 107 bp downstream and, like R501X, stops protein translation within the first filaggrin repeat (**Fig. 3a**). Mutation 2282del4 creates a *Dra*III restriction enzyme site; we used this to screen ichthyosis vulgaris families and control samples (**Fig. 3f**; **Table 1**). This mutation segregated in ten of our ichthyosis vulgaris families (**Fig. 2**). Of the eight 'sporadic' cases of clinically significant ichthyosis vulgaris in which family history was not available, four were homozygous for R501X and the remaining four were R501X/2282del4 compound heterozygotes. By a combination of restriction digest and sequencing, we found that the mutations in all four isolated compound heterozygotes were present in *trans* (data not shown). Notably, we studied part of the US family previously reported to show significant linkage to the *FLG* locus[15] using freshly obtained high-quality DNA required for analysis of exon 3. The severely affected individuals in family 7 were compound heterozygous for R501X/2282del4 (**Fig. 2**), consistent with the linkage data previously reported[15]. The semidominant mode

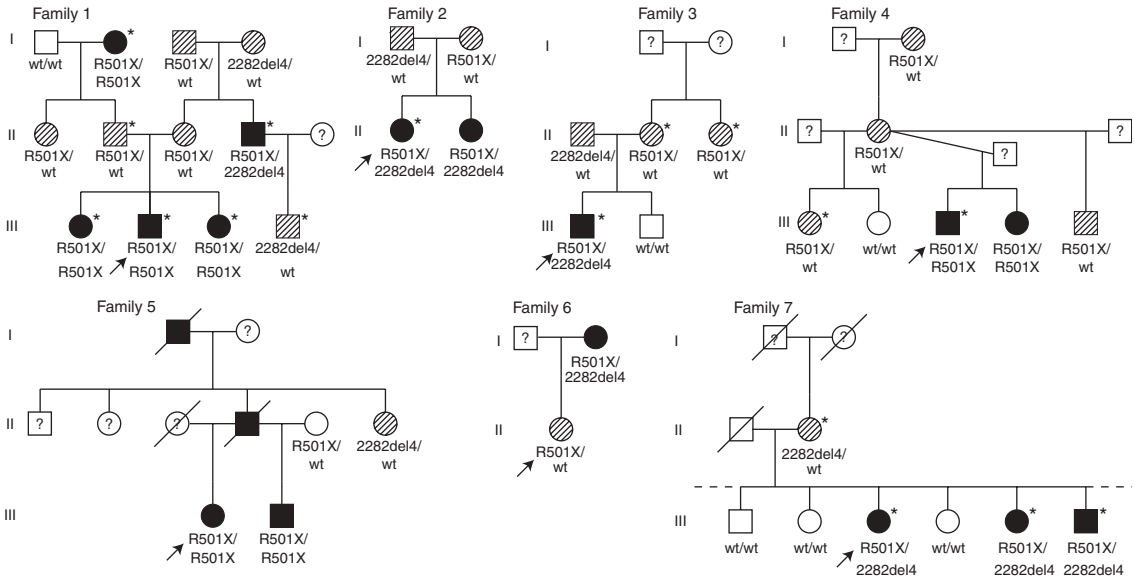

Figure 2 Pedigrees of ichthyosis vulgaris families studied. Roman numerals refer to generations, and individuals within a generation are numbered from left to right, as per convention. Families 1–3 are Irish, families 4–6 are Scottish and family 7 is of European American ancestry[15]. Filled symbols refer to the marked ichthyosis vulgaris presentation; cross-hatched symbols refer to the very mild ichthyosis vulgaris presentation. The genotypes for the two mutations R501X and 2282del4 are shown. Note that wt/wt refers only to the regions screened and does not preclude other sequence changes in the central regions of *FLG* exon 3. The histological absence of a granular layer was confirmed in the proband of family 4 (homozygous R501X, see **Fig. 4**) and all three compound heterozygotes in family 7, as previously reported[15]. In contrast, a heterozygous carrier of 2282del4 with the mild ichthyosis vulgaris phenotype (family 7, II:2) had a histological granular layer[15]. Only two people with no discernible ichthyosis vulgaris phenotype were found to have a filaggrin mutation: individual II:5 in family 5, who carries R501X; and individual II:1 in family 7, who is an obligate carrier of R501X. Atopy was prevalent in the seven families shown here: * denotes individuals with dermatologist-diagnosed atopic dermatitis ('eczema'). In addition, five individuals had asthma (two R501X heterozygotes, two R501X homozygotes and one R501X/2282del4 compound heterozygote). This is consistent with the previously reported high incidence of atopic disease in association with ichthyosis vulgaris[1,28]. wt, wild-type.

of inheritance is best exemplified in family 1 where there are multiple examples of ichthyosis vulgaris patients with very mild presentation as well as examples of R501X homozygotes and R501X/2282del4 compound heterozygotes with the full ichthyosis vulgaris phenotype. In our series of families, there were only two individuals who were heterozygous for a null mutation (both R501X) and had no obvious phenotype (families 5 & 7; **Fig. 2**). On the basis of these small numbers, the penetrance in heterozygotes seems to be about 90%, however, this may be an overestimate due to ascertainment bias. We found the allele frequency for 2282del4 in the Irish, Scottish and

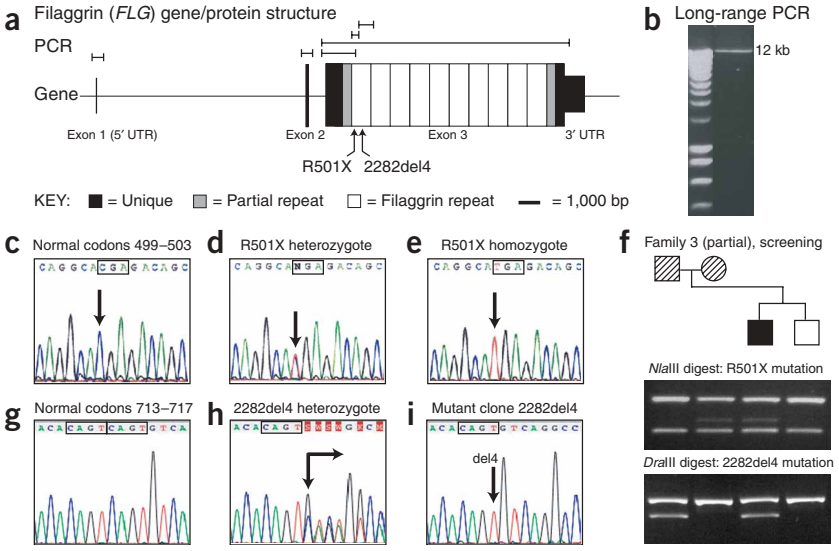

Figure 3 *FLG* mutation detection and confirmation. (**a**) Schematic diagram of *FLG*, annotated to show the corresponding protein structure. Exon 1 consists of a short 5′ UTR sequence. Exon 2 and the 5′ end of exon 3 encode the profilaggrin N-terminal domain. The remainder of exon 3 consists of 10–12 repeats of approximately 1 kb, each encoding a filaggrin peptide separated by linker sequences, followed by a short unique coding sequence and the 3′ UTR. The positions of PCR fragments used here and of the two null mutations, R501X and 2282del4 in repeat 1 of exon 3, are shown. (**b**) Long-range PCR product from genomic DNA covering exon 3 and therefore all the filaggrin repeats. (**c**) Normal sequence from filaggrin repeat 1 in exon 3, corresponding to codons 499–503. (**d**) The same region of the *FLG* as seen in **c** showing heterozygous transition mutation 1501C→T resulting in nonsense mutation R501X. (**e**) The same region of *FLG* as in **c** showing a homozygous mutation resulting in a nonsense codon, R501X. (**f**) Confirmation of mutation R501X by *Nla*III restriction digest and 2282del4 by *Dra*II restriction digest from some members of family 3. (**g**) Normal sequence from filaggrin repeat 1 in exon 3, corresponding to codons 713–717. (**h**) The same region of *FLG* as in **g** showing overlapping peaks owing to a heterozygous deletion mutation, 2282del4. (**i**) The same region of the *FLG* as in **g**, derived from a mutant clone confirming mutation 2282del4.

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Table 1 Population screening for FLG mutations

Population	Allele frequency R501X	Allele frequency 2282del4
Irish	0.041 ($n = 97$)	0.005 ($n = 91$)
Scottish	0.021 ($n = 145$)	0.012 ($n = 166$)
European American	0.024 ($n = 124$)	0.011 ($n = 133$)
Combined	0.027 ($n = 366$)	0.01 ($n = 390$)

Allele frequencies of *FLG* mutations R501X and 2282del4 as determined by restriction digestion of DNA samples obtained from anonymous unrelated individuals. The ichthyosis vulgaris status of these individuals was not known, and owing to ethical constraints, mutation carriers identified could not be contacted or examined.

European American population samples to be ~0.01 (**Table 1**). Using the determined allele frequencies, and assuming mildly affected heterozygotes and severely affected homozygotes, the maximum combined two-point lod score for families 1–7 (**Fig. 2**) was 8.11 at $\theta = 0$.

We subjected skin biopsy material from an R501X homozygote (proband, family 4) to histological and ultrastructural analysis. We found the granular layer to be absent by conventional histology (**Fig. 4a**,**b**), and electron microscopy showed complete absence of keratohyalin (**Fig. 4c**,**d**). Immunohistochemistry showed that an epitope conserved in all filaggrin repeat peptides was completely absent in the R501X homozygote (**Fig. 4e**,**f**). In contrast, an epitope in the N-terminal domain of profilaggrin, encoded by sequences upstream of filaggrin repeat 1, was still present, albeit in an abnormal, diffuse distribution (**Fig. 4g**,**h**). Immunohistochemical analysis of an R501X/2282del4 compound heterozygote gave identical results (data not shown). This confirmed that either R501X or 2282del4 results in complete loss of filaggrin peptide production. Functionally, these are null alleles for filaggrin, although a severely truncated N-terminal portion of the pro-protein seems to be made. We also confirmed the loss of filaggrin epitopes in keratinocytes cultured from a sporadic R501X homozygote (**Supplementary Fig. 1** online). In addition, we confirmed the complete absence of processed filaggrin in skin biopsy material from the proband in family 4, also an R501X homozygote, by protein blotting (**Supplementary Fig. 2** online).

As profilaggrin is the major component of keratohyalin granules, this explains the absent granular layer associated with the more severe cases of ichthyosis vulgaris[15] (**Fig. 4**). The presence of a truncated profilaggrin peptide in ichthyosis vulgaris epidermis (**Fig. 4h**) is consistent with previous studies demonstrating that a peptide containing the unique N-terminal domain and a small amount of filaggrin sequence is stable *in vitro*[20]. In normal epidermis, the N-terminal Ca^{2+}-binding domain is cleaved from profilaggrin by a proprotein convertase and subsequently localizes to different cell compartments, including the nucleus[21,22]. Similar processing of the truncated mutant polypeptide may occur in ichthyosis vulgaris epidermis.

Here we have shown that in three populations of European descent, ichthyosis vulgaris seems to be predominantly caused by

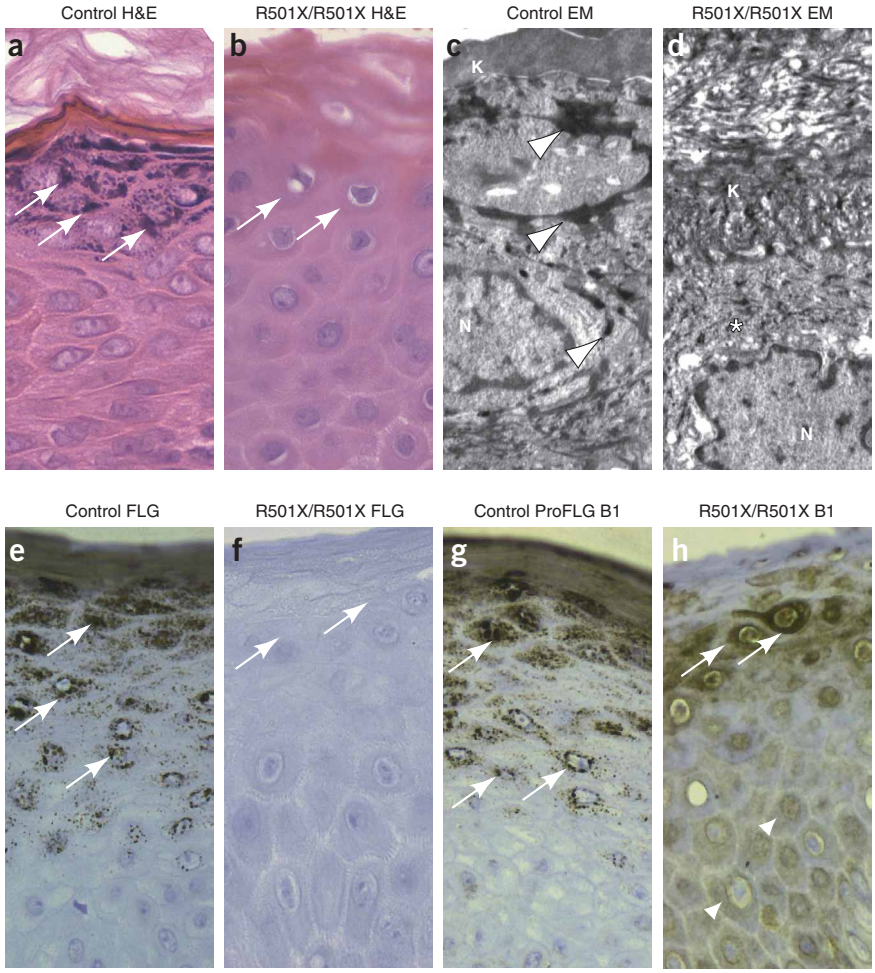

Figure 4 Morphological features of filaggrin-null ichthyosis vulgaris. (**a**) Skin biopsy from a normal (nonichthyotic) control. Hematoxylin and eosin staining shows keratohyalin granules in the granular layers of the epidermis (arrows). (**b**) The proband in family 4 shows complete absence of keratohyalin granules. Degenerating nuclei in the uppermost living layers (arrows) show that this is where keratohyalin granules should be found. (**c**) Transmission electron microscopy of a normal individual shows keratohyalin granules (arrowheads). N, nucleus; K, keratinized material in stratum corneum. Original magnification: ~5,600×. (**d**) Electron microscopy shows complete absence of keratohyalin granules (*) in the proband in family 4. The stratum corneum is not fully cornified (K), indicative of an epidermal barrier defect. N, nucleus. Original magnification: ~5,600×. (**e**, **f**) Immunohistochemical staining using monoclonal antibody against the repeat region of filaggrin. In the normal control (**e**), keratohyalin granules are strongly stained (arrows). The proband in family 4 (**f**) shows complete absence of staining in the upper suprabasal layers (arrows). This demonstrates that no filaggrin peptides are produced in patients homozygous for R501X. (**g**,**h**) Immunohistochemical staining using polyclonal antibody against the N-terminal domain of profilaggrin. Staining of a normal control (**g**) shows prominent staining of keratohyalin granules (arrows). Staining the proband in family 4 (**h**) shows no granular staining, but unlike the filaggrin repeat epitope (**f**), there is diffuse residual staining. This is more pronounced in the upper suprabasal cells (arrows), but there is some patchy cytoplasmic staining throughout the epidermis (arrowheads).

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two frequent null mutations in *FLG*, leading to loss of filaggrin production and impaired epidermal barrier formation. In our ichthyosis vulgaris families, most R501X mutations are in linkage disequilibrium with the same 156-bp allele of a microsatellite in intron 2 of *FLG* (data not shown). Further analysis of polymorphisms near *FLG* will determine the approximate age of the mutations.

Regarding the inheritance pattern and incidence of ichthyosis vulgaris, the very subtle heterozygote phenotype probably does often not come to clinical attention unless specifically sought, as was the case here. Assuming a combined null allele frequency of ~0.037 (**Table 1**) and a pronounced heterozygote phenotype, then 1 in 14 people would have ichthyosis vulgaris, which is clearly not the case. With this allele frequency, 1 in 730 should be homozygous or compound heterozygous and have marked ichthyosis vulgaris. The subtlety of the heterozygote phenotype, combined with incomplete penetrance and seasonal variation[2], probably explains the reported incidence of 1 in 250 (ref. 1). With these high mutant allele frequencies, families with ichthyosis vulgaris will also frequently seem to have dominant or pseudodominant inheritance with reduced penetrance (**Fig. 2**). By DNA analysis, polymorphism in the number of filaggrin repeats has been observed in humans (10–12 repeats)[6] and mice (12–20 repeats)[23]. We did not observe this using long-range PCR but have not analyzed large numbers of controls by this technically demanding method. Nevertheless, it is possible that a heterozygote for a null mutation might carry an expanded exon 3 on the other allele, lessening the overall effect of the mutation. This might explain the phenotypically normal heterozygotes seen in families 5 and 7 (**Fig. 2**). Owing to their relatively high population frequencies, filaggrin null mutations may themselves be modifying factors in other ichthyotic skin conditions, including congenital ichthyoses, Netherton syndrome or disorders due to defects in suprabasal keratins, in which intra- and interfamilial phenotypic variation is well documented[24–27]. The association of ichthyosis vulgaris with the atopic diathesis is well established; 37–50% of people with ichthyosis vulgaris have atopic diseases[1,28], and roughly 8% of atopic dermatitis patients have classical features of ichthyosis vulgaris[1,29]. Thus, filaggrin may be a factor in very common skin disorders known to have a major genetic component.

METHODS

Affected individuals and phenotypes. Blood samples were obtained with informed consent from 15 families with ichthyosis vulgaris and from normal ethnically matched controls. Experiments comply with all principles of the Helsinki Accord.

Long-range PCR for *FLG* exon 3. All primer sequences are listed in **Supplementary Table 1**. Primers FilLR2F and FilLR1R were used to amplify approximately 12 kb of the *FLG* gene (including all of exon 3 and therefore all the repeat domains) from genomic DNA using the Expand Long Template PCR System, Buffer 3 (Roche Diagnostics) and double-strength dNTP (0.5 mM each). A 'hot start' was performed with 1 unit Expand Long Template enzyme mix (Roche). Reactions were amplified using the following extended PCR program: one cycle of 92 °C for 5 min 1; ten cycles of 92 °C for 10 s, 49 °C for 30 s and 68 °C for 6 min; and 28 cycles of 92 °C for 10 s, 49 °C for 30 s, and 68 °C for 6 min, with an additional 10 s added to each 68 °C extension per cycle; followed by one cycle of 68 °C for 10 min.

R501X mutation analysis. A shorter PCR fragment was designed to amplify approximately 1.5 kb of *FLG* for mutation analysis of R501X. Primers FilF3 and RPT1P6 were used in High Fidelity PCR buffer (Roche) containing 1.5 mM $MgCl_2$, 4% DMSO and 1 unit High Fidelity thermostable DNA polymerase mix (Roche). Reactions were amplified under the following conditions: one cycle of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 57 °C for 1 min and 72 °C for 2 min; and one cycle of 72 °C for 5 min. Mutation R501X creates a new *Nla*III restriction enzyme site; this was used to confirm the mutation and screen control samples. Primers FilH1F3 and RPT1P6 were used to amplify 312 bp of genomic DNA using PCR buffer (Promega) containing 1.5 mM $MgCl_2$, 4% DMSO and 1 unit *Taq* polymerase mix (Promega). Reactions were amplified as follows: one cycle of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C for 1 min; followed by one cycle of 72 °C for 5 min. PCR products were digested with 5 units *Nla*III for 4 h at 37 °C. Digests were resolved on 3% agarose gels.

2282del4 mutation analysis. A PCR fragment amplifying 811 bp of genomic DNA was amplified with primers RPT1P7 and RPT2P1 using PCR buffer (Applied Biosystems) containing 1.5 mM $MgCl_2$, 4% DMSO and 1 unit *Taq* polymerase mix (Promega). PCR amplification conditions were one cycle of 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 57 °C for 45 s and 72 °C for 1 min 30 s followed by one cycle of 72 °C for 5 min. Mutation 2282del4 creates a new *Dra*III restriction enzyme site, which was used to screen samples for this mutation. PCR products were digested with 5 units *Dra*III for 4 h at 37 °C. Digests were resolved on 2% agarose gels. A PCR fragment from a heterozygous individual was cloned into vector pCR2.1 (Invitrogen). Clones were screened by *Dra*III digestion and sequenced to confirm the 4-bp deletion.

Histology and electron microscopy. Routine hematoxylin and eosin staining was performed to evaluate morphologic features of each specimen. Immunoperoxidase staining of frozen and paraffin-embedded sections utilized the Envision system (DakoCytomation). Antibodies used were mouse monoclonal 15C10 against an epitope in the C-terminal portion of the human filaggrin repeat unit (15C10; Novocastra) and rabbit polyclonal antiserum B1 raised against the N terminus of profilaggrin[20]. For transmission electron microscopy, skin samples from patients were fixed in half-strength Karnovsky's fixative (containing 2.5% glutaraldehyde and 2% formaldehyde) then in 1.3% osmium tetroxide and processed using standard methods, largely as described previously[30].

Calculation of lod scores. We calculated lod scores with the MLINK algorithm of LINKAGE version 5.1 using a semidominant model of the disease, in which heterozygotes were assigned a mild phenotype with 90% penetrance, and homozygotes or compound heterozygotes were assigned as a severe phenotype with 100% penetrance. The combined mutant allele frequency was assumed to be 0.037 (**Table 1**). Recalculation with 50% penetrance in heterozygotes still yielded a highly significant maximum combined lod score of 7.08 at $\theta = 0$.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

This paper is dedicated to the memory of the late P.M. Steinert, who named filaggrin. Thanks to the patients and their families for their participation which made this research possible and to K. Johnston for clinical assistance. Thanks to the following at Ninewells Hospital and Medical School: J. Hands, N. Joy and C. Black, Molecular Genetics Laboratory, for DNA extraction and storage; A. Cassidy, G. Scott and G. McGregor, DNA Analysis Facility, for DNA sequencing and genotyping support; A. Grant and G. Milne, Pathology Department, for pathology support; and J. Mcfarlane, Epithelial Genetics Group, for clerical assistance. Thanks to M. Greenway, National Centre for Medical Genetics, Our Lady's Hospital for Sick Children, Crumlin, Dublin 12, Ireland for providing Irish control samples. This work was supported by a Wellcome Trust Senior Research Fellowship (W.H.I.M.), US National Institutes of Health/National Institute of Arthritis and Musculoskeletal and Skin Diseases (R.B.P.), and the Odland Endowed Research Fund (P.F., R.B.P.), as well as grants from The Dystrophic Epidermolysis Bullosa Research Association (W.H.I.M.), The Pachyonychia Congenita Project (F.J.D.S.) and The British Skin Foundation (F.J.D.S., W.H.I.M.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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