Unique and Recurrent Mutations in the Filaggrin Gene in Singaporean Chinese Patients with Ichthyosis Vulgaris

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Filaggrin is an abundant protein of the outer epidermis that is essential for terminal differentiation of keratinocytes and formation of an effective barrier against water loss and pathogen/allergen/irritant invasion. Recent investigations in Europe and Japan have revealed null mutations in the filaggrin gene (*FLG*) as the underlying cause of ichthyosis vulgaris (IV), a common skin disorder characterised by dry skin, palmar hyperlinearity and keratosis pilaris. Following the development of a strategy for the comprehensive analysis of *FLG*, we have identified five unique mutations and one recurrent mutation in Singaporean Chinese IV patients. Mutation 441delA is located in the profilaggrin S100 domain, whereas two additional frameshift mutations, 1249insG and 7945delA, occur in the first partial filaggrin repeat ("repeat 0") and in filaggrin repeat 7, respectively. Both nonsense mutations Q2147X and E2422X are found in filaggrin repeat 6, whereas R4307X was found on one of the longer size variant alleles of *FLG*, within duplicated repeat 10.2. Mutation E2422X, previously found in a single Dutch patient, was found in one Singaporean IV patient and at a low frequency in Asian population controls. Our study confirms the presence of population-specific as well as recurrent *FLG* mutations in Singapore.

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INTRODUCTION

Ichthyosis vulgaris (IV; OMIM #146700) is one of the most common monogenic skin disorders, reported to affect 1 in 250 English school children (Wells, 1966). The disorder is characterized by dry, scaly skin, palmar hyperlinearity, and keratosis pilaris (Sybert *et al.*, 1985; Smith *et al.*, 2006), and can be aggravated by climate. Other allergic conditions, importantly including atopic dermatitis (AD), atopic asthma and rhinitis, are also commonly manifested by IV patients (Irvine and McLean, 2006; Irvine, 2007).

In 1985, Sybert *et al.* demonstrated that profilaggrin and filaggrin were reduced or absent in five IV patients. The filaggrin gene (*FLG*) is located on chromosome 1q21 within a cluster of genes that make up the EDC—the epidermal differentiation complex (Mischke *et al.*, 1996). Profilaggrin consists of three exons (Presland *et al.*, 1992)—exon 1 (15 bp) and exon 2 (159 bp) contain the 5′-untranslated region and start codon, respectively. Exon 3 is unusually large (12.7–14.7 kb) and codes for most of the polyprotein profilaggrin, which consists of 10–12 tandem repeats of the 37-kDa filaggrin peptide. Profilaggrin is stored within keratohyalin granules in the granular layer of the epidermis and proteolytically cleaved to filaggrin subunits when keratinocytes undergo terminal differentiation. Subsequently, filaggrin binds to keratin intermediate filaments within the keratinocytes, as the cells collapse and flatten into squames; transglutaminases also work to cross-link other cornified envelope precursor proteins such as involucrin to form an impermeable epidermal barrier. It has also been shown that filaggrin subunits are ultimately proteolyzed within the stratum corneum into hygroscopic amino acids and derivatives thereof, which help retain epidermal moisture (Rawlings and Harding, 2004).

Although filaggrin expression was known to be reduced or absent in IV patients for 20 years, the route to uncovering the direct genetic cause of IV was challenging because it was very difficult to conduct routine and effective sequencing of *FLG* due to its sheer size. The *FLG* gene is also highly repetitive and polymorphic, which prevents sequencing of the entire gene by conventional methods. In 2006, the precise molecular basis of IV was finally uncovered by the identification of null mutations in *FLG* (Smith *et al.*, 2006). Since IV was known to be associated with a high incidence of AD (Kuokkanen, 1969; Tay *et al.*, 2002), the link between

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Abbreviations: AD, atopic dermatitis; FLG, *filaggrin gene; IV, ichthyosis vulgaris*

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filaggrin-null mutations and AD was quickly established (Palmer *et al.*, 2006). Reduced or absent filaggrin expression chronically exposes the skin to irritants that can lead to inflammation of skin, resulting in AD (Irvine, 2007). In some populations, up to 50% of children with moderate to severe AD carry *FLG* mutations and defects in *FLG* also predispose AD patients to asthma and related allergic phenotypes (Irvine and McLean, 2006).

This laboratory has recently reported methods for the comprehensive analysis of *FLG* (Sandilands *et al.*, 2007). Three different sizes of *FLG* alleles were identified containing 10, 11, or 12 filaggrin repeats. By overlapping PCR and unidirectional deletion strategies, *FLG* was fully sequenced, and a total of 15 null mutations in *FLG* in the European, Japanese and Chinese populations have been identified to date (Nomura *et al.*, 2007; Sandilands *et al.*, 2007). Interestingly, common mutations in the European population differ from those in the Oriental populations studied so far.

Singapore is an immigrant South-East Asian city-state with a population of 4.5 million consisting of Chinese (75.6%), Malays (13.6%), Indians (8.7%), and other ethnic groups (2.1%). The 1-year prevalence of IV in 2002 was reported to be 8% in Singaporean school children (Tay *et al.*, 2002). Similar to many developed European countries, AD cases are also on the rise in Singapore; a study conducted on 2,363 students in 2002 reported a prevalence of 20.8% (Tay *et al.*, 2002). In this study, we aimed to identify the *FLG* mutations that underlie IV in this Asian population to facilitate future studies of AD in this geographical region.

RESULTS

Case reports

All patients, adults of Chinese Singaporean ethinicity, were referred to outpatient dermatological clinics at the National Skin Centre, Singapore for the main complaint of dry skin, with varying accompanying symptoms of eczema. The diagnosis of IV was established on the clinical basis of finding extensive semi-adherent scaling, which was characteristic, a history of onset early in life; as well as a positive family history of a similar affliction. These criteria were chosen to increase the yield of a positive result on genetic testing. Palmar hyperlinearity was seen in all patients (Figure 1, Figure S1). Interestingly, keratosis pilaris was not seen at all. IV was graded in terms of severe or mild, based on the degree and extent (acral and truncal sites versus acral sites only respectively) of scaling (Figure 1). Four out of six patients with severe IV also had AD based on the widely recognized diagnostic criteria (Hannifin and Rajka, 1980). None of the patients with mild IV had active AD. All subjects were examined by a single dermatologist.

Unique *FLG* mutations in Singaporean Chinese IV patients

We carried out full sequencing of the *FLG* in eight unrelated Singaporean Chinese IV patients and detected mutations in six individuals (Figures 3 and 4). A total of six mutations were detected (Table 2)—five mutations are previously unreported and one mutation was identified in a single Dutch IV patient from an earlier study (Sandilands *et al.*, 2007).

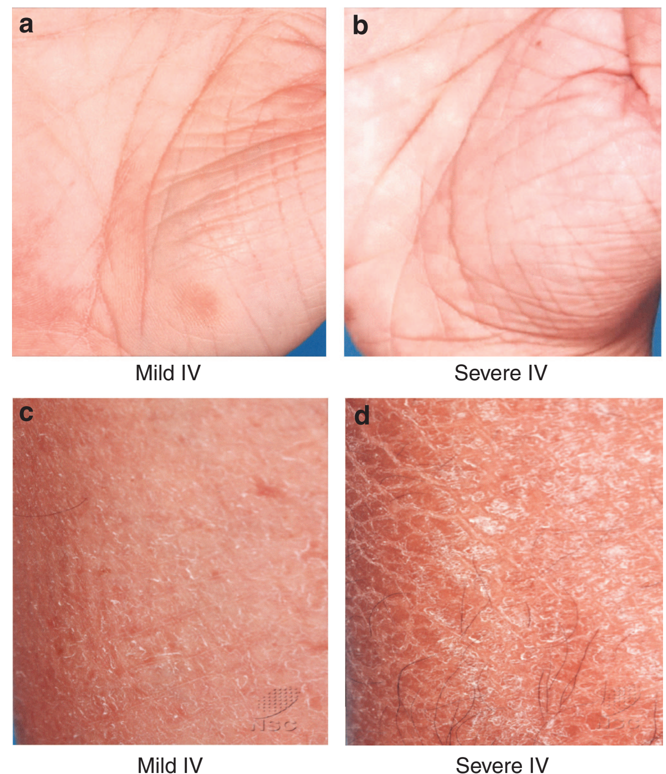

Figure 1. Clinical features of patients with IV. (**a**) Palmar hyperlinearity seen in patient 3 heterozygous for *FLG* mutation 7945delA. (**b**) Due to the semi-dominant inheritance pattern of IV, a more marked hyperlinearity can be seen on the palms of patient 8, who is a compound heterozygote for *FLG* mutations 441delA and 1249delG. (**c**) Fine scaling on limbs was also present in patient 3. (**d**) In contrast, prominent scaling on the trunk and limbs was seen in patients diagnosed with severe IV.

We identified a mutation within the S100 domain of profilaggrin, 441delA, which was detected in patient 8 and resulted in the introduction of a premature stop codon at position 193. In patients 5 and 8, mutation 1249insG was found in the first partial filaggrin repeat (termed ''repeat 0'') preceding the first complete filaggrin repeat, leading to a downstream premature stop codon at position 418. In repeat 6, two different nonsense mutations were detected in patients 4 and 6. Mutation E2422X and Q2417X mutations resulted from a single nucleotide base substitution of G>T or C>T at positions 7,249 and 7,264, respectively. Interestingly, mutation E2422X was previously reported in a Dutch IV patient (Sandilands *et al.*, 2007) and we also detected one heterozygous carrier in a cohort of 164 normal controls from China. Frameshift mutation 7945delA was detected in filaggrin repeat 7 in patients 3 and 5. Patient 2 harbored a nonsense mutation in repeat 10.2, the first mutation to be reported in one of the duplicated repeats found within the longer size variant alleles of *FLG* (Sandilands *et al.*, 2007). All mutations were confirmed by direct sequencing of PCR products. In addition, we further confirmed the presence of frameshift mutations (441delA, 1249insG, 7945delA) by sizing of fluorescently labeled PCR products and the three nonsense mutations (Q2417X, E2422X, R4307X) were also confirmed

by restriction digestion of PCR products. No *FLG* mutations were found in patients 1 and 7 (Table 2).

Screening of 100 Singaporean and 160 Chinese normal controls for the six mutations identified in Singaporean Chinese IV patients was also performed. With the exception of one case of E2422X found in a Chinese control, none of the other mutations were detected in the controls.

Absent or reduced profilaggrin expression in IV patients

Preliminary analysis of skin biopsies from all eight patients was conducted using hematoxylin and eosin staining. The appearance of keratohyalin granules was greatly reduced in the granular layer of all patients except patient 1 (Figure 2). To detect a change in filaggrin levels, we carried out immunohistochemical analysis of the skin biopsies with monoclonal antibody 15C10, which identifies an epitope conserved in all or most filaggrin repeats. Filaggrin staining in Patient 1 was comparable to that of normal skin (not shown). In contrast, filaggrin staining was greatly reduced in patients 2, 3, 4, and 6; barely detectable staining was observed in patients 5 and 8 (Figure 2), and staining was completely absent in patient 7 (not shown).

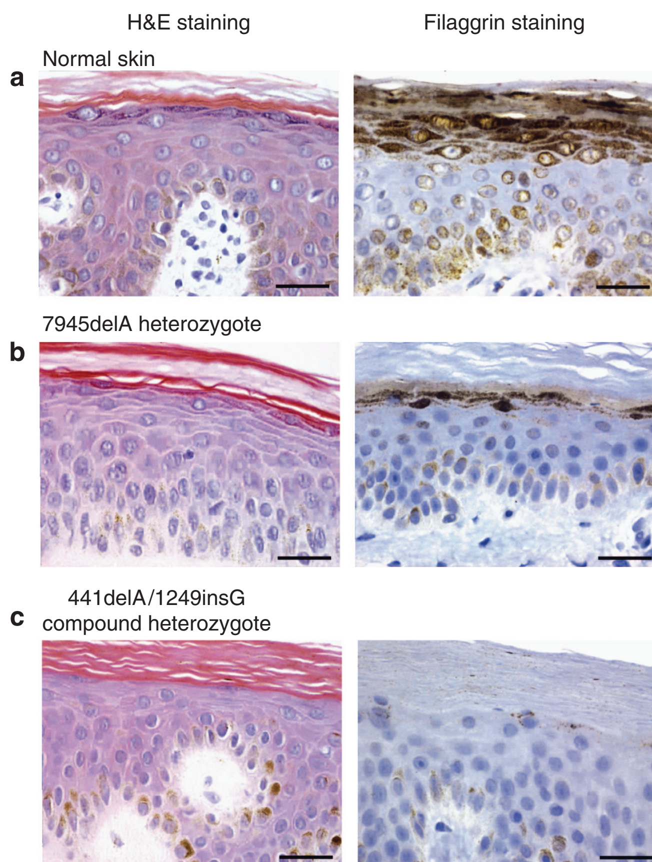

Figure 2. Histological features of patients with IV. All left panels show hematoxylin and eosin (H&E) staining and right panels show immunohistochemical staining with the 15C10 monoclonal antibody against filaggrin repeats in skin biopsies. (**a**) Keratohyalin granules are clearly visible in normal control skin after H&E staining. Immunohistochemical staining detects filaggrin repeats readily in 4–5 granular cell layers and throughout the stratum corneum. (**b**) The presence of a heterozygous 7945delA *FLG* mutation in patient 3 leads to reduced keratohyalin granules and filaggrin staining in the granular layer. The stratum corneum is also thickened. Patients 2, 4, and 5 show similar histochemical features. (**c**) Patient 8, who is a compound heterozygote for 441delA/1249insG *FLG* mutations, has barely detectable keratohyalin granules and filaggrin staining in the granular layer. The stratum corneum is severely thickened. Patient 6 shows similar histochemical features. Bars = 50 μm.

DISCUSSION

Profilaggrin is an unusually large (12.7–14.7 kb) and repetitive gene that has proved to be very challenging to sequence for detection of pathogenic mutations. Recently, comprehensive re-sequencing primers have been developed to allow complete routine analysis of the entire *FLG* (Sandilands *et al.*, 2007).

In this report, we detected six mutations in Singaporean Chinese IV patients, of which five have not been seen in other populations previously studied (Gruber *et al.*, 2006; Marenholz *et al.*, 2006; Smith *et al.*, 2006; Barker *et al.*, 2007; Nomura *et al.*, 2007; Sandilands *et al.*, 2007; Stemmler *et al.*, 2007). Given the similar ancestry among the Singaporean Chinese, Chinese, and Japanese, we were surprised that the mutations previously detected in those populations were not found in our study cohort. The Chinese and Japanese populations share a common mutation 3321delA that is absent in the European population but interestingly, this was not found in our Singaporean Chinese patients despite being found in 3.6% of Chinese controls (Sandilands *et al.*, 2007). It may be that this mutation is less common in Southern Asian populations. With the known mutations now numbering more than 20, it is possible that *FLG* is constantly under evolutionary mutation pressure, so that every population will have a unique set of *FLG* mutations.

The five unique mutations reported here were not detected in any of the 260 normal control samples, therefore these mutations are probably rare. This could also explain why these mutations were not previously reported in the Japanese and Chinese patients studied previously (Nomura *et al.*, 2007; Sandilands *et al.*, 2007). Alternatively, these mutations may also have arisen quite recently amongst the Singaporean Chinese. Interestingly, both the 1249insG and 7945delA frameshift mutations were detected in two out of eight unrelated IV patients; therefore, a larger cohort of IV patients will be useful in assessing the prevalence of these mutations in Singaporean Chinese IV patients. It will also be of considerable interest to screen Singaporean and Chinese AD case series for the presence of these mutations as well as those previously shown to be prevalent in other Asian populations (Nomura *et al.*, 2007; Sandilands *et al.*, 2007); however, we do not currently have access to such a study cohort.

Mutation E2422X was first reported in a Dutch IV patient (Sandilands *et al.*, 2007). It is intriguing to find the identical mutation in a Singaporean Chinese patient as well as a single case in a Chinese control individual. As this mutation was not detected in other European populations nor was it recurrent in the Dutch, it was classified as a rare mutation. Upon further investigation, we were able to identify Chinese ancestry in the Dutch IV patient. Therefore, our present data

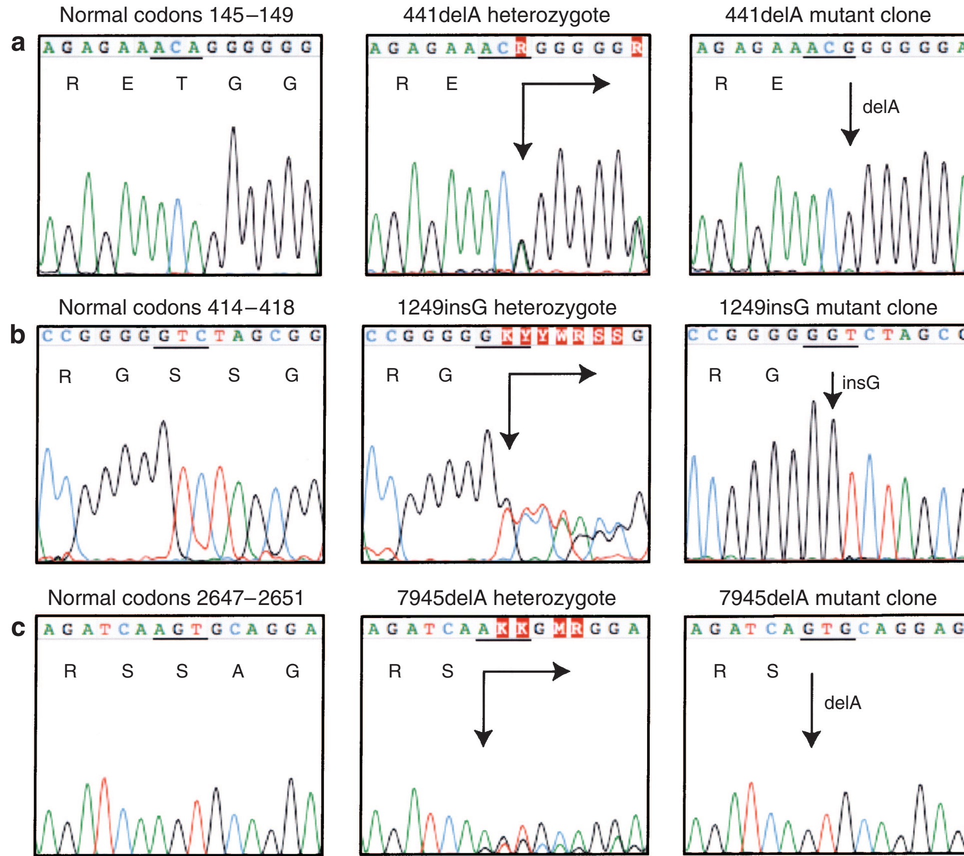

Figure 3. Frameshift mutations of patients with IV. (**a**) Direct DNA sequencing of specific *FLG* PCR products. Left panel shows the normal control sequence from the *FLG* S100 domain corresponding to codons 145–149. Middle panel shows the detection of a heterozygous 441delA mutation in patient 8. Right panel confirms the 441delA mutation by cloning and sequencing of the mutant allele. (**b**) Left panel shows the normal control sequence from *FLG* partial repeat (''repeat 0'') corresponding to codons 414–418. Middle panel shows the identification of a heterozygous 1249insG mutation detected in patients 6 and 8. Right panel confirms the 1249insG mutation by cloning and sequencing of the mutant allele. (**c**) Left panel shows the normal control sequence from *FLG* repeat 7 corresponding to codons 2647–2651. Middle panel shows the detection of a heterozygous 7945delA mutation detected in patient 6. Right panel confirms the 7945delA mutation by cloning and sequencing of the mutant allele.

suggests that this mutation may be present at low levels in some Asian populations. Ideally, all detected mutations thus far should be screened in different population samples to achieve a more complete picture of the *FLG* mutations in different ancestral groups, but this can be time consuming unless high-throughput screening methods for each mutation can be developed.

Among the eight IV cases studied, patients 5 and 8 are compound heterozygotes for *FLG* mutations and immunohistochemical analysis of skin biopsies showed filaggrin to be nearly absent in the granular layer, whereas patients 2, 3, 4, and 6 who are heterozygous for *FLG* mutations showed reduced filaggrin staining compared with the control (Figure 2). In view of this, it is clearly useful to stain the skin biopsies, when available, for filaggrin to predict the likely number of filaggrin mutations carried by an IV patient. However, it has become apparent that all premature termination codon mutations in *FLG* lead to destabilization of profilaggrin, as observed with human mutations (Sandilands *et al.*, 2007). Thus, it is not possible to carry out precise quantification of the amount of filaggrin repeats in the epidermis of a patient and use this information as a predictive tool to pinpoint the location of mutations along the *FLG* gene.

Immunohistochemical studies of skin biopsy from patient 1 revealed normal levels of filaggrin; therefore, it was not surprising that no *FLG* mutations were detected. As IV and X-linked ichthyosis share similar clinical manifestations (Cuevas-Covarrubias *et al.*, 1998) and this patient is male, we considered the possibility that this individual might harbor a mutation in the steroid sulfatase gene (Shapiro *et al.*, 1978). Subsequently, all 10 exons of the steroid sulfatase gene were fully sequenced but no mutation was identified, thus excluding X-linked ichthyosis. Upon further investigation of the family history, the patient's daughter was found to be similarly affected, suggestive of dominant inheritance. This family therefore appears to have a form of ichthyosis clinically similar to IV, but where immunohistochemical and molecular analysis tend to exclude a filaggrin defect.

Patient 7 exhibited a severe IV phenotype and showed complete absence of filaggrin staining, but no mutation was detected in the exon 3. Further re-sequencing also confirmed that the patient had no detectable mutations in exons 1, 2, or

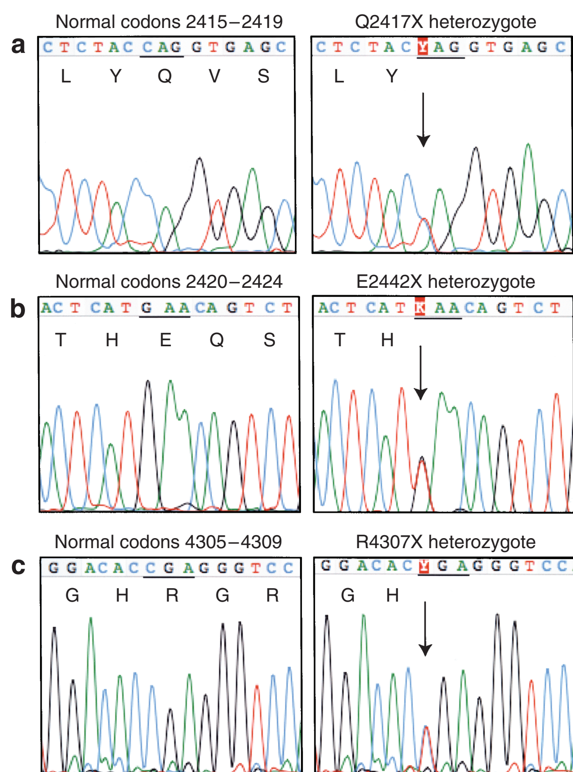

Figure 4. Nonsense mutations of patients with IV. (**a**) Direct DNA sequencing of specific *FLG* PCR products. Left panel shows the normal control sequence from the *FLG* repeat 6 corresponding to codons 2415–2419. Right panel shows a heterozygous transition 7249C>T resulting in nonsense mutation Q2147X in patient 6. (**b**) Left panel shows the normal control sequence from *FLG* repeat 6 corresponding to codons 2420–2424. Right panel shows a heterozygous transversion 7264G>T resulting in nonsense mutation E2422X in patient 4. (**c**) Left panel shows the normal control sequence from *FLG* repeat 10.2 corresponding to codons 4305–4309. Right panel shows a heterozygous transition 12919C>T resulting in nonsense mutation R4307X in patient 2.

the splice sites. Thus, it is possible that patient 7 has a mutation in another unknown gene that plays a critical role in the regulation of filaggrin expression, although we also feel it is possible that a mutation has been missed due to the size and complexity of this repetitive gene.

Our study brings the total number of *FLG* mutations reported to date to twenty. The reason why *FLG* mutations are so prevalent is unclear although it has been proposed that balancing selection conferring a heterozygote advantage might explain the persistence and independent emergence of *FLG*-null mutations in different populations (Nomura *et al.*, 2007; Sandilands *et al.*, 2007). It has also been speculated that in individuals carrying one or more *FLG*-null mutation, ''natural vaccination'' may have permitted the better survival of such heterozygotes during ancient pandemics, due to a stronger background immunity built up by increased trans-epidermal transfer of pathogenic antigens (Irvine and McLean, 2006). Further work on animal models carrying *FLG*-null mutations will be essential to validate this point.

With the emergence of so many different *FLG*-null mutations, more genetic studies on larger numbers of patients will allow us to identify mutation hotspots. Some mutations may also deserve closer examination to study any possible effects on disease penetrance. Current studies report no difference in phenotype between IV patients with *FLG* mutations located in different repeats, but decreased penetrance in AD susceptibility has been reported for certain *FLG*-null mutations (Sandilands *et al.*, 2007). Further *FLG* mutation analysis in different populations will allow us to uncover the full spectrum of mutations and assess their contributions to skin disease and healthcare globally.

MATERIALS AND METHODS

Clinical material

Blood samples were obtained from eight independent Singaporean Chinese patients diagnosed with IV and genomic DNA was isolated according to standard procedures. Skin biopsies (3-mm) from the patients were also embedded in formalin and sectioned for immunohistochemistry analysis. In all cases, we followed the Singapore Institutional Review Board/Domain Specific Review Board guidelines, which are in full accordance with the Declaration of Helsinki Principles, and obtained informed written consent from the participants or their parents where the patients were below 21 years of age. DNA from 100 Singaporean and 164 Chinese anonymous, unselected population controls were also included in the study.

Filaggrin genotyping

FLG mutation analysis and allele size variant determination by comprehensive screening of *FLG* in these IV patients was performed using primers and conditions that have been previously reported (Sandilands *et al.*, 2007). Further specific details are available on request. All PCR products were sequenced using an ABI PRISM 3730 genetic analyzer (Applied Biosystems, Foster City, CA). Mutation E2422X was also screened by restriction digestion as reported previously (Sandilands *et al.*, 2007). Previously unreported mutations were confirmed with additional screening methods.

High-throughput screening for 441delA, 1249insG, and 7945delA

To enable high-throughput screening for the three mutations on control samples, a multiplex fluorescent PCR was used. 50 ng of DNA was amplified in a 10-μl reaction with 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 5 pmol forward primer, 5 pmol reverse primer, 4% (v/v) DMSO, and 0.5 U AmpliTaq Gold polymerase (Applied Biosystems). The primers used to screen for each mutation and PCR conditions are listed in Table 1. The combined PCR products were diluted 1:60 and sized against ROX-500 size markers according to the manufacturer's recommended protocol (Applied Biosystems). Expected sizes for wild-type and mutant alleles are listed in Table 1.

Mutation screening for Q2417X

The Q2417X mutation creates an additional *Bfa*I restriction. A 185-bp PCR product was amplified in a 25-μl reaction containing 1.5 mM $MgCl_2$, 10 pmol forward primer 5′-CCACACGTGGCCGGTCAGCA-3′, 10 pmol reverse primer 5′-GTCCTGACCCTCTTGGGACGT-3′, 0.25 mM of each dNTP, 4% (v/v) DMSO, and 1 U high-fidelity polymerase mix (Roche, Penzberg, Germany). PCR amplification conditions were as follows: one cycle of 94 °C (5 minutes) followed by 35 cycles of 94 °C (30 seconds), 64 °C (30 seconds), 72 °C (45 seconds),

Table 1. Primers and conditions for *FLG* mutation screening by fluorescent PCR

Mutation	Primer pairs	Label	Annealing temperature (°C)	Wild-type allele (bp)	Mutant allele (bp)
441delA	Forward 5′-GTTTCTTGCTGATAATGTGATTCTGTCT-3′ Reverse 5′-ACTAGATTCATGCCTTTTCCC-3′	HEX	58	390	389
1249insG	Forward 5′-GTTTCTTGGGCAAGCTTCATCTGCAGTC-3′ Reverse 5′-CTTGGTGGCTCTGCTGTCTCA-3′	FAM	62	156	157
7945delA	Forward 5′-GTTTCTTCCCAGGACAAGCAGGAACT-3′ Reverse 5′-GTCTTCTGAATGTCCCTCAT-3′	FAM	60	441	440

FLG, filaggrin gene.
Note: The sequence GTTTCTT was added to the unlabeled forward primer to promote plus-A addition for easier allele calling.

Table 2. *FLG* profiles of eight Singaporean Chinese IV patients

Patient	IV severity	Staining of filaggrin repeats	Mutation(s) detected	Repeat Number	Ethnicity of other cases where found
1	Severe	Normal	—	—	NA
2	Severe	Reduced	R4307X	10.2	—
3	Mild	Reduced	7945delA	7	—
4	Mild	Reduced	E2422X	6	Dutch, Chinese
5	Severe	Nearly absent	1249insG, 7945delA	0, 7	—
6	Severe	Reduced	Q2417X	6	—
7	Severe	Absent	—	—	NA
8	Severe	Nearly absent	441delA, 1249insG	S100 domain, 0	—

FLG, filaggrin gene; IV, ichthyosis vulgaris; NA, not available.

and a final extension cycle of 72 °C (5 minutes). A 5-µl volume of PCR product was incubated with 2.5 U of *Bfa*I (New England Biolabs, Ipswich, MA) in a 20-ml reaction volume at 37 °C overnight and resolved on 3% (w/v) agarose gels. The wild-type allele resolved as an uncut 185-bp fragment, whereas the mutant allele gave fragments of 132 and 53 bp.

Mutation detection and screening for R4307X

100 ng of DNA was amplified in a 25-µl reaction containing 1.5 mM $MgCl_2$, 10 pmol forward primer 5′-CTCATCATGCAGAGAATTCCT CTG-3′, 10 pmol reverse primer 5′-CTCCAGTACTGGGCCCAGC-3′, 0.25 mM of each dNTP, 4% (v/v) DMSO, and 1 U of high-fidelity enzyme (Roche). PCR amplification conditions were as follows: one cycle of 94 °C (5 minutes) followed by 35 cycles of 94 °C (30 seconds), 58 °C (30 seconds), 72 °C (45 seconds), and a final extension cycle of 72 °C (5 minutes). Mutation R4307X creates an additional *Dde*I site; 5 µl of PCR product was incubated with 2.5 U of *Dde*I (New England Biolabs) in a 20-µl reaction volume at 37 °C overnight and resolved on 3% (w/v) agarose gels. The wild-type allele resolved as 172, 140, 102, and 36-bp fragments, whereas the mutant allele gave fragments of 172, 140, 51, 51, and 36 bp.

Steroid sulfatase mutation analysis

All exons of the steroid sulfatase gene were amplified by PCR and directly sequenced using the previously reported methods (Liao *et al.*, 2007).

Immunohistochemistry

Immunoperoxidase staining of paraffin-embedded sections with the Envision system (DakoCytomation, Hamburg, Germany) used the mouse monoclonal 15C10 antibody (Novocastra, Newcastle, UK), which binds to an epitope in the C-terminal portion of the human filaggrin repeat unit. Antigen retrieval was performed by heating sections under pressure for 10–15 minutes in 10 mmol l^{-1} citrate buffer, pH 6. Normal skin samples were stained as controls.

CONFLICT OF INTEREST

Irwin McLean has filed patents relating to genetic testing and therapy development aimed at the *FLG*.

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SUPPLEMENTARY MATERIAL

Figure S1. Hyperlinearity observed in IV patients at low magnification.

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