

Analyses of *FLG* mutation frequency and filaggrin expression in isolated ichthyosis vulgaris (IV) and atopic dermatitis-associated IV

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Summary

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Conflicts of interest

None declared.

The first three authors contributed equally to this paper.

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Background Ichthyosis vulgaris (IV; OMIM 146700) is a very common inherited skin disorder. Loss-of-function mutations in the filaggrin gene (*FLG*) have been identified as the cause of IV. In a previous study, we found that the percentage of *FLG* null mutations was lower in IV associated with atopic dermatitis (AD) than in IV not associated with AD (isolated IV). We speculated that some clinical manifestations of IV in patients with AD are not induced by *FLG* mutations.

Objectives In order to clarify this issue, we collected 21 IV pedigrees, 33 patients with sporadic isolated IV and 116 patients with AD-associated IV to analyse *FLG* mutation frequency and filaggrin expression in isolated IV and AD-associated IV.

Methods A comprehensive sequencing of the *FLG* gene in all patients was performed using an overlapping polymerase chain reaction (PCR) strategy. We also studied the immunohistochemistry of profilaggrin/filaggrin protein expression in the skin and measured the mRNA expression using real-time PCR in seven patients, including one patient with IV harbouring the mutation c.3321delA, two patients with AD-associated IV harbouring c.3321delA and c.6834del5, and four patients with AD-associated IV without *FLG* mutations.

Results The percentage of mutations in the *FLG* gene was 74% and 43% in patients with isolated IV and patients with AD-associated IV, respectively. Immunohistochemical staining revealed that profilaggrin/filaggrin peptides were remarkably reduced in the epidermis of all the patients. All the patients with either AD or IV showed lower *FLG* mRNA expression compared with the normal control.

Conclusions These results indicate that factors other than *FLG* gene mutations can downregulate profilaggrin/filaggrin expression, leading to the ichthyosiform phenotype in the context of AD.

Ichthyosis vulgaris (IV; OMIM 146700) is a common inherited skin disorder characterized by dry skin, palmar hyperlinearity and keratosis pilaris.¹ Past reports indicate that 2.5–37% of patients with atopic dermatitis (AD) have clinical evidence of IV.¹ We found that 29 (11%) patients had clinical evidence of coexisting IV in a cohort of 261 patients with AD.² *FLG* null mutations were present in 48% of the 29 patients with AD-associated IV, which was much lower than the percentage of *FLG* null mutations in 21 patients with IV without AD (62%).^{2,3} Considering these results, we speculated that there might be differences in *FLG* null mutations in patients with

isolated IV (i.e. not associated with AD) and those with AD-associated IV.

In this study, we collected 21 Chinese families with IV, 33 patients with sporadic isolated IV and 116 patients with AD-associated IV and sequenced the *FLG* gene.^{4,5} In addition, the frequencies of *FLG* mutations in patients with isolated IV and patients with AD-associated IV were compared. We also studied the immunohistochemistry of profilaggrin/filaggrin protein expression in the skin and measured the mRNA expression using real-time polymerase chain reaction (PCR) in seven patients.

Materials and methods

For detailed information on the study population and their clinical features see Supporting information (Data S1, Tables S1 and S2, Figure S1).

Skin biopsy

A skin biopsy was taken from a normal control and seven patients, including one patient with IV harbouring the mutation 3321delA, one patient with AD-associated IV harbouring c.6834del5, one patient with AD-associated IV harbouring 3321delA and four patients with AD-associated IV without *FLG* mutations. The biopsy sample was obtained from the thigh of the normal control and from the extensor of the leg of the patient with IV with 3321delA; in the other six patients with AD and IV, skin biopsies were obtained from active AD lesional sites on the lower limbs.

Immunohistochemistry

For immunohistochemistry, we used mouse monoclonal antibody filaggrin from Novocastra Laboratories Ltd (Newcastle upon Tyne, U.K.) in a working dilution of 1 : 50 and the high-temperature antigen unmasking technique according to the manufacturer's instructions.

Measurement of mRNA expression using quantitative real-time polymerase chain reaction

Total RNA was extracted from skin using the trizol–chloroform–isopropanol extraction method. The mRNA levels of the *FLG* gene were measured by quantitative real-time PCR using

SYBR Premix Ex Taq Green PCR Mix (Takara, Dalian, China) in a Light Cycler 480 real-time PCR machine (Roche Diagnostics, Shanghai, China).

Results

FLG mutations in patients with isolated ichthyosis vulgaris (IV) and patients with atopic dermatitis-associated IV

A comprehensive sequencing of the entire *FLG* coding sequence in probands from 21 Chinese families with IV and 33 patients with sporadic isolated IV was conducted. Two novel mutations, namely, 441-442delAG and Q4492X (Figure S1b,c), and five previously reported mutations, 3321delA, Q1790X, 5757del4, S2706X and K4671X, were identified. Eleven known mutations were found in the 116 patients with AD-associated IV (Table 1). Three hundred and one unrelated healthy controls were screened at the locus of the 15 *FLG* mutations found in these patients. Three *FLG* mutations (3222del4, 3321delA and K4671X) were found in 12 controls.⁵

The compound genotypes for all *FLG* variants were significantly associated with IV [odds ratio (OR) 60.21, 95% confidence interval (CI) 26.56–136.46, $P < 0.001$] and AD-associated IV (OR 101.22, 95% CI 9.20–36.71, $P < 0.001$).

Differences in frequency of *FLG* mutations in patients with isolated ichthyosis vulgaris (IV) and AD-associated IV

The frequency of *FLG* mutations was 74% vs. 43% in patients with isolated IV and AD-associated IV, respectively (OR 3.77,

Table 1 Association of *FLG* mutations with ichthyosis vulgaris (IV) and atopic dermatitis (AD)-associated IV

FLG mutation	Isolated IV (n = 54), n	AD + IV (n = 116), n	AD + IV vs. IV			
			χ^2	P-value	OR	95% CI
441-442delAG	1		2.15	0.14	–	–
478insA		1	0.47	0.49	–	–
3222del4		1	0.47	0.49	–	–
3321delA	26	23	14.41	< 0.001	3.76	1.86–7.58
Q1070X		1	0.47	0.49	–	–
4026delT		1	0.47	0.49	–	–
Q1790X	1		0.47	0.49	–	–
5757del4	1	3	0.000	1.000	1.40	–
6834del5		1	0.47	0.49	–	–
7945delA		1	0.47	0.49	–	–
Q2397X		2	0.94	0.33	–	–
Q2417X		2	0.94	0.33	–	–
S2706X	1		2.15	0.14	–	–
Q4492X	1		2.15	0.14	–	–
K4671X	9	20	0.01	0.93	0.96	–
Combined ^a	40	50	14.19	< 0.001	3.77	1.85–7.68

OR, odds ratio; CI, confidence interval; –, insufficient data for reliable statistical analyses. ^aCombination of the mutations above.

95% CI 1.85–7.68, $P < 0.001$). The percentage of patients with isolated IV with the 3321delA mutation was significantly higher than the patients with AD-associated IV (48% vs. 20%, $\chi^2 = 14.4$, $P < 0.001$) (Table 1).

Immunohistochemistry

Biopsies from seven patients, including one patient with IV harbouring the mutation 3321delA, one with AD-associated IV harbouring c.6834del5, one with AD-associated IV harbouring 3321delA, four patients with AD-associated IV without FLG mutations and one normal control without FLG mutations, were analysed. All patients showed lower FLG protein expression compared with the normal control. The four patients with AD-associated IV without FLG mutations all presented with a decreased FLG protein expression in various degrees (Fig. 1a–h).

Real-time reverse transcription–polymerase chain reaction analysis of FLG mRNA expression in the skin

All patients with either AD or IV showed lower FLG mRNA expression compared with the normal control. Two patients harbouring the mutation 3321delA presented with the most significant reduction of FLG mRNA expression, while the patient harbouring c.6834del5 showed a higher level of FLG mRNA expression compared with patients with AD-associated IV without FLG mutations (Fig. 2).

Discussion

IV is a genetic skin disorder causing dry, scaly skin, with a prevalence of approximately 1 in 250–1000.¹ In 2006, Smith *et al.*⁶ first reported that two FLG mutations (R501X and

2282del4) were associated with IV in European populations. In the current study, we identified seven mutations in patients with isolated IV, of which 441–442delAG and Q4492X have not been reported previously. Five FLG mutations had previously been reported by other studies, but only within Asian populations.^{2,3,7,8} The novel mutation 441–442delAG is located in the S100 domain of exon 3. This frameshift mutation generated a pretermination codon six codons downstream of the deletion site, and severely diminished the production of free filaggrin in the epidermis. The other novel mutation Q4492X, which is located in filaggrin repeats 10·2, may also result in a severe filaggrin deficiency, despite the synthesis of a truncated profilaggrin peptide, but we did not obtain a functional explanation, as biopsy material from the patients of this family was not available. We found one compound heterozygous and two homozygous mutations in our cases, who showed a much more severe phenotype than that of their parents (Figure S1d–f), which is consistent with the reported semidominant pattern of inheritance.⁶

Oji *et al.*⁹ evaluated a cohort of 26 German patients with IV and found FLG mutations in 96%. In Chinese families with IV in a previous study, FLG mutations were present in 100%.¹⁰ In 21 patients with sporadic isolated IV, we found 13 patients harbouring FLG mutations (62%).⁸ However, we found that only 48% of 29 patients with AD-associated IV had FLG null mutations.⁷ In this study, we found that FLG null mutations were present in 74% of 54 patients with isolated IV and 43% of 116 patients with AD-associated IV. All six patients with AD with or without FLG mutations were demonstrated to suffer from epidermal barrier dysfunction in that decreased expression of FLG at the mRNA and protein level was observed in all of them. We speculate that FLG mutations may not be the main cause of the IV phenotype in patients with AD. Other mutations within the epidermal differentiation complex

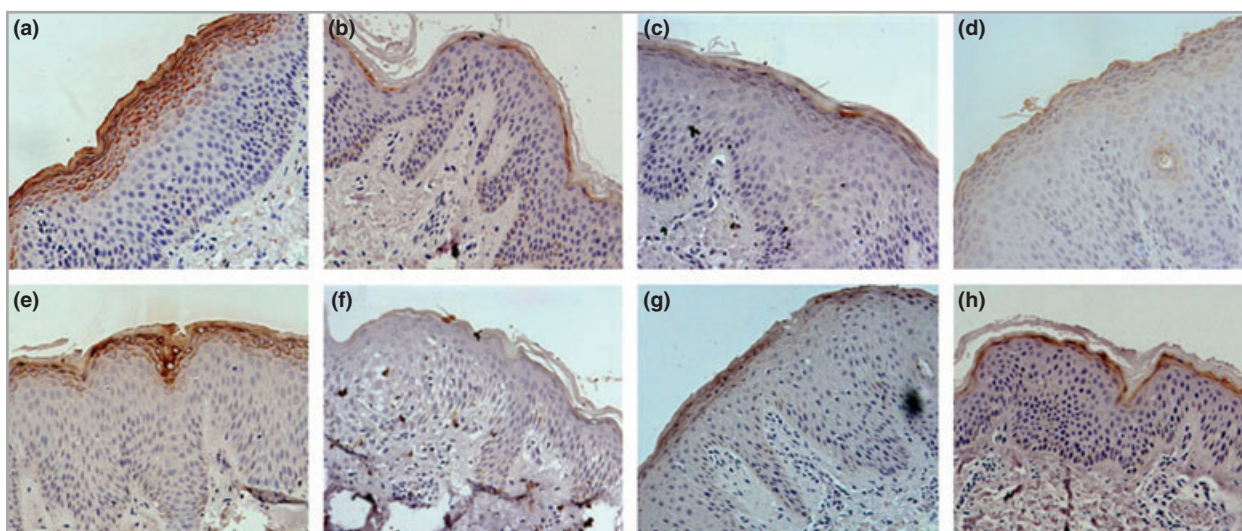


Fig 1. Filaggrin staining in ichthyosis vulgaris (IV) and/or atopic dermatitis (AD) skin. (a) Normal control with wild-type FLG; (b) patient with IV with the 3321delA mutation. (c–f) patient with AD-associated IV with wild-type FLG; (g) patient with AD-associated IV with the 3321delA mutation; (h) patient with AD-associated IV with the 6834del5 mutation. Original magnification: $\times 200$.

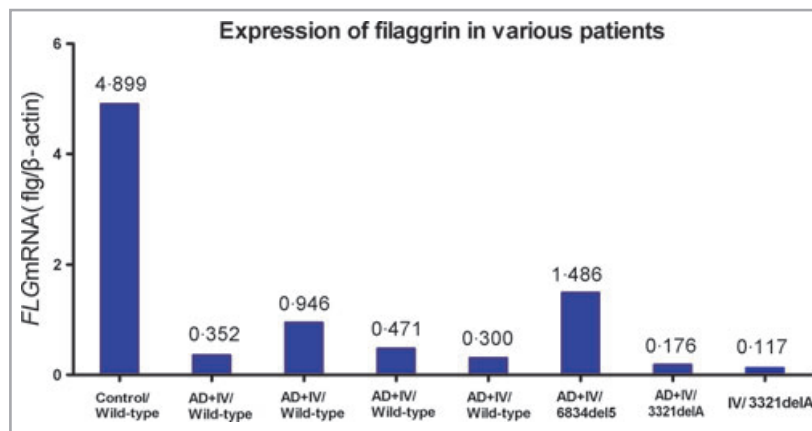


Fig 2. Real-time reverse transcription–polymerase chain reaction analysis of FLG mRNA expression in ichthyosis vulgaris (IV) and/or atopic dermatitis (AD) skin. FLG mRNA expression was reduced in patients with isolated IV and AD-associated IV with or without FLG mutations.

or chronic inflammation may result in the deficiency of epidermal structural proteins at the epithelial barrier, which can lead to the ichthyosiform phenotype occurring in the context of AD. These results may reflect the fact that interleukin (IL)-4 and IL-13, T helper 2 cytokines that are usually increased in AD skin, downregulate filaggrin expression in the epidermis.¹¹

What's already known about this topic?

- FLG mutations are the cause of ichthyosis vulgaris (IV) and the strongest genetic risk for atopic dermatitis (AD).
- T helper 2 cytokines and tumour necrosis factor- α may reduce filaggrin expression.

What does this study add?

- The frequency of FLG mutations in isolated IV is much higher than in AD-associated IV.
- Factors other than FLG gene mutations can downregulate profilaggrin/filaggrin expression, leading to the ichthyosiform phenotype in the context of AD.

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Supporting Information

Additional Supporting Information may be found in the online version of the article at the publisher's website:

Data S1. Study population and clinical features.

Figure S1. Clinical features and two novel null mutations in FLG. (a) Pedigrees of 21 families including 17 families with FLG null mutations. (b) Heterozygous 441-442delAG mutation. (c) Heterozygous transition mutation 13474C>T resulting in nonsense mutation Q4492X. (d–f) Clinical features of members of family 15 showing a much more severe phenotype in the daughter compared with her parents.

Table S1. Clinical data of 116 patients with atopic dermatitis and ichthyosis vulgaris.

Table S2. SCORAD values of 116 patients with both atopic dermatitis and ichthyosis vulgaris.