

FLG mutation p.Lys4021X in the C-terminal imperfect filaggrin repeat in Japanese patients with atopic eczema

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Summary

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Conflicts of interest

W.H.I.M. has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene.

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Background Mutations in the gene encoding filaggrin (FLG) have been shown to predispose to atopic eczema (AE).

Objectives Further to establish population genetics of FLG mutations in the Japanese population and to elucidate effects of FLG mutations to filaggrin biosynthesis in skin of patients with AE.

Methods We searched for FLG mutations in 19 newly recruited Japanese patients with AE. We then screened 137 Japanese patients with AE and 134 Japanese control individuals for a novel mutation identified in the present study. In addition, we evaluated FLG mRNA expression by real-time reverse transcription–polymerase chain reaction and profilaggrin/filaggrin protein expression by immunohistochemical staining in the epidermis of the patients carrying the novel mutation.

Results We identified a novel FLG nonsense mutation c.12069A>T (p.Lys4021X) in one patient with AE. Upon further screening, p.Lys4021X was identified in four patients with AE (2.9% of all the patients with AE). In total, there are at least eight FLG variants in the Japanese population. Here we show that about 27% of patients in our Japanese AE case series carry one or more of these eight FLG mutations and these variants are also carried by 3.7% of Japanese general control individuals. There is a significant statistical association between the eight FLG mutations and AE (χ^2 $P = 6.50 \times 10^{-8}$). Interestingly, the present nonsense mutation is in the C-terminal incomplete filaggrin repeat and is the mutation nearest the C-terminal among previously reported FLG mutations. Immunohistochemical staining for filaggrin revealed that this nonsense mutation leads to remarkable reduction of filaggrin protein expression in the patients' epidermis.

Conclusions We clearly demonstrated that FLG mutations are significantly associated with AE in the Japanese population. The present results further support the hypothesis that the C-terminal region is essential for proper processing of profilaggrin to filaggrin.

Filaggrin is a protein essential to skin barrier function. Mutations in FLG, the gene encoding profilaggrin/filaggrin, have been demonstrated as the underlying cause of ichthyosis vulgaris (IV; OMIM 146700) and have been shown to be an important predisposing factor for atopic eczema (AE).^{1–4} The presence of population-specific FLG mutations in Europeans, Chinese-Singaporeans, Japanese and Taiwanese has been reported.^{3,5–9} Recently, it was clarified that FLG mutations were found in approximately 25% of Japanese patients with AE.^{6,8}

Materials and methods

We searched for FLG mutations in 19 newly recruited Japanese patients with AE. All these patients had been diagnosed with AE based on widely recognized diagnostic criteria.¹⁰ Initially, using genomic DNA, patients with AE were screened for seven FLG mutations previously identified in the Japanese population by restriction enzyme digestion, fluorescent polymerase chain reaction (PCR) and/or direct DNA sequencing as described previously.⁸ Subsequently, for the patients with AE without

any known FLG mutation, we sequenced the entire coding region of FLG. The medical ethics committee of Hokkaido University Graduate School of Medicine approved all the studies, which were conducted according to the Declaration of Helsinki Principles. The participants or their legal guardians gave written informed consent.

Results

This sequencing revealed a novel nonsense mutation c.12069A>T (p.Lys4021X) in repeat 11 (imperfect filaggrin

repeat) of one patient with AE (Fig. 1a–c). The nucleotide change was not detected in 50 unrelated, healthy Japanese individuals (100 alleles).

Subsequently, we screened for the newly identified FLG mutation p.Lys4021X in all 137 Japanese patients with AE we had collected to date and 134 unrelated Japanese control individuals. The 118 patients with AE and 134 control individuals were identical to those in a previous study,⁸ and the data except for those on p.Lys4021X were reported by Nomura *et al.*⁸ We identified p.Lys4021X in four patients with AE (2.9% of all the patients with AE) in our Japanese AE cohort.

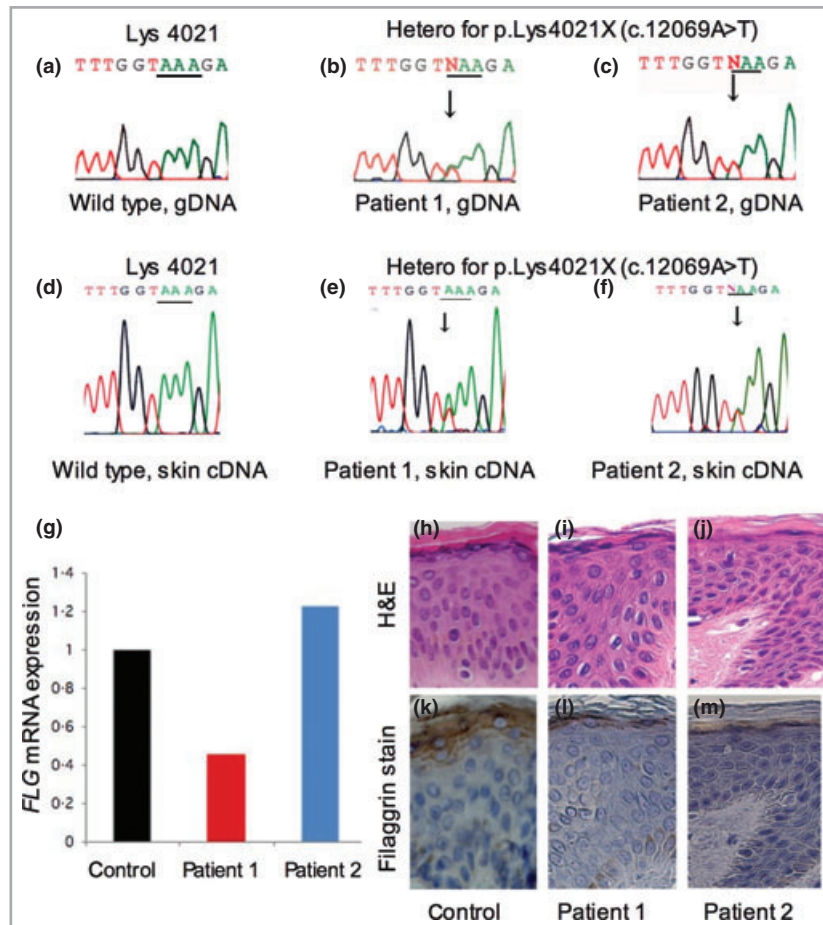


Fig 1. FLG mutation analysis and expression of p.Lys4021X mutant alleles. (a–c) Identification of the FLG mutation. Direct sequence analysis of FLG was performed on genomic DNA from peripheral leucocytes. (a) Normal control sequence from filaggrin repeat 11 in exon 3. (b, c) A heterozygous transition mutation c.12069A>T was identified in patient 1 (b) and patient 2 (c), resulting in p.Lys4021X. (d–f) Direct sequence analysis of FLG cDNA from mRNA expressed in skin samples from the back. (d) Normal control cDNA sequence derived from filaggrin repeat 11 in exon 3. (e, f) Expression of mRNA derived from both wild-type alleles and mutant alleles was confirmed in patient 1 (e) and patient 2 (f). The expression level of mRNA derived from the mutant allele was lower than that from the wild-type allele in patient 1 (e), although expression levels of mRNA from both mutant and wild-type alleles were roughly equal in patient 2 (f). (g) Real-time reverse transcription–polymerase chain reaction analysis of FLG mRNA expression in the skin. FLG expression was reduced in patient 1, but not in patient 2: patient FLG mRNA expression/control FLG mRNA expression = 0.46 for patient 1 and 1.23 for patient 2. mRNA expression of FLG in patients 1 and 2 was not significantly different from that in control skin. (h–m) Histological features of patients 1 and 2: (h–j) haematoxylin and eosin (H&E) staining; (k–m) immunohistochemical staining using antifilaggrin monoclonal antibody against an epitope conserved in all filaggrin repeat peptides. Patient 1 (i) and patient 2 (j) showed a lack of granular layers in the epidermis, where only a small amount of a basophilic substance, which resembled keratohyaline granules, was occasionally present. In contrast, normal control skin (h) had abundant keratohyaline granules in the granular layers. A marked reduction in staining for filaggrin was seen in the epidermis from both patient 1 (l) and patient 2 (m), relative to the strong staining in normal control skin (k).

It was confirmed that these four patients with AE carry no other known FLG mutations. None of the control individuals had the p.Lys4021X mutation. The four patients with AE with the newly discovered mutation – three women and one man – were aged 12–31 years, and all four patients had severe AE symptoms. There was no specific clinical feature of AE characteristic to the four patients. None of the four patients had apparent clinical features or a family history of IV.

We investigated FLG mRNA expression by real-time reverse transcription (RT)-PCR and sequencing, and studied profilaggrin/filaggrin protein expression in the skin by immunohistochemistry in two of the patients with AE harbouring p.Lys4021X. Real-time RT-PCR analysis revealed that mRNA expression of FLG was not reduced significantly (Fig. 1g). The expressed mRNA included messages derived from both the wild-type alleles and the mutant alleles (Fig. 1d–f). However, histopathological examinations of the patients' skin showed reductions in keratohyaline granules in the granular

layers (Fig. 1h–j). Immunohistochemical staining revealed that profilaggrin/filaggrin peptides were remarkably reduced in the patients' epidermis (Fig. 1k–m).

Eight FLG mutations including the present mutation p.Lys4021X have been identified in the Japanese population (Fig. 2). Case–control association analyses were performed for FLG mutations in Japanese patients with AE and normal controls using Pearson χ^2 statistics, as previously described.² The FLG genotype data in the Japanese AE case series and ethnically matched population control series are summarized in Table 1. All alleles were observed to be in normal Hardy–Weinberg equilibrium. Here we demonstrate that about 27% of the patients in our Japanese AE case series carry one or more of the eight FLG mutations (combined minor allele frequency = 0.150, $n = 274$) and these variants are also carried by 3.7% of Japanese control individuals (combined minor allele frequency = 0.019, $n = 268$). There is a statistically significant association between the eight FLG mutations and AE

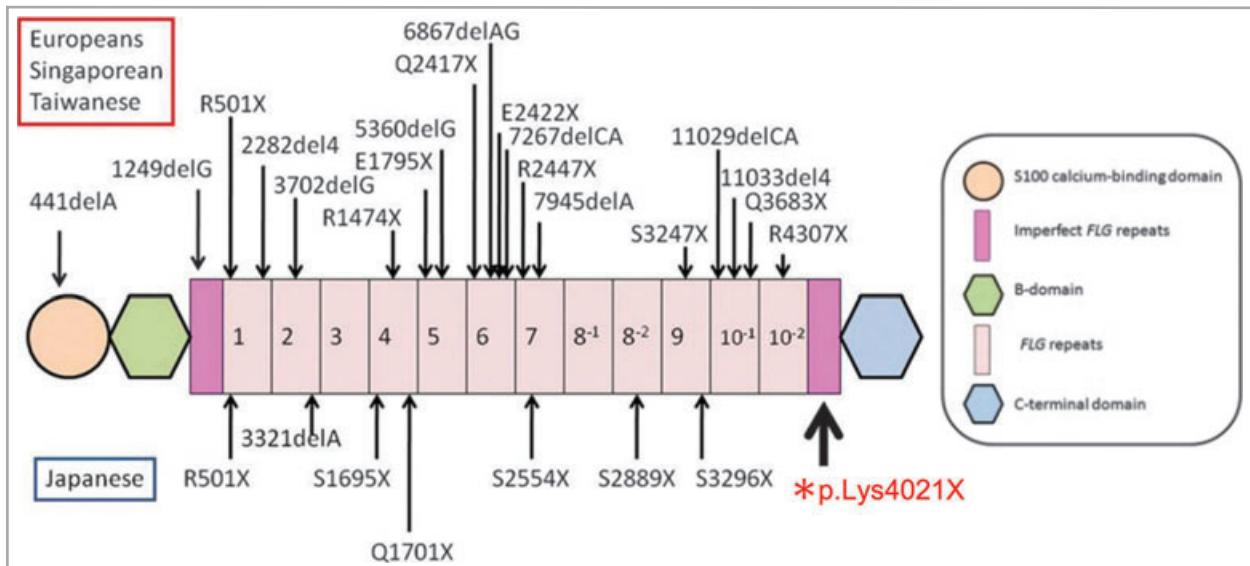


Fig 2. The present and previously reported FLG mutations are shown in a scheme of profilaggrin peptide. Some individuals have a duplication of the 8th and/or 10th filaggrin repeat(s). Duplicated filaggrin repeats are represented as 8⁻¹, 8⁻², 10⁻¹ and 10⁻². *Indicates the present mutation p.Lys4021X. This mutation is the nearest to the C-terminus domain among all the reported mutations and is located in the C-terminal incomplete filaggrin repeat downstream of all the filaggrin repeats.

Table 1 Atopic eczema case–control association analysis for FLG null variants in Japan

	R501X		3321delA		S1695X		Q1701X		S2554X		S2889X		S3296X		K4021X		Combined	
Genotypes	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases
AA	134	137	133	131	133	137	134	134	133	129	132	122	134	132	134	133	129	96
Aa	0	0	1	6	1	0	0	3	1	8	2	15	0	5	0	4	5	33
aa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Total	134	137	134	137	134	137	134	137	134	137	134	137	134	137	134	137	134	137

For combined genotype, $\chi^2 = 29.218$, $P = 6.50 \times 10^{-8}$; Fisher's exact test odds ratio = 9.94 (95% confidence interval 3.77–26.2, $P = 2.35 \times 10^{-8}$).

(χ^2 $P = 6.50 \times 10^{-8}$). Moreover, AE was manifested in heterozygous carriers of these FLG mutations with a Fisher's exact test odds ratio for AE of 9.94 (95% confidence interval 3.77–26.2, $P = 2.35 \times 10^{-8}$), suggesting a causal relationship between FLG mutations and AE.

Discussion

Filaggrin is synthesized initially as profilaggrin, an approximately 500-kDa polypeptide that contains two imperfect filaggrin-repeat domains flanking 10–12 essentially identical filaggrin repeats.¹¹

Previous studies reported that FLG truncation mutations in both filaggrin repeats 1 and 7 lead to a severe filaggrin deficiency, despite the synthesis of a short N-terminal profilaggrin peptide.^{1,3,12} The present mutation p.Lys4021X is in the C-terminal incomplete filaggrin repeat, and the truncation site is the nearest to the C-terminal among FLG mutations identified to date (Fig. 2). The longest truncated profilaggrin peptide containing all the complete filaggrin repeats may theoretically be produced from the mutant allele. However, our immunohistochemical staining revealed that profilaggrin/filaggrin peptides were remarkably reduced in the epidermis of the patients with p.Lys4021X, even though FLG mRNA expression was not reduced significantly and expressed mRNA included messages derived from both the wild-type alleles and the mutant alleles. These results suggest that the truncated profilaggrin peptides are degenerated and are not processed to filaggrin peptides even when the mutation site is in the C-terminal incomplete filaggrin repeat.

In conclusion, we have identified another prevalent FLG mutation in the Japanese population. We have also shown that about 27% of Japanese patients with AD carry one or more FLG mutations. The present nonsense mutation p.Lys4021X in the C-terminal incomplete filaggrin repeat leads to filaggrin deficiency and our results further support the hypothesis that the C-terminal region is essential for proper processing of profilaggrin to filaggrin peptides.

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