

BRIEF COMMUNICATION

Comprehensive screening for a complete set of Japanese-population-specific filaggrin gene mutations

M. Kono¹, T. Nomura², Y. Ohguchi², O. Mizuno², S. Suzuki², H. Tsujiuchi¹, N. Hamajima³, W. H. I. McLean⁴, H. Shimizu² & M. Akiyama¹

¹Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya; ²Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo; ³Department of Healthcare Administration, Nagoya University Graduate School of Medicine, Nagoya, Japan; ⁴Centre for Dermatology and Genetic Medicine, Colleges of Life Sciences, Medicine, Dentistry & Nursing, University of Dundee, Dundee, UK

To cite this article: Kono M, Nomura T, Ohguchi Y, Mizuno O, Suzuki S, Tsujiuchi H, Hamajima N, McLean WHI, Shimizu H, Akiyama M. Comprehensive screening for a complete set of Japanese-population-specific filaggrin gene mutations. *Allergy* 2014; DOI: 10.1111/all.12369.

Keywords

allergic rhinitis; filaggrin; hay fever; mutation; real-time PCR.

Correspondence

Masashi Akiyama, Department of Dermatology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan.
Tel.: 81-52-744-2314
Fax: 81-52-744-2318
E-mail: makiyama@med.nagoya-u.ac.jp

Accepted for publication 27 December 2013

DOI:10.1111/all.12369

Edited by: Stephan Weidinger

Abstract

Mutations in *FLG* coding profilaggrin cause ichthyosis vulgaris and are an important predisposing factor for atopic dermatitis. Until now, most case-control studies and population-based screenings have been performed only for prevalent mutations. In this study, we established a high-throughput *FLG* mutation detection system by real-time PCR with a set of two double-dye probes and conducted comprehensive screening for almost all of the Japanese-population-specific *FLG* mutations (ten *FLG* mutations). The present comprehensive screening for all ten *FLG* mutations provided a more precise prevalence rate for *FLG* mutations (11.1%, $n = 820$), which seemed high compared with data of previous reports based on screening for limited numbers of *FLG* mutations. Our comprehensive screening suggested that population-specific *FLG* mutations may be a significant predisposing factor for hay fever (odds ratio = 2.01 [95% CI: 1.027–3.936, $P < 0.05$]), although the sample sizes of this study were too small for reliable subphenotype analysis on the association between *FLG* mutations and hay fever in the eczema patients and the noneczema individuals, and it is not clear whether the association between *FLG* mutations and hay fever is due to the close association between *FLG* mutations and hay fever patients with eczema.

Mutations in *FLG*, the gene-coding profilaggrin/filaggrin, are an important predisposing factor for atopic dermatitis (AD) (1) and are significantly associated with asthma with AD, mainly in the European population (2–4). The prevalence of *FLG* mutations in AD patients seems to be increasing (5).

The presence of population-specific *FLG* mutations has been reported in both Europeans and Asians (1, 6, 7) and is a serious obstacle to *FLG* mutation screening in each population. We established a real-time polymerase chain reaction (PCR)-based rapid detection system for Japanese-population-specific *FLG* mutations and performed high-throughput *FLG* screening on 820 residents in a rural area of Japan.

Subjects and methods

The subjects were 820 residents (284 males and 536 females) aged 39–90 years in Yakumo, a rural town in Hokkaido,

Japan (8) (Data S1 and Table S1). The participants were requested to answer a questionnaire on health and daily lifestyle at the occasion of the health checkup. Patients with hay fever were defined as individuals reported to have had frequent episodes of all three symptoms of watery eyes, running nose, and sneezing. Patients with asthma were defined as individuals reported to have had a history of asthma, which was diagnosed by physicians. This study was approved by the Ethics Review Committee of Nagoya University Graduate School of Medicine. Ten *FLG* mutations have been identified in the Japanese population, nine of them found by our group (7). We have already performed the sequencing of all the coding regions of *FLG* for more than 30 Japanese families with ichthyosis vulgaris, to identify Japanese-specific *FLG* mutations comprehensively. We expect that screening for these ten mutations can detect almost all Japanese *FLG* mutation carriers. Thus, the present *FLG* mutation screening addressed those ten *FLG* mutations (Table 1). Real-time PCR-based genotyping

Table 1 *FLG* genotypes in the present cross-sectional study

Genotype	p.Arg501X	c.3321delA	p.Ser1695X	p.Gln1701X	p.Ser2554X	p.Ser2889X	p.Ser3296X	p.Lys4022X	p.Q1790X	c.441-442delAG	Combined
AA	820 (1.000)	810 (0.988)	820 (1.000)	818 (0.998)	806 (0.983)	785 (0.957)	816 (0.995)	796 (0.971)	816 (0.995)	820 (1.000)	729
Aa	0 (0)	10 (0.012)	0 (0)	2 (0.002)	14 (0.017)	35 (0.043)	4 (0.005)	24 (0.029)	4 (0.005)	0 (0)	89
aa	0	0	0	0	0	0	0	0	0	0	2*
Total	820	820	820	820	820	820	820	820	820	820	820
Allele freq.(a) (%)	0.0	0.61	0.0	0.12	0.85	2.13	0.24	1.46	0.24	0.0	5.65
HWE† test (chi-square test, <i>P</i> -value)	n.a.	0.861	n.a.	0.972	0.805	0.532	0.944	0.671	0.944	n.a.	n.a.

In genotype data columns, numbers are actual measurement data and actual genotype rate in parentheses.

*These data mean compound heterozygote.

†Hardy–Weinberg equilibrium.

of the *FLG* mutations was performed with TaqMan probe genotyping assay (Data S1 and Table S1).

Results and discussion

Of the 820 participants, 89 individuals were heterozygous for one of the ten *FLG* mutations, and two individuals were compound heterozygous for two of the ten mutations. The distribution of genotypes is shown in Table 1. A total of 91 individuals were carriers of one or two of the ten *FLG* mutations. Thus, the mutant allele frequency was 0.057, and the carrier rate was 0.111 in the present study. To confirm the reliability of the present real-time PCR-based rapid detection system of *FLG* mutations, the accuracy of genotyping was confirmed by direct sequencing or PCR-restriction fragment length polymorphism (RFLP) of samples obtained from all carriers and selected noncarriers of the null mutations. As a result, the presence of *FLG* mutations was confirmed by direct sequencing or PCR-RFLP in all 93 alleles in which the mutations were detected by the real-time PCR-based system. We performed direct sequencing or PCR-RFLP on 220 alleles (11 individuals, ten mutations) in which no mutation was detected by the real-time PCR-based system, and we confirmed that no mutation was found in any of the studied alleles. These results clearly indicate that the present real-time PCR-based rapid detection system for the Japanese-population-specific *FLG* mutations is a highly reliable screening method for population-specific *FLG* mutations.

In Yakumo town, the prevalence rate of *FLG* mutations was found to be relatively high (11.1%) compared with the data of previous reports (2, 3). We do not know the exact reason, but one possibility is that we studied all the Japanese-population-specific *FLG* mutations that we have detected for the last 8 years in the Japanese population, whereas the prevalence rates of *FLG* mutations in European studies were mostly evaluated from the data of studies on only a few prevalent mutations, for example, screening only for R501X and 2282del4, or for the five prevalent mutations in the European population. If only the seven mutations reported in the previous study in 2009 (9) had been used in the present study, the prevalence rate of *FLG* mutations in Yakumo town would have been only 7.7%. In light of this, the higher prevalence rate of 11.1% in the present study might be a reasonable value. On the other hand, we cannot exclude the possibility that the present subjects have skewed genetic backgrounds regarding *FLG* mutations, because many Yakumo residents are descendants of immigrants who moved to Yakumo from a limited area of Nagoya city in central Japan.

According to the present data from a clinical questionnaire given to 816 Yakumo residents, 63 individuals reported having had frequent episodes of watery eyes, runny nose, and sneezing, and they were considered to be putative hay fever patients. Of the 91 individuals with *FLG* mutations, 12 individuals were putative hay fever patients (13.2%), and of the 725 individuals without *FLG* mutations, 51 were putative hay fever patients (7.0%). Thus, *FLG* mutations were significantly associated with putative hay fever (odds ratio = 2.01 [95% CI: 1.027–3.936, *P* < 0.05]; Table 2).

Table 2 Prevalence of history of asthma, watery eyes, and runny nose ($n = 820$) for the total group and the subgroups of individuals with the combined genotype ($n = 91$) and without the combined genotype ($n = 729$)

History	All individuals% (n)	Individuals with <i>FLG</i> mutations% (n)	Individuals without <i>FLG</i> mutations% (n)	OR	95% CI	<i>P</i> -value
Asthma	6.6 (54/815)	10 (9/90)	6.2 (45/725)	1.68	0.792–3.561	0.129
Watery eyes	20.8 (170/817)	22 (20/91)	20.7 (150/726)	1.08	0.638–1.833	0.431
Runny nose	25.9 (211/816)	24.2 (22/91)	26.1 (189/725)	0.90	0.544–1.502	0.403
Watery eyes and runny nose	9.1 (74/816)	14.3 (13/91)	8.4 (61/725)	1.81	0.954–3.451	0.056
Putative hay fever (watery eyes, runny nose, sneezing)	7.7 (63/816)	13.2 (12/91)	7 (51/725)	2.01	1.027–3.926	0.038

Concerning asthma, of the 90 individuals with *FLG* mutations, nine individuals had asthma history (10.0%), and of the 725 individuals without *FLG* mutations, 45 had asthma history (6.2%). Thus, asthma history was not significantly associated with *FLG* mutations (odds ratio = 1.68 [95% CI: 0.792–3.561, $P = 0.129$]; Table 2). This result, that is, nonsignificant association of general asthma with *FLG* mutations, is consistent with previous data by case-control study (4, 10). However, in the present study, we think that the association between the *FLG* mutations and asthma was maybe not significant, due to the reduced sample size and power.

Weidinger et al. (11) reported that, independent of eczema, *FLG* mutations confer a substantial risk for allergic rhinitis. Contrasting reports suggest that the significant association observed between *FLG* mutations and hay fever may be due to the close association between *FLG* mutations and patients with both hay fever and eczema (10, 12). As for asthma, the significant association between the *FLG* mutations and asthma was thought to be due to the close association between *FLG* mutations and asthma patients with eczema (10–12), although there is a report that suggests a significant association between *FLG* mutations and eczema-free asthma patients (13). In light of this, we tried to perform subphenotype analysis to clarify the connection between *FLG* mutations and hay fever/asthma in the eczema patients and the noneczema individuals. However, in these subphenotype analyses, the sample sizes were too small, and we were unable to obtain reliable data (Data S2). Thus, it is not clear whether the association between *FLG* mutations and hay fever in the present study is due to the close association between *FLG* mutations and hay fever patients with eczema.

A number of case-control studies have addressed the association of *FLG* mutations with asthma and hay fever (2–4, 10, 11). However, the number of studies among the general population is limited, and previous studies were performed

only for prevalent *FLG* mutations in each population. In contrast, in the present study, we performed comprehensive screening for almost all the population-specific *FLG* mutations in the general population. In light of this, our present results are noteworthy when we discuss the significance of *FLG* mutations in the pathogenesis of hay fever.

Author contributions

MK, NH, WHIM, HS, and MA contributed study conception and design. MK, TN, NH, YO, OM, SS, HT, and NH contributed data acquisition and analysis. MK, NH, HS, and MA contributed data interpretation. MK and MA wrote the manuscript, and MK, TN, NH, HS, and MA revised it critically for important intellectual content. All authors approved the final version of the manuscript.

Funding

Grants-in-Aid for Scientific Research (A) 23249058 (to MA) and (C) 24591646 (to MK) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflicts of interest

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The sequence of assay probes/primers.

Data S1. Materials and methods.

Data S2. Results: subphenotype analysis.

References

- McAleer MA, Irvine AD. The multifunctional role of filaggrin in allergic skin disease. *J Allergy Clin Immunol* 2013;**131**:280–291.
- Rodriguez E, Baurecht H, Herberich E, Wagenpfeil S, Brown SJ, Cordell HJ et al. Meta-analysis of filaggrin polymorphisms in eczema and asthma: robust risk factors in atopic disease. *J Allergy Clin Immunol* 2009;**123**:1361–1370.
- van den Oord RA, Sheikh A. Filaggrin gene defects and risk of developing allergic sensitisation and allergic disorders: systematic review and meta-analysis. *BMJ* 2009;**339**:b2433.
- Osawa R, Konno S, Akiyama M, Nemoto-Hasebe I, Nomura T, Nomura Y et al. Japanese-specific filaggrin gene mutations in Japanese patients suffering from atopic eczema and asthma. *J Invest Dermatol* 2010;**130**:2834–2836.

5. Thyssen JP, Linneberg A, Johansen JD, Carlsen BC, Zachariae C, Meldgaard M et al. Atopic diseases by filaggrin mutations and birth year. *Allergy* 2012;**67**:705–708.
6. Nomura T, Sandilands A, Akiyama M, Liao H, Evans AT, Sakai K et al. Unique mutations in the filaggrin gene in Japanese patients with ichthyosis vulgaris and atopic dermatitis. *J Allergy Clin Immunol* 2007;**119**:434–440.
7. Nemoto-Hasebe I, Akiyama M, Nomura T, Sandilands A, McLean WH, Shimizu H. FLG mutation p.Lys4021X in the C-terminal imperfect filaggrin repeat in Japanese patients with atopic eczema. *Br J Dermatol* 2009;**161**:1387–1390.
8. Itou S, Goto Y, Suzuki K, Kawai S, Naito M, Ito Y et al. Significant association between methylenetetrahydrofolate reductase 677T allele and hyperuricemia among adult Japanese subjects. *Nutr Res* 2009;**29**:710–715.
9. Nomura T, Akiyama M, Sandilands A, Nemoto-Hasebe I, Sakai K, Nagasaki A et al. Prevalent and rare mutations in the gene encoding filaggrin in Japanese patients with ichthyosis vulgaris and atopic dermatitis. *J Invest Dermatol* 2009;**129**:1302–1305.
10. Schuttelaar ML, Kerkhof M, Jonkman MF, Koppelman GH, Brunekreef B, de Jongste JC et al. Filaggrin mutations in the onset of eczema, sensitization, asthma, hay fever and the interaction with cat exposure. *Allergy* 2009;**64**:1758–1765.
11. Weidinger S, O'Sullivan M, Illig T, Baurecht H, Depner M, Rodriguez E et al. Filaggrin mutations, atopic eczema, hay fever, and asthma in children. *J Allergy Clin Immunol* 2008;**121**:1203–1209.
12. Marenholz I, Nickel R, Ruschendorf F, Schulz F, Esparza-Gordillo J, Kerscher T et al. Filaggrin loss-of-function mutations predispose to phenotypes involved in the atopic march. *J Allergy Clin Immunol* 2006;**118**:866–871.
13. Li M, Chen X, Chen R, Bao Y, Yao Z. Filaggrin gene mutations are associated with independent atopic asthma in Chinese patients. *Allergy* 2011;**66**:1616–1617.

Data S1

Subjects

The subjects were residents (39 years or older) of Yakumo, a rural town in Hokkaido, Japan, who attended a health checkup program in 2005 or 2006 (8). Of these, 820 residents (284 males and 536 females) aged 39 to 90 years who agreed to have their residual blood used for DNA analysis and whose DNA samples were successfully extracted were included in the present study. Genotype analysis was performed for 820 individuals. However, some individuals did not answer one or more questions. Thus, the total numbers of individuals included in each statistical test were not equal.

This study was performed according to *The Declaration of Helsinki Principles* and the ethics policies of the institution. The participants were requested to answer a questionnaire on health and daily lifestyle on the occasion of a health checkup. Written informed consent to provide lifestyle information and residual blood for DNA analysis was obtained.

Real-time PCR-based genotyping of *FLG* mutations

Ten *FLG* mutations have been identified in the Japanese population, 9 of which were found by our group (7). We have already performed sequencing of all the coding regions of *FLG* for more than 30 Japanese families with ichthyosis vulgaris, to comprehensively

characterize Japanese-specific *FLG* mutations. We expect that screening for these 10 mutations can detect almost all Japanese *FLG* mutation carriers. Thus, in the present *FLG* mutation screening, we studied the 10 *FLG* mutations (Table 1). Real-time PCR-based genotyping of the *FLG* mutations was performed with TaqMan probe genotyping assay. Genomic DNA was extracted from buffy coat conserved at -80 °C using BioRobot EZ1 (Qiagen, Tokyo, Japan). Real-time PCR-based genotyping of the *FLG* mutations was performed with TaqMan probe genotyping assay according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). To detect an allele of each mutation, a set of two TaqMan probes labeled with a fluorescent dye (FAM or CAL Fluor Orange 560) and a quencher dye (BHQ-1), and sequence-specific forward and reverse primers were synthesized by Biosearch Technologies (Novato, CA). The sequence of assay probes/primers is shown in Table S1. Real-time PCR was performed with a LightCycler 480 system II 384 plate (Roche Diagnostics) in a final volume of 5 µl containing 2x LightCycler 480 Probes Master (Roche Diagnostics), 200 nM probes for wild type and mutant each, and 500 nM forward and reverse primers each, with 5 ng genomic DNA as the template. The thermal conditions were the following: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 60 s and 72 °C for 1 s, with a final cooling at 40 °C for 30 s. Endpoint fluorescence was measured for each sample well. Afterward, genotyping was performed using endpoint genotyping analysis of LightCycler 480

software.

Data S2

Sub-phenotype analysis

We performed sub-phenotype analysis to clarify the association between FLG mutations and hay fever in the eczema patients, and the association between FLG mutations and hay fever in the non-eczema individuals. Unfortunately, for the association between FLG mutations and hay fever in the eczema patients, the sample size of individuals with both hay fever and eczema was so small that we were unable to perform statistical analyses. For the association between FLG mutations and hay fever in the non-eczema individuals, sub-phenotype analysis was possible. It showed that even in the non-eczema individuals, hay fever was significantly associated with FLG mutations (odds ratio=2.53 [95% CI: 1.148-5.576, $p=0.022$]). However, in this sub-phenotype analysis, the number of hay fever cases was very small and we were unable to conclude that hay fever is associated with FLG mutations independently of eczema.

As for asthma, we also performed sub-phenotype analysis to clarify the association between FLG mutations and asthma in the eczema patients, and the association between FLG mutations and asthma in the non-eczema individuals. No significant association was found between FLG mutations and asthma in the eczema patients (odds ratio=1.83 [95%

CI: 0.507-6.570, $p=0.350$]), or between FLG mutations and asthma in the non-eczema individuals (odds ratio=1.53 [95% CI: 0.616-3.804, $p=0.355$]).”

Table S1. The sequence of assay probes/primers

Mutation	Primer/Probe	Sequence
c.1501C>T	FLG_1501.F	GCACTGGAGGAAGACAAGGATC
	FLG_1501.R	TCCTCATTTTCGTGTTTGTCTGC
	FLG_1501C_wild	FAM-AGGCACGAGACAGCTCCAGGC-BHQ1
	FLG_1501T_mut	CFO-CAGGCATGAGACAGCTCCAGGC-BHQ1
c.5084C>G	FLG_5084.F	ACCAGCAGTCAGCAGACAGC
	FLG_5084.R	CTGACTCTTCTGAGTGTCCCTCG
	FLG_5084C_wild	FAM-CCACAGACTCAGGCACTGGGC-BHQ1
	FLG_5084G_mut	CFO-CCACAGACTGAGGCACTGGGC-BHQ1
c.5101C>G	FLG_5101.F	GTCAGCAGACAGCTCCACAGAC
	FLG_5101.R	CTGACTCTTCTGAGTGTCCCTCG
	FLG_5101C_wild	FAM-TGGGYGCAGACAAGATTCATCTG-BHQ1
	FLG_5101T_mut	CFO-CACTGGGYGCAGATAAGATTCATCTG-BHQ1
c.7661C>G	FLG_7661.F	GCTCCAGGCACTCAGGGTC
	FLG_7661.R	TGGATCCCCAGTTCCTGCT
	FLG_7661C_wild	FAM-CCCTCTGATTGTCCCTGGMCC-BHQ1
	FLG_7661G_mut	CFO-CCCTCTCATTGTCCCTGGMCC-BHQ1
c.8666_7CC>GA	FLG_8666-7.F	CTCAGGGTCGCGTCACC
	FLG_8666-7.R	CTCTCAGAGTCTTCTGAATGTCCCT
	FLG_8666-7CC_wild	FAM-CCTGGCTMACACTGGATCCCTG-BHQ1
	FLG_8666-7GA_mut	CFO-TCCTGGCTMACACTTCATCCCTG-BHQ1
c.9887C>A	FLG_9887.F	TGGTGGACAGGCTGCATC
	FLG_9887.R	AAGCYTGTCCACGCGGA
	FLG_9887C_wild	FAM-ACAGGCAAGATCAAGTCCAGGAGAGA-BHQ1
	FLG_9887A_mut	CFO-TGAACAGGCAAGATAAAGTCCAGGAGAG-BHQ1
c.12064A>T	FLG_12064.F	AGACACTCTCAGCACGGAAGTGT
	FLG_12064.R	GCCACATAAACCTGGGTCCTTA
	FLG_12064A_wild	FAM-CCTTGGATGATCTTTACCAAACGCACT-BHQ1
	FLG_12064T_mut	CFO-CCTTGGATGATCTTAACCAAACGCACT-BHQ1
c.441_2delAG	FLG_441-2.F	AAGGGAATAAGGGAAGATCCAAGA
	FLG_441-2.R	CTTAGCCTCTTCTATTGTCTCCTAATCT
	FLG_441-2AG_wild	FAM-CAAGAGAAACAGGGGGGAAAAGGC-BHQ1
	FLG_441-2del_mut	CFO-CAAGAGAAACAGGGGGGAAAAGGCA-BHQ1
c.3321delA	FLG_3321.F2	AGGCCATGGACAGGATGG
	FLG_3321.R2	CTCACCTGGTAGATGAAAGACCCT
	FLG_3321A_wild2	FAM-CGTGACTGGTCAGGGGGAAGGTC-BHQ1
	FLG_3321del_mut2	CFO-ACGTGACTGGTCGGGGGAAGGT-BHQ1
c.5368C>T	FLG_5368.F	CACAGGGCCCAGCACTG
	FLG_5368.R	ACCGATTGCTCATAGTGGGATC
	FLG_5368C_wild	FAM-AGGAAGACAAAGATCCCGCCACG-BHQ1
	FLG_5368_mut	CFO-AGGAAGATAAAGATCCCGCCACGAG-BHQ1

CFO: CAL Fluor Orange 560