ORIGINAL ARTICLE

Filaggrin loss-of-function mutations, atopic dermatitis and risk of actinic keratosis: results from two cross-sectional studies

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Abstract

Background Common loss-of-function mutations in filaggrin gene (*FLG*) represent a strong genetic risk factor for atopic dermatitis (AD). Homozygous mutation carriers typically display ichthyosis vulgaris (IV) and many have concomitant AD. Previously, homozygous, but not heterozygous, filaggrin gene mutations have been associated with squamous cell carcinomas.

Objective The first objective was to examine the association between *FLG* mutations and actinic keratosis (AK). The second objective was to investigate the occurrence of AK in patients with IV and AD, respectively.

Methods *FLG* mutation status in patients with AK was compared with controls from the general population. Furthermore, based on nationwide data from Danish registers, we compared the risk of AK in patients with IV, AD and psoriasis, respectively.

Results The prevalence of homozygous *FLG* mutations was significantly higher in the AK group (n = 4, 0.8%) in comparison with the control group (n = 18, 0.2%), whereas the prevalence of heterozygous *FLG* mutations was lower. In hospital registry data, patients with AD exhibited an increased risk of AK than did psoriasis controls (adjusted OR 1.46; [95% CI 1.12–1.90]), whereas no difference in risk was observed between patients with IV and AD.

Conclusions This study indicates an increased susceptibility to AK in individuals with homozygous, but not heterozygous, *FLG* mutations and in patients with AD compared to psoriasis. Whether a reduction or absence of epidermal filaggrin could contribute to the susceptibility to AK in patients with IV and AD is unknown and additional research is needed to further explore this relationship.

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Declaration of interests

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Introduction

Approximately 10% of Northern Europeans carry a loss-of-function mutation in filaggrin gene (*FLG*) (heterozygous), whereas <0.5% carry two mutations (compound heterozygous or homozygous).[1] These mutations cause a reduction, or complete absence of epidermal filaggrin and its degradation products,[2] representing a well-known genetic risk factor for atopic dermatitis (AD).[3,4] Homozygous *FLG* mutation carriers display clinical ichthyosis vulgaris (IV), characterized by xerotic and scaly skin, as well as palmar and plantar hyperlinearity,[1,5] with an increased propensity to develop AD.

Filaggrin is degraded to its constituent amino acids, including histidine, which is further deiminated enzymatically to trans-urocanic acid (tUCA), a chromophore that provides protection against ultraviolet B (UVB) radiation.[6] Some, but not all studies, have demonstrated that epidermal deficiency of filaggrin may lead to reduced endogenous protection against UV exposure, potentially increasing the risk of UVB-induced DNA damage and skin cancer.[7–9] Yet, a small Danish patient-based, case–control study that included 13 heterozygous mutation carriers found no association between *FLG* mutations and basal cell carcinomas (BCCs),[10] and a Danish general population cohort showed a borderline lower risk of non-melanoma skin cancer (NMSC) in *FLG* mutation carriers.[11] In contrast, homozygous, but not heterozygous, *FLG* mutation status was significantly associated with squamous cell carcinomas (SCCs) in a Danish case–control study.[8]

Besides *FLG* mutations, acquired factors may also reduce epidermal filaggrin levels.[2] For example, Th-2 cytokine generation in AD downregulates filaggrin in both lesional and non-lesional skin,[12] and chronic topical glucocorticoid use reduces epidermal filaggrin.[13] While suberythemal medical UV therapy can improve the skin barrier and upregulate filaggrin,[14] chronic UVB irradiation will also increase accumulated UVB doses, at least in theory increasing the risk of UVB-induced DNA damage.[2,12] The above factors, together with use of immunosuppressants and possibly detection bias,[15–17] may explain the higher prevalence of NMSC that has been observed in adult Danish and Swedish patients with AD.[18–20] Yet, collectively, the evidence for an association between AD and NMSC is not established, and published results have been inconclusive.[18–24]

Importantly, it is still unclear whether actinic keratoses (AKs), premalignant intra-epidermal skin lesions that can progress into SCCs, occur more frequently in individuals with *FLG* mutations or AD. AKs typically develop on irradiated skin, and their incidence correlates linearly with the individual's cumulative UVB exposure.[25,26] A recent Dutch study found no association between AD and AK,[27] but a Danish questionnaire study in dermatitis patients shows that homozygous mutation carriers (16.7%) had a higher prevalence of self-reported AKs than heterozygous (9.0%) and wild-type carriers (2.2%).[28] In this study, we investigated the relationship between AKs and *FLG* mutation status, as well as the occurrence of AKs in hospital-diagnosed patients with IV and AD, respectively, hypothesizing that individuals with a primary or secondary epidermal filaggrin deficiency would have a higher risk of AK than controls.

Materials and methods

Two separate sets of data were assessed for this study. The first study compared the prevalence of *FLG* mutations in a cohort of patients with AKs in the general population. The second part of the study was a register-based epidemiological study that evaluated the risk of AKs in patients with IV and AD, vs. non-atopic controls, while also taking certain, potential confounding factors into consideration.

Filaggrin gene mutation status and actinic keratosis

For the first part of the study, approval was obtained from The Danish Research Ethics Committee (approval H-4-2011-145). The sample size calculations for the number of biopsies needed were primarily based on heterozygous mutations. An estimated sample size of 470 biopsies was required to be able to detect an OR of 1.5 with 80% power. In total, 500 anonymous and consecutively archived formalin-fixed, paraffin-embedded blocks of biopsy-verified AKs were retrieved from the Department of Pathology, Herlev Hospital, Copenhagen, Denmark. A pathologist (EB) confirmed the diagnosis of AK on a haematoxylin and eosin-stained microscopic slide. Following this, a 3-mm punch biopsy of the cellular lesion area was obtained from the paraffin-embedded tissue. After overnight digestion with proteinase K, genomic DNA was extracted using silica-based magnetic particles (QIAsymphony DNA Mini Kit on the QIAsymphony SP workstation; Qiagen, Hilden, Germany), according to manufacturer's instructions. The DNA concentration of the samples was measured using a NanoDrop (Nanodrop Products, Wilmington, DE, USA). A total of 481 (96%) of the AK samples were successfully genotyped by a suspension array-based, allele-specific polymerase chain reaction (Luminex, Austin, TX, U.S.A.) previously described for the three most common loss-of-function mutations in *FLG* (GenBank NM_002016.1: c.1537C>T, c.2318_2321del and c.7375C>T, commonly designated as R501X; 2282del4; and R2447X). Compound heterozygous mutation carriers were categorized as having homozygous mutation genotype as the two mutations nearly always occur on different alleles.[29] The methodology for genotyping such tissue samples is described in detail elsewhere.[8,30] The information on *FLG* mutation status from the AK cohort was compared with pooled data from two filaggrin genotyped cohorts from the adult general population in Denmark, the Inter99 study cohort and Health 2006 study cohort, comprising a total of 6,784 and 3,346 individuals, respectively.[31,32]

Ichthyosis vulgaris, atopic dermatitis and actinic keratosis

For the second part of the study, approval was obtained from by the Danish Data Protection Agency (ref. 2007-58-0015, int. ref. GEH-2014-018, I-Suite 02736). Review of an ethics committee is not required for register studies in Denmark. In Denmark, information on health utilization in the entire population is consecutively registered in nationwide registers and made available for research purposes. The registers contain information on all hospital diagnoses (coded as the International Classification of Diseases [ICD] codes), medication use (coded as Anatomical Therapeutic Chemical classification [ATC] codes), including hospital procedures such as phototherapy and pharmacological treatment (coded as procedure [SKS] codes) and vital statistics.[33] A unique personal identification number, which is assigned to all Danish citizens at immigration or birth, allows for unambiguous, individual-level cross-linkage of administrative registers.

All Danish residents with a hospital diagnosis of IV (ICD-10 Q800), AD (ICD-10L20) and psoriasis (ICD-10L40), respectively, between 1 January 1997 and 31 December 2012 were identified for the study cohorts. Only subjects >30 years of age at the date of inclusion were included in the study cohorts. Cases with both a diagnosis of IV and AD (n = 29) were attributed to the IV population. Patients with both AD and psoriasis (n = 303) were deleted from the cohort. The occurrence of AK was identified in the three study populations by searching for diagnostic codes for AK (ICD-10L570) recorded in the same study period. The AK occurrence in the IV group was compared with AD, and the AK occurrence in the AD group was compared with psoriasis. Patients with psoriasis were selected as a relevant control population for AD in this study, because it is a chronic, relapsing inflammatory skin disease, thereby reducing the risk of surveillance bias. Furthermore, they often receive phototherapy, topical glucocorticoids and other immunosuppressants similar to patients with AD. Therefore, psoriasis should provide a more appropriate control group than randomly sampled individuals from the general population.

Covariates

Phototherapy was identified by SKS codes BNGA and BNGD, including UVA, UVB, photo-chemotherapy (PUVA) and X-ray therapy. Photodynamic therapy and laser therapy were not included. The following codes were used for the identification of pharmacotherapy: systemic immunosuppressants (ATC code group L04) and systemic corticosteroids (ATC code group H02). As a measure of healthcare consumption, the total number of dermatology clinic visits (all ICD codes beginning with 'L' and codes for IV) was identified and categorized into one, two, or three or more visits.

Statistical analysis

Pearson's chi-square and Fisher's exact tests of independence were used to compute two-sided P-values in analysis of filaggrin genotyped data. Population characteristics in patients with IV, AD and psoriasis were described with means and standard deviations (SDs) for continuous variables and frequencies and percentages for categorical variables. We used multivariable logistic regression to compute odds ratios (ORs). $P < 0.05$ was considered statistically significant, and results were reported with 95% confidence intervals (CIs), where applicable. All analyses were performed using SAS statistical software version 9.4 (SAS Institute Inc. Cary, NC, USA) and STATA software version 11.0 (StataCorp, College Station, TX, USA).

Results

Homozygous *FLG* mutation status was associated with actinic keratoses

A total of 481 patients with biopsy-verified AKs were genotyped for *FLG* mutation status, as were 9112 controls from the adult general population. In total, 36 (7.5%) of AK patients had a loss-of-function mutation in *FLG*, of whom four (0.8%) were homozygous mutation carriers. Loss-of-function mutations were found in 853 (8.6%) individuals in the control group, of which 18 (0.2%) were homozygous carriers (Table 1). The allele counts are presented in Table S1. Homozygous mutation carriers had increased risk of AK compared with wild types (Fisher's exact test yielded a P-value of 0.017), whereas no association was found for heterozygous mutation carriers compared with wild types (Pearson's chi-square test yielded a P-value of 0.150).

Prevalence of actinic keratosis in three patient populations

Between 1 January 1997 and 31 December 2012, a total of 159; 5923 and 22 301 Danish citizens 30 years or older received a hospital (inpatient or ambulatory) diagnosis of IV, AD or psoriasis, respectively. Mean (SD) age at first diagnosis was 56.8 (17.1), 46.7 (13.8) and 55.4 (14.3) years, respectively. There was a slight male predominance in the IV group (57.2%) and female predominance (59.1%) in the AD group. Phototherapy and systemic immunosuppressants were more frequently used in the

Table 1 *FLG* mutation status in patients with AK compared with controls

FLG mutations* (number of cases)	AK (n = 481)	Controls (n = 6616)	OR (95% CI)	P-value
Wild type	446 (92.7)	9112 (91.4)	—	—
Heterozygous	31 (6.4)	835 (8.4)	0.75 (0.52–1.09)	0.150
Homozygous	4 (0.8)	18 (0.2)	4.63 (1.56–13.75)	0.017

Chi-squared test was used to compare heterozygous mutation carriers with wild-type mutation carriers.
Fisher's exact test was used (cell number <5) to compare homozygous mutation carriers with wild-type mutation carriers.
*The investigated *FLG* mutations were R501X, 2282del4 and R2447X.
AK, actinic keratosis; CI, confidence interval; *FLG*, filaggrin gene; OR, odds ratio.

Table 2 Characteristics of patients with ichthyosis vulgaris, atopic dermatitis and psoriasis

	Ichthyosis vulgaris (*n* = 159)	Atopic dermatitis (*n* = 5923)	Psoriasis (*n* = 22 301)
Mean (SD) age (years)	56.8 (17.1)	46.7 (13.8)	55.4 (14.3)
Women (%)	68 (42.8)	3501 (59.1)	11 370 (51.0)
Men (%)	91 (57.2)	2422 (40.9)	10 931 (49.0)
Phototherapy†	10 (6.3)	737 (12.4)	2979 (13.4)
Immunosuppressant therapy*	74 (46.5)	3570 (60.3)	13 773 (61.8)
Number of dermatology visits			
1	68 (42.8)	2616 (44.2)	9299 (41.7)
2	32 (20.1)	1337 (22.6)	4886 (21.9)
3 or more	59 (37.1)	1970 (33.3)	8116 (36.4)
Actinic keratosis	5 (3.1)	73 (1.2)	329 (1.5)

*Include systemic immune suppressant agents in ATC category L04 and systemic corticosteroids.
†Include UVA, UVB, PUVA therapy. Photodynamic therapy and laser therapy are not included.
SD, standard deviation.

AD and psoriasis groups compared with the IV group. The total numbers of observed cases of AK in the various patient groups were as follows: 5 (3.1%) in IV, 73 (1.2%) in AD and 329 (1.5%) in psoriasis, respectively (Table 2).

In fully adjusted logistic regression analysis, IV was non-significantly associated with AK (adjusted OR 1.31; [95% CI 0.50–3.40], $P = 0.585$) when compared with AD. On the other hand, AK was significantly associated with AD compared with psoriasis in both age- and sex-adjusted (OR 1.42; [95% CI 1.09–1.85], $P = 0.009$) and fully adjusted analyses (adjusted OR 1.46; [95% CI 1.12–1.90], $P = 0.006$).

Table 3 Association between actinic keratosis and ichthyosis vulgaris and atopic dermatitis

	Age and sex adjusted			Fully adjusted*		
	OR	95% CI	*P*-value	aOR	95% CI	*P*-value
IV vs AD	1.46	0.57–3.73	0.433	1.31	0.50–3.40	0.585
AD vs Psoriasis	1.42	1.09–1.85	0.009	1.46	1.12–1.90	0.006

*Adjusted for age, sex, immunosuppressant therapy, phototherapy and number of dermatology visits.
AD, atopic dermatitis; IV, ichthyosis vulgaris; aOR, adjusted odds ratio; CI, confidence interval; OR, odds ratio; SD, standard deviation.

Discussion

Main findings

In a large random histological sample of AKs, we found a higher prevalence of homozygous, but not heterozygous *FLG* mutations when compared to general population controls. Then, in nationwide Danish hospital registers, we showed that AKs occurred more frequently in patients with AD compared with psoriasis. The risk of having AKs was similar in patients with IV and AD.

Interpretation

Homozygous, but not heterozygous, *FLG* mutation carriers had an increased occurrence of AKs albeit only few homozygous mutation carriers were identified. One possible explanation is that complete, but not partial, filaggrin deficiency may allow excessive transcutaneous penetration of carcinogenic UVB.[34] The absence of tUCA in the stratum corneum of individuals with homozygous *FLG* mutations could explain the observed increase in AK risk. The prevalence of heterozygous mutation carriers in fact was lower in the AK sample when compared to the general population cohort, arguing against a biological correlation between reduced epidermal filaggrin levels and risk of AK. Although some studies have shown differences in skin barrier functions (such as trans-epidermal water loss and skin pH) between heterozygous *FLG* mutation carriers and wild types, others have demonstrated similar skin barrier functions in these two genotypes, in possible support of our findings.[35–37] Interestingly, a recent Danish questionnaire-based study found a dose-dependent positive association between *FLG* mutations and self-reported AK in patients with dermatitis.[28]

While it is likely that individuals with homozygous mutation status are more susceptible to chronic solar irradiation due to a deficiency in tUCA, we cannot rule out that the difference could instead be explained by the generous use of phototherapy to treat inflamed skin, prescription of glucocorticoids, immunosuppressants and/or an altered sun-seeking behaviour in the large subgroup with concomitant AD. However, the previously observed, positive association between homozygous *FLG* mutations and SCC supports a true association with the presence of AK,[8] as at least 60% of SCCs arise from pre-existing AKs[38] and as both SCCs and AKs are associated with cumulative UVB exposure. Collectively, these data suggest that homozygous *FLG* mutation carriers could have an increased occurrence of AKs, but this study could not determine with certainty whether the increase is due to inherited epidermal filaggrin deficiency, prior UVB exposure, pharmacological therapy, detection bias and/or other unrecognized factors.

In a nationwide hospital cohort, we found a positive association between AD and AK, although a recent study from the Netherlands did not find an association.[27] Despite the belief that genetic differences are expected to be negligible, the two study populations indeed differed. Hence, our data were based on AD patients, followed in a Danish hospital, while the Dutch study was general population-based. Moreover, our patients with AD were more likely to have been examined for AKs by dermatologists, and they apparently suffered from more chronic and severe AD. These factors are important as chronic skin inflammation in AD as well as use of topical corticosteroids may lead to (further) reductions in filaggrin,[12] and impaired cell-mediated immunity could further interfere with immunosurveillance of malignant and premalignant cells.[2] Furthermore, *FLG* mutations are associated with a severe course of AD characterized by an early onset, persistent disease and atopic comorbidity.[39,40] These patients often require treatment with potent topical glucocorticoids or immunosuppressants, which again could interfere with the immunosurveillance.[39] Moreover, oral glucocorticoids, cyclosporine and azathioprine can increase the risk of skin malignancies.[15–17]

Strengths and limitations

The filaggrin genotyped data in the first part of the study were not likely biased by confounding factors, as we investigated the distribution of genetic mutations and the samples were randomly selected. However, cases of AK may have occurred in individuals in the general population controls and thereby polluting the control population. This would have biased the results towards the null, and it is likely that the precise *FLG* mutation-AK association may be more pronounced than reported in our study. A diagnosis of AK is normally based on clinical grounds alone but in doubtful cases, in particular when suspecting SCC, a punch biopsy is taken. Therefore, the AK cases in the filaggrin genotyped cohort were sampled from biopsies and may represent a clinically more severe subtype of AK.

The high accuracy of the Danish registers utilized in the second part of the study allows for large-scale, nationwide analyses in which selection bias and recall bias are minimized. However, due to the observational design of our study, we could not establish a firm causative link to filaggrin deficiency, and it remains possible that our results could be explained in part by shared environmental or lifestyle factors. Decreased expression of *FLG* has been observed in psoriatic skin as well, possibly diluting the effect of epidermal filaggrin levels in our study.[41] We did not have access to information regarding level of sun exposure, tendencies to use sun-protective measures, skin colour and ethnicity, all of which could act as confounders in the current study. However, the Danish population is predominantly of Caucasian descent, and therefore, extrapolation of results to patients of other ethnicities must be carried out with caution. While surveillance bias is a strong concern when examining the co-occurrence of two dermatological diseases, we addressed this issue by adjustment for the number of dermatology clinic visits, and used patients with psoriasis as a control population. Moreover, we did not have access to information on total number of AKs per individual or their anatomical localization. Lastly, the cohort of IV patients might have been underpowered to detect a statistically significant difference and as the total number of AK cases was low, the results should be interpreted with caution.

Conclusion

We showed an association between homozygous, but not heterozygous, *FLG* mutations and AK in a filaggrin genotyped cohort. We failed to replicate a higher occurrence of AKs in hospital-diagnosed patients with IV compared to patients with AD. However, hospital-diagnosed patients with AD had a higher occurrence of AK, compared with hospital-diagnosed patients with psoriasis. While complete epidermal filaggrin deficiency could influence the susceptibility of UV-induced cell damage, it is impossible to firmly conclude on causality based upon these observational data as other plausible explanations exist and the results may be biased. Our findings are primarily hypothesis generating, and additional studies are needed to further explore this possible link.

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Supporting information

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

Table S1 Allele counts for each *FLG* mutation in the filaggrin genotyped data.