

Loss-of-Function Mutations in the *Filaggrin* Gene and Allergic Contact Sensitization to Nickel

Natalija Novak^{1,2}, Hansjörg Baurecht³, Torsten Schäfer⁴, Elke Rodriguez⁵, Stefan Wagenpfeil³, Norman Klopp², Joachim Heinrich², Heidrun Behrendt⁵, Johannes Ring⁶, Erich Wichmann², Thomas Illig² and Stephan Weidinger^{5,6}

Allergic contact dermatitis is one of the most frequent dermatological problems affecting 7% of the general population. Impaired skin barrier function facilitates the penetration of contact allergens and irritants into the epidermal layer and is regarded as an important cofactor promoting the process of allergic contact sensitization. Filaggrin is crucial for the maintenance of the skin barrier function. Loss-of-function mutations within the *filaggrin* (*FLG*) gene are associated with skin barrier diseases such as ichthyosis vulgaris and atopic eczema (AE). To assess the impact of *FLG* on allergic contact sensitization and plausible intermediate traits, the two prevalent *FLG* mutations R501X and 2282del4 were typed in 1,502 individuals of the KORA C population-based cohort with extensive dermatologic phenotyping. Associations of *FLG* mutations with AE could be replicated. Strong associations were seen with dry skin, palmar hyperlinearity, and keratosis pilaris. In addition, an association with contact sensitization to nickel and contact sensitization to nickel combined with intolerance to fashion jewelry, but not with other contact allergens, was observed. From these data, we conclude that a genetically determined *FLG* deficiency manifests as dry skin and features of ichthyosis vulgaris. In addition, *FLG* deficiency may also represent a risk factor for contact sensitization to allergens.

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INTRODUCTION

Recently, null mutations in the filaggrin (*FLG*) gene were identified as the cause of ichthyosis vulgaris (Smith *et al.*, 2006), one of the most common disorders of keratinization. In the meantime, *FLG* mutations have also been established as strong genetic risk factors for atopic eczema (AE) (Barker *et al.*, 2006; Marenholz *et al.*, 2006; Morar *et al.*, 2007; Palmer *et al.*, 2006; Ruether *et al.*, 2006; Sandilands *et al.*, 2006; Stemmler *et al.*, 2006; Weidinger *et al.*, 2006a,b). Ichthyosis vulgaris and AE are characterized by dry and scaly skin and show many overlapping characteristics as well as association with each other (Okulicz and Schwartz, 2003). A hallmark of both diseases is an impaired skin barrier with profound changes in skin physiology such as increased

epidermal water loss, shift in pH, low hydration, and changes in molecular composition of the stratum corneum (Cork *et al.*, 2006; Proksch *et al.*, 2006).

The main function of filaggrin is to aggregate keratin filaments leading to keratinocyte compaction and formation of the stratum corneum (Candi *et al.*, 2005). It is thereby crucial for engineering and maintaining the barrier function of the skin. Two *FLG* mutations, R501X and 2282del4, have been shown to be prevalent in the white European population (Sandilands *et al.*, 2007), and it has been suggested that reduction or loss of filaggrin expression leads to a disturbed barrier formation, which manifests as varying degrees of dry skin, ichthyosis vulgaris, and/or eczema (McLean and Hull, 2007). In addition, it might allow an increased passage of antigens, allergens, and chemicals through the epidermis and thereby facilitate allergic sensitization. This would explain additional associations observed for asthma, sensitization, and increased IgE levels in the context of AE (Irvine and McLean, 2006). However, the functional consequences and exact phenotypic characteristics caused by *FLG* mutations are not completely understood, and the hypothesis of a “dry or defective barrier” in *FLG* mutation carriers remains to be proven (Irvine, 2007).

Allergic contact dermatitis (ACD) is one of the most frequent dermatological problems affecting about 7% of the general population (Nielsen *et al.*, 2001; Heine *et al.*, 2004). The current disease model proposes that it develops based on the interactions between genetic and environmental factors. Some of these environmental factors have been defined as the

¹Department of Dermatology and Allergy, University of Bonn, Bonn, Germany; ²Department of Epidemiology, GSF-National Research Center for Environment and Health, Neuherberg, Germany; ³Institute for Medical Statistics and Epidemiology IMSE, Technical University Munich (TUM), Munich, Germany; ⁴Institute of Social Medicine, Medical University Schleswig-Holstein, Campus Lübeck, Germany; ⁵Division of Environmental Dermatology and Allergy, GSF-National Research Center for Environment and Health and ZAUM-Center for Allergy and Environment, Technical University Munich, Munich, Germany and ⁶Department of Dermatology and Allergy Biederstein, Technical University Munich (TUM), Munich, Germany

Correspondence: Dr Natalija Novak, Department of Dermatology and Allergy, University of Bonn, Sigmund-Freud-Str 25, Bonn 53105, Germany. E-mail: Natalija.Novak@ukb.uni-bonn.de

Abbreviations: ACD, allergic contact dermatitis; AE, atopic eczema; FLG, filaggrin

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nature and concentration of hapten, time and frequency of the allergen exposure, and the status of skin damage (Bryld *et al.*, 2004). As haptens have to cross the horny layer of the skin to interact with cutaneous dendritic cells, which are capable of presenting the haptenized peptides to T cells, skin barrier disruption plays a central role in the pathophysiology of contact dermatitis (Proksch *et al.*, 2006).

Here we aim to investigate the potential role of *FLG* mutations in allergic contact sensitization and to work out phenotypic characteristics of *FLG* mutation carriers using a large population-based sample of adults with extensive dermatological phenotyping, including patch testing of 25 standard allergens.

RESULTS

In this study, both *FLG* mutations previously identified to result in a deficiency of filaggrin expression were genotyped in 1,502 German adults. The clinical characteristics of the study population are outlined in Table 1. The genotyping success rate was 99.1% for R501X and 98.7% for 2282del4. Observed genotype frequencies of both polymorphisms did not deviate significantly from expected frequencies under

the Hardy-Weinberg equilibrium assumption. Genotypes were distributed equally between male and female individuals.

In this cross-sectional German population, the 2282del4 variant was present in 5.7% of adults and the frequency of carriers of R501X was 2.2%, resulting in a combined carrier frequency of 7.5% (Table 2).

Strong *FLG* effect on AE and stigmata of atopic constitution independently from AE

The known association between *FLG* mutations and AE could be replicated in the KORA C population. We estimated an odds ratio (OR) of 6.78 (95% confidence interval (95% CI) 2.76–16.64) for the combined genotype. No independent associations were seen with total IgE levels and for type 1 sensitization despite association with sensitization to cat allergen ($P=0.031$) and a trend for association with sensitization to mite allergen ($P=0.060$). In patients suffering from any atopic disease (that is, allergic rhinoconjunctivitis AE or asthma), linear regression on log-transformed IgE values showed a strong association ($P=0.009$).

FLG-null alleles showed strong associations with palmar hyperlinearity (R501X, $P=3.4 \times 10^{-4}$; 2282del4, $P=4.3 \times 10^{-14}$; combined genotype (R501X and 2282del4), $P=7.2 \times 10^{-15}$), dry skin (2282del4, $P=1.8 \times 10^{-5}$; combined genotype, $P=10^{-4}$), and keratosis pilaris (2282del4, $P=0.0018$; combined genotype, $P=10^{-4}$). No associations were seen with Dennie-Morgan infraorbital fold or Herthoge's sign.

Association of R501X and 2282del4 with contact allergy to nickel

Data on patch tests and contact dermatitis in the KORA C cohort have been published previously (Schafer *et al.*, 2001). Briefly, a total of 1,141 valid patch tests were analyzed and 40.0% of the subjects exhibited at least one positive reaction (Table 1). For association analysis with *FLG* mutations, a total of 1,133 individuals with genotype data and valid patch tests were analyzed. No associations were seen with self-reported adverse skin reactions to fashion jewelry, fragrances, or occupational allergens. Analysis of the traits contact sensitization against at least one allergen and contact sensitization against one or more allergens did not yield significant associations. In contrast, we observed a trend toward an association with actual patch test reactions to nickel and a significant association with actual patch test reactions to

Table 1. Population characteristics of KORA C (n=1,502)

	KORA C (n=1,502)	
	Data available	
Male gender	1,499	738 (49.1%)
Mean age ($\pm$ Std)	1,502	47.2 (13.2)
Mean total IgE ($\pm$ Std)	1,480	137.6 (262.7)
Total IgE > 100	1,480	498 (33.7%)
Type 1 sensitization	1,502	747 (49.7%)
Atopic eczema	1,501	56 (3.7%)
<i>Intermediate traits</i>		
Palmar hyperlinearity	1,358	109 (8.0%)
Dry skin	1,495	246 (16.5%)
Keratosis pilaris	1,485	178 (11.9%)
Dennie-Morgan fold	1,347	323 (24.0%)
Herthoge's sign	1,405	284 (20.2%)
<i>Contact sensitization and contact allergy</i>		
Valid patch test results	1,160	1,141 (98.4%)
≥ 1 contact sensitization	1,141	448 (39.3%)
≥ 2 contact sensitization	1,141	151 (13.2%)
Contact sensitization to nickel	1,141	149 (13.1%)
Contact sensitization to nickel and intolerance of fashion jewelry	1,141	59 (5.2%)
Contact sensitization to fragrance mix	1,141	181 (15.9%)
Contact allergy to fragrance mix	1,141	36 (3.2%)

Table 2. Frequency of filaggrin-null alleles R501X and 2282del4 in KORA C

	R501X	2282del4	Combined genotype
AA	1,455 (96.9%)	1,397 (93.0%)	1,354 (90.2%)
Aa	33 (2.2%)	85 (5.7%)	112 (7.5%)
aa	—	—	2 (0.1%)

nickel combined with reported intolerance to fashion jewelry (OR=4.04, 95% CI=1.35–12.06, $P=0.0125$) (Table 3). When the analysis was restricted to women only, the genotype effect was significantly stronger (OR=5.49; 95% CI=1.63–18.45, $P=0.006$) (Table 3).

DISCUSSION

Genetic modifications in EDC genes have been shown to be associated with a deficient skin barrier as seen in chronic eczematous skin diseases such as AE. On the basis of these findings, the question whether genetic modifications determining for dry skin and disturbed skin barrier function might also predispose to the manifestation of other chronic eczematous skin diseases needs to be addressed. In this study, we observed a slight association of the loss-of-function mutations R501X and 2282del4 in the *FLG* gene with clinical manifest sensitization against nickel(II) sulfate evaluated by patch testing, and a more pronounced association with positive patch testing for nickel in combination with a history of adverse skin reactions to jewelry. In contrast, no associations were seen with contact sensitizations to other allergens.

The impact of a particular genetic predisposition on the manifestation of allergic contact sensitizations is still controversially discussed. Results of twin studies provided some evidence for a genetic predisposition (Moss *et al.*, 1985). Furthermore, based on the observation of a strong upregulation of IL-16, which induces chemotactic responses of CD4⁺ T cells in delayed-type hypersensitivity reactions in animal models (Yoshimoto *et al.*, 2000), the association of ACD with a promoter polymorphism in the *IL16* gene has been shown in a case-control study (Reich *et al.*, 2003). In addition,

association of a polymorphism in the gene region encoding for *N*-acetyltransferase, an enzyme that catalyzes the local metabolism of the arylamine *p*-phenylenediamine, one of the most potent contact sensitizers, with ACD has been shown (Westphal *et al.*, 2000a, b; Nacak *et al.*, 2006). In this context, for a subcollective of older female patients, association of a polymorphism in the gene region encoding manganese superoxide dismutase (MnSOD), one of the most important antioxidant enzymes within the skin, with ACD has been described (Brans *et al.*, 2005; Yasui and Baba, 2006). In contrast, other studies strongly support the hypothesis that environmental influences represent the major risk factors affecting the manifestation of ACD (Bryld *et al.*, 2004). Interestingly, in our cohort, the observed association between nickel contact allergy and *FLG* null alleles was strikingly stronger when analysis was restricted to female study participants only. It seems plausible that, in female *FLG* mutation carriers, a more frequent nickel exposure contributes to the manifestation of contact allergy. In view of the results of these studies and combined with our findings, it might be speculated that the development of hyperreactivity to some contact sensitizers is promoted by a genetic predisposition, whereas other sensitizations will develop in response to environmental factors alone.

We were able to show that, independently from the presence of manifest AE, *FLG* mutations are strongly associated with dry skin, palmar hyperlinearity, and keratosis pilaris, which are all related to low hydration of the skin and specific skin composition. It would be interesting to study further which factors might determine the manifestation of atopic skin disease in patients with a genetically impaired skin barrier function. In contrast, total IgE levels were

Table 3. Results of association analysis

Phenotype	R501X				2282del4				Combined genotype			
	OR	95% CI	P-value		OR	95% CI	P-value		OR	95% CI	P-value	
Atopic eczema	6.25	1.55	25.22	0.0100	4.86	1.77	13.33	0.0021	6.78	2.76	16.64	3.0×10^{-5}
Palmar hyperlinearity	5.26	2.35	11.79	3.4×10^{-4}	8.87	5.31	14.80	4.3×10^{-14}	7.89	4.91	12.69	7.2×10^{-15}
Dry skin	0.69	0.16	3.05	NS	3.66	2.02	6.63	1.8×10^{-5}	2.95	1.70	5.11	0.0001
Keratosis pilaris	2.04	0.73	5.74	NS	2.74	1.46	5.15	0.0018	2.72	1.56	4.75	0.0004
Dennie-Morgan fold	1.45	0.46	4.56	NS	0.88	0.45	1.77	NS	0.96	0.52	1.74	NS
Herthoge's sign	0.60	0.12	3.08	NS	0.80	0.35	1.81	NS	0.66	0.31	1.42	NS
Contact sensitization to ≥ 1 contact allergen	1.14	0.45	2.90	NS	1.09	0.60	1.98	NS	1.11	0.66	1.86	NS
Contact sensitization to ≥ 2 contact allergens	0.44	0.06	3.34	NS	1.01	0.39	2.65	NS	0.88	0.37	2.11	NS
Contact sensitization to nickel	1.33	0.34	5.15	NS	2.36	1.03	5.44	0.043	1.91	0.91	4.04	NS
Contact sensitization to nickel and intolerance of fashion jewelry	1.44	0.25	8.27	NS	5.96	1.53	23.30	0.010	4.04	1.35	12.06	0.0125
Contact sensitization to nickel and intolerance to fashion jewelry (women only)	1.64	0.26	10.49	NS	7.83	1.72	35.76	0.0079	5.49	1.63	18.45	0.0060
Contact sensitization to fragrance mix	1.63	0.50	5.36	NS	0.46	0.14	1.55	NS	0.89	0.38	2.06	NS
Contact sensitization to fragrance mix and intolerance of fragrances	2.57	0.50	14.44	NS	0	0	∞	NS	0.80	0.17	3.72	NS

NS, not significant.

associated with *FLG*-null alleles in the presence of atopic diseases only.

Nickel is the most frequent cause of hypersensitivity in the general population (Hostynek, 2006). The induction and elicitation of nickel contact allergy is dependent on the amount of nickel per skin unit area present in the epidermis (Menne *et al.*, 1987; Fischer *et al.*, 2007). It might be speculated whether the disturbed skin barrier due to filaggrin deficiency enlarges the amount of nickel in the epidermis and thereby in particular predisposes to sensitizations to nickel, but not to other contact allergens tested in this study. Conversely, it has to be considered that nickel hypersensitivity might be expected to be less dependent on stratum corneum barrier integrity because the barrier is often violated by piercing. It is of note that the association of *FLG* mutations with nickel allergy described here was not related to a higher prevalence of nickel sensitizations in atopic individuals (Heine *et al.*, 2004; Spiewak, 2005) as a kind of secondary effect but occurred with the same frequency in nickel-sensitized individuals without atopy. An important question that arises from this data is whether people with barrier defects have an increased rate of allergic contact sensitivity to haptens in general.

It has to be considered that the association with nickel contact allergy, but not with sensitization to other contact allergens observed here might be due to different case numbers and therefore different study power, as the group of nickel-sensitized patients in our collective presented one of the largest subgroups. However, we suggest that a genetically determined skin barrier deficiency might confer an increased risk of developing allergic contact sensitization depending on the type of the contact allergen and sites and magnitude of exposure. Thus, a reduced level of filaggrin caused by a genetic predisposition might be more relevant in areas of constitutively lower filaggrin expression, for certain allergens and in individuals with high exposure.

However, the data presented here only give a first hint that patients suffering from filaggrin deficiency might also be at an increased risk for allergic sensitization against nickel. This hypothesis still needs further evaluation and confirmation in additional studies.

Taken together, our data on intermediate traits such as skin dryness, aid in narrowing down the phenotype associated with *FLG*-null alleles, and the preliminary data on the association of loss-of-function mutation in the *FLG* gene with specific contact sensitization shed some light on possible further consequences of filaggrin deficiency in ACD.

MATERIALS AND METHODS

Study population

The population-based study platform KORA ("Study Platform for Cooperative Health Research in the Region of Augsburg;" <http://www.gsf.de/KORAN/en>) and the KORA C Allergy Study have been described in detail previously (Schafer *et al.*, 2001; Weidinger *et al.*, 2004). Briefly, the "KORA Allergy Study" is based on a random, cross-sectional sample stratified for age and sex studied in 1994/1995 ($n=4,356$) (Table 1). This study was approved by the Ethics Committee of the Bavarian Medical Association (Bayerische

Landesärztekammer) and the Bavarian commissioner for data protection and privacy (Bayerischer Datenschutzbeauftragter) and was performed in adherence to the Declaration of Helsinki Principles. Consent was obtained from all patients. In the sera of these subjects, specific IgE antibodies to timothy grass and birch pollen, cat, *Cladosporium herbarum*, and house dust mite (*Derma-Dermatophagoides pteronyssinus*) were measured using the Pharmacia CAP-FEIA System (Pharmacia Diagnostics, Uppsala, Sweden). Results for specific IgE antibodies were considered positive if they exceeded the detection limit of 0.35 kU/l. For KORA C, we aimed to recruit an enriched sample of 1,600 subjects out of the 4,178 individuals who had valid IgE results. Subjects were selected, stratified by age and sex, so as to provide 50% with and 50% without specific IgE to at least one of the allergens tested, and, furthermore, so that within these groups 50% had reported symptoms of atopic diseases such as allergic rhinoconjunctivitis, asthma, or AE. Finally, 1,537 subjects participated, with DNA samples available from 1,502 subjects. All subjects received a full dermatologic examination by physicians of the Department of Dermatology and Allergy, Technical University Munich. In addition, all subjects received a conventional lancet skin prick test and total as well as specific IgE measurements (enzyme immunoassay: CAP-FEIA; Pharmacia Diagnostics) using a more detailed sample of common allergens (grass, birch, rye, mugwort pollen, *Alternaria*, *Cladosporium*, cat, dog, and *Dermatophagoides pteronyssinus*). Patch tests using a standard panel of 25 allergens (Hermal Kurt Hermann GmbH & Co, Reinbeck, Germany) were performed as recommended by the International Contact Dermatitis Research Group (ICDRG) and described by Schafer *et al.* (2001) with reading after 72 hours. Subjects were asked whether they had experienced adverse skin reactions to certain products, and, if so, whether they could link these to specific things such as fashion jewelry or fragrances (results are shown in the Table S1).

Phenotype definitions

Actual cases of AE were diagnosed using the UK diagnostic criteria (Williams *et al.*, 1994). Subjects were classified as suffering from asthma or allergic rhinoconjunctivitis if they reported a physician's diagnosis of asthma or allergic rhinoconjunctivitis. Specific sensitization was defined to be present if at least one of the specific IgE antibodies was positive (CAP RAST class ≥ 1 , corresponding to ≥ 0.35 kU/l). In addition, specific and total IgE values were analyzed quantitatively.

Features of ichthyosis vulgaris and intermediate traits such as dry skin, hyperlinearity of palms and soles, keratosis pilaris, Dennie-Morgan infraorbital fold, and Herthoge's sign were identified on a clinical basis, graded using a 4-point scale (0, none; 1, mild; 2, moderate; 3, severe) and considered to be present if graded 2–3.

Valid patch tests were available from 1,141 individuals. Only definitive positive (+); (++) and (+++) and negative (0) results were considered, whereas irritative and questionable (+/?) results were excluded. Association analysis with the following traits was performed: (a) monosensitized (contact sensitization to 1 of the contact allergens tested, including nickel), (b) polysensitized (contact sensitization to >1 contact allergens), and (c) sensitized against nickel-(II) sulfate. In addition, "contact sensitization to nickel and intolerance of fashion jewelry" was defined as history of intolerance of fashion jewelry with actual patch reactions to nickel

and “contact allergy to fragrances” as history of intolerance of fragrances and actual reactions to fragrance mix.

Genotyping

Genotyping for R501X and 2282del4 was performed using the MassARRAY system (Sequenom, San Diego, CA) as described previously (Weidinger *et al.*, 2006a). Briefly, genomic DNA was amplified by PCR using HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). The genotyping assay was carried out by using 5 ng of genomic DNA. PCR primers were used at 167 nM final concentrations for a PCR volume of 6 µl. The PCR condition was 95 °C for 15 minutes for hot start, followed by denaturing at 95 °C for 30 seconds, annealing at 56 °C for 30 seconds, extension at 72 °C for 1 minute for 45 cycles, and finally incubation at 72 °C for 10 minutes. PCR products were first treated with shrimp alkaline phosphatase (Amersham, Freiburg, Germany) for 20 minutes at 37 °C to remove excess dNTPs and afterward for 10 minutes at 85 °C to inactivate shrimp alkaline phosphatase. Thermo Sequenase (Amersham) was used for the base extension reactions. Extension primers were used at a final concentration of 5.4 µM in 10 µl reactions (Table 1). The base extension reaction condition was 94 °C for 2 minutes, followed by 94 °C for 5 seconds, 52 °C for 5 seconds, and 72 °C for 5 seconds for 40 cycles. All reactions (PCR amplification, base extension) were carried out in a Tetrad PCR thermal cycler (MJ Research, Waltham, MA). The final base extension products were treated with SpectroCLEAN resin (Sequenom) to remove salts in the reaction buffer. This step was carried out with a Multimek 96-channel autopipette (Beckman Coulter, Fullerton, CA), and 16 µl of resin–water suspension was added into each base extension reaction, making the total volume 26 µl. After a quick centrifugation (2,000 r.p.m., 3 minutes) in an Eppendorf Centrifuge 5810, 10 nl of reaction solution was dispensed onto a 384 format SpectroCHIP (Sequenom) prespotted with a matrix of 3-hydroxypicolinic acid by using a SpectroPoint nanodispenser (Sequenom). A modified Bruker Biflex matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Sequenom) was used for data acquisition from the SpectroCHIP. Genotyping calls were made in real time with MASSARRAY RT software (Sequenom).

Statistical analysis

Patch test results have been kept blinded from the genotype results. Descriptive statistics for quantitative and qualitative values are given by mean ± SD and relative frequencies or absolute numbers, respectively. Deviation from the Hardy–Weinberg equilibrium was tested with PROC ALLELE in SAS/Genetics for both *FLG*-null mutations. To test for associations with binary traits, logistic regression models were applied with age, sex, and genotype as independent variables. In addition, associations with IgE levels were evaluated using a linear regression model on log-transformed IgE values with the same covariates. Backward variable selection was applied for both regression analysis. Results are given for the estimates of genotype effects after variable selection. The *P*-values are two-sided and subject to a significance level of 0.05. ORs are given with 95% CIs and two-sided *P*-values in parentheses. For all analyses, SAS statistical software package version 9.1 was used.

As shown previously (Smith *et al.*, 2006; Sandilands *et al.*, 2007), the mutations are in *trans*- and biochemically equivalent. Therefore, in addition, a combined genotype can be analyzed, where AA

designates homozygous wild type for *FLG*-null variant, Aa heterozygous for either of the common *FLG*-null alleles R501X or 2282del4, and aa homozygous or compound heterozygous for the *FLG*-null alleles R501X or 2282del4. Thus, “combined genotype” analyzed in this study means that both prevalent *FLG* mutations are taken into account.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Frequency of positive patch test results in 1,141 adults by allergen and gender (Schafer *et al.*, 2001); * in petrolatum if not given otherwise.

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