Rapid publication

Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations

Stephan Weidinger, MD,[a,b] Thomas Illig, PhD,[c] Hansjörg Baurecht, PhD,[d] Alan D. Irvine, MD,[e] Elke Rodriguez, PhD,[b] Amalia Diaz-Lacava, PhD,[f] Norman Klopp, PhD,[c] Stefan Wagenpfeil, PhD,[d] Yiwei Zhao, MD,[g] Haihui Liao, MD,[g] Simon P. Lee, PhD,[h] Colin N. A. Palmer, PhD,[h] Claudia Jenneck, MD,[i] Laura Maintz, MD,[i] Tobias Hagemann, MD,[i] Heidrun Behrendt, MD,[b] Johannes Ring, MD, PhD,[a,b] Markus M. Nothen, MD,[j] W. H. Irwin McLean, DSc, PhD,[g] and Natalija Novak, MD[c,h] *Munich, Neuherberg, and Bonn, Germany, Dublin, Ireland, and Dundee, United Kingdom*

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease with a strong genetic background. One of the characteristic features of AD and causative factor for the disease is an impaired epidermal skin barrier based on a primary defect of epidermal differentiation.

Objectives: Recently, 2 loss-of-function mutations (R501X and 2282derl4) in the filaggrin gene *(FLG)* that cause ichthyosis vulgaris, one of the most common inherited skin disorders of keratinization, have been reported to be strong predisposing factors for AD.

Methods: We evaluated the association of the loss-of-function mutations R501X and 2282del4 within the *FLG* gene in a large collection of 476 well-characterized white German families with AD by using the transmission-disequilibrium test.

Results: Our family-based approach revealed prominent associations between the 2 loss-of-function *FLG* mutations and AD, as previously observed in a traditional Mendelian linkage analysis and case-control cohort analysis approach. In addition, we observed associations of the *FLG* mutations in particular with the extrinsic subtype of AD, which is characterized by high total serum IgE levels and concomitant allergic sensitizations. Furthermore, *FLG* mutations are significantly associated with palmar hyperlinearity in patients with AD, which represents a shared feature of AD and ichthyosis vulgaris.

Conclusion: Together these data implicate that *FLG* is the first really strong genetic factor identified in a common complex disease.

Clinical implications: These findings underline the crucial role of the skin barrier in preventing allergic sensitization.

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Key words: *Atopic dermatitis, skin barrier, epidermal differentiation complex, polymorphisms, filaggrin*

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease caused by complex interactions between multiple susceptibility genes and environmental factors.[1] Its high familial occurrence and a concordance rate of about 80% among monozygotic twins and 20% among dizygotic twins[2,3] indicate a substantial genetic component. However, the inheritance pattern of atopic diseases, such as asthma, rhinitis, or AD, is complex and does not follow an obvious Mendelian mode.[4] Moreover, the dissection of multifactorial genetic traits like AD is hampered by considerable phenotypic heterogeneity and incomplete penetrance.[5] Nevertheless, in the past decade, significant progress has been made in the field of AD genetics with the identification of numerous loci and candidate genes linked and associated with AD.[6-8] Unfortunately, a high percentage of genetic studies on AD have

From [a]the Department of Dermatology and Allergy Biederstein, [b]the Division of Environmental Dermatology and Allergy, GSF-National Research Center for Environment and Health and ZAUM-Center for Allergy and Environment, and [d]the Institute for Medical Statistics and Epidemiology IMSE, Technical University Munich; [c]the Department of Epidemiology, GSF-National Research Center for Environment and Health, Neuherberg; [e]the Department of Pediatric Dermatology, Our Lady's Hospital for Sick Children, Crumlin, Dublin; [f]the Institute for Medical Biometry, Informatics, and Epidemiology, [i]the Department of Dermatology, and [j]the Department of Genomics, Life and Brain Center, University of Bonn; and [g]the Epithelial Genetics Group, Human Genetics Unit, Division of Pathology and Neuroscience, and [h]the Population Pharmacogenetics Group, Biomedical Research Centre, University of Dundee.

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Reprint requests: Stephan Weidinger, MD, Department of Dermatology and Allergy Biederstein, Technical University Munich, Biedersteiner Str. 29, 80802 Munich, Germany. E-mail: weidinger@lrz.tum.de.

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Abbreviations used
AD: Atopic dermatitis
FLG: Filaggrin gene

reported conflicting results, and only very few genetic associations have been successfully replicated in other cohorts. However, replication is fundamental for deciding that an observed association is not due to chance.[9]

In part, this irreproducibility can be attributed to inaccurate phenotyping. Only a clear-cut definition of the phenotype allows the subanalysis of different subtypes of AD, such as extrinsic or intrinsic forms, mild and severe variants, and AD in infancy versus chronic persistent courses or courses with a late-type onset in adulthood, which might all be influenced by different genetic modifications.

While in the past years research on the genetics of AD has focused on candidate genes with effects on innate and adaptive immunity, recently attention has been drawn to the epithelial barrier, and it has been suggested that in AD the epidermal barrier might be impaired by a primary defect of epidermal differentiation.[10] Naturally, the epidermal skin barrier represents an effective protective shield that maintains, to some degree, resistance to environmental agents, such as allergens, microbes, or various irritants. A compromised skin barrier with an enhanced transepidermal water loss and reduced hydration of the skin is a characteristic feature of patients with AD. Further on, a higher pH in the epidermis of patients with AD is suspected to affect the activity of enzymes in the lamellar lipid matrix of the stratum corneum, which are involved in ceramide synthesis and epidermal differentiation.[11]

Supporting this view of a crucial role of the skin barrier in AD, genome-wide linkage analysis has shown a significant linkage signal with AD on chromosome 1q21.[7] This region contains the human epidermal differentiation complex.[12] The epidermal differentiation complex harbors several genes characterized by common structural features, such as a central region of short tandem peptide repeats, that encode proteins involved in the formation of the cornified cell envelope, such as small proline-rich region proteins, S100A proteins, and late envelope proteins, many of which are overexpressed in the skin of patients with AD.[10,13] During the epidermal differentiation process, there is increasing expression of keratin proteins, which form the intermediate filament cytoskeleton on epithelial cells. Similarly, expression of desmosomal proteins increases as keratinocytes differentiate upward and outward in the epidermis, firmly anchoring the keratin network to cell membranes. Filaggrin, which consolidates the keratin filaments into dense bundles at the granular layer–stratum corneum boundary, represents an integral part of the epidermis and is crucial for the development of the cornified envelope to engineer and maintain the barrier function of the uppermost layer of the skin.[14] Very recently, it has been reported that 2 loss-of-function mutations of the filaggrin gene *(FLG)* associated with ichthyosis vulgaris[15] are strong predisposing factors for AD.[16] These *FLG*-null mutations, R501X and 2282del4, led to deficiency of filaggrin peptides in the upper part of the epidermis in homozygote and compound heterozygote patients. In addition to the fact that ichthyosis vulgaris, which is one of the most common inherited skin disorders of keratinization, is frequently associated with AD, patients with AD share some clinical features with patients with ichthyosis vulgaris, such as dry scaly skin, keratosis pilaris, and palmar hyperlinearity, which, because of their relevance in AD, have been incorporated into the basic diagnostic criteria for AD.[17] The observed association of *FLG* mutations with AD support the view that these mutations contribute to the impaired skin barrier in patients with AD and therefore represent an important factor determining the severity and course of AD. To confirm these recent results, we aimed to replicate these associations in a large collection of 476 white German families with AD and to identify subtypes of AD in which these mutations might be of major relevance.

METHODS

Study population

For family-based association analysis, we used 476 complete white German parent-offspring trios for AD who had been recruited in Munich and Bonn, Germany. All subjects had completed a standardized questionnaire, which, next to demographic data, included the basic allergy questions of the European Community Respiratory Health Survey.[18] Both total and specific IgE antibodies against common environmental allergens (grass, birch pollen, cat, and house dust mite) were measured with the help of an enzyme immunoassay (CAP-FEIA, Pharmacia, Uppsala, Sweden or Immulite and DPC Biermann, Bad Nauheim, Germany). Specific sensitization was defined to be present if at least one of the specific IgE antibodies was positive (CAP RAST class $\geq$1, corresponding to $\geq$0.35 kU/L). Subjects were classified as having asthma or allergic rhinoconjunctivitis when they reported a physician's diagnosis of asthma or rhinoconjunctivitis, and AD was diagnosed on the basis of a skin examination by experienced dermatologists using the UK diagnostic criteria for AD.[19] In addition, stigmata of atopic constitution were evaluated.[20] Extrinsic AD was defined as AD with concomitant sensitization and IgE levels of 150 kU/L or greater.[21] IgE levels were considered increased at values of greater than 100 kU/L. All study methods followed the Declaration of Helsinki protocols and were approved by the ethics committee of the ''Bayerische Landesärtzekammer'' Munich and the ethics committee of the University of Bonn. Written informed consent was obtained from all participants before the beginning of the study.

Genotyping

Genomic DNA was extracted from leukocytes with a commercial DNA isolation kit (Gentra Systems, Minneapolis, Minn), according to the manufacturer's recommendation. Genotyping for R501X was performed with the MassARRAY system (Sequenom, San Diego, Calif), as described recently.[22] Briefly, genomic DNA was amplified by means of PCR with 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

TABLE I. PCR primers used for genotyping of *FLG* variants

SNP ID	Direction	PCR primer	Extension primer
R501X	Forward	ACGTTGGATGCTGGAGGAAGACAAGGATCG	ATGCCTGGAGCTGTCTC
	Reverse	ACGTTGGATGATGGTGTCCTGACCCTCTTG	
2282del4	DEL4.F2	TCCCGCCACCAG CTCC	
	DEL4.F1	6-FAM-GTGGCTCTGCTGATGGTGA	

Genotyping for R501X was performed with the MassARRAY system (Sequenom, San Diego, Calif), and 2282del4 mutation was typed by using fluorescent PCR, as described previously.[14,21]

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 first were treated with shrimp alkaline phosphatase (Amersham, Freiburg, Germany) for 20 minutes at 37°C to remove excess deoxyribonucleoside triphosphates and afterward for 10 minutes at 85°C to inactivate shrimp alkaline phosphatase. Thermo Sequenase (Amersham, Freiburg, Germany) was used for the base extension reactions. Extension primers were used at a final concentration of 5.4 μM in 10-μL reactions (Table I).[14,21] 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 and base extension) were carried out in a Tetrad PCR thermal cycler (MJ Research, Waltham, Mass). The final base extension products were treated with SpectroCLEAN resin (Sequenom, San Diego, Calif) to remove salts in the reaction buffer. This step was carried out with a Multimek 96-channel autopipette (Beckman Coulter, Fullerton, Calif), and 16 μL of resin-water suspension was added to each base extension reaction, making the total volume 26 μL. After a quick centrifugation (2000 rpm for 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 acquisitions from the SpectroCHIP. Genotyping calls were made in real time with MASSARRAY RT software (Sequenom).

The 2282del4 mutation was typed by sizing a fluorescently labeled PCR fragment on an Applied Biosystems 3100 or 3730 DNA sequencer, as described previously.[15,22]

Ten-microliter PCR reactions were carried out with primers DEL4.F2 and DEL4.R1 in AmpliTaq Gold buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems), 10 nmol of each deoxyribonucleoside triphosphate, and 1 unit of AmpliTaq Gold DNA polymerase. Reactions were amplified as follows: 94°C (12 minutes) for 1 cycle; 94°C (15 seconds), 58°C (30 seconds), and 72°C (45 seconds) for 30 cycles; and 72°C (5 minutes) for 1 cycle. Fragments were diluted 1:60 and sized against ROX-500 size markers, according to the manufacturer's recommended protocol (Applied Biosystems). The wild-type allele was 199 bp, and the 2282del4 allele was 195 bp. All primers are shown in Table I.

Statistical analysis

Descriptive statistics for quantitative and qualitative values were calculated with SAS version 9.1 (SAS Institute, Cary, NC) and are given as means ± SD and relative frequencies or absolute numbers, respectively. Hardy-Weinberg equilibrium was tested by using the χ^2 goodness-of-fit test. Evidence of associations with the binary traits AD, asthma, total serum IgE level (cutoff median), and allergic sensitization was evaluated by using the classical transmission-disequilibrium test implemented in Haploview 3.2. Odds ratios measuring how much more often the variant is transmitted and the wild-type allele is nontransmitted as opposed to the variant being nontransmitted and the wild-type allele being transmitted were calculated by using SAS version 9.1. An additional case-control analysis for parents with AD versus parents without AD from the study trios was calculated by using multiple logistic regression analyses with AD as dependent and sex and mutant allele (yes/no) for either polymorphism as independent variables.

Any *P* values given are 2-sided, subject to a global significance level of 5%, and not adjusted for multiple comparisons. However, most association results remained significant after applying the Bonferroni correction for multiple testing (Bonferroni-corrected significance level: 0.05/21 = 0.0024). Power analyses for transmission-disequilibrium tests concerning AD were calculated with nQuery Advisor version 5.0 by using the McNemar test of equality of paired proportions with a 5% two-sided significance level and the following values as observed in the family data: (1) difference in proportions of carrier allele transmitted and nontransmitted (7%) and (2) proportion of discordant transmissions (ie, the number of transmissions of carrier allele and nontransmission of the wild-type allele or vice versa) related to the total number of parents in the family dataset (16%).

TABLE II. Phenotype characteristics of all individuals (parents and affected offspring) included in analyses

	Trios (n = 476)	
	Parents	Offspring
Male sex (%)	476 (50.0)	196 (41.1)
Mean ± SD age, y	54.71 ± 10.27	22.12 ± 10.76
Median IgE, kU/L	42.0	253.0
AD (%)	90 (9.6)*	476 (100)
Extrinsic AD (%)	59 (6.4)*	397 (83.4)*
Intrinsic AD (%)	15 (1.6)*	79 (16.6)*
Early-onset AD, <2 y (%)	–	285 (62.0)*
Asthma (%)	94 (11.3)*	146 (31.6)*
Rhinitis (%)	278 (33.3)*	269 (58.2)*
Sensitization (%)	278 (33.3)*	352 (79.8)*
Palmar hyperlinearity (%)	ND	113 (47.7)*

ND, Not done.
*Relative proportions are calculated without considering missing values.

RESULTS

The clinical characteristics of the study population are outlined in Table II. The average genotyping success rate for both polymorphisms was 97.8%. Analysis of Mendelian inheritance within the families revealed no major errors. The 2282del4 variant was present in 15.76% of offspring, and the frequency of carriers of R501X was 8.8%, with a combined carrier frequency of 22.75%

TABLE III. Frequency of *FLG*-null alleles in parents and offspring

	R501X		2282del4		Combined genotype	
Genotype	**Parents**	**Children**	**Parents**	**Children**	**Parents**	**Children**
AA	877 (94.30%)	424 (91.18%)	848 (89.08%)	401 (84.24%)	781 (83.98%)	360 (77.25%)
Aa	53 (5.70%)	41 (8.82%)	103 (10.82%)	72 (15.13%)	145 (15.59%)	96 (20.60%)
Aa	–	–	1 (0.11%)	3 (0.63%)	4 (0.43%)	10 (2.15%)

AA, Wild-type/wild-type *FLG* genotype for R501X and 2282del4 variants; *Aa*, heterozygous genotype for either R501X or 2282del4; *aa*, homozygous R501X or 2282del4 genotype or compound heterozygous genotype.

TABLE IV. Association analysis of *FLG*-null alleles in the trio cohort

	R501X		2282del4		Combined genotype Aa	
Trait	**T:U**	***P* value**	**T:U**	***P* value**	**T:U**	***P* value**
AD	42:11	2.2×10^{-5}	73:30	2.2×10^{-5}	101:37	5.1×10^{-8}
Allergic sensitization	36:05	1.3×10^{-6}	56:25	6.1×10^{-4}	79:26	2.3×10^{-7}
Total IgE level	30:03	2.6×10^{-6}	53:20	.0001	69:19	9.8×10^{-8}
Asthma	16:03	.003*	26:09	.004*	34:10	.0003
Extrinsic AD	40:07	1.5×10^{-6}	62:27	.0002	88:30	9.3×10^{-8}
Intrinsic AD	04:02	NS	11:03	.03*	13:07	NS
Palmar hyperlinearity	16:01	3.0×10^{-4}	24:05	.0004	35:06	5.9×10^{-6}

Combined genotype Aa, Heterozygous genotype for either R501X or 2282del4; *T:U*, transmitted:untransmitted; *NS*, not significant.
*Not significant after Bonferroni correction for multiple testing.

(Table III). In our cohort we did not observe any homozygotes for R501X, but we did observe 3 homozygotes for 2282del4 and 10 compound heterozygotes. Two of the individuals heterozygous for 2282del4 and 6 of the individuals heterozygous for both polymorphisms exhibited asthma in addition to AD, and all compound heterozygotes were characterized by increased total IgE levels and allergic sensitization.

Results of association analysis are summarized in Table IV. Both *FLG* gene variants were significantly overtransmitted to AD-affected offsprings. The strongest association was seen for the combined Aa genotype (presence of either R501X or 2282del4 variant, $P = 5.1 \times 10^{-8}$), pointing to a dominant risk for AD conferred by both alleles. Corresponding odds ratios were 3.64, 2.43, and 2.73 for R501X, 2282del4, and the combined genotype, respectively. Power calculations showed that with our cohort of 476 families, a significance level of .05, and a rate of discordant pairs of 0.156, a difference in proportions of 0.042 for the combined genotype can be detected with a statistical power of 90%. Additional analysis of affected versus unaffected parents confirmed a strong association of both mutations with AD ($P < .0001$ for combined genotype, Table V).

Although a number of phenotypic and genetic studies suggested parent-of-origin effects, in particular maternal effects in AD,[23] we did not observe such effects for R501X and 2282del4 by using a previously described analysis method.[24]

Both *FLG* variants also showed significant association with total serum IgE levels ($P = 9.3 \times 10^{-8}$ for combined genotype), with geometric means for serum IgE of 177.7 kU/L in wild-type/wild-type versus 298.9 kU/L in carriers

TABLE V. Case-control analysis of parents with AD versus parents without AD from the study trios, with logistic regression analyses controlled for sex showing the increased risk of the mutant allele for AD

SNP	**Variable**	***P* value**	**OR (95% CI)**
2282del4	Mutant allele	.0013	2.5 (1.4-4.3)
R501X	Mutant allele	<.0001	4.1 (2.2-7.9)
Combined genotype	Mutant allele	<.0001	3.3 (2.1-5.4)

SNP, Single nucleotide polymorphism; *OR*, odds ratio.

of either allele, and with allergic sensitization ($P = 2.3 \times 10^{-7}$ for combined genotype). Further on, consistent with the observation of Palmer et al[16] that these *FLG*-null alleles are important risk factors for asthma in association with AD, both variants showed significant association with asthma as a secondary trait ($P = .0003$ for combined genotype). With 270 (274 and 288, respectively) families with offspring also with asthma successfully genotyped for the combined genotype (R501X and 2282del4, respectively), the observed statistical power to detect a difference in transmission and nontransmission proportions of the carrier allele of 9% (4% and 6%, respectively; as observed in our data) when the proportion of discordant transmission is 16% (7% and 12%, respectively; as observed in our data) was 97% (79% and 84%, respectively).

Along with these observations, a highly significant transmission distortion was seen for the extrinsic variant of AD ($P = 9.3 \times 10^{-8}$ for combined genotype). In contrast, no association was seen with intrinsic AD. In our cohort of families with AD, these *FLG* variants were revealed to be also associated with the presence of palmar

hyperlinearity in patients with AD ($P = 5.4 \times 10^{-6}$ for combined genotype), which is considered a strong sign for atopic constitution.[20] No correlation between the presence of *FLG*-null mutations and an early onset of AD was observed.

DISCUSSION

Genetic factors appear to be important in the multifactorial pathogenesis of AD, and in recent years there have been many reports of ''significant'' associations.[4] However, most initial findings were not replicated by subsequent association studies, which is of utmost importance to demonstrate a true relationship between a genetic marker and a disease.[25] Notably, the associations between the 2 loss-of-function *FLG* mutations and AD observed by Palmer et al,[16] using traditional Mendelian linkage analysis and case-control cohort analysis approaches, are revealed to be as prominent in our family-based analysis. These observations were strengthened by an additional analysis of unaffected versus affected parents, which confirmed the association of the *FLG* mutations with AD. Thus our results add value by reinforcing the role of *FLG* mutations in AD, which are the first really strong genetic factors identified in this common complex disease. In essence these mutations act in a low-penetrance Mendelian fashion, showing codominant transmission in families.

In addition, our results further support the hypothesis that an impaired skin barrier is an important prerequisite for subsequent allergic sensitization and avails the manifestation of additional allergic respiratory diseases in patients with AD. Functional clues for a crucial role of the skin barrier in the development of sensitizations have already been provided in the past with the help of tape-stripping experiments in mice, which showed that epicutaneous sensitization through barrier-disrupted skin enhances T_H2 cytokine expression within the skin.[26-28] In line with these findings are observations in children with AD who were exposed to topical emollients containing peanut protein. These children showed a significantly increased risk of peanut sensitization,[29] indicating a close relation between the integrity of the skin barrier and the development of skin-mediated sensitizations.

It might be speculated whether disturbance of the epidermal barrier caused by *FLG* variants facilitates allergic sensitization and predisposes to the manifestation of respiratory atopic disease, which classifies the extrinsic variant of AD. The fact that we observed strong associations with allergic sensitization, increased total IgE levels, and extrinsic AD, but not with the intrinsic variant of AD, points to a stringent correlation between FLG status and allergic sensitization. Some of our individuals with intrinsic AD did carry the *FLG* mutations; however, the overall number of intrinsic AD cases was rather low. Further investigations in additional cohorts are necessary to confirm these intriguing preliminary observations.

Although the epidermal barrier matures rapidly after birth, percutaneous absorption of topically applied substances is higher in early infancy.[30] Combined with a markedly higher surface area/weight ratio in infants, a genetically determined dysfunction of the epidermal skin barrier was expected to be of higher relevance in children with a manifestation of AD, in particular during the first 2 years of life. However, the presence of *FLG*-null mutations was not correlated with an early onset of AD in our study cohort.

As mentioned above, the 2 *FLG*-null mutations investigated in this study have been identified originally as a cause of ichthyosis vulgaris, a keratinization disorder that exhibits strong association with AD.[31] In our cohort of families with AD, these *FLG* variants were revealed to be also associated with the presence of palmar hyperlinearity in patients with AD ($P = 5.4 \times 10^{-6}$ for combined genotype), which is characteristic for ichthyosis vulgaris but also considered a strong sign for atopic constitution,[20] thereby providing a further genetic link between these 2 diseases.

Taken together, in view of the data presented here, a genetically determined disruption of the epidermal skin barrier should be regarded as a key event in the pathogenesis of AD and as a considerable risk factor for the development of subsequent sensitizations and respiratory diseases in a subgroup of patients, which might pave the way for a severe and long-lasting atopic career.[32]

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