

Combined occurrence of filaggrin mutations and *IL-10* or *IL-13* polymorphisms predisposes to atopic dermatitis

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Although filaggrin mutations are presently believed to play a key role in AD development, it is also obvious that immunological factors involved in acquired immune response are important for allergic inflammation development. The study reveals association between *FLG* mutations, *IL-10* and *IL-13* polymorphisms and AD development. The obtained results highlight the role of interactions between innate and adaptive immune responses in pathogenesis of the disease.

Abstract:

Background: Although filaggrin mutations are presently believed to play a key role in the development of atopic dermatitis (AD), obviously also immunological factors involved in acquired immune response are important for the development of allergic inflammation.

Objective: To assess the frequency of *FLG* mutations and the polymorphisms 590 C/T in the *IL-4* gene, -1082A/G in the *IL-10* gene and -1055C/T in the *IL-13* gene in patients with AD and their correlations between severity of AD and asthma.

Methods: R501X and 2282del4 *FLG* mutations and *IL-4*, *IL-10* and *IL-13* polymorphisms were assayed in 163 patients with AD of Polish origin.

Results: In the Polish patients with AD, the prevalence of *FLG* mutations was higher in patients with AD than in the controls

and 2282del4 *FLG* mutation was more frequent than R501X, and it was associated with a 6-fold higher risk for AD development ($P < 0.001$; OR: 5.76), moderate or severe disease course, early onset of asthma and palmar hyperlinearity. Significant interactions between the 2282del4 *FLG* mutation and the CT genotype for *IL-13* or GG genotype for *IL-10* and a higher risk for developing AD were demonstrated.

Conclusion: *FLG* mutation, alone and in combination with certain *IL-10* or *IL-13* polymorphisms, enhances the risk for the development of AD in the Polish population.

Key words: asthma – atopic dermatitis – filaggrin mutations – *IL-10* polymorphism – *IL-13* polymorphism

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Introduction

Atopic dermatitis (AD) is a recurrent, inflammatory skin disease (1). Multiple genes are involved in the genetic background of AD, roles of which are variably established in different populations (2).

Despite these observations, genetic defects unequivocally responsible for AD development are not known. Based on the performed studies, associations between AD and genes located on chromosome 5q31-33 encoding Th2-derived cytokines were observed (2). Their real role in AD pathogenesis was explained by the fact that the presence of certain polymorphisms results in enhanced synthesis of IgE. Although it was proven that some polymorphisms in genes encoding Th2-derived cytokines occur more frequently in patients with AD, their impact on the clinical course of the disease, type of skin lesions or response to treatment has not been established (1,2).

The studies on the role of polymorphisms in genes encoding *IL-4* and *IL-10* in AD pathogenesis were conducted in many populations. Although some studies showed positive correlations, others declined these relationships, stressing the difficulties in the

interpretation of the obtained results and necessity for further studies (2–5).

Previous investigations also concerned the role of polymorphisms in the *IL-13* gene in AD development. It was found that polymorphic variants in the promoter region of *IL-13* are linked with higher transcription activity of the gene that leads to enhanced synthesis of IL-13 cytokine and development of allergic and inflammatory disorders (1). Analysing the -1055C/T *IL-13* polymorphism, the association between the occurrence of a T allele and increased expression of IL-13, high IgE serum levels, asthma, atopy and eczema was noted (6).

A significant breakthrough in understanding of AD pathogenesis was the result of studies on the role of filaggrin gene (*FLG*) mutations (R501X and 2282del4) in the development of the disease (7). The authors (7,8) unambiguously showed the link between these two mutations and the presence of AD and its specific clinical phenotype, especially dry skin, keratosis pilaris and palmar hyperlinearity. These observations prove an important role of impaired epidermal barrier function, not only in

pathogenesis of AD but also in determining its clinical picture. Based on these observations, it has been recently postulated that immunological disturbances and allergy development are secondary phenomena to genetically determined defects of the epidermal barrier (9).

The studies performed in Scottish, German and Dutch populations showed not only the links between *FLG* mutations and AD, but also their connections with extrinsic AD types and high IgE serum levels (10,11). Other studies revealed a link between these genetic defects and asthma development, however, only in the subjects with AD (12). The frequency of *FLG* mutations and their link with AD were also assessed in other European populations, among others in Irish and British (8,10,13–16). Studies were also conducted in non-European populations, and analogue to R501X and 2282del4, i.e. 3321delA and S2554X, mutations was identified (17). To our knowledge, there have been no similar studies performed in Eastern European population of patients with AD by now and our study is the first one.

Although *FLG* mutations are presently believed to play a key role in AD development, it is obvious that immunological factors especially involved in acquired immune response also play an important role in allergic inflammation. Thus, studying links on the genetic level between these two pathogenetic pathways in AD, we assessed this hypothesis and determined the frequencies of *FLG* mutations and *IL-4*, *IL-10*, *IL-13* genes polymorphisms in the Polish population and ascertained whether the combined occurrence of these genetic disturbances influences AD development and determines its clinical severity. The obtained results demonstrate for the first time genetic interactions between impaired function of epidermal barrier and acquired immunity.

Table 1. Dermographics of patients with AD

Feature	Controls (N = 204)		Patients with AD (N = 163)	
	N	%	N	%
Men	92	45.1	66	40.5
Women	112	54.9	97	59.5
AD severity				
Mild	NA	NA	78	48.4
Moderate	NA	NA	66	41.0
Severe	NA	NA	17	10.6
Asthma	NA	NA	44	26.9
Allergic rhinitis	NA	NA	79	48.5
Allergic conjunctivitis	NA	NA	32	19.6

NA, not applicable.

Methods

The study included 163 (97 women, 66 men) patients with AD in a mean age of 11.3 years old (age range 1–42 y.o.) and 204 healthy volunteers (112 women, 92 men), age matched. The subjects from the control group had no allergic disease or medical history as well as no familial allergies. All subjects were exclusively of Eastern European/Polish descent. All individuals gave written informed consent before entry into the study. The experimental plan was approved by the Local Ethics Committee of the Medical University of Lodz and was conducted according to the Declaration of Helsinki principles.

Diagnosis of AD was established by two independent and experienced dermatologists based on clinical picture, according to criteria proposed by Hanifin and Rajka (18). The severity of AD assessed by Rajka and Langeland scale (19) was defined as mild, moderate and severe. Detailed clinical characteristics of the patients with AD are presented in Table 1.

In all the patients and volunteers, the presence of the following polymorphisms -590C/T *IL-4*, -1082A/G *IL-10*, -1055C/T *IL-13* and frequency of R501X and 2282del4 mutations in *FLG* gene were assessed.

Genotyping for -590C/T *IL-4*, -1082A/G *IL-10*, -1055C/T *IL-13* polymorphisms

Genomic DNA was prepared from peripheral blood leucocytes using a genomic DNA isolation kit (DNA-Gdansk, II SC, Poland). Analysis of polymorphic variants in the promoter regions of the *IL-4*, *IL-10* and *IL-13* genes was performed by PCR and RFLP using the appropriate restriction enzyme (digestion for 2 h) (20,21). Primer sequences of the used oligonucleotides and restriction enzymes are shown in Table 2. PCR was carried out in a total volume of 25 µl containing 40 ng of genomic DNA, 10 pmol of each primer (Eurogentec, Seraing, Belgium), 200 µmol of dNTPs, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂ and 1 U of Taq polymerase (QIAGEN GmbH, Hilden, Germany). PCR conditions were as follows: 4 min 95°C (denaturation), 35 cycles: 30 s 94°C, annealing step 45 s at 58°C for *IL-4*, 61.8°C for *IL-10*, 55°C for *IL-13* and 40 s 72°C, final extension at 72°C for 7 min. The PCR amplifications were performed in a MultiGene TC9600, (Labnet International Inc, Edison, NJ, USA). The amplification products were treated with restriction enzymes. The digested PCR products were analysed by electrophoresis on 10% polyacrylamide gels stained with ethidium bromide.

Identification of R501X and 2282del4 mutations in *FLG*

Identification of R501X and 2282del4 mutations in *FLG* was performed by PCR and subsequent restriction analysis described by Smith et al. (22).

Table 2. Sequences of oligonucleotides and restriction enzymes used for identification of *IL-4* -590 C/T, *IL-10* -1082 A/G, *IL-13* -1055 C/T polymorphisms

Polymorphism	Oligonucleotides sequences	PCR products	Restriction enzyme	Restriction fragments
<i>IL-4</i> -590 C/T	5'-TGGGGAAGATAGAGTAATA-3' 5'-TAAACTTGGGAGAACATGGT-3'	195 bp	<i>Ava</i> II	Allele C (177 bp and 18 bp), allele T (195 bp)
<i>IL-10</i> -1082 A/G	5'-TCTTACCTATCCCTACTTC-3' 5'-CTCGCTGCAACCCAAGTGGC-3'	139 bp	<i>Mn</i> I	Allele A (139 bp), allele G (106 bp and 33 bp)
<i>IL-13</i> -1055 C/T	5'-ACTTCTGGGAGTCAGAGCCA-3' 5'-TACAGCCATGTCGCCCTTT CCTGCTCTCCGTC-3'	377 bp	<i>Hpy</i> 99 I	Allele C (340 bp and 37 bp), Allele T (377 bp)

Underlined and bold text indicated mismatched nucleotides, which were introduced in order to obtain the restriction site.

Table 3. The association between *IL-10* and *IL-13* polymorphisms and atopic dermatitis development

		Control group		AD group		<i>P</i>	Odds ratio	-95%CI	+95%CI
		<i>N</i>	%	<i>N</i>	%				
<i>IL-10</i>	GG	48	23.5	34	20.9	0.816	1	(Reference)	
	GA	89	43.6	75	46.0	0.525	1.190	0.696	2.034
	AA	67	32.8	54	33.1	0.655	1.138	0.645	2.006
<i>IL-13</i>	CC	145	71.1	98	60.1	0.076	1	(Reference)	
	CT	56	27.5	60	36.8	0.043	1.585	1.016	2.475
	TT	3	1.5	5	3.1	0.224	2.466	0.576	10.556

Statistical analysis

Chi-square tests were used to detect a significant deviation in genotype frequency from the Hardy–Weinberg equilibrium. The associations between different dichotomic variables and independent variables were assessed by logistic regression analysis. Possible interactions between independent variables (*FLG* mutations and cytokines' polymorphisms) were also tested in the model of logistic regression analysis.

The odds ratio (OR) and 95% confidence intervals (CI) were calculated with the use of the logistic regression model. STATISTICA 6.0 software (Statsoft, Tulsa, OK, USA) was employed to perform analyses. *P* values lower than 0.05 were considered as statistically significant.

Results

Genotyping for -590 C/T *IL-4*, -1082 A/G *IL-10* and -1055C/T *IL-13* polymorphisms.

Distribution of the genotypes for -1082 A/G *IL-10* and -1055C/T *IL-13* polymorphisms was consistent with a Hardy–Weinberg equilibrium in AD and the control group, while for -590 C/T *IL-4* this was not consistent. The genotype distribution for the analysed *IL-10* polymorphism did not differ between AD and the control group (Table 3). The CC genotype for the -1055 C/T *IL-13* polymorphism was observed in 98 patients with AD (60.1%) and in 145 control subjects (71.1%) (*P* = 0.076). The frequency of the CT genotype for the *IL-13* polymorphism was higher in patients with AD (36.8%) than in controls (27.5%) and was linked with a slightly increased risk for AD development (OR = 1.585) (Table 3).

No significant association between the examined *IL-4*, *IL-10* and *IL-13* polymorphisms and severity of AD, clinical symptoms or early asthma onset was found (*P* > 0.05 for all comparisons).

Frequency of R501X and 2282del4 mutations in *FLG*

FLG R501X and 2282del4 mutations were analysed in 163 patients with AD and 204 healthy controls. In the AD group, we observed five heterozygotes (3.1%) for the R501X mutation, whereas in the control group 4 heterozygotes (2%) were present (*P* = 0.499) (Table S1). Heterozygotes for the 2282del4 mutation were observed in nine controls (3.1%) and in 29 (17.9%) patients with AD, while five patients with AD were homozygous (*P* < 0.001). Combined allele frequency for the two *FLG* mutations was 19.1% of patients with AD, compared to 6.4% of healthy subjects (*P* < 0.001). The occurrence of the 2282del4 mutation increases the risk for AD development almost sixfold (OR = 5.76) and the combination of the two variants – 4- fold (OR = 4.2) (Table S1).

The association between *FLG* mutations and AD severity, its clinical symptoms and asthma

Analysing the association between AD severity and the occurrence of *FLG* mutations, we found that carriers with the 2282del4 mutation are at higher risk (OR = 2.34) of developing a moderate or severe type of AD (*P* = 0.034) (Table S2).

In the studied AD cohort, we found that either the presence of the 2282del4 mutation (OR = 11.34) or the combination of two *FLG* mutations (OR = 10.54) is associated with the early onset of asthma, i.e. below 3 y.o. (*P* = 0.02 for two comparisons). No association between the presence of any *FLG* mutation and early (<2 y.o) AD onset was found (*P* > 0.05).

In patients with the 2282del4 *FLG* mutation, we observed almost an 11-fold higher risk for palmar hyperlinearity occurrence (OR = 10.62; *P* < 0.05) whereas such an association was not found for R501X (*P* > 0.05).

Combined effect of the R501X and 2282del4 filaggrin mutations and -590 C/T *IL-4*, -1082 A/G *IL-10* and -1055C/T *IL-13* polymorphisms

Analysing the risk for AD development, we found a significant interaction between the 2282del4 *FLG* mutation and the analysed polymorphisms for *IL-10* and *IL-13*. Homozygotes or heterozygotes *FLG* mutation carriers who also carry the CT genotype for *IL-13* have higher risk for AD development (OR = 6.381) than patients with the same *FLG* mutation but with a different *IL-13* genotype (Table S3). We also found a significant relationship between the 2282del4 mutation and the *IL-10* polymorphism, i.e. homozygotes or heterozygotes *FLG* mutation carriers with a GG genotype for the *IL-10* polymorphism have higher risk of developing moderate to severe AD (OR = 10.833) than patients with this *FLG* mutation but a different -1082 A/G *IL-10* genotype (Table S3). We stratified our population according to the *FLG* 2282del mutation in the model of logistic regression analysis. Interactions are clearly visible, as in the stratified cohort the *P* values for *IL-13* and *IL-10* signals become more significant (Table S3).

We found no analogue correlations between the R501X *FLG* mutation and any of the studied cytokines' polymorphisms.

Discussion

Genetic studies in patients with AD are focused on identifying predisposing factors for disease development. As AD was much considered as an immunological disease, sequence variations in proinflammatory cytokines like *IL-4*, *IL-10* and *IL-13* were given a great deal of attention. Association of *IL-10* polymorphism with AD was not found (2,3) although Sohn et al. (4) did show a link between polymorphisms in the promoter region of *IL-10* and AD in children. Also Lacy et al. (5) did show the association between the TGAC *IL-10* haplotype and high IgE serum levels in patients with AD. However, in our study, no correlation between the analysed *IL-10* polymorphism and AD development was found.

Previous studies demonstrated that occurrence of polymorphic variants in the *IL-13* gene is linked to higher transcription activity, enhanced *IL-13* synthesis and development of inflammatory diseases (1). The results obtained by us also indicate a weak association between the -1055C/T *IL-13* polymorphism and AD development in the Polish population. The CT genotype was shown as a predisposing factor for AD. Moreover, we found that in carriers with the C allele the risk for AD development is slightly

lower. These observations are in line with Glen et al. (23). Two consistent studies conducted in Polish AD cohorts indicate that the -1055C/T *IL-13* polymorphism is important in AD development. Similar results were also found by other authors (2).

Currently, AD pathogenesis is considered to be a result of complex interactions between genetic, immunological and environmental factors (24). Mutations in *FLG* proved to be the foundation for an impaired barrier function and AD development (22,25,26). However, McGrath and Uitto (27) point out that there are other molecular factors involved in AD pathogenesis. In the study by Stemmler et al. (16), combined occurrence of R501X and 2282del4 mutations was found in 5.1% of healthy subjects and 8.7% of patients with AD. In our study, combined occurrence of the two mutations was 6.4% in healthy subjects. Of note, most mutations involved the 2282del4 *FLG* mutation in which 3.1% of our patients with AD were homozygous and 17.9% heterozygous. Because R501X is infrequent in Polish patients with AD, we found no association with this mutation. Therefore, the genetic background of the disease in the Polish population may be distinct compared to other European populations (7,10).

Stemmler et al. (16) showed an association between *FLG* mutations and early onset of AD, while such a correlation was not proven by Weidinger et al. (10) which is in line with our results. Our study showed the connection between palmar hyperlinearity and the 2282del4 mutation. Lack of association with ichthyosis vulgaris and *FLG* mutations in our group probably results from the clinical characteristics of the examined patients. Ichthyosis vulgaris was present only in two cases; however, they were the individuals with homozygous 2282del4 mutations and palmar hyperlinearity.

R501X and 2282del4 *FLG* mutations are the risk factors for severe AD and asthma development (7,10,14,28–30). The presence of the 2282del4 mutation in our patients increased the risk over 2-fold for moderate or severe AD. Moreover, we found significant associations between the 2282del4 *FLG* mutation and early onset of asthma (<3 years of age).

Although recently published data indicate a clear pathogenetic link between *FLG* mutations and AD, such a relationship is observed only in approximately 30% of patients with AD (10,14). Howell et al. (31) showed that in active skin lesions of *FLG* mutation carriers, one can observe lower levels of filaggrin expression compared to the uninvolved skin. This observation indicates that a heterozygous *FLG* mutation is not enough to cause a complete inhibition of protein synthesis. Only bi-allelic null mutations are responsible for the lack of filaggrin expression in the skin (31). Hence, this suggests that other factors determine filaggrin expression and proper function of the epidermal barrier. In addition, it was shown that in skin biopsies a decreased expression of filaggrin was accompanied by simultaneously increased expression of IL-4 and IL-13 (31). An

impaired epidermal barrier may be partially associated with the genetic makeup for the inflammatory response and influx of Th2-derived cytokines to cause disease. These observations are interesting in the context of the results obtained by us. We found that increased risk for AD development exists in the subjects who simultaneously carry a 2282del4 *FLG* mutation and certain *IL-10* or *IL-13* gene polymorphisms. The 2282del4 mutation carriers also having the heterozygous -1055C/T *IL-13* polymorphism have a 6-fold higher risk for AD development compared to the 2282del4 carriers having a different -1055 C/T *IL-13* genotype makeup. Similarly, AD 2282del4 carriers with a homozygous GG genotype for the -1082 A/G *IL-10* polymorphism have over 10-fold higher risk of developing AD than carriers having a different *IL-10* genotype. These results indicate that interactions exist between filaggrin, component of the epidermal barrier, and IL-10 and IL-13, involved in acquired immunity, mediating inflammatory response. In concordance, our further results (data not published) show that serum levels of IL-10 are significantly higher in GG genotype compared to patients with AD who have either GA or AA genotypes. We also observed statistically higher serum levels of IL-10 and IL-13 in all patients with AD than in the control group. However, the differences in IL-13 serum levels did not depend on the *IL-13* genotype. Our observations are in line with Leung (28) who suggested that an impaired epidermal barrier and a secondary inflammatory response result from mutual interactions between insufficient filaggrin expression and enhanced Th2-derived response. A similar theory is also given by O'Regan et al. (32) who highlight the role of interrelations between innate and acquired immune responses in AD development.

Concluding, although many published data unequivocally indicate the association between *FLG* mutations and AD, other factors, both immunological and environmental, also play key roles in the pathomechanism of the disease. The presence of *FLG* mutations causes impairment of the epidermal barrier and facilitates allergen penetration through the epidermis; however, participation of acquired immunity is necessary to develop allergic inflammation.

Based on the available published data and our own results, we presume that further complex studies on the mutual roles and interactions between genetic, immunological and environmental factors in AD pathogenesis are absolutely necessary and this concept is in line with the theory suggested by Leung (28).

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

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

Table S1. The association between the occurrence of *FLG* mutations and atopic dermatitis.

Table S2. The association between *FLG* mutations and severity of atopic dermatitis.

Table S3. Interactions between 2282del4 *FLG* mutation (stratifying variable) and 1055C/T *IL-13* and 1082A/G *IL-10* polymorphism in the logistic regression analysis.

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Table 4. The association between the occurrence of *FLG* mutations and atopic dermatitis.

		Control group		AD group		OR*	-95%CI	+95%CI	p
		N	%	N	%				
R501X	AA	200	98.0	158	96.9	1.58	0.42	5,99	0,499
	Aa	4	2,0	5	3.1				
	aa	0	0.0	0	0.0				
2282del	AA	195	95.6	129	79.0	5.76	2.67	12,40	<0,001
	Aa	9	4.4	29	17.9				
	aa	0	0.0	5	3.1				
combined	AA	191	93,6	127	77,8	4.20	2.14	8,23	<0,001
	Aa	13	6,4	31	19,1				
	aa	0	0,0	5	3,1				

*OR was calculated by the AA versus combined Aa+aa genotypes.

Table 5. The association between *FLG* mutations and severity of atopic dermatitis.

		mild AD		moderate and severe AD		OR*	-95%CI	+95%CI	p
		N	%	N	%				
R501X	AA	74	94.9	82	98.8	0.226	0.025	2.064	0.187
	Aa	4	5.1	1	1.2				
	aa	0	0	0	0				
2282del	AA	67	85.9	60	72.0	2.374	1.068	5.280	0.034
	Aa	8	10.3	21	25.6				
	aa	3	3.8	2	2.4				
combined	AA	65	83.3	60	72.0	1.887	0.877	4.059	0.104
	Aa	10	12.8	21	25.6				
	aa	3	3.8	2	2.4				

*OR was calculated by the AA versus combined Aa+aa genotypes.

Table 6. Interactions between 2282del4 FLG mutation (stratifying variable) and 1055C/T IL-13 and 1082A/G IL-10 polymorphism in the logistic regression analysis.

2282del(Aa and aa) by	P	OR*	-95%CI	+95%CI
IL-13 (CC)	0.016	1	(reference)	
IL-13 (CT)	0.004	6.381	1.801	22.607
IL-13 (TT)	-	-	-	-
IL-10 (AA)	0.008	1	(reference)	
IL-10 (GG)	0.002	10,833	2,401	48,882
IL-10 (GA)	0.866	1,204	0,140	10,323

-: OR can not be calculated due to lack of observations

*OR was calculated by the AD vs control group for IL-13 or by the moderate/severe AD vs mild AD for IL-10