

Association of *Filaggrin* loss-of-function-mutations with atopic dermatitis and asthma in the Early Treatment of the Atopic Child (ETAC) population

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Many candidate gene studies for atopic dermatitis (AD) and associated phenotypes have been conducted so far, but replication of significant results has been a major problem. Two loss of function polymorphisms *FLG* *R501X*- and *2282del4*, in the *Filaggrin* (*FLG*) gene encoding for an epidermal barrier protein were recently identified. They were reported to be predisposing factors for AD and concomitant asthma. Several groups confirmed the initial results in independent populations. The aim of this study is to further investigate the importance of these *FLG* variants in the development of AD and subsequent asthma symptoms in pre-school children, we investigated children and parents of the Early Treatment of the Atopic Child (ETAC)-trial. We genotyped 496 children and 488 parents of the ETAC population for the two *FLG* variants, evaluating an association by family based analysis (transmission disequilibrium test). We found a highly significant association of the *FLG* null variants *R501X*- and *2282del4* with AD (combined genotype $p < 0.0001$) and asthma (combined genotype $p < 0.0001$). The replication and its statistical significance underlines the importance of the *FLG* polymorphisms and the importance of the skin barrier function in the development of AD and subsequent asthma.

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Allergic diseases are among the most frequent chronic diseases in childhood in industrialized countries. Typically atopic manifestations follow a certain sequence, called the atopic march, beginning with atopic dermatitis (AD) in early infancy, followed by food allergy, asthma and the development of allergic rhinitis (1). This pattern suggests a common aetiology for different atopic diseases (2).

A major risk factor for the development of allergic disease is a positive family history, demonstrating the importance of genetic factors in the disease aetiology. While most publications focused on candidate genes with effects on innate

and adaptive immunity, recent publications disclosed the effect of candidate genes promoting epicutaneous sensitization (3). A report by Palmer et al. (4) showed that loss-of-function mutations in the *Filaggrin* gene (*FLG*) play an important role in the development of AD and asthma.

Filaggrin plays an essential role in the formation of the skin barrier, by consolidating the keratin filaments into dense bundles, thus being crucial for development of the cornified cell envelope, which maintains the barrier function of the uppermost layer of the skin (5). Smith et al. (6) could show that loss or reduction of this

structural protein leads to varying degrees of impaired cornification.

The *FLG* gene is located on the human chromosome 1q21, within the epidermal differentiation complex, a complex composed of more than 30 genes all involved in terminal differentiation of the epidermis (7). Genome-wide linkage analysis has shown a significant linkage of childhood AD to this region (8).

In the present study, we evaluated an association between the *FLG* *R501X* and *2282del4* variants and AD in a large Caucasian population, the Early Treatment of the Atopic Child (ETAC) population. Additionally, we analysed the association of *FLG* variants with asthma in pre-school children.

Methods

Study population

The ETAC population has been described previously in detail (9). Briefly, 795 infants, diagnosed with eczema during their first 2 yr of life were enrolled in a randomized, double-blind, placebo controlled trial on the efficacy of Cetirizine treatment in the prevention of asthma. Infants between 1 and 2 yr of age from 12 European countries and Canada were enrolled. All children had a positive family history of atopy. The medication trial lasted for 18 months, the study population was followed up for another 18 months. Three hundred and ninety-eight children received Cetirizine twice a day for the period of 18 months, 397 received a placebo. Blood samples for the determination of total and specific IgE against grass pollen, cow's milk, egg, house dust mites and cat, were obtained at four time points.

Phenotype definition

The diagnosis of eczema was made according to standard criteria in the presence of a chronic or chronically relapsing pruritic dermatitis with the typical morphology and distribution [Hanifin and Rajka (10)]. Asthma was defined as three episodes separated by at least 7 days, either of nocturnal cough with sleep disturbance lasting for at least three consecutive nights, three episodes of wheezing or a combination of the two symptoms.

Genotyping

For DNA analysis blood of 496 children and 488 parents, having remained throughout the

whole investigation period in the trial, was accessible. Genomic DNA was extracted from peripheral blood leukocytes according to standard protocols. All individuals were genotyped for the loss of function variants *R501X* and *2282del4* in the *Filaggrin* gene as described previously (11).

Statistical analysis

The sib transmission disequilibrium test (TDT) of the ASPEX software was used for statistical analysis.

Results

During the 36 months of follow up 69% of the children developed asthma. All genotype frequencies were found to be consistent with Hardy-Weinberg equilibrium. The TDT was performed in children and parents to test for associations with the *2282del4*- and *R501X*-variants.

Allele frequencies for the *2282del4*-variant were 5.2%, for the *R501X*-variant were 3.1%, either variant (combined allele frequency) was found in 8.3%. The TDT analysis showed highly significant associations of the *R501X*- ($p = 0.0014$) and of the *2282del4*-variants ($p = 0.00022$), with AD, respectively. The level of significance was increased in the analysis of the combined genotype ($p < 0.0001$, Table 1).

Also, associations for the particular variants and the combined genotype with asthma showed high significance (*R501X* $p = 0.0036$; *2282del4* $p = 0.0014$; combined $p < 0.0001$). To evaluate a parent-of-origin effect in AD and asthma, we conducted the TDT analysis with regard to paternal and maternal transmission (see Table 2). A significant difference between paternal and maternal transmission was not identified.

Discussion

Recently, the *R501X* and the *2282del4* variants in the *FLG* gene have shown to be associated with AD and asthma, a finding which was unambiguously replicated in six independent study populations (4, 11–14). Using a family-based association analysis, we also demonstrate in the ETAC population highly significant associations of the two *FLG* variants with AD and asthma, confirming the previous results. The family-based study design allowed us to investigate a maternal effect which has frequently been reported in the susceptibility for allergic disease (15). We did not observe an excess of maternal transmission compared to paternal transmission. We conclude

Table 1. Association analysis of the *FLG* null alleles

Trait	<i>R501X</i>		<i>2282del4</i>		Combined genotype	
	T:U	p value	T:U	p value	T:U	p-value
Atopic dermatitis	20:4	0.0014	33:9	0.00022	51:12	<0.0001
Asthma	14:2	0.0036	22:5	0.0014	36:7	<0.0001

T, transmitted; U, untransmitted; combined genotype, *R501X* and/or *2282del4*.

Table 2. Association analysis of paternal and maternal transmission of *FLG* null variants

	Paternal		Maternal	
	T:U	p value	T:U	p-value
Atopic dermatitis				
<i>2282del4</i>	16:5	0.022	14:1	0.00013
<i>R501X</i>	12:1	0.0017	7:2	0.10
Combined genotype	27:5	0.0001	19:2	0.0002
Asthma				
<i>2282del4</i>	10:2	0.038	9:0	0.00045
<i>R501X</i>	6:0	0.016	7:1	0.038
Combined genotype	16:1	0.00015	15:1	0.00022

T, transmitted; U, untransmitted; combined genotype, *R501X* and/or *2282del4*.

that there is no marked parent-of-origin-effect of the investigated mutations on the risk of AD.

We demonstrate that *FLG* variants are already strongly associated with asthma symptoms in pre-school children aged 4–5 yr. Only children with AD but without any asthma symptoms were included in the ETAC trial, our results therefore strongly support the notion that *FLG* variants in children with AD predispose to the development of asthma. The *FLG* allele frequencies in the ETAC population (*R501X* and/or *2282del4*: 8.3%) were similar to those reported in other Caucasian populations previously (4, 11, 13, 14, 16). Higher allele frequencies were observed by Palmer et al. in a cohort ascertained in an AD clinic (combined allele frequency 33%). Also Barker et al. could find a higher combined allele frequency (27%) in an adult Caucasian population with persistent AD. These differences may be caused by different phenotype composition and a variable disease severity between the populations. A large number of associations of genetic variants with allergic disease could not be replicated in independent studies (17). The strength of association replicated by many groups including our study is therefore particularly remarkable.

Smith et al. (6) were the first to identify two *FLG* variants *R501X* and *2282del4*, both leading to a complete loss of the processed filaggrin product. In 15 families, these null alleles were

shown to be the predominant cause of ichthyosis vulgaris. The association of ichthyosis vulgaris with atopic diathesis is well established, so they proceeded to further investigations into an association between *FLG* variants and atopic disease. Palmer et al. (4) identified *FLG* variants as a predisposing factor for the development of AD, finding a highly significant association between AD and either variant. In this study, there also emerged a significant association of these variants with asthma occurring in the context of AD. Other atopy-associated phenotypes were not investigated.

Weidinger et al. (12) evaluated an association of *FLG* null alleles with AD, allergic sensitization and asthma in a German population of 476 families. The family based approach revealed strong associations between the *FLG* variants and AD in particular with extrinsic AD. However, after correcting for multiple testing they were not able to show a significant association of the tested variants with asthma.

By comparing *FLG* null variants in an adult AD population (n = 163) to an ethnically matched population (n = 1463). Barker et al. (13) found strong evidence for *FLG* null alleles being an indicator for a poor prognosis in AD, and predisposing to a persistence into adulthood.

Marenholz et al. (11) could replicate strong associations between *FLG* null alleles and eczema as well as concomitant asthma in a large European population (GENUFAD) and a large German birthcohort (MAS), both well characterized. They showed that only in the presence of eczema these mutations predisposed to asthma, allergic rhinitis and allergic sensitization. Finding that *FLG* variants predispose equally to the atopic and nonatopic forms of AD, they suggested that eczema may occur independently of allergic sensitization.

Recently, Palmer et al. (18) showed an association of common *FLG* null variants with the severity of asthma. Although they found that individuals with asthma and *FLG* null alleles were more likely to suffer also from eczema and individuals with eczema tended to have more severe asthma, the association of *FLG* null variants and disease severity of asthma was independent of the eczema status in the tested population (n = 874).

Marenholz et al. (11) have estimated that 11.1% of eczema cases in the German population are attributable to *FLG* loss of function variants. Palmer et al. (4) could not find these variants in Africans or Asians. Nomura et al. (19) published an association of two novel mutations (3321delA and S22554X) with AD in a Japanese popula-

tion, indicating that in future there might be more genetic variants identified, affecting the epidermal skin barrier, thereby leading to the development of atopic disease. Shortly after Sandilands et al. (20) fortified this hypothesis by identifying three additional rare null mutations (R2447X, S3247X, 3702delG) in an Irish population. They stated that filaggrin-related AD would consist of both prevalent and rare risk alleles.

Recently, another gene affecting the epidermal integrity and function was identified, the collagen gene (COL29A1) (21). Söderhäll et al. also showed a strong association of COL29A1 variants with AD. Investigations on an association of COL29A1 variants with the development of asthma and checking for combined effects of filaggrin null variants and COL29A1 variants will enlighten furthermore the pathophysiologic role of the epidermis in the development of AD and asthma.

We were able to replicate the previously reported association of *FLG* variants with AD and associated asthma in pre-school children in a family-based analysis with a very high level of significance. Our findings further support the role of the epidermal barrier in allergic disease.

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