INVESTIGATIVE REPORT

Analysis of SPINK 5, KLK 7 and FLG Genotypes in a French Atopic Dermatitis Cohort

Thomas HUBICHE,1,4 Cécile GED,2–4,5 Antoine BENARD,1,4 Christine LÉAUTÉ-LABRÈZE,1 Ken McELREAVEY,6 Hubert de VERNEUIL,2–4,5, Alain TAÏEB2–4,5 and Franck BORALEVI,1,4

1Service de Dermatologie, CHU Bordeaux, Hôpital St André; 2Laboratoire de Biochimie; 3Service d’Information Médicale, CHU Bordeaux, Hôpital Pellegrin; 4Université V. Segalen Bordeaux 2; 5INSERM, U876, Bordeaux, and 6EA1533 Reproduction, Fertility and Populations, Institut Pasteur, Paris, France

The role of a genetically impaired epidermal barrier as a major predisposing factor in the pathogenesis of atopic disorders is currently under closer investigation. Variants on three candidate genes (SPINK5, KLK7 and FLG) have been associated with atop dermatitis. A functional relevance has already been established for filaggrin variants, but not for SPINK5 and KLK7 polymorphisms. The objectives of this study were to confirm the association between SPINK5, KLK7, FLG variants and atopic dermatitis and to assess how variants influence selected phenotypic traits. This cross-sectional study was carried out over 20 months in 99 children and adults with atopic dermatitis (median age 7 years). The following items were analysed: SCORAD, TEWL, ichthyosis vulgaris, presence of asthma, total IgE serum levels. The SPINK5 E420K SNP, the KLK7 4bp insertion polymorphism and the filaggrin mutants (R501X and 2282del4) were analysed as described previously. The control group for genetic analysis was recruited in an ethnically matched, phenotypically anonymous cohort (n=102). The allelic frequencies were 0.525 for SPINK5, 0.26 for KLK7 polymorphisms, 0.101 and 0.075 for 2282del4 and R501X FLG mutants, respectively. The association of atopic dermatitis with filaggrin variants was confirmed, but not that of SPINK5 or KLK7 polymorphisms. SCORAD and TEWL measurements were not influenced by any of the variants. The SPINK5 polymorphism was associated with high IgE serum levels (p=0.011). Abnormal barrier genes do not influence the severity of atopic dermatitis. The SPINK5 gene polymorphism may modulate systemic immune effects favouring the IgE response to atopens. TEWL does not allow the characterization of subsets of patients with or without abnormal barrier genes. **Key words:** atopic dermatitis; filaggrin; KLK7; SPINK5; TEWL.

(Accepted April 18, 2007.)


Thomas Hubiche, Service de Dermatologie, Hôpital Saint André, 1 rue Jean Burguet, FR-33075 Bordeaux Cedex, France. E-mail: thomas.hubiche@chu-bordeaux.fr

A skin barrier defect has been proposed as a primary event in the pathogenesis of atopic disorders based primarily on clinical and allergy testing observations as well as morphological and biochemical data in infantile atopic dermatitis (AD) (1). The integrity of the stratum corneum is essential to prevent epidermal water loss, but also to limit the penetration of aeroallergens, infectious organisms or toxic chemicals (2). An underlying skin barrier defect in predisposed individuals could lead in the first months of life to epicutaneous sensitization to atopens, which, in a second step, may trigger a Th2 immune response in a subset of those individuals. Since this hypothesis has been put forward, genome-wide scans have shown several loci associated with the AD phenotype, especially in the epidermal terminal differentiation cluster on chromosome 1 (3). In parallel, the candidate gene approach has linked several polymorphisms or mutations affecting genes involved in the skin barrier structure or function to AD. SPINK5 encodes LEKTI, a protease inhibitor that is defective in Comel-Netherton syndrome. AD has been associated with a polymorphism in exon 14 (E420K) in one British and two Japanese studies (4–6). The relevance of this single nucleotide polymorphism (SNP) in AD pathogenesis is based mainly on the phenotype of Netherton’s syndrome, which associates immediate skin and mucosal allergic features, ichthyosis, AD-like features and developmental anomalies. The involvement of this polymorphism in AD itself is still debated. A polymorphism in the human kallikrein 7 gene (KLK7) characterized by a 4-bp insertion in the 3’UTR has been reported in a British AD cohort (7). KLK7 encodes the protease kallikrein 7, also named stratum corneum chymotryptic enzyme (SCCE), which is involved in the desquamation process by degrading corneodesmosomes (8). According to Vasilopoulos et al. (7), this polymorphism may result in a gain of function of the protease, leading to a premature degradation of the corneodesmosomes and subsequent defect of the skin barrier. The functional relevance of this polymorphism has, however, not yet been demonstrated.

Recently Palmer et al. (9) showed that two independent loss-of-function genetic variants (R501X and 2282del4) in the gene encoding filaggrin (FLG) are associated with AD. Filaggrin is essential in the formation of the stratum corneum barrier (2). The same mutations were first identified as causative of ichthyosis.
vulgaris (10). In several Caucasian-based cohorts, these mutations are associated both with AD, influencing both severity and early onset of the disease, and asthma in the context of AD (9, 11–16).

Our study analyses simultaneously the three different genotypes in the same AD cohort including infants, children and adults. Our primary objective was to analyse the association between gene variants and AD, and to assess simultaneously skin barrier function using trans-epidermal water loss (TEWL) assay, scoring of atopic dermatitis (SCORAD) index (severity), and Th2 skewing (total IgE serum levels). Since AD is a multifactorial disease, it can be assumed that environmental influences combined with genetic background produce the AD phenotype (17). In this context, the assessment of a potential stratum corneum gene effect influencing barrier dysfunction, clinical severity of AD or associated asthma was our secondary objective.

METHODS

This cross-sectional study was carried out in the Department of Dermatology at Bordeaux University Hospital between December 2004 and September 2006. All patients met the criteria for AD of the UK Working Party (18) and a clinical diagnosis of ichthyosis vulgaris was made by a dermatologist (TH, FB, CL, AT). An ethnically matched control group made of phenotypically anonymous subjects served as control for allele frequencies (KM).

Procedures

A complete dermatological examination was performed by a trained dermatologist and ichthyosis vulgaris features (palmar hyperlinearity, xerosis, scaling on legs) were noted. Information about familial and personal atopic history was obtained using a standardized questionnaire. Physician-diagnosed asthma was registered. Disease severity was assessed using the SCORAD index (19, 20). After written informed consent, blood sample was obtained for genetic testing and IgE measurement.

Trans-epidermal water loss measurement (TEWL)

TEWL measurements (g m⁻² h⁻¹) were performed with a vapometer (Delfin Technologies Ltd, Kuopio, Finland) following published guidelines (21). In particular, the measurements were performed at constant temperature and moisturizers were avoided in the previous 24 h. The probe was applied on the medium part of uninvolved volar forearm.

Total IgE serum levels

The total IgE serum level was determined using the Pharmacia CAP system (Pharmacia Diagnostics, Uppsala, Sweden). Total IgE serum levels higher than 150 kU l⁻¹ were classified as abnormal.

Genetic analysis

DNA was extracted using the Wizard genomic kit (Promega, Lyon, France). The genotypes were determined by restriction fragment length polymorphism (RFLP) analysis (E420K SNP of the SPINK5 gene and R501X mutation of the FLG gene) or by fluorescent fragment size analysis (4 bp insertion of the KLK7 gene and 4 bp deletion of the FLG gene) using an automated gene analyser and the Gene mapper software (Applied Biosystems 3130). The RFLP analyses were performed as described by Walley et al. (4) and Palmer et al. (9). The fragment size analysis techniques were adapted from Palmer et al. (9). The following sequences were used as amplifiers: 5'- TGG AAT TGT GAG GAT TTC ACA G -3' (forward SPINK5), and 5'- CAC CAG AAT CAT GAT CTC TG TGG ATC -3' (reverse SPINK5); 5'- CTC ACT GAC TCT TCT CCA GCA C -3' (forward KLK7); 5'- TCC GCC CAC CAG CTC C -3' (forward FLG) and 5'- TG GCC CTG CTG ATG GTG A -3' (6FAM-labelled reverse KLK7); 5'- GCC TAC GCC GCA GCA GCA C -3' (forward R501X) and 5'- GCC GAT GGG ATC GAC TCA -3' (reverse FLG R501X). Polymerase chain reaction (PCR) conditions were as follows: 100 ng genomic DNA, 2 mmol/l MgCl₂, 200 μmol/l each dNTP, 200 nmol/l each amplifier, 1X Taq Gold buffer and 1 unit Taq Gold enzyme (Perkin Elmer, France). Cycling conditions were as follows: 10 minutes at 95°C (initial denaturation) then 25 cycles (KLK7 and FLG 2282del4) or 35 cycles (SPINK5 and FLG R501X) including denaturation (30 sec at 94°C) annealing (30 sec at 58°C, 60°C or 65°C for SPINK5, FLG and KLK7, respectively), extension (30 sec at 72°C) and a final extension step (5 min at 72°C). PCR products were run on a 1.5% agarose gel prior to RFLP or fragment size analyses. Amplicon sizes were 304, 177–184, 312 and 193–197 bp for FLG, SPINK5, KLK7 and R501X, respectively. Restriction digests using NlaIII (FLG, SPINK5 and KLK7) and HphI (SPINK5) were performed on 1/10 of the PCR product during 2 h at 37°C according to the supplier’s instructions (New England Biolabs, Saint Quentin Yvelines, France) and run on a 3% agarose gel. The FLG R501X and the SPINK5 variant alleles exhibited a new restriction site for NlaIII and HphI enzymes, respectively. For fragment size assays, 6FAM-labelled PCR products (KLK7 and FLG 2282del) were diluted 1/2 to 1/40 and the Genescan Rox 350 fluorescent marker was added prior to analysis on the automated gene analyser.

Statistical analysis

The prevalence estimates are reported as proportions with their 95% confidence intervals (CI). Differences of prevalence according to different patient’s characteristics were compared using Pearson’s χ² test or Fisher’s exact test (according to group size). In order to increase the potency of statistical analyses, for each gene studied, the alleles were gathered in two groups, either wild-type or variant. The variant allele group, patients had one (heterozygous status) or two (homozygous or compound heterozygous) mutant alleles. Quantitative variables were reported as medians or means, depending on their distribution. Comparisons were made using t-test or Wilcoxon rank test. Statistical analysis used SAS software, version 8.2 (SAS Institute Inc., Cary, North Carolina, USA). A p-value <0.05 was considered statistically significant.

RESULTS

Characteristics of the cohort

Ninety-nine patients with atopic dermatitis (34 females, 65 males) were enrolled. The median age was 7 years, ranging from 2 months to 68 years old. The clinical characteristics of the patients are summarized in Table 1. The samples used as population controls belong to a phenotypically anonymous cohort of French
adult men, which has already been tested in population studies concerning atopic dermatitis (9). The frequency of AD is known to be approximately 5% in the general population (22).

Allelic frequencies of candidate genes

The allelic frequencies of the \( \text{SPINK5} \)-SNP, the \( \text{KLK7} \)-4bp insertion, R501X and 2282del4 \( \text{FLG} \) variants in the AD group and in the control group are shown in Table II. Three homozygous (22282del4/2282del4) and one compound heterozygous (2282del4/R501X) patients were identified. The allelic frequencies of \( \text{FLG} \) 2282del4 and R501X mutants were higher in the AD group than in the control group (\( p < 0.001 \) and \( p = 0.004 \), respectively). The apparent linkage disequilibrium between 2282del4 and R501X mutants needs further analysis on a larger population of mutants. No association between AD and \( \text{SPINK5} \)-SNP or \( \text{KLK7} \)-4bp insertion was found according to allele frequencies (Table II) or genotypes (data not shown).

Assessment of an additive gene effect on asthma, IgE, SCORAD and TEWL

In order to test the hypothesis of a phenotypic influence of the addition of compound gene variants/polymorphisms, we compared the frequency of associated asthma, mean SCORAD index, mean TEWL readings, respectively, with the presence of either zero, one, two or three variants of the studied genes (Table IV). No significant difference was found between the four classes of variants; however an increased prevalence of asthma was a non-significant trend in patients with compound variant genotypes.

DISCUSSION

Our data confirm the association between AD and \( \text{FLG} \) mutants, but fail to establish a correlation between AD and either \( \text{SPINK5} \)-SNP or \( \text{KLK7} \) 4bp insertion polymorphisms.

The allele frequency for \( \text{FLG} \) R501X variant was 0.075 in the AD cohort and 0.025 in the control group (\( p = 0.004 \)). The allele frequency of the 2282del4 variant was 0.101 in the patients with AD cohort and 0.010 in

| Table I. Characteristics of the patients with atopic dermatitis (AD) |
|--------------------------|-----------------|-----------------|-----------------|
| Group size               | AD group        | Children <15 years | Adults >15 years |
| Available data (n)       | 99              | 99               | 59               | 40               |
| Median age (years) (IQ)  | 99              | 7 (2.0–23.0)     | 3                | 30               |
| Sex ratio (% male)       | 99              | 65%              | 71%              | 60%              |
| Mean SCORAD (CI)         | 89              | 24 (20.8–28.0)   | 23               | 25               |
| Mean TEWL, (g·m⁻²·h⁻¹) (CI) | 60          | 12.4 (10.5–15.4) | 13.4             | 11.4             |
| Median total IgE, kU·l⁻¹ (IQ) | 72          | 565.0 (57.5–3991.0) | 138.5          | 2888             |
| Asthma prevalence, % (CI)| 51              | 41.2 (27.7–54.7) | 39               | 42               |

SCORAD: scoring atopic dermatitis; TEWL: trans-epidermal water loss; CI: 95% confidence interval; IQ: interquartile interval.

| Table II. Allelic frequencies in atopic dermatitis (AD) and population controls |
|--------------------------|-----------------|-----------------|
| AD genotypes             | Frequency of the variant allele |
|                         | AD              | Population controls | p-value | OR (95%CI) |
| \( \text{SPINK5} \)      |                 |                  |         |            |
| wt                       | E420K           | K420K            | 0.525   | 0.520<sup>a</sup> | 0.94 | 1.02 (0.95–1.11) |
| 19/99                    | 56/99           | 24/99            |         |            |
| \( \text{KLK7} \)        |                 |                  |         |            |
| wt                       | ins/wt          | ins/ins          | 0.26    | 0.22<sup>b</sup> | 0.33 | 1.26 (1.13–1.40) |
| 53/99                    | 40/99           | 6/99             |         |            |
| \( \text{FLG} \)         |                 |                  |         |            |
| wt                       | del/wt          | del/del          | 0.101   | 0.010<sup>c</sup> | <0.001 | 11.7 (5.4–25.3) |
| 82/99                    | 14/99           | 3/99             |         |            |
| wt                       | R501X           | X501X            | 0.075   | 0.025<sup>c</sup> | 0.004 | 3.2 (2.1–4.7) |
| 84/99                    | 15/99           | 0/99             |         |            |

<sup>a</sup>SPINK5 and KLK7 genotypes were determined in the same population controls previously analysed for FLG variants (9). <sup>b</sup>FLG allele frequency was extracted from (9). <sup>c</sup>CI: confidence interval; OR: odds ratio; wt: wild-type; E420K, ins/wt, del/wt, and R501X are heterozygous genotypes; K420K, ins/ins, del/del, and X501X are homozygous mutant genotypes.

Acta Derm Venereol 87
Table III. Correlations between phenotype and genotype

<table>
<thead>
<tr>
<th></th>
<th>SPINK5 genotype</th>
<th>KLK7 genotype</th>
<th>FLG genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>variant</td>
<td>wt</td>
</tr>
<tr>
<td>Median age at AD onset (months) (IQ)</td>
<td>3 (2–30)</td>
<td>6 (2–12)</td>
<td>6 (4–12)</td>
</tr>
<tr>
<td>Mean SCORAD (CI)</td>
<td>29.4</td>
<td>23.2</td>
<td>25.7</td>
</tr>
<tr>
<td>Mean TEWL (CI)</td>
<td>14.4</td>
<td>11.9</td>
<td>13.0</td>
</tr>
<tr>
<td>High IgE patients (prevalence)</td>
<td>n = 4</td>
<td>n = 47*</td>
<td>n = 28</td>
</tr>
<tr>
<td>Asthmatic patients (prevalence)</td>
<td>n = 4</td>
<td>n = 19</td>
<td>n = 11</td>
</tr>
</tbody>
</table>

Mean TEWL values are expressed as gm⁻²h⁻¹. High IgE is defined as IgE > 150 kU.l⁻¹. Prevalence is the ratio: number of high IgE (or asthmatic) patients with a given genotype/total number of patients with the same genotype.

*p = 0.011. Other p-values were non-significant.

CI: 95% confidence interval; IQ: interquartile interval; AD: atopic dermatitis; TEWL: trans-epidermal water loss; SCORAD: scoring atopic dermatitis; wt: wild-type genotype; variant: heterozygous, homozygous mutant or compound heterozygous (FLG) genotypes.

Table IV. Analysis of compound gene variants effect on selected phenotypic traits

<table>
<thead>
<tr>
<th>Number of gene variants</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean TEWL (95% CI)</td>
<td>11.8 (8.6–19.5)</td>
<td>12.7 (8.8–16.5)</td>
<td>12.9 (9.9–15.8)</td>
<td>9.5 (5.4–13.5)</td>
</tr>
<tr>
<td>Mean SCORAD (95% CI)</td>
<td>27.2 (13.9–40.5)</td>
<td>27 (21.3–32.7)</td>
<td>21.3 (16.0–26.6)</td>
<td>24.2 (13.9–34.4)</td>
</tr>
<tr>
<td>High IgE patients (prevalence)</td>
<td>n = 1 (1/5)</td>
<td>n = 22 (22/28)</td>
<td>n = 23 (23/31)</td>
<td>n = 5 (5/8)</td>
</tr>
<tr>
<td>Asthmatic patients (prevalence)</td>
<td>n = 1 (1/5)</td>
<td>n = 9 (9/22)</td>
<td>n = 10 (10/20)</td>
<td>n = 3 (3/4)</td>
</tr>
</tbody>
</table>

0 is wt genotype for the 3 genes. 1, 2 and 3 are heterozygous or homozygous for 1, 2 and 3 genes, respectively.

Prevalence is the ratio: number of high IgE (or asthmatic) patients with 0, 1, 2 or 3 gene variants/total number of patients with the same number of gene variants.

*p-values were non-significant.

TEWL: trans-epidermal water loss; SCORAD: scoring atopic dermatitis; CI: 95% confidence interval; IQ: interquartile interval.

As shown recently in larger cohorts (12, 13, 16), a tendency for a younger age at onset of AD was noted in patients with a FLG variant. This may suggest that infants with defective barrier functions are more likely to develop atopen-induced skin lesions because of early epicutaneous sensitization (25). In a German cohort, filaggrin variants have been described as associated with high total serum IgE levels (11) suggesting a link between a genetically impaired epidermal barrier and Th2 skewing. The low number of FLG mutants in our cohort decreased the power of statistical analysis and could not confirm this finding.

Our results did not confirm the association between AD and the KLK7 polymorphism (7). In a similar size cohort, these authors reported an allelic frequency of 0.56 in the AD group compared with 0.43 in the control group. This is in contrast with our findings, which show a much lower allelic frequency and no significant difference between allelic frequencies in AD and controls (0.26 and 0.22, respectively). This difference could be due to a marked genetic heterogeneity between the two populations. Nevertheless, KLK7 is located on 19q13.3 and previous genome-wide scans have not found a linkage with AD at this locus (23, 26). Furthermore, increased mRNA or KLK7 proteolytic activity in AD...
No significant association was found in our study between the number of gene variants and TEWL, disease severity, serum IgE level or onset of asthma. However, our results for asthma, showing an increase in asthma prevalence according to the number of gene variants, even though not statistically significant, suggest that it would be useful to increase the database to address this issue in more depth. *FLG* variants are a predisposing factor for the clinical subtype of asthma that occurs in the context of existing AD. Asthma prevalence was lower in the ichthyosis vulgaris group (10%) than in the AD cohorts (49%) studied by Palmer et al. (9).

This indeed suggests the involvement of other factors, including genetic influences, in the onset of asthma. The speculation on the role of *SPINK5* in the onset of asthma is based on reported associations between *SPINK5* SNP and asthma in patients with AD (4, 34). However, data on the association of *SPINK-5* SNP with isolated asthma are conflicting (34, 35), leading to consider that this genotype may only influence the onset of asthma in patients with AD (35). Thus, larger studies are needed to validate the concept that both *SPINK5* polymorphism and *FLG* variants are prognostic factors for asthma onset in children with AD.

In summary, the recent Copernican revolution, which has centred the pathophysiology of AD on skin itself, leads now to other questions (38). The patient with loss of function *FLG* mutations and who does not develop AD belongs to a subset that needs to be investigated as a priority in order to gain a better understanding of the steps beyond stratum corneum defects, which probably involve a dysregulated cross-talk between environmental irritants or pathogens and innate barrier immune/inflammatory responses. The abnormal barrier genes, such as *FLG* mutants, have a clear permissive effect on the early inflammatory steps that characterize infantile eczema. They are found at overall identical frequencies across age groups of patients with AD, suggesting that this cutaneous barrier defect has priming effects on disease expression, but affects also the chronicity of the disease. If abnormal barrier genes increase the accessibility of irritants and atopens to the innate and adaptive immune system, this effect needs to be better apprehended through methods more sensitive than the TEWL measurements obtained in this study, which failed to detect differences between carriers and non-carriers, and among carriers of one or several genetic variants. However, abnormal barrier genes do not seem, in isolation, to influence the severity of the AD phenotype. In the atopic diathesis, skin constitution is probably more important than previously thought, and skin should thus be considered as a primary objective for prevention (39). However, newer therapies, still badly needed in established disease, will have to address, in addition, other constitutional factors triggering non-remitting inflammation and mucosal allergy, which so far remain largely unknown.
ACKNOWLEDGEMENT
The authors thank Cécile Chemin for expert technical support.

REFERENCES


