

# Serine protease inhibitor lymphoepithelial Kazal type-related inhibitor tends to be decreased in atopic dermatitis

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## Abstract

**Background** A pathogenic role of serine protease inhibitor lymphoepithelial Kazal type-related inhibitor (LEKTI) in atopic dermatitis (AD) is currently in intense debate. Analyses of an association between genetic polymorphisms of *SPINK5* and atopic diseases revealed contradictory results. Herein, we assessed the role of LEKTI in AD at an expressional and functional level.

**Methods** The expression of LEKTI and its inhibitory capacity was measured by real-time polymerase chain reaction and hydrolytic activity assay, respectively, in keratinocyte cell cultures of three AD patients in comparison to cultures of healthy individuals (5×) and Netherton (NS) patients (3×).

**Results** Expression of LEKTI was significantly decreased in AD vs. healthy volunteers. Due to reduced protease inhibition, trypsin-like hydrolytic activity in AD was slightly increased, although not significantly.

**Conclusions** Even though the number of investigated subjects was small and hydrolytic activity was only slightly increased, the results denote that LEKTI might be diminished in AD. The study also disclosed the necessity of functional analyses in addition to genetic investigations to gain further and more detailed insights into the role of LEKTI in AD.

## Keywords

allergic asthma, atopic eczema, atopy, Netherton syndrome, SPINK5

## Conflicts of interest

None declared.

## Introduction

*SPINK5*, the serine protease inhibitor of Kazal type 5, is currently under intensive investigation as a candidate gene for genetic polymorphisms at the chromosome 5q31 cytokine cluster in atopic dermatitis (AD).<sup>1</sup> *SPINK5* is well known as the defective gene causing Netherton syndrome (NS).<sup>2</sup> It encodes the serine protease inhibitor lymphoepithelial Kazal type-related inhibitor (LEKTI) that is involved in epidermal barrier function and immunity. LEKTI has shown to inhibit several tryptic enzymes such as trypsin, subtilisin A, plasmin, cathepsin G, and neutrophil elastase.<sup>3</sup> In the skin, LEKTI is expressed within the most differentiated viable layers of the epidermis, in particular within the granular layer, where critical biochemical and morphological changes occur during terminal differentiation.<sup>4</sup> There, the inhibitory control of two proteases is of particular interest, namely

kallikrein 5/stratum corneum trypsin-like enzyme (KLK5/SCTE) and kallikrein 7/stratum corneum chymotrypsin-like enzyme (KLK7/SCCE). These two tryptic enzymes lead to degradation of intercellular adhesion molecules such as desmoglein 1 and desmoplakin, and can cleave profillagrin.<sup>5</sup> In NS, LEKTI is markedly diminished due to mutations in *SPINK5*, leading to a reduced protease inhibition, and subsequently to an increased protease activity that cause an abnormal desquamation due to undamped desmosomal degradation.<sup>6</sup> Since patients with NS exhibit atopic manifestations with an atopic dermatitis like rash, high serum IgE levels, and multiple food allergies, it has been speculated that LEKTI may be involved in the pathogenesis of AD.<sup>1</sup> Herein, we investigated LEKTI expression and its inhibitory activity in keratinocyte cell cultures obtained from patients with AD.

## Materials and methods

### Patients

Screening of the cell culture stocks of the Center for Allergy and Environment, Division of Environmental Dermatology and Allergy, Munich provided access to cell lines obtained from 3 patients with AD, 3 with NS and 5 healthy volunteers (normal human keratinocytes, NHK). Each individual has given informed consent initially when the biopsy was taken.

### Cell culture

Following unfreezing, keratinocytes were grown and expanded for one or two passages on feeder layer using standard keratinocyte growth medium. Before experiments were undertaken, keratinocytes were grown for one passage without feeder layer in low-calcium KSM medium containing standard supplementations (Invitrogen, Carlsbad, CA, USA). Since LEKTI is expressed at the granular layer, differentiation of cultured keratinocytes was induced by the addition of 1.2 mM calcium chloride<sub>2</sub> over a 3-day period.

### Screening for Glu420→Lys polymorphism

Total DNA was extracted from cell lines of the three atopic individuals by the use of QIAamp mini kit (Qiagen, Hilden, Germany). Exon 14 of *SPINK5* was amplified by using the same primers as published by Walley *et al.* (forward: 5'-TGCAATTGTGAGGATTTTCACAG-3'; reverse: 5'-CCTGAA CATGATCTGTGGATC-3').<sup>7</sup> The expected 304-bp fragment was isolated by gel electrophoresis and commercially sequenced (Sequiseive, Vaterstetten, Germany).

### Real-time polymerase chain reaction for LEKTI expression

Total RNA was isolated by peqGOLD RNAPure kit (peqLab, Erlangen, Germany), reverse transcribed by First Strand cDNA Synthesis Kit for real-time polymerase chain reaction (RT-PCR, AMV; Roche, Penzberg, Germany), and amplified using SYBR Green ER<sup>TM</sup> qPCR SuperMix for ABI PRISM® (Invitrogen) with primers specific for the first 268 bps of LEKTI (sense primer: 5'-CGCTAGCAACATGAAGATAG-3' and anti-sense primer: 5'-CTAGAAGCATTTCGGTGGACGATGGAGGG-3'). Values were expressed relative to levels in the same cells of  $\beta$ -actin (RT-PCR Primer and Control Set, Invitrogen) using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

### Indirect LEKTI inhibitory activity assay (hydrolytic activity assay)

The inhibitory activity of LEKTI was determined indirectly by measuring the trypsin-like hydrolytic activity in homogenates of keratinocytes according to a modified protocol by Komatsu *et al.*<sup>8</sup> In short, a synthetic peptide containing 7-amino-4-methyl-coumarin (Boc-Phe-Ser-Arg-AMC; MP Biomedicals, Santa Ana, USA) was used as substrate in a reaction mixture, containing 1 mg cell

homogenate, 20  $\mu$ L of *N,N*-dimethylformamide, 0.48 mL of 0.1% Triton X-100 (w/w), 0.35 mL of 0.2 mol per L Tris-HCl buffer (pH 8.0). The reaction mixtures were incubated at 37 °C for 2 h, and released AMC was measured using a fluorescence spectrophotometer (BMG LABTECH, Offenburg, Germany) at excitation/emission spectra of 355/460 nm. The activity was calculated in relation to a standard reference AMC (Sigma, Munich, Germany) curve as nmol released AMC per mg protein and per minute using the linear part of a trend line from the resulting curves. Each assay was performed in duplicates.

### Statistics

Values were expressed by their mean and standard error of the mean. The significance of the difference was calculated by a two-tailed Student's *t*-test with values of  $P < 0.05$  being considered to be significant.

## Results

### Patient's characteristics

The AD keratinocytes derived from non-lesional skin of three AD patients with clinically manifested AD and highly elevated serum IgE levels above 5000 IU/mL. All three AD patients exhibited single nucleotide polymorphism (SNP) 1258G→A in exon 14 causing amino acid change Glu420→Lys. In addition, they showed polymorphisms 1221-50G→A in intron 13 and 1302+19G-A in intron 15.

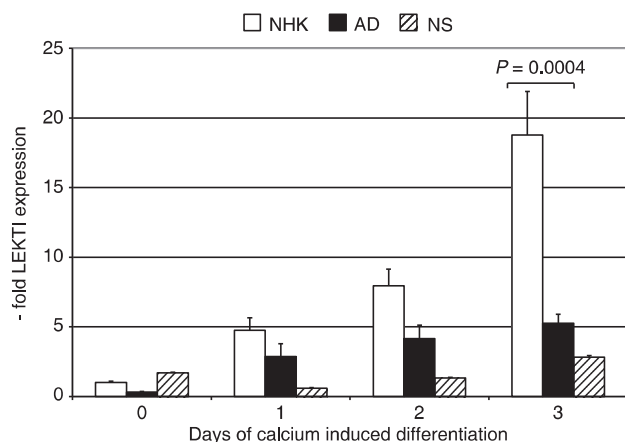
The three NS patients exhibited the following nonsense mutations in *SPINK5*: patient 1: homocytous 1432-13G→A mutation (intron 15, 7th domain); patient 2: heterocytous compound defect with mutations 354delTTGT (exon 5, 2nd domain) and 1432-13G→A (intron 15, 7th domain); patient 3: 316delGA mutation (exon 5, 2nd domain) in one allele; a potential mutation in the second allele has so far not been detected and may be located within one of the intron sequences that have not yet been analysed.

### LEKTI expression

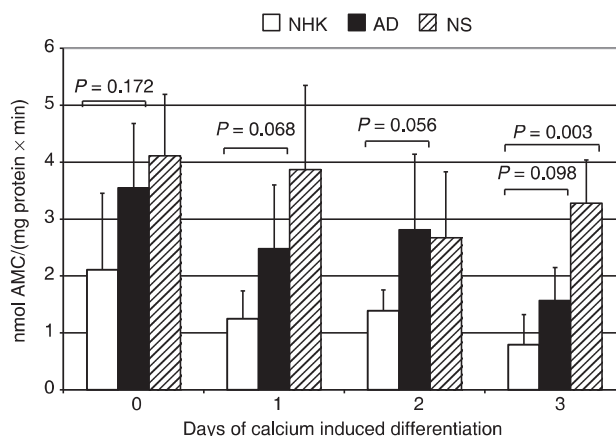
LEKTI expression in NHK increased with progress of differentiation in high calcium-containing media (Fig. 1), whereas no increase occurred in NS, consistent with LEKTI insufficiency due to mutations in *SPINK5*. In AD keratinocytes, LEKTI expression did increase with progress of differentiation, but to a significant lower extent than in NHK ( $P = 0.0004$  at day 3).

### Inhibitory activity of LEKTI

The trypsin-like hydrolytic activity assay performed gave a reciprocal measurement of the inhibitory activity of LEKTI, meaning that high hydrolytic activity was consistent with low LEKTI inhibition and vice versa. As expected, the hydrolytic activity in NHK was low due to high inhibition of functional LEKTI in contrast to the high hydrolytic activity in NS-keratinocytes with deficiency in LEKTI ( $P = 0.003$  at day 3; Fig. 2). AD keratinocytes demonstrated a trend towards an increased hydrolytic activity



**Figure 1** LEKTI expression measured by RT-PCR in NHK (5×) and keratinocytes from individuals with AD (3×), and NS (3×), each cultured for 3 days in differentiation medium containing 1.2 mmol CaCl<sub>2</sub>. As expected, LEKTI expression in NHK is increasing with progress of differentiation, whereas no increase was seen in NS. In AD, LEKTI is also increasing with progress of differentiation, but to significant lower level than NHK (shown are mean values with standard deviation).



**Figure 2** Hydrolytic activity of trypsin as a reciprocal measurement of serine protease inhibitory activity of LEKTI in NHK (5×) and keratinocytes from individuals with AD (3×), and NS (3×), each cultured for 3 days in differentiation medium containing 1.2 mmol CaCl<sub>2</sub>. Trypsin cleaves AMC from a substrate protein and the former is assayed fluorometrically. As expected, the hydrolytic activity in NHK is low due to high inhibition through active LEKTI (white bars), whereas in NS hydrolytic activity is high due to missing LEKTI inhibition (dashed bars). In AD (black bars), hydrolytic activity has the tendency to be higher than in NHK corresponding to a reduced inhibitory activity of LEKTI (shown are mean values with standard deviation from 5 × NHK, 3 × AD, and 3 × NS, each assay performed in duplicates).

consistent with a decrease in LEKTI inhibition even the differences in mean values were not significantly altered with *P*-value being 0.172, 0.068, 0.0559 and 0.098 at days 0, 1, 2 and 3, respectively.

## Discussion

In NS, the defective inhibitory regulation of serine protease inhibitor LEKTI results in an increased protease activity in the stratum corneum that accelerates degradation of desmosomal and intercellular adhesion molecules, and finally leads to over-desquamation of corneocytes and severe skin barrier defects.<sup>2,4-6,8</sup> Since NS shares several features of AD, it has been a speculation for several years that LEKTI might be involved in the pathogenesis of AD.<sup>1</sup> Recent research has focused on a putative association between genetic polymorphisms within *SPINK5* and atopic diseases, so far with contradictory results for both AD and asthma. Most studies looked at the same SNP in *SPINK5* causing amino acid change Glu420→Lys that has been initially found to be associated with AD.<sup>7</sup> Several groups were able to confirm this association,<sup>9-12</sup> whereas others were unable to find any association at all.<sup>13-15</sup>

Herein, we approached the role of *SPINK5*/LEKTI on AD at mRNA and functional protein levels. The results of our RT-PCR analyses demonstrated that LEKTI expression is significantly reduced in keratinocytes from our atopic patients in comparison to NHK. Consistently, the hydrolytic activity of AD keratinocytes was slightly increased which can be explained by a diminished inhibitory protease activity due to less available LEKTI. Since all of our three AD patients investigated did exhibit the Glu420→Lys polymorphism, one might speculate that this *SPINK5* polymorphism goes along with a reduction in LEKTI expression concomitant

with a slightly increased protease activity leading to reduced desmosomal cleavage in the upper epidermal layers and subsequent to impaired keratinization and abnormal desquamation. It is noteworthy that the reduction of LEKTI and increased protease activity were already detectable in non-lesional skin. Although only be minimally altered, these biological effects may be enough to trigger AD. One can speculate that in lesional skin the reduction of LEKTI may be much more obvious and lead to a significant higher decrease of protease inhibition, but unfortunately keratinocyte cultures from lesional atopic skin were not available for our study to prove it.

Our results add to the immunohistochemical findings by Bitoun *et al.* and Ong *et al.* who both observed an abnormal decreased patchy staining pattern of LEKTI in the uppermost spinous and granular layers in specimens of AD, whereas in normal skin a continuous cytoplasmic staining pattern is expected.<sup>4,16</sup> Even though their and our expression analyses point to a decrease of LEKTI in AD, we can not exclude the possibility that the increase in proteolytic activity results not only from a reduction of LEKTI, but also from an increase in kallikreins, which have recently been shown to be elevated in the stratum corneum and serum of AD patients.<sup>17</sup> In this context, *KLK7* is of particular interest being predominantly enhanced in AD skin. Moreover, just like in *SPINK5*, a polymorphism in *KLK7* has recently been found that is in debate of being associated with AD.<sup>12,13</sup>

In conclusion, our results add to the theory that LEKTI is involved in the pathogenesis of AD at least in AD patients exhibiting polymorphism Glu420→Lys. Nevertheless, multiple other factors such as kallikreins contribute to the development of AD lesions and it will be a matter of future investigations to clarify a more detailed connectivity of such factors.

### Acknowledgements

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